EXAGGERATED CLIMATE WARMING ON THE ASSUMPTION OF UNIFORM ATMOSPHERIC CO_2 CONCENTRATION

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Abstract. Based on the observed atmospheric CO_2 concentration data from the GAW (Global Atmosphere Watch) stations, we constitute a dynamical heterogeneous dataset of atmospheric CO_2 concentration that varies monthly within the regional climate model domain around China referring to the heterogeneity of CO_2 concentration in different economic regions and land use types. By running RegCM4-CLM3.5 climate model with this dataset, the climate warming rate was simulated and was compared with the control run with uniform CO_2 concentration. The simulation study shows that CO_2 greenhouse effect might have been aggrandized about 10% in the prevailing climate simulation due to improper assumption of uniform atmospheric CO_2 concentration. The effect of CO_2 itself (changing atmospheric transmissivity) is not the main reason for the reduction of temperature. The change of atmospheric CO_2 concentration affects the CO_2 partial pressure between air and interior of plant cells, and then the land plants adjust to this change by altering their stomatal conductance, which affects the water evapotranspiration from plant leaf to atmosphere consequently. On the one hand, this effect affects environmental temperature through the evaporation cooling, and on the other hand, the evaporated moisture alters the air humidity and influence the amount of low cloud by diffusion effect. This phenomenon is especially obvious in summer when the plants grow vigorously. The change of low cloud amount changes the short-wave radiation to the ground, which leads to the change of temperature.
Keywords: *dynamic heterogeneous distribution of CO_2, greenhouse effect, climate simulation, radiation budget and cloud feedback, physiological forcing effect*

Introduction

Climate model is the most popular tool in routine climate prediction and climate change study because of its capability of handling the greenhouse effect quantitatively with various greenhouses gasses such as CO_2, CH_4 etc, which are closely related to human activities (Kiehl and Ramanathan, 1983; Szu-cheng and Liou, 1983; Smith et al., 2017). Unfortunately, the spatio-temporal heterogeneity of CO_2 concentration in the atmosphere is not taken into consideration by the prevailing climate models, either global climate model (GCM) or regional one (RCM). For instance, in the prevailing GCM of CAM3.0, the volumetric mixing ratio of CO_2 was assumed to 355 ppmv (Collins et al., 2004). While in the popular mesoscale model MM5, the mixing ratio of CO_2 was 330 ppmv (Dudhia et al., 1998). The latest regional climate model RegCM3.1 and RegCM4.0 issued by ICTP

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(Abdus Salam International Centre for Theoretical Physics) has considered the CO_2 concentration variation from 1750 to 2100 (Elguindi et al., 2010), but seasonal, monthly and even daily CO_2 concentration variations were not taken into consideration. Besides, within the model domain, the CO_2 concentration was still assumed to be uniform. The greenhouse effect plays the most important role in climate warming; the untrue atmospheric CO_2 concentration in climate model should twist simulation (or prediction) accuracy.

China is a large country and covers several different climate zones. The vegetation types are diverse, and the source (or sink) of CO_2 varies seasonally. The intense human activities and the uneven population density cause heterogeneous CO_2 concentration distribution around China (Zhou et al., 2008; Zhao and Sun, 2014). Besides, the economic development level from east to middle and west of China is different, and the phenology from south to north of China is different as well, which causes CO_2 concentration heterogeneities around China (Liu et al., 2011). The same is true for other big countries, particularly for the big developing countries.

The baseline atmospheric CO_2 concentration and $\delta^{13}C$ observation have proved that the annual and daily periodic variation of photosynthesis and respiration of the terrestrial ecosystems affects the CO_2 concentration significantly (Liu et al., 2011). The large-scale ecological engineering construction around China, such as the Nature Forest Protection, Three-North Protection Forest Belt Construction, and Replacing Farm Land with Forest (or Grass) Engineering etc., changes CO_2 source (or sink) structure and then CO_2 concentration heterogeneities (Lin and Li, 1998).

Changing atmospheric CO_2 concentration was the primary driver of global climate change even in the early Eocene (Anagnostou et al., 2016). So, the global network for CO_2 concentration observation is set around the world gradually. The preliminary observation data shows that CO_2 concentration is not uniform, but it shows significant temporal and spatial heterogeneity. The constant CO_2 concentration used in the prevailing climate model is not proper, and there is a lack of work on climate simulation under heterogeneous atmospheric CO_2 concentration at the moment. Therefore, we think it is necessary to study the possible climate changes that may arise from the heterogeneous distribution of CO_2 concentration relative to the uniform distribution and to reveal the mechanism of these changes, which may provide a theoretical basis for improving the climate simulation. Based on this considerations, we collected CO_2 concentration observation data around China, considering possible CO_2 sources (or sinks) formation by social economic development factors, human activity and vegetation types etc., and configured a monthly varied dataset of heterogeneous CO_2 concentration within the model domain, which was used to run the regional climate model RegCM4-CLM3.5 for 108 months from March 2000 to February 2009. The model outputs were compared with the control run in the same period and with constant CO_2 concentration. On this basis, we discussed the regional climate effect caused by the heterogeneous CO_2 concentration. Furthermore, according to the order of annual mean radiant flux, cloud feedback effect and CO_2 physiological forcing effect, the possible mechanism of heterogeneous CO_2 concentration affecting the temperature change is gradually explored from outside to inside.

Materials and methods

The dataset formation

According to the CO_2 emission inventory issued by IPCC and the actual situation in China (Lin and Li, 1998; IPCC AR4, 2007), the factors that cause CO_2 concentration

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heterogeneity include energy production, industrial emission, land use and land cover variation, and population density. The above factors are simplified to two categories: 1) factors related to industrial emission and economy development, 2) LULC variation related to agriculture and forestry. The details of dataset formation are illustrated in the *Appendix* after the article.

Combining with the temporal and spatial variation, monthly heterogeneous atmospheric CO_2 concentration dataset in the model domain can be obtained (*Fig. 1*). The distributional pattern is reasonable as thinking about the plant phenological period, economic development level, and as well the population density etc. The CO_2 concentration is high in the big city area and the East Coast of China. The annual variation with a high value in winter and lower value in summer is clear. The more details in winter, which is rather higher CO_2 concentration occurrs in the forested area caused by respiration of the woody plants since the photosynthesis is weaker in this season. From May to July, however, CO_2 concentration is lower in the land than that over the sea because most plants are in their vigorous growing season and assimilate more CO_2. Statistics carried on within the model domain shows that the amplitude of yearly variation curve of the CO_2 concentration varied for different LULC types (*Fig. A3* in the *Appendix*). The correlation coefficient between the fitted curve and the observation data is 0.78. The area-weighting averaged value of CO_2 concentration in the model domain was 382.5×10^{-6}, a rather popular value of the present atmospheric CO_2 concentration cited frequently by climate change researchers.

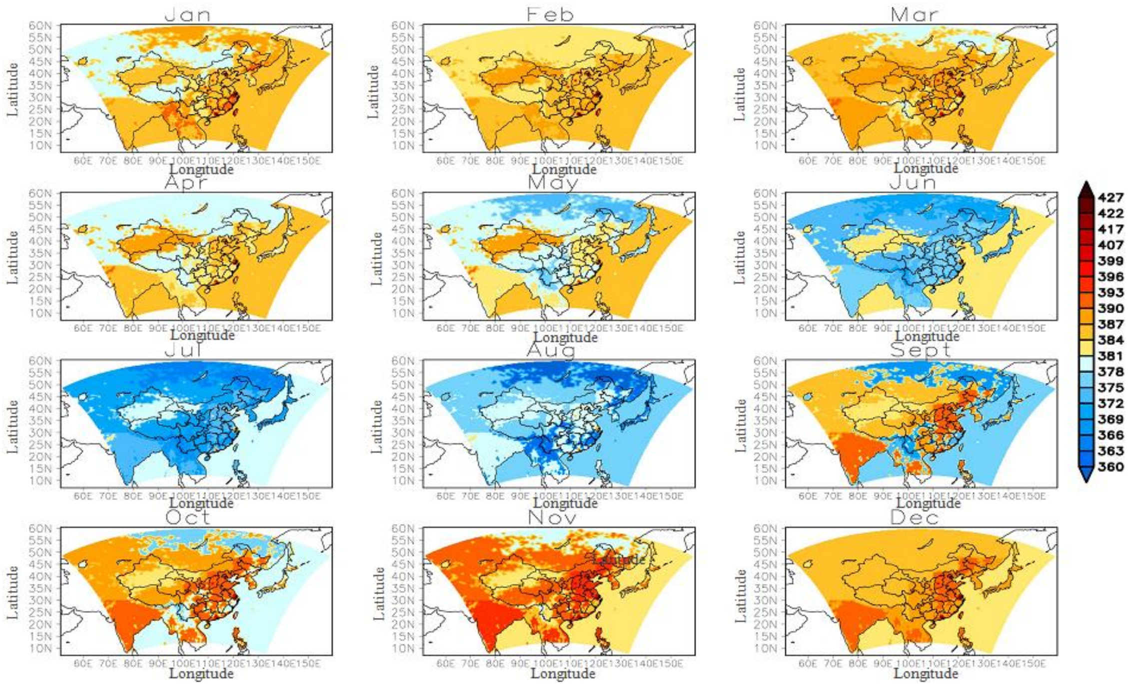

Figure 1. *Monthly dynamical heterogeneous atmospheric CO_2 concentration dataset within a regional domain around China (units: ppmv). The dataset is based on the baseline concentration data of atmospheric CO_2 observed from the GAW stations, considering the heterogeneous distributional characteristics of CO_2 concentration among different economic regions and land use types. The details of dataset formation are illustrated in the Appendix after the article*

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We emphasize that the dynamic and heterogeneous CO_2 concentration distributional pattern presented here is not perfect, nor is it the unique one. With the increasing atmospheric baseline stations, the temporal and spatial heterogeneity of CO_2 concentration will be understood more clearly. It is just to remind on the dynamics and heterogeneity of the atmospheric CO_2 concentration only.

Model description and numerical simulations

The regional climate model (RCM) used here is the version of RegCM4-CLM3.5 issued by ICTP in June 2010 (Elguindi et al., 2010). Compared with the previous version, RegCM4 incorporates a new land surface model of CLM3.5, which is from NCAR's Global Climate System Model CCM4 (Oleson et al., 2008). CLM3.5 is welcomed warmly by modelers because it can describe the land surface in more details, particularly in the carbon cycling process of land surface to reflect the feedback mechanism between the biosphere and climate system (Cox et al., 2000; Friedlingstein et al., 2006). Besides, RegCM4 also incorporates a sea surface temperature prediction module, which is essential to improve momentum flux in the sea-atmosphere interaction layer and is important to the present simulation study since one-third of the model domain is the ocean.

The horizontal grid is the same with the CO_2 concentration dataset shown by *Figure A.2* in the *Appendix*, vertically, the atmosphere is divided into 18 layers; the model top is set at 5 hPa level. Physical parameterization scheme is as usual (Elguindi et al., 2010). A 12-grid exponential relaxing boundary is used in the lateral boundaries of the model domain. The land surface process is modeled by CLM3.5. The time integration step for land surface and upper atmosphere is 600 and 150 s respectively. Radiation module is updated every 30 min; the lateral boundary condition is updated every 6 h using NCEP reanalysis data.

Besides the NECP reanalysis data, the OISST sea surface temperature (SST) data is also used in the model run. The model was run 110 months from January 1, 2000 to March 1, 2009. The first two-month data was removed since spin-up period and the left data of 108 months were used to analyze the effects of dynamic heterogeneous CO_2 concentration on the climate change.

The model system was run for three CO_2 concentration scenarios (*Table 1*).

Table 1. *Three scenarios of atmospheric CO_2 concentration*

Name of scenarios	Atmosphere CO_2 concentration × 10^{-6}	Distributional pattern of CO_2	Comments
Sce1	280	Uniform and constant	Pre-industry
Sce2	382.5	Uniform and constant	CO_2 concentration at present published by IPCC
Sce3	382.5	Dynamic and heterogeneous	CO_2 concentration at present published by IPCC

Sce1 represents the pre-industry stage when the atmospheric CO_2 concentration is 280 ppmv, and Sce2 is the averaged status of Sce3, with which a constant CO_2 concentration of 382.5 ppmv was used, Sce3 refers to a particular model run using a dynamic and heterogeneous atmospheric CO_2 concentration dataset as mentioned above. The comparison study was carried out first between Sce1 and Sce2 (Sce2 minus Sce1,

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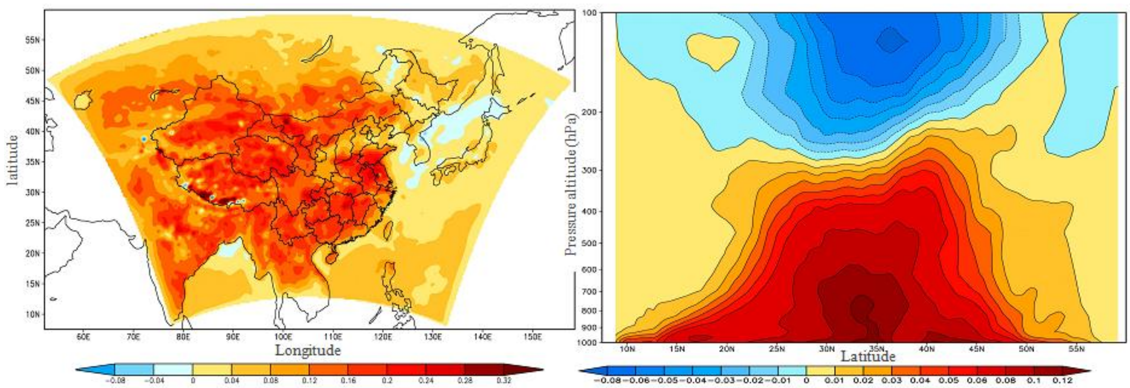
hereafter referred as EXP1) to test the model capability. Later on, we compared the difference of model outputs between Sce2 and Sce3 (Sce3 minus Sce2, hereafter referred as EXP2) to understand what the climate change will be if we use actual dynamic and heterogeneous CO_2 concentration.

Results

Comparison between Sce1 and Sce2 (EXP1)

Figure 2 gives out averaged temperature difference at 2-m high of EXP1. As the atmospheric CO_2 concentration increased from 280 ppmv to 382.5 ppmv, the temperature increased (*Fig. 2a*) clearly, particularly in the continent, where the increment is more than 0.12 °C. Over the sea, it is less than 0.08 °C, and the mean value in the domain is 0.11 °C. Considering the integrate time is about 10 years; this value is in good agreement with IPCC AR4's reports (2007). There are small areas in the Sea of Japan, northeast of China, and Bay of Bengal where temperature decrease, but the amount is small; in general, the CO_2 increase does cause surface layer warming.

Figure 2b is a latitude-high section showing vertical structure of temperature difference. The warming occurs in the lower part of the atmosphere below a level of 300h Pa. Above this level, the temperature difference shows negative. It had been reported that the greenhouse effect warms the troposphere and cool the stratosphere as compensation (Govindasamy and Caldeira, 2000). The model is acceptable in simulating greenhouse effect as atmospheric CO_2 concentration increased.



(a) (b)

Figure 2. *Averaged value of temperature difference in the EXP1 (units: centigrade), (a) 2-m high temperature variation; (b) latitude-high section of the temperature difference*

Comparison between Sce2 and Sce3 (EXP2)

Figure 3 shows the temperature difference of EXP2. As compared with the status of uniform CO_2 concentration, the temperature was decreased in most areas of the model domain, which means we had aggrandized the greenhouse effect by assuming uniform and stable CO_2 concentration in the climate model. The most significant temperature reduction happened in the middle, west and northeast of China's continent and the north of India. The decrement is -0.03~-0.15 °C. There are also some areas where the temperature increased including middle part of Inner Mongolia, The triangle of Beijing-Tianjin-Tangshan and the Delta Economic Zone of Yangtze River, the southeast coast

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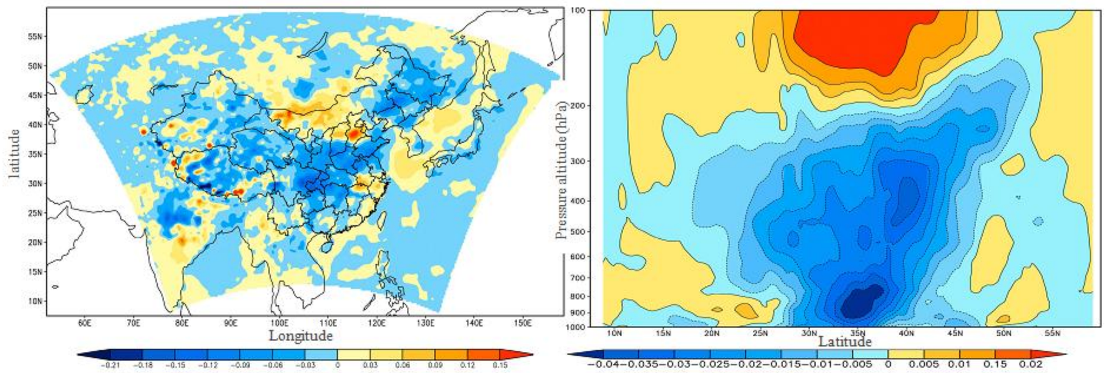
of China and the southeast of India etc. These areas are recognized as developed areas where the population density is high and human activities are intense. The average temperature of the whole domain is reduced by -0.01 °C. The White Paper of China's Policy and Action on Climate Chang reported that in average, the temperature was increased by 1.1 °C during the past 100 years (from 1908 to 2007) in China or 0.11 °C in every decade, the temperature reduction of 0.01 °C accounts for about 10% of this value. In other words, because of the uniform and stable CO_2 concentration assumption in the climate model, the greenhouse effect of CO_2 was aggrandized about 10% in the prevailing climate modeling.

The latitude-high section of the temperature difference in EXP2 was shown in *Fig. 3b*. An opposite shape occurred as compared with EXP1 (*Fig. 2b*). In the lower level below 250 hPa, the temperature decreased, while above this level, the temperature increased. The most significant temperature difference occurred in the middle latitude between 30-40°N, and the values are rather small in the lower or higher latitude. Again, a negative feedback mechanism of the stratosphere to compensate the troposphere is clear.

The statistics were carried out for each LULC types (*Table 2*) in every economic zone in EXP2. The woodland and grassland show temperature reduction with larger values in the 2-class economic zone, and the urban and barren area show temperature increase except in the 3rd economic zone, where the value is -0.46. Water body causes temperature reduction as well since it sequestrates CO_2 from the atmosphere.



(a) (b)

Figure 3. *Averaged value of temperature difference in the EXP2 (units: centigrade), (a) 2-m high temperature variation; (b) latitude-high section of the temperature difference*

Table 2. *Average temperature reduction caused by dynamic and heterogeneous CO_2 concentration in different economic regions and land use types (×10-2 °C)*

	Woodland	Grassland/Crop	Urban	Barren land (desert, permanent ice and snow)	Water
1-class	-0.48	-1.27	1.41	2.56	
2-class	-1.92	-3.37	0.17	0.12	
3-class	-0.46	-0.53	NaN	-0.46	
Water					-0.34

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Discussion

Radiation budget

Table 3 shows the comparison of various radiation elements, cloud amount and the temperature difference in EXP2. The statistics were carried out on clearly and cloudy day separately. Whenever the total cloud fraction is larger than 0.01, it is classified as a cloudy day. Otherwise, it is a clear day.

Table 3. *Bias of atmospheric radiative flux and cloud amount in the Exp2*

Items	Clear day	Cloudy day
Income shortwave (SW) radiation flux at the top layer of the model ($\times 10^{-2}$ W·m^{-2})	0.4	-2.0
SW radiation forcing by cloud	-2.4	
Out longwave radiation (OLR) in clear day	-1.2	-0.4
Longwave radiation forcing by cloud	-0.8	
Energy budget	-1.6	
Surface longwave radiation flux ($\times 10^{-2}$ W·m^{-2})	-0.1	-3.2
Out longwave radiation at the surface	-2.0	-3.8
Atmospheric radiation flux ($\times 10^{-2}$ W·m^{-2})	0.5	1.2
Cloud amount ($\times 10^{-2}$ percentage)	Total cloud amount 0.09	
	High cloud amount -0.04	
	Middle and low cloud amount 0.13	
Temperature difference at 2-m high (×10-2 °C)	0. 1	-1.3

In clear days, compared with Sce2, Sce3 gives a temperature increment of 0.1×10^{-2} °C, which is mainly caused by the increment SW radiation in the top layer (0.4×10^{-2} W/m^2), reduction of OLR (1.2×10^{-2} W/m^2) and the increase of atmospheric absorption of LR (0.5×10^{-2} W/m^2), though the income surface SW radiation is reduced (0.1×10^{-2} W/m^2). The atmospheric radiation penetration is not the main reason to cause the temperature reduction in Sce3.

In cloudy days, however, Sce3 gives a temperature reduction of -1.3×10^{-2} °C, which is mainly caused by the reduction of income SW radiation (-2.0×10^{-2} W/m^2), the negative energy budget (-1.6×10^{-2} W/m^2), reduction of income SW radiation at the surface (-3.2×10^{-2} W/m^2) and reduction of radiation forcing by cloud (-2.4×10^{-2} W/m^2) though atmospheric absorption is increased ($1.2\ \times10^{-2}$ W/m^2). The statistics for all cloudy day shows that high cloud amount reduced -0.04% in Sce3, but the total cloud amount increased 0.09% on average and the middle and low cloud increased 0.13%. The increased cloud can capture more LW radiation in the atmosphere, but it resists more shortwave radiation getting into the atmosphere, the integrated effect is to cause cooling (Andrews et al., 2012; Forster et al., 2015). So the cloud is the main reason to reduce greenhouse effect in dynamic and heterogeneous CO_2 concentration scenario.

Cloud amount variations

The latest studies showed that temperature reduction is mainly due to cloud sensitivity to the CO_2 concentration variation (Gregory and Webb, 2008; Andrews and Forster, 2008; Williams et al., 2008), and the cloud feedback mechanism is the main

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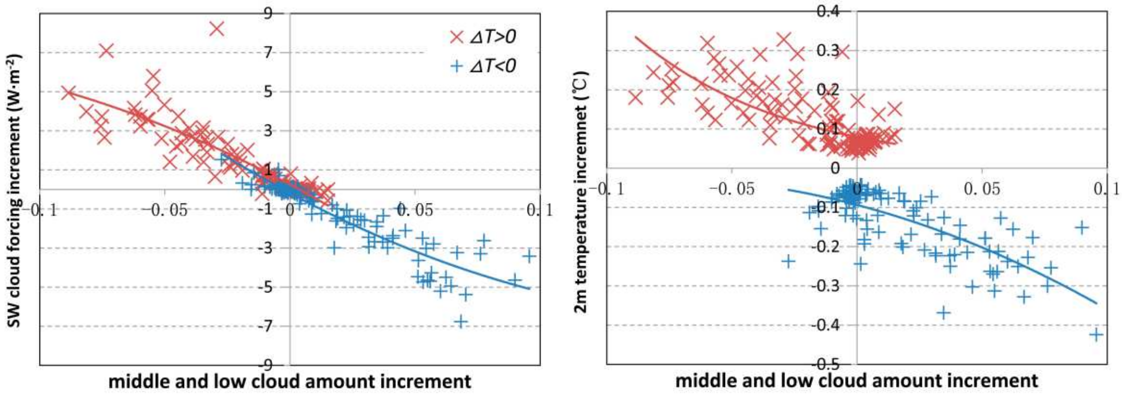
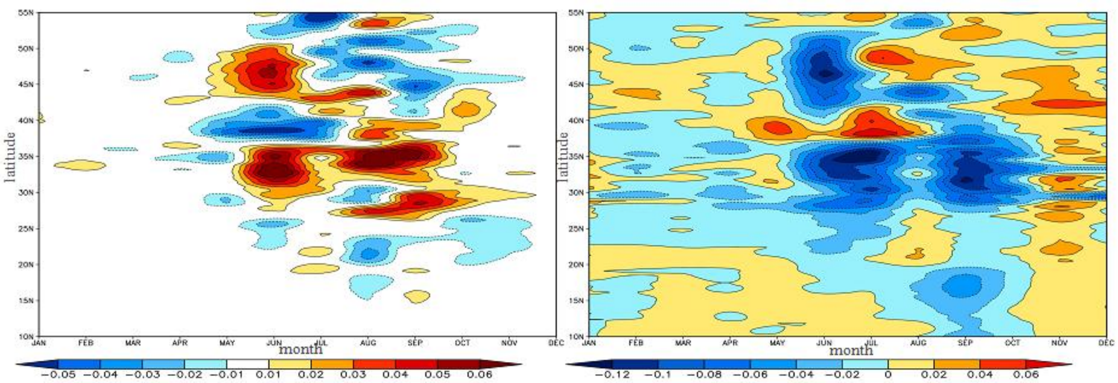
reason that causes difference among climate models (Bony et al., 2006; Caldwell et al., 2013). The cloud amount variation in EXP2 (Sce3 minus Sce2) is further analyzed by plotting all simulated examples to show the relationship among cloud amount, SW radiation and temperature reduction since high cloud is of less importance, and only the middle and low cloud data were used (*Fig. 4*). All the numerical grids either of temperature increase (red cross) or decrease (blue cross) are accounted though the former is of less number than the later. *Figure 4a* shows that with increased cloud amount, both SW radiation and temperature reduction increased, the correlation coefficient reaches to -0.87 in temperature increased grid and -0.91 in temperature decreased grid. This means middle and low cloud increase is the main reason to reduce temperature in Sce3.



a **b**

Figure 4. *Scatter diagram showing the relationship between middle and low cloud amount increment and (a) shortwave radiative forcing increment and (b) 2 m high temperature reduction*

The close relationship between cloud amount and temperature reduction can be illustrated by latitude-time section. *Figure 5* also shows the temporal and spatial variability of cloud amount and the temperature difference between Sec2 and Sce3.



(a) (b)

Figure 5. *latitudinal mean-month section of (a) low cloud amount variation and (b) temperature difference (units: centigrade) in EXP2*

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The most significant difference occurred in a middle latitude zone between 30-50°N during the summer from May to October. Wherever and whenever the middle and low cloud amount is increased (decreased), the temperature decreased (increase), and a negative correlation is clear.

Physiological mechanism

With the causality of CO_2 concentration increasing and plant physiological adaptation being noticed widely, the consequence to the cloud formation, the radiation forcing and temperature reduction are also reported frequently (Lambert and Chiang, 2007; Lambert et al., 2011). Our simulation study shows that in summer, the CO_2 concentration is rather lower, and the plant stomata conductance increases physiologically to adapt insufficient CO_2 supply (Sellers, 1996; Sellers et al., 1997; Betts et al., 1997), which causes rather stronger plant transpiration and adds more water vapor into the atmosphere (Boucher et al., 2009; Joshi and Gregory, 2008; Abe et al., 2015). Moreover, this phenomena is more significant in the growing seasons of summer in the East Asian monsoon climate zone and in the middle latitude where the vegetation is dense. The plant transpiration cools the plant canopy and then the lower part of the atmosphere, which stimulates the formation of convective cloud (Joshi et al., 2008; Doutriaux-Boucher et al., 2009). In winter, however, most plants are in hibernation period, and the biological vigor is weaker, so plants show less sensitive to the outside CO_2 concentration variation.

The present climate model and the relevant land surface model output affected by various plant physiological factors shows such ratiocination. *Figure 6* illustrates regularity of some physiological parameters. Since most plants are vigorous in summer in the East Asian monsoon climate zone, the statistics were carried on the data in summer (June–November) only. The geographical difference can be classified into three regions based on *Figure 6*. To the south of 25°N, the averaged stomata resistance (a) shows an increase, so the canopy transpiration (b), canopy evaporation (c) and 2-m high air humidity (d) show a decrease, which consequences the increase of absorbed radiation (e) and the 2-m high temperature (f). Between 25 to 40°N, all the curve's phases were reversed, because of the increased stomata conductance, the canopy transpiration, canopy evaporation and 2-m high air humidity increased, which causes increased cloud amount and decreased absorbed radiation and 2-m high temperature. To the north of 40°N, the curve tends to less amplified; it consists of the rather flat stomatal conductance.

In general, the lower CO_2 concentration always occurs in the growing season and the densely vegetative area, and the plant responses with larger stomatal conductance, which leads to more canopy evaportranspiration, higher air humidity, and increment of the middle-low cloud. It is the main reason causing the temperature reduction in the growing season and vegetative areas. Since the vegetative area covers a rather larger portion of the model domain, the weighting averaged value of temperature reduced. This physiological mechanism can be extended to the diurnal variation of CO_2 concentration as well, the periodic diurnal variation of CO_2 concentration just like the yearly one. In the daytime, when the plant is in its vigorous period and the CO_2 concentration is lower, which causes less greenhouse effect and mitigate warming, in the night. The CO_2 concentration is high because of the plant respiration, but the greenhouse effect is weaker. Unfortunately, this paper has not incorporated diurnal

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heterogeneity of CO_2 concentration into the modeling, but it is expected the temperature reduction could be even larger if it is considered.

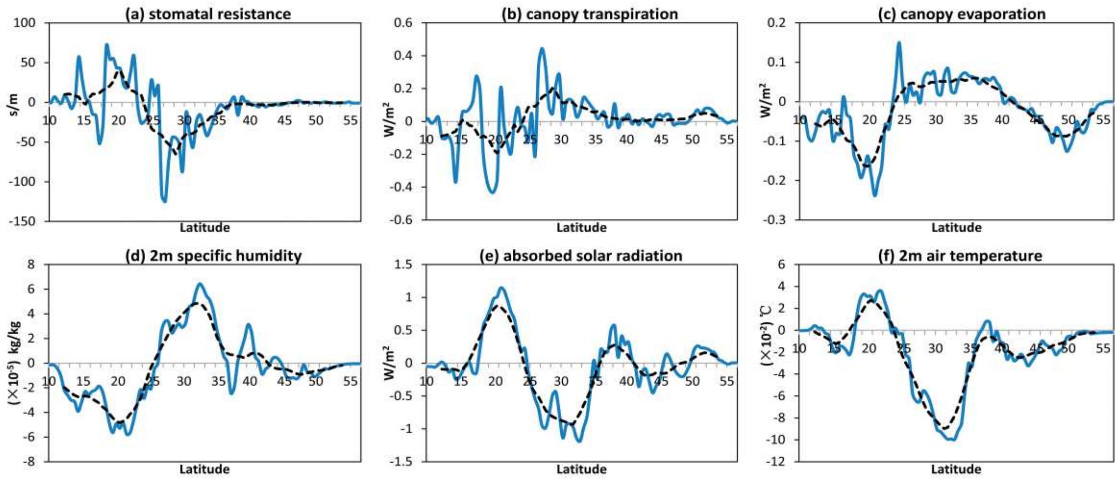

Figure 6. *Zonal mean increment change of (a) stomatal resistance, (b) canopy transpiration, (c) canopy evaporation, (d) 2 m specific humidity, (e) absorbed solar radiation, (f) 2 m high air temperature in the EXP2, the dashed line means the five-latitude moving average*

For proving the physiological mechanism that dynamic and heterogeneous CO_2 concentration causes less climate warming, a supplement model run was conducted, in which the land surface model CLM3.5 was replaced with BATS in EXP2 to remove any physiological effect in Sce3 since BATS uses constant plant parameters (Elguindi et al., 2010). The simulated temperature reduction is shown in *Figure 7*. Compared with *Figure 3b,* there is no significant temperature reduction in the troposphere in middle latitude, rather, there is a slight temperature increase, and the absolute values are much smaller as compared with *Figure 3b*.

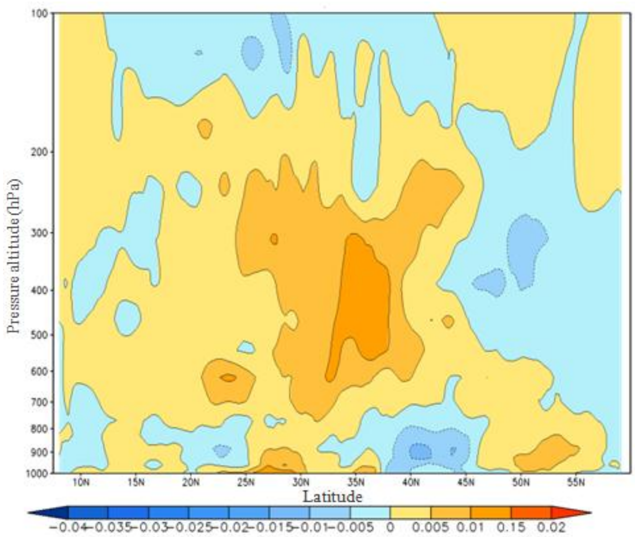

Figure 7. *Latitude-high section of the temperature difference (units: centigrade) from the supplement run, same with EXP2 but replace the land surface model CLM3.5 with BATS in Sce3*

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Conclusions

1) The uniform atmospheric CO_2 concentration data used in the prevailing climate modeling is improper, the greenhouse effect of CO_2 might be aggrandized about 10% because of the data flaw.

2) The simulation study reveals the main mechanism that dynamic and heterogeneous CO_2 concentration reduces temperature increment. The variety of atmospheric CO_2 concentration influences on the CO_2 partial pressure between atomosphere and internal plant cell first. The land plants adapt to this change by altering their stomatal conductance, which affects the water evepotranspiration from plant canopy to the atmosphere, and the evaporated moisture increases air humidity then the formation and amount of low cloud. And the relevent evaporation cooling accelerates cloud formation as well. This mechanism is significant in summer when the most plants are in vigorous growing period and in the middle latitude where vegetation coverage is high.

3) With the increasing atmospheric baseline stations with CO_2 concentration observation, the temporal and spatial heterogeneity of CO_2 concentration will be clearer in the future, which should be used in the climate modeling to improve the accuracy of climate change prediction.

Because the dataset and model regions in this work are targeted at China, so the conclusion of the fourth part of the article (Analysis on the simulation results) is specific to the region of China. But the mechanism of the impact of the CO_2 physiological effects on the climate change revealed in the fifth part of the article (Mechanism analyses) is universal. So we believe that in any region of the world, the climate effects that take into account the heterogeneity of CO_2 concentration are similar, but the sensitivity of temperature change may be different. Therefore, it is very meaningful to carry out relevant research by using global climate model and CO_2 concentration data of satellite detection.

In addition, it is expected that if the diurnal variation of atmospheric CO_2 concentration is taken into consideration, the atmospheric CO_2 concentration will be lower when the physiological activity of the plant is strong during the day, which may lead to the stronger physiological forcing effect and the greater amplitude of cooling, which is another issue worth studying.

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APPENDIX

Details of the dataset formation

The two-dimensional horizontal heterogeneity was assigned according to the different categories, and each category has its own monthly variation curve. The grid dataset of heterogeneous CO_2 concentration suitable for numerical simulation is shown in *Fig. A1*. The domain center was set at 35°N, 105°E, and the grid resolution was 60 km. In addition, the longitudinal and latitudinal grid were 120 and 90 respectively. Lambert map project scheme was used in the model.

Appendix 1

Economy development consideration

The model domain was divided roughly into three different economic zones (*Fig. A.1*): 1) to the east of 110°E China, heavy industrial northeast of China, north of Indian plain (altitude is lower than 1000 m), Burma, Vietnam and Philippine etc., the first category was classified as the most economically developed area where the population density is high, and CO_2 emission is the highest; 2) The China's territory

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within 100～110°E, and 20～38°N, Japan, Korea peninsula, Taiwan and Hainan Island of China etc. This category was classified by the following considerations: the middle of China is less developed as compared with the east of China with rather backward science and technology. The CO_2 emission of unit GDP is higher, but the population density is rather lower than that in the east of China. So the total CO_2 concentration was classified as the second category, and Japan, Korea and Taiwan are with high population density. But compared with the first class area, the manufacture configuration is more reasonable, energy efficiency is high and the advanced high-tech utilization is more popular, so the CO_2 emission from unit land is lower than those in the first class area; 3) The left area was classified as the third class, where the CO_2 emission and CO_2 concentration was assumed to be least, because the population is sparse and the economy is backward compared with above two classes. The water body itself is CO_2 sink to absorb CO_2 from the atmosphere. The CO_2 emission over the water body by human activity was assumed to be zero, so it is classified separately as the fourth class. Based on the above assumption, the model domain area was classified with different CO_2 concentration as shown in *Figure A1*.

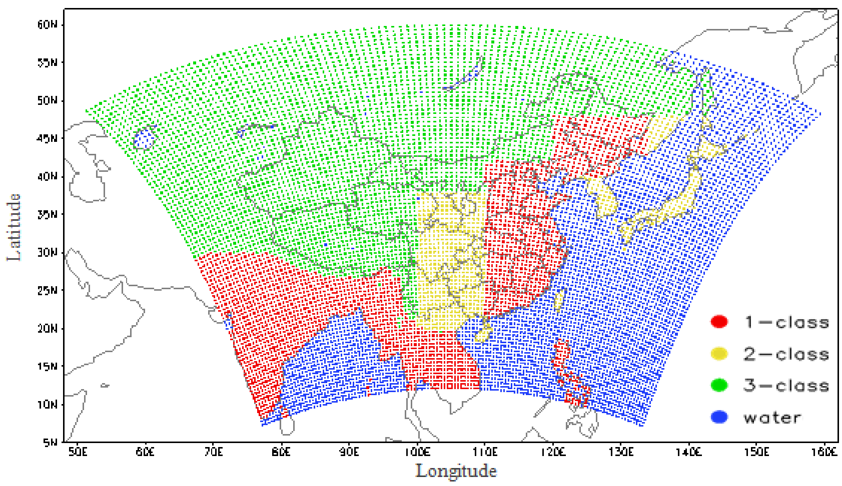

Figure A1. *Distributional pattern of CO_2 concentration classified according to economic development and population density*

Appendix 2

LULC variation consideration

The LULC was classified into 5 categories to reflect their differences in CO_2 sequestration capability according to the IPCC's relevant document (Busche, 2006). Those are forest, grass (or crop), urban, water, and barren land (include rock and Gobi, vegetation-free land, ice and snow). The original data was from the 1 km resolution LULC data derived from EOS/MODIS satellite information (hereafter called IGBP data). The 1-km resolution data of the land surface model BATS (Biosphere-Atmosphere Transfer Scheme; http://edcdaac.usgs.gov/glcc/glcc.html) was also used to compensate missing data in the mainland territory of China, which was from the NOAA's AVHRR dataset and commonly called GLCCD data. The 1-km resolution was then projected on the model domain to form a new dataset with a specific LULC type of the highest portion in each grid square only (*Table A1*), which is a benefit to differ the

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CO_2 sequestration capability of different LULC types. Since the big city has a special function in CO_2 emission, the grids are defined as city whenever the city portion in a grid square exceed 20%. The LULC difference in the model domain is shown in *Figure A2.*

Table A1. *Merging scheme of multi-types and high-resolution land use data based on the IGBP and GLCCD*

ID	LULC type classification	IGBP (2004)	GLCC (1992~1993)
1	Woodland	Evergreen needle-leaf forests Evergreen broadleaf forests Deciduous needle-leaf forests Deciduous broadleaf forests Mixed forests Closed shrublands	Evergreen needle-leaf tree Deciduous needle-leaf tree Deciduous broadleaf tree Evergreen broadleaf tree Mixed Woodland Forest/Field mosaic Evergreen shrub
2	Grassland/Crop	Open shrublands Woody savannas Savannas Grasslands Croplands Cropland/Natural vegetation mosaics Permanent wetlands	Crop/mixed farming Short grass Tall grass Tundra Irrigated Crop Bog or marsh Deciduous shrub
3	Urban	Urban and built-up lands	
4	Barren land	Snow and Ice Barren	Desert Semi-desert Ice cap/glacier
5	Water	Water bodies	Inland water Ocean Water and Land mixture

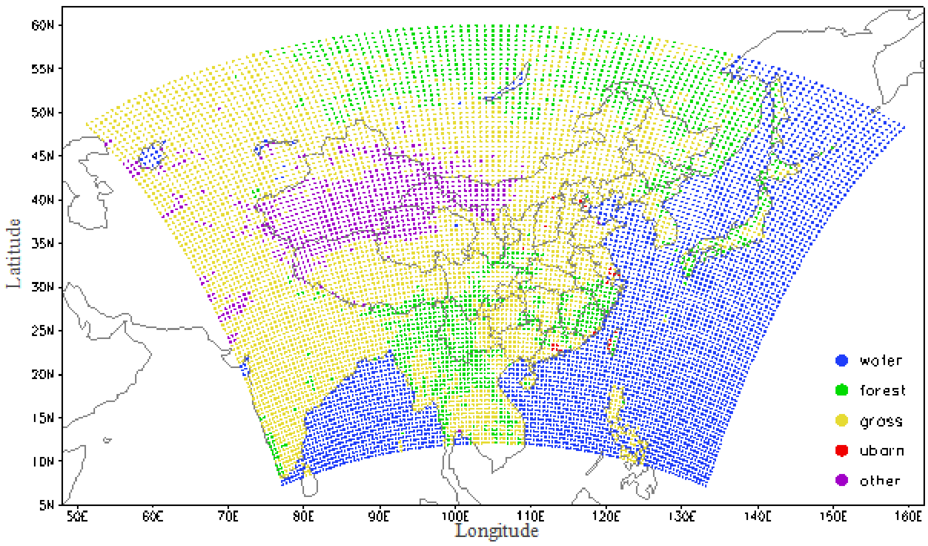

Figure A2. *LULC classification for building the dynamical heterogeneous atmospheric CO_2 concentration data*

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Appendix 3

Dynamics of the atmospheric CO_2 concentration

Intra-year variation of CO_2 concentration for each LULC type was formed by referring to the baseline observation data, including the global atmospheric baseline stations, regional background stations, field and/or city temporal data logging (Wu et al., 2005; Cheng et al., 2003; Zhou et al., 2007; Wang et al., 2003). The data from the barren area (Waliguan Station) and ocean showed the smaller amplitude of CO_2 concentration variation (*Table A2*), and these data were used to present the large-scale background data. While those from forest, grass and urban areas showed larger amplitude, and the CO_2 concentration were affected by biological activities and local emission, which presents the local heterogeneity of CO_2 concentration. In general, the CO_2 concentration is higher in the east of China than in the west of China because of the intense human activity and higher population density.

Table A2. *Information of referenced stations for atmospheric CO_2 concentration*

LULC type	Name of the station	Latitude (N)	Longitude (E)	Altitude /m	Vegetation type	CO_2 concentration /10^{-6}	Data collection year
Woodland	Longfen Mountain	44°44’	127°36’	310	Subsidiary forest	365±20	2003
	Changbai Mountain	44°08’	128°06’	740	Broad leaf-Korea Pine forest	370±20	2003
	Suyukou in Ningxia Autonomous Region	38°44’	105°55’	1950	Northwest arid area	297±16	2006
Grassland /Crop	Linan	30°18’	119°45’	130	Agricultural mixing	380±15	2003
	Xinglong	40°24’	117°30’	940	shrubs	375±15	2000
	Haibei	37°37’	101°17’	3400	Short grass and wetland shrubs	315±20	2004
	Wulanchabu	41°47’	111°53’	1450	Typical grassland	330±18	2006
	Dashuiqugou of Ningxia Autonomous Region	38°39’	105°57’	1330	Hungriness grassland	335±20	2006
Urban	Beijing	38°58’	116°22’	30	Urban	400±40	2002
	Shang Dianzi	40°39’	117°07’	290	Sub-urban area	375±25	2003
	Huaian	30°16’	118°59’	40	Middle-side city	400±30	2006
	Hefei	31°52’	117°17’	30	Reservoir close to city	390±30	2008

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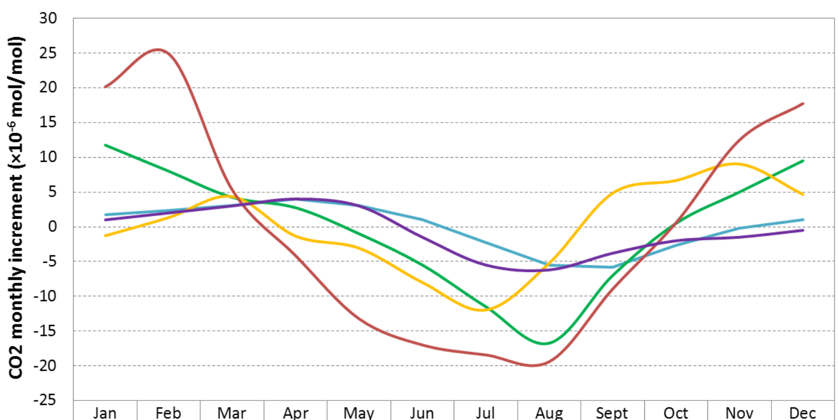
	Wuxi	31°25’	44°44’	10	Lake-side rural area	405±20	2003
Other	Waliguan	36°18’	100°54’	3820	Hungriness grass	375± 5	2004
Water	Ocean				Oceanic boundary layer	375± 5	2004

Supposing the CO_2 concentration of each LULC type varies regularly in a same economic zone, the average value of five LULC categories in four different economic zones are listed in *Table A3*. These values are used to feed the model at the initial state but vary as time of model integration (see next section).

Table A3. *Model initial data of CO_2 concentration for different economic regions and land use types*

	Woodland	**Grassland/Crop**	**Urban**	**Barren land (desert, permanent ice and snow)**	**Water**
1-class	380	386	410	388	
2-class	378	384	405	386	
3-class	376	382	400	384	
Water					383

The dynamic characteristics of CO_2 concentration were divided into two parts, the inter-annual variation and monthly variation in a specific year. The former followed the IPCC and reported values (ftp://crgd.atmos.uiuc.edu/pub/post-sres/A2-conc.txt) from 2000 to 2009. The later was assigned by thinking of growing period for different plants and the phenology in different climate zones. The monthly differential values of CO_2 concentration from the average for five LULC types were listed and graphed in *Figure A3*.



	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
water	1.7	2.4	3.1	4	3.1	1	-2.2	-5.5	-5.8	-2.7	-0.2	1
forest	11.8	8.0	4.3	2.8	-1.0	-5.5	-11.5	-16.8	-7.0	0.5	5.0	9.5
grass	-1.3	1.3	4.3	-1.3	-3.1	-8.0	-12.0	-5.2	4.8	6.7	9.0	4.7
urban	20.1	24.9	5.3	-4.1	-13.2	-17.0	-18.4	-19.4	-8.9	0.6	12.5	17.7
other	1	2	3	4	3	-1.5	-5.5	-6.2	-3.8	-2	-1.5	-0.5

Month

Figure A3. *Monthly atmospheric CO_2 concentration increment for different land use types*

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The seasonal CO_2 concentration variation is clear, and particularly in the urban area, forest and grass. The average value of the amplitude of CO_2 concentration variation curve in the forest is about 10 ppmv, and those in urban area is 20 ppmv. The later reflects the intense human activity in the big cities such as house heating in winter, car running and other vehicles, over the water body and in the barren area, however, the amplitude is much smaller.

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SPATIAL DISTRIBUTION OF RIVER BASIN SUSTAINABILITY INDICATORS IN TRANSITION REGION OF NORTHEASTERN BRAZIL

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Abstract. Sustainability indicators were applied in river basins of a state in Northeastern Brazil, aiming to outline and support effectively sustainable actions of water resources management. Twelve indices and three indicators of Sustainability focused on socioeconomic, hydrological and institutional issues were calculated for six hydrographic regions, quantitatively and qualitatively. Partial scales for all indices related the calculated values to levels of performance (Very High, High, Medium, Low, and Very Low). Posteriorly the indices were grouped in global scales, constructed with levels of water sustainability for all indicators. The results have shown an overall intermediate performance in the assessed river basins, indicating the need to adopt priority measures in the hydrological dimension, especially in relation to groundwater; attention to primary sanitation, with regard to public supply; reduction in water demand and waste; implementation of management tools; consolidation and support to river basin committees. The spatialization of water sustainability indicators enabled a clearer perception and an unmistakable assessment of each river basin surveyed.
Keywords: *water resources, decision-making, indicators, sustainability, river basin*

Introduction

The imbalance between water availability and demand causes its scarcity, which has become one of the most pressing problems in the world (Peterson and Schoengold, 2008). This situation may worsen due to population growth, global climate change and deterioration of water quality (Qu et al., 2013). Demand for water is expected to increase by more than 40% by 2050. By 2025, about 1.8 billion people will live in countries or regions where water is scarce, and two-thirds of the earth may live in a condition where the supply of clean water does not meet its demand (UN, 2015; Ross, 2017).

In Brazil, water demand has grown significantly in the last decades due to the economic development process, the increase of population clusters and the quantification, increasingly grounded, of environmental needs (Carvalho and Curi, 2013). Although Brazil has an abundance of water, it is poorly distributed in relation to the demographic density of the country, with 80% of the water being in the Amazon region and a severe scarcity existing in the Northeastern region (ONU, 2007). However, in addition to misdistribution, Brazil faces more severe issues, such as permanent contamination and waste of the remaining clean water (Bragatto et al., 2012).

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According to Oelkers et al. (2011), one of the key solutions to this global water crisis is better management of this valuable natural resource. The literature reports that, as the complexity of issues related to water resources has increased, there have been extensive studies to combine the concept of sustainability with water management matters (Loucks e Gladwell, 1999; Loucks et al., 2000; Starkl and Brunner, 2004; Mays, 2006; Policy Research Initiative, 2007). An integrated view of water is essential because it can add social, economic, environmental and institutional aspects to all management processes (Juwana et al., 2010), contributing to understand the evolution of the water system and its influences, that is, the achievement of a sustainable management of water resources (Sun et al., 2016).

According to the OECD (2003, p. 19), "water is the perfect example of a sustainable development challenge – encompassing environmental, economic and social dimensions." The sustainable management of water resources, therefore, implies not only the indefinite continuation of physically and biologically stable systems, but also concern for the other dimensions of sustainable development, such as the economic efficiency of water use, the equitable distribution of the costs and benefits of water resource developments and participatory approaches to policy-making and decision-taking (Ioris et al., 2008).

Sustainability indicators have become relevant tools for the integrated planning and management of water resources (Hooper, 2010). Very useful in decision-making, they enable the simplification of information on complex phenomena and the identification of primary demands (Barros and Silva, 2012). The adoption of indicators to assess and monitor progress towards sustainable development is highly recommended by scientists (Moldan et al., 2012; Cornescu and Adam, 2014; Bolcárová and Kološta, 2015), policy developers (UN, 2007), financial institutions (OECD, 2014; WWAP, 2003), governments (OSE, 2008), business sector (WBCSD, 2000) and nongovernmental organizations (WWF, 2010), as they are means to assess the level of satisfaction of several criteria, helping to translate abstract concepts into measurable parameters (Lee and Huang, 2007).

In Brazil, several authors have used indicators to analyze and propose suggestions aimed at enabling water management in an integrated and sustainable method in river basins (He et al., 2000; Pompermayer et al., 2007; Chaves and Alipaz, 2007; Ioris et al., 2008, Vieira and Studart, 2009; Magalhães Júnior, 2010; Carvalho et al., 2011; Maynard et al., 2017), fact also occurs in river basins in several countries, according to studies carried out by UNESCO (2008), Ioris et al. (2008), Catano et al. (2009), Cortés et al. (2012), Pellicer-Martínez and Martínez-Paz (2016).

Currently, society is seeking a broader debate on sustainable development in terms of rational use and valuation of natural resources. Therefore, discussing aspects related to water management in river basins by using indices and indicators in order to enable a more integrated and sustainable water management brings relevant contributions to the current scenario.

This article it is proposed to a holistic assessment of river basins through indicators that measure how water management is progressing in the perspective of sustainability in a strategic region of Brazil, located between the semiarid Northeastern region and the Amazon region.

As a background, specifically regarding water resources in Maranhão, the area used to perform this study, Dias (2018) points out that there is a notable gap in literature on water resource management, which presents an unfavorable framework regarding the

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implementation of management instruments. There are no studies that address the issue of water sustainability in the State of Maranhão.

Thus, this study also intends to help fill a specific gap in the Northeastern part of Brazil, where there is little literature related to integrated management of water resources aiming at the sustainability of river basins.

Taking into consideration these arguments and the relevance of this issue in the context of water management and sustainability, this article aims to diagnose, through indicators, the water sustainability of river basins in the State of Maranhão, in Northeastern Brazil, and to substantiate the decision-making process for the integrated management of water resources.

Materials and methods

Characterization of the study area

The study was conducted in the State of Maranhão, which is located in the Northeastern part of Brazil, between the coordinates of 02° to 10° south latitude and 44° to 48° west longitude. Covering an area of 331,935,507 km², Maranhão has approximately 7 million inhabitants, being the fourth most populous state in Northeastern Brazil (IBGE, 2017).

It is located in a transition area between the Amazon (wet) and Northeastern (semiarid) regions, favoring great annual rainfall contrasts. The portion of Legal Amazon in Maranhão covers an area equivalent to 80% of the territorial area of the State, about 264,000 km². The region of transition between the Amazon and Cerrado biomes is characterized by a high diversity of ecosystems and biodiversity. (Silva et al., 2016).

The State of Maranhão is divided into twelve hydrographic regions, of which six were selected for this research: Parnaíba, Tocantins, Gurupi, Munim, Mearim and Itapecuru (*Fig. 1*).

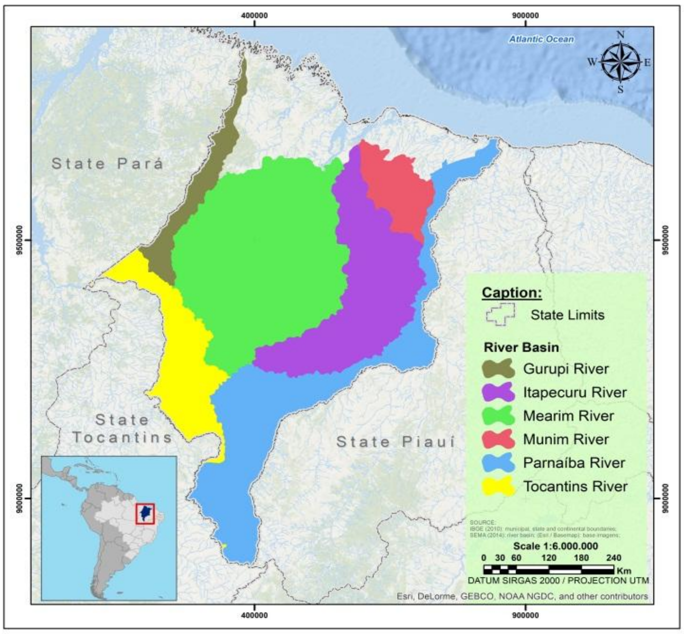

***Figure 1.** Geographical location of the river basins researched*

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Frame 1 shows some demographic characteristics of the river basins studied.

Frame 1. *Demographic characteristics of the river basins studied in this research. (Source: IBGE, Censo Demográfico, 2010; NUGEO/UEMA, 2011)*

Basin	Area (km²)	Population	% over the State area	Population density in the basin (inhabitant/km²)	No. of cities in the basin
Tocantins River	30,665.15	498,105	9.24%	16.24	23
Parnaíba River	66,449.09	717,723	20.02%	10.80	39
Gurupi River	15,953.91	178,302	4.81%	11.18	12
Mearim River	99,058.69	1,681,307	29.84%	16.97	83
Munim River	15,918.04	320,001	4.79%	20.10	27
Itapecuru River	53,216.84	1,019,398	16.03%	19.16	57

Selecting indicators and setting indices

The selection process was initially based on an extensive bibliographical research on indices and indicators applied to water resources, aiming at meeting the need to evaluate water sustainability conditions in the basins of Maranhão.

Based on the information required to compose each indice and indicator and the information available in the river basins studied, we have decided to use the methodology elaborated by Vieira (1999) and modified by Campos et al. (2014). Thus, based on these authors, we have chosen to apply the following indicators:

(a) Indicator of Potentiality, Availability and Demand (IPAD), to assess information regarding potentiality and availability of the water from the river basin, as well as the capacity to meet demands; (b) Indicator on Water Resources Management (IWRM), to reflect how entities are implementing it and instruments of the water resources policy; and (c) Water Use Efficiency Indicator (WUEI), to inform the conditions of environmental sanitation in the river basin and the level of efficiency of public utilities in the distribution of the captured water. These sustainability indicators were applied at the river basin level, which is the water resources management unit. Chaves and Alipaz (2007) highlight that this consideration is important, since the assessment of water resource sustainability cannot be limited by jurisdictional boundaries.

The process of choosing indicators also considered whether they meet the four criteria of sustainability: social, economic, environmental and institutional, as described by Pires et al. (2017). In this evaluation, the three indicators IPAD, IWRM and WUEI meet most sustainability criteria. Thus, the chosen indicators can be applied to reliably diagnose the sustainable use and water management in selected river basins. In addition, they are interesting tools that allow us to see some of the multiple aspects of water management and use from specific angles.

The following criteria were taken into consideration to define the indices constituting each indicator: (a) relevance (ability to translate the phenomenon); (b) local adherence (ability to capture the phenomenon produced or that can be transformed at the local level); (c) availability (coverage and timeliness of data); and (d) ability to allow time comparisons (Campos et al., 2014).

Frame 2 shows the indices selected for each indicator.

Frame 2. *Indicators and their respective indices selected for this research. (Based on Campos et al., 2014)*

Indicator	Indice	Description
IPAD Indicator of Potentiality, Availability and Demand	Activation of Potentialities **(IAP)**	Relation between availability and potentiality
	Use of Potentialities **(IUP)**	Relation between demand and potentiality
	Use of Availabilities **(IUA)**	Relation between demand and availability
IWRM Indicator on Water Resources Management	River Basins Committees **(IRBC)**	Existence and scope of operations
	Grant **(GI)**	Level of implementation of the grant
	Collection **(CI)**	Level of implementation of the collection
WUEI Water Use Efficiency Indicator	Households Supplied by Wells **(IHSW)**	Percentage of households supplied by wells in relation to the total number of households
	Households Supplied by a Water Supply System **(IHSWSS)**	Percentage of households supplied by a water supply system in relation to the total number of households
	Sewer Connections **(ISW)**	Percentage of households with a sewage network or a septic tank in relation to the total number of households
	Sewage Treatment **(IST)**	Percentage of households with sewage treatment in relation to the total number of households
	Solid Waste Treatment **(ISWT)**	Percentage of households with waste collection in relation to the total number of households
	Water Loss in the Network **(IWLN)**	Average percentage of physical losses (leaks) and billed losses (illegal connections)

Data collection and calculation of indices

The information used in this study was obtained by bibliographic research. To determine the IPAD, the values of availabilities, potentialities and surface water demands were obtained from the most recent report prepared by the Geoenvironmental Nucleus of the State University of Maranhão (NUGEO, 2010), entitled "Estimation of demand and water availability of river basins in Maranhão". The determination of demands by river basin has taken into consideration human supply (urban and rural), industry, irrigation and livestock (*Frame 3*).

Frame 3. *Potentialities, availabilities, and surface water demands, by river basin, in the State of Maranhão. (Source: NUGEO, 2010)*

Basin	Potentialities (h^3/year)	Availabilities (h^3/year)	Demands (h^3/year)
Parnaíba River	3828.41	1475.17	288.38
Munim River	4098.95	269.8	66.44
Itapecuru River	6599.97	1112.55	306.64
Tocantins River	11692.44	2412.15	432.4
Mearim River	13971.85	665.21	290.47
Gurupi River	5970.17	1263.94	27.62

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Information on availability, potentialities and underground water demands were based on data from Brasil (2009) and Santos (2010). In the calculation of the water demands, we have considered human supply (urban and rural), industry, irrigation, livestock, agricultural production and environmental demands (*Frame 4*).

Frame 4. *Potentialities, availabilities, and underground water demands, by river basin, in the State of Maranhão. (Source: Brasil, 2009; Santos, 2010)*

Basin	**Potentialities (h^3/year)**	**Availabilities (h^3/year)**	**Demands (h^3/year)**
Parnaíba River	9030	1107.92	2076.08
Munim River	3120	183.85	220.87
Itapecuru River	1550	219.54	267.68
Tocantins River	500	81.11	150.08
Mearim River	3490	639.16	656.18
Gurupi River	2510	90.85	299.72

The data necessary for the calculations of the indices composing the WUEI were obtained on the platforms of the Brazilian Institute of Geography and Statistics (IBGE, 2010) and the National Information System on Sanitation (Sistema Nacional de Informações Sobre Saneamento - SNIS) for the reference year of 2010.

Data on total households, households supplied by wells, households supplied by a water supply system, households supplied by a sewage network or septic tank, households with sewage treatment, households with waste collection and information on water losses in the supply network were gathered by municipality (see *Appendix*). The final indice, by river basin, was calculated from the average among municipalities that compose the basin.

Currently SNIS has the most comprehensive data on the sanitation sector in Brazil, constituting the largest and most important information tool on water services, collection and treatment of sewage since 1995, as well as solid waste management since 2002 (SNIS, 2015). Thus, the information in this study is very relevant regarding the reliability of its data.

Information on Grant, Collection and Committees required to compose the IWRM was obtained from the Superintendency of Water Resources of the State Department of Environment and Natural Resources of Maranhão (SEMA), the agency responsible for water resources management in Maranhão.

Partial scales

According to *Frame 2,* the indices constituting IPAD are the following: IAP, IUP and IUA. These indices show that regions with IUA > 1 are under situations of exhaustion of availability (Fernandes, 2002).

Vieira (1999) states that due to natural physical limitations, the maximum IUP value would be equal to 0.8. Thus, IUP values > 0.7 would indicate a critical situation of use of water resources in a river basin.

The IAP indice, on the other hand, would represent the level of efficiency of the water resources of a basin, being larger as they are closer to 1 (Fernandes, 2002).

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Thus, the partial scale for all indices relates the calculated values to levels of performance (Very High, High, Medium, Low, and Very Low). For example, a IUA value greater than 1, a IUP value greater than 0.7 and a IAP value closer to 0 were related to “Very Low” performance levels; a IUA and a IUP equal to zero and a IAP closer to 1 are related to “Very High” performance levels.

These indices were calculated by river basin considering superficial and underground waters, which allows us to observe the relations of the demands with availabilities and potentialities. Vieira and Gondim Filho (2006) strongly recommend that these indicators are spatially implemented in terms of surface and ground potentiality.

The indices that make up the IWRM (IRBC, GI, CI) are subjective and determined from the analysis of their application to the hydrographic basin being studied. The partial scales for these indices are qualitative (*Frame 5*).

For the indices that make up the WUEI (IHSW, IHSWSS, ISW, IST, ISWT, IWLN), the partial scales (also from Very High to Very Low) are related to percentages ranging from 0 to 100%. For example, an indice with a value equal to 60% is classified as “Medium”, while another with a value equal to 25% is considered “Low”. These indices were calculated for all the cities that compose the river basins.

Frame 5. *Partial scales for IWRM indices. (Source: Campos et al., 2014)*

LEVEL	DESCRIPTION
Indice of River Basins Committees (IRBC)	
Very High	The committee is well articulated and has a high rate of problem solving in the basin
High	The committee has been operating for a few years and has a mean rate of problem solving in the basin
Medium	The committee has been recently set up and has a low rate of problem solving in the basin
Low	The committee has been proposed by law and is being set up
Very Low	There is no action for creating a committee in the basin
Grant Indice (GI)	
Very High	The grant has been implemented, it is very well supervised and there is a high reduction in water consumption
High	The grant has been implemented and the level of inspection and reduction in water consumption is medium
Medium	The grant has been implemented and the level of inspection and reduction in water consumption is low
Low	The grant has been proposed by law and is being implemented
Very Low	There is no action for deploying a grant in the basin
Collection Indice (CI)	
Very High	The collection has been implemented, a high amount is being collected and there is a high level of development in the basin
High	The collection has been implemented, a significant amount is being collected and there is a good level of development in the basin
Medium	The collection has been recently implemented and involves a deficit
Low	The collection has been proposed by law and is being implemented
Very Low	There is no action for deploying a collection in the basin

Global scales

When the indices are grouped, global scales are constructed with levels of water sustainability for all indicators in order to allow a better understanding of the value obtained for each indicator. The levels of the scales are qualitative, varying according to

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the values obtained when grouping the indices. This grouping can occur by considering the average of the indices (IPAD) and (WUEI), or by joining the management instruments (IWRM) (*Frame 6*).

After obtaining the indicators and respective level of water sustainability for each basin surveyed, the indicators IPAD, IWRM and WUEI were spatially distributed. For the development of maps, we used ArcGIS 10.3 software (ESRI, 2011), a GIS (Geographic Information System) tool that allows the manipulation of geospatial, matrix and vector databases.

Frame 6. *Global scale for the indicators used in this research. (Adapted from Campos et al., 2014)*

LEVEL	**IPAD and WUEI** **Average (%)**	**IWRM** **Joint Indices (J)**
Very High	80 ≤ M	Committee, grant and collection in full operation in the basin, generating a high reduction in demand
High	60 ≤ M < 80	Committee, grant and collection operating for a few years, generating little reduction in demand
Medium	40 ≤ M < 60	Committee, grant and collection (one or more) recently implemented, but with operational problems
Low	20 ≤ M < 40	Committee, grant and collection (one or more) proposed by law, in the process of installation
Very Low	M < 20	There is no action to apply a committee, a grant and a collection in the basin (one or more)

Results

Indicator on water resources management - IWRM

The assessment of the performance indicator for the water resources management system can be observed in *Table 1*.

Table 1. *Classification of the indices composing IWRM*

Basin	Indice of River Basins Committees (IRBC)	Grant indice (GI)	Collection indice (CI)
Parnaíba River	Very Low	Medium	Very Low
Munim River	Medium	Medium	Very Low
Itapecuru River	Very Low	Medium	Very Low
Tocantins River	Very Low	Medium	Very Low
Mearim River	Medium	Medium	Very Low
Gurupi River	Very Low	Medium	Very Low

Water use efficiency indicator – WUEI

Table 2 shows classification of the indices composing WUEI.

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Table 2. *Classification of the indices composing WUEI*

BASIN	Indices					
	IHSW	**IHSWSS**	**ISW**	**IST**	**ISWT**	**IWLN**
Mearim River	11.67	13.08	47.64	16.73	15.22	50.30
	VL	**VL**	**M**	**VL**	**VL**	**M**
Itapecuru River	9.53	14.06	14.67	7.77	6.90	55.72
	VL	**VL**	**VL**	**VL**	**VL**	**M**
Parnaíba River	14.48	73.92	17.01	9.72	8.53	48.69
	VL	**A**	**VL**	**VL**	**VL**	**M**
Tocantins River	11.85	18.45	20.76	10.22	14.64	30.15
	VL	**VL**	**L**	**VL**	**VL**	**L**
Munim River	15.40	9.82	13.50	5.25	4.19	67.59
	VL	**VL**	**VL**	**VL**	**VL**	**A**
Gurupi River	17.94	16.95	28.31	11.50	12.91	26.14
	VL	**VL**	**L**	**VL**	**VL**	**L**

Indices: IHSW - Indice of Households Supplied by Wells; IHSWSS - Indice of Households Supplied by a Water Supply System; ISW - Indice of Sewer Connections; IST - Indice of Sewage Treatment; ISWT - Solid Waste Treatment; IWLN - Indice of Water Loss in the Network. Classification: (VH), Very High; (H), High; (M), Medium; (L), Low; (VL), Very Low

Indicator of potentiality, availability and demand – IPAD

The application of sustainability indices for this indicator has shown the low utilization of existing water resources in all river basins surveyed (*Table 3*).

Table 3. *Classification of the indices composing IPAD*

BASIN	SURFACE			UNDERGROUND		
	IAP	**IUP**	**IUA**	**IAP**	**IUP**	**IUA**
Parnaíba River	0.39	0.08	0.20	0.12	0.23	1.87
	L	**VH**	**H**	**VL**	**H**	**VL**
Munim River	0.07	0.02	0.25	0.06	0.07	1.20
	VL	**VH**	**H**	**VL**	**VH**	**VL**
Itapecuru River	0.17	0.05	0.28	0.14	0.17	1.22
	VL	**VH**	**H**	**VL**	**VH**	**VL**
Tocantins River	0.21	0.04	0.18	0.16	0.30	1.85
	L	**VH**	**VH**	**VL**	**H**	**VL**
Mearim River	0.05	0.02	0.44	0.18	0.19	1.03
	VL	**VH**	**M**	**VL**	**VH**	**VL**
Gurupi River	0.21	0.00	0.02	0.04	0.12	3.30
	L	**VH**	**VH**	**VL**	**VH**	**VL**

Indices: IAP - Indice of Activation of Potentialities; IUP - Indice of Use of Potentialities; IUA - Indice of Use of Availabilities. Classification: (VH), Very High; (H), High; (M), Medium; (L), Low; (VL), Very Low

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Water sustainability

The assessment of the sustainability indicators used in this research is described in *Table 4*.

Table 4. *Classification of water sustainability indicators*

BASIN	IWRM	WUEI	IPAD	
			sur	**und**
Mearim River	M	L	H	L
Itapecuru River	M	L	VH	M
Parnaíba River	M	L	VH	M
Tocantins River	M	VL	VH	L
Munim River	M	L	VH	M
Gurupi River	M	L	VH	VL

Indicators: IWRM - Indicator on Water Resources Management; WUEI - Water Use Efficiency Indicator; IPAD - Indicator of Potentiality, Availability and Demand; sur - surface; und - underground. Classification: (VH), Very High; (H), High; (M), Medium; (L), Low; (VL), Very Low

All basins of the state showed a "Medium" level of sustainability for IWRM (*Fig. 2*).

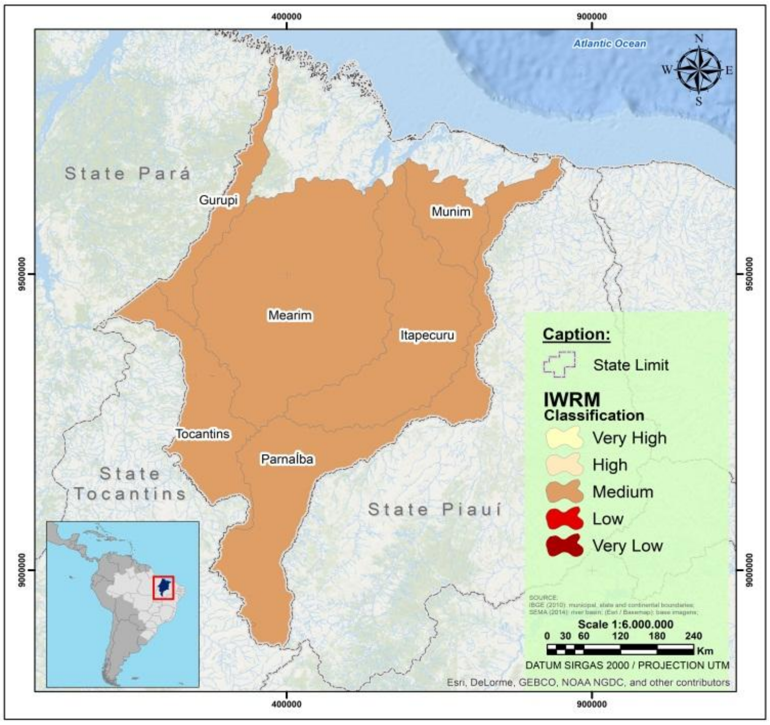

Figure 2. *Spatial distribution of IWRM*

The WUEI showed a sustainability level ranging from "very low" to "low" in the state river basins (*Fig. 3*).

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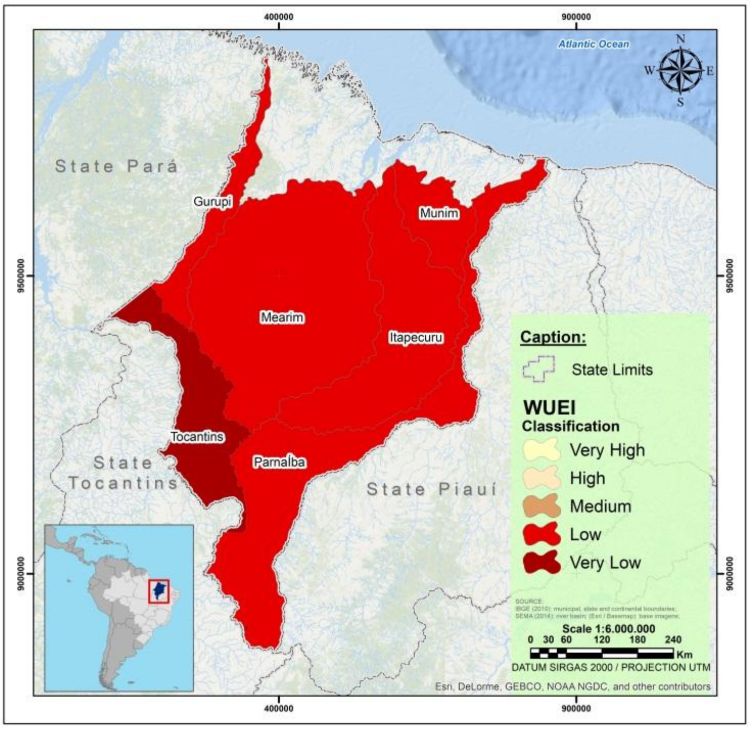

Figure 3. *Spatial distribution of WUEI*

On the global scale, for surface water the IPAD showed a sustainability level ranging from "Very High" to "High" (*Fig. 4*), depicting surplus availabilities in relation to demands and indicating that water resources are not fully available in the basins studied.

Figure 4. *Spatial distribution of IPAD, for surface water*

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The global scale of IPAD for groundwater showed sustainability levels varying from "very low" to "low" and "medium" (*Fig. 5*), due to demands higher than the available water resources, which means that the potential of the basins is not fully activated. Despite these results, the indissociability of surface and underground waters should be noted, as well as the importance of groundwater in maintaining the flow of the Maranhão rivers that have perennial characteristics.

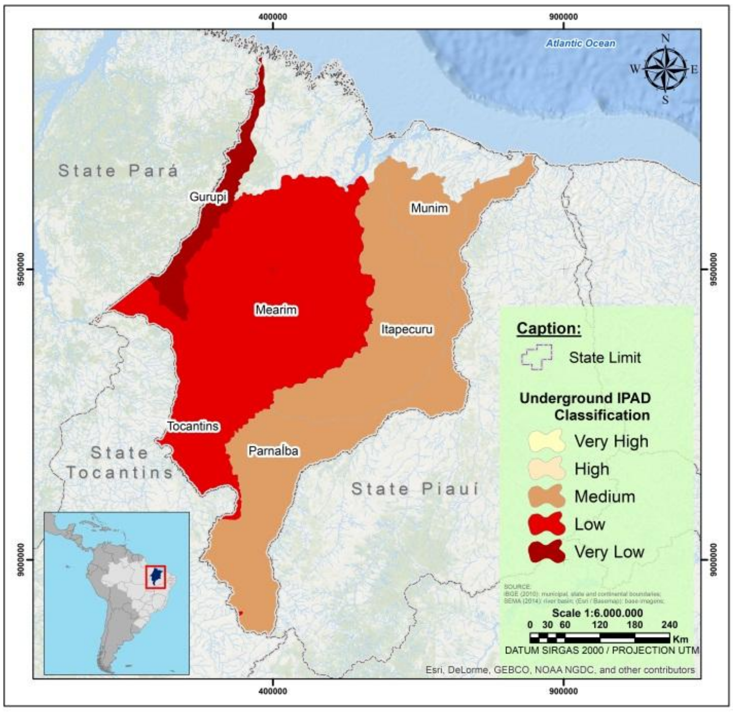

Figure 5. *Spatial distribution of IPAD, for groundwater*

Discussion

About IWRM, the grant indice had a "medium" level in all basins in Maranhão because this instrument is implemented, but it has a low level of inspection and reduction in water consumption. The collection indice has shown a "very low" level in all basins because the collection for the use of water is only established in the state policy of water resources (Brazilian Law no. 8.149/2004). The effective implementation of this instrument would require legal regulation, but there is still no state action in this regard in any of the basins studied in this research. The indice of river basins committees has shown a "very low" level for the Parnaíba, Tocantins, Itapecuru and Gurupi river basins, since none of these has a committee; and a "medium" level for the Mearim and Munim river basins, because they are the only ones that have a recently installed committee, but both have a low rate of problem solving.

Despite the average level of sustainability obtained in the IWRM, the shallow institutionalization of management instruments (grant with problems in implementation undergoing a bureaucratic process, poor inspection, collection for the use of nonexistent water) is clear.

Only two committees have been created so far in the state, which have little or no economic and political sustainability, indicating the existence of issues and fragilities in

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their effective performance and resulting in the low performance of these organizations. The river basin committees which are members of the National System of Water Resources Management play a relevant role in water management. The effective performance of these entities implies in the democratization of water resources management and in sharing the power to decide. This requires effort from the public authorities to share power, and effort from users and civil society to share responsibilities.

The IHSW and IHSWSS indices, which make up the water use efficiency indicator, were “very low”, indicating little coverage of water supply (including by wells).

According to a diagnosis carried out by the National Information System on Sanitation - SNIS, approximately 83% of the Brazilian population is served by a water supply system (Brazil, 2016). Although it appears to be a positive percentage, 17% do not have access to this basic utility, which represents more than 35 million people, mainly concentrated in the Northern and Northeastern regions of the country (Araújo et al., 2016).

Almost all basins studied have shown “very low” ISW and IST levels, except for the Tocantins and Gurupi river basins, whose ISW was “low”. These data show a poor service of collection and sewage treatment. In the calculation of IST, for example, we have observed that in some cities there were no data available on sewage treatment or, when this type of treatment did not occur in the region being studied, the percentage was equal to zero. It should be noted that the National Plan for Basic Sanitation (Plano Nacional de Saneamento Básico - PLANSAB) considers as an appropriate sanitary sewage service not only the presence of a sewage collection and treatment network, but also the use of a septic tank. This fact may have contributed to increasing the level of the ISC indicator, since it also considers the households served by septic tank, the main type of treatment adopted in the cities of Maranhão.

It is important to note that the percentage of cities in Maranhão that has appropriate systems of sewage treatment, with effective removal of organic load, is minimal. Most cities only implement sewage removal in urban regions, with in natura discharge in surface waters, or preliminary treatment, facts that may have contributed to the low level obtained in the IST indicator. According to ANA (2017), the lowest levels of organic load removal from sewage are found especially in the Northern and Northeastern regions of the country. Of the 5,570 cities, 70% remove at most 30% of the organic load generated.

The ISWT indicator also reached a “very low” level in all the basins surveyed, indicating a deficiency in solid waste collection services. The collection of waste in general is not a guarantee that this waste will receive proper treatment, because despite the goal of closing all dumps in Brazil by 2014 as established by the National Policy on Solid Waste, Brazilian Law no. 12.305/2010, Maranhão is now the second State of Brazil with the largest number of dumps, totaling 250 in operation that are responsible for receiving daily almost three thousand tons of waste (ABRELPE, 2017).

The inappropriate disposal of solid waste has become a worldwide issue and can cause damages to the environment, particularly water pollution. This type of pollution can change the characteristics of the aquatic environment through leachate percolation associated to rainwater and springs in the waste disposal sites (Pires et al., 2016).

As for the IWLN indice, which measures the percentage of physical and billed losses, the level shown in the Mearim, Itapecuru and Parnaíba river basins was “medium”. In the Tocantins and Gurupi river basins it was “low” and in the Munim

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river basin it was “high”, above 60%. Elevated levels of loss in water distribution systems have been reported in Northeastern Brazil, including in Maranhão, reaching 60% and 70% (Maranhão, 2014; Araújo et al., 2016).

Except for the basins with low indices, the others are above the national average, which is 37.57% (Brazil, 2016). In addition, all the basins assessed are above the level of water loss considered acceptable by Cambrainha and Fontana (2015), which is 10%.

A survey released by IBNET (International Benchmarking Network for Water and Sanitation Utilities) showed that, in terms of water loss, Brazil ranks 20th in a ranking of 43 countries (Instituto Trata Brasil, 2015). According to the institute, around 6.5 billion cubic meters of treated water were lost in Brazil in 2013, which amounts to a financial loss of 8.015 billion Reais.

The elevated level of loss of water resources observed in this research indicates the fragility and precariousness of the sanitation system and of the companies that operate water supply services in the regions studied. This also results in many problems, not only economic and social, but mainly environmental, since this loss increases the need for exploration of surface and underground sources of water (Morais et al., 2010).

The the low degree of sustainability of WUEI revealed alarming sanitation conditions, such as low coverage of supply by public utilities, poor or non-existent sewage collection and treatment, incipient waste collection and significant losses of water in the distribution network, which reflects the current reality of Maranhão although the data and information refer to 2010.

As for the IPAD, the Indice of Use of Availabilities - IUA for surface waters ranges from 0.02 to 0.028 and 0.44, resulting in “Very High”, “High” and “Medium” levels, respectively. A “Medium” level of sustainability was observed in the Mearim river basin, being the greatest demand considered for irrigation. The “High” level was observed in the Itapecuru river basin, in which the higher demand is mainly used for urban supply. The “Very High” level was seen for the Gurupi river basin, which presents demands for multiple uses, such as urban and rural supply, industry, irrigation and livestock. The assessment of this indicator revealed surplus availabilities in relation to demands, meaning there is no unmet demand.

The indice of use of underground availability evidences demands higher than availability, which results in a “Very Low” level of sustainability in all basins analyzed. Therefore, there is a situation of exhaustion of availability, with repressed demands in all basins, which would imply the need to increase supply by drilling more wells or adopting other measures such as demand rationalization. Currently, the situation is even more critical as demand has been growing mainly for urban supply, industrial use and irrigation.

Specifically, with respect to groundwater, Costa (1994) already observed that its availability was, in general, lower than the total demand in most regions of Northeastern Brazil. Vieira and Gondim Filho (2006), using potentiality, availability and demand indicators in river basins in Northeastern Brazil, classified the groundwater of the Itapecuru, Mearim and Tocantins basins as little sustainable due to the high demands in these regions. Recent articles show that groundwater resources are under increasing pressure in developing regions and in other regions that are more crucial to economic development (Mukherjee et al., 2015; Watto and Mugera, 2015).

The “Very High” IUP levels for surface waters and the “Very High” and “High” IUP levels for underground waters indicates a comfortable situation regarding the use of potentiality of water resources in all basins.

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The IAP indice, on the other hand, showed that water resources are not efficiently available in all basins, especially in relation to groundwater, for which the level obtained in all the basins was “Very Low”. As for surface water, the IAP levels ranged from “Very Low” to “Low”, meaning that the basins potential is not fully activated.

Relevant information was observed in this research by assessing the IPAD for 2010. On the other hand, it is necessary to calculate such indice with updated data to allow comparisons and to obtain a more current picture on the balance of demand, availability and potentialities of the river basins in the State of Maranhão.

Currently, in the case of surface water, it is already possible to identify conflicts over the use of water in some regions of Maranhão, especially in times of water scarcity. In the Parnaíba river basin, for example, conflicts in the southern region of the State are related to the use of water for irrigation. In the Mearim river basin, in addition to demands for irrigation, there is a growing use of water for intensive and semi-intensive fish farming, associated with illegal abstractions and effluent releases without the authorization from the water resources management authority. In this basin there are also problems with floods, especially in the Pindaré and Grajaú rivers, two important tributaries of the Mearim river. In the Itapecuru river basin, the demand for urban supply stands out because the Italuís system, which supplies a large part of the metropolitan region of São Luís, the state capital, will have its uptake flow doubled by the end of 2018. In addition, deforestation, silting and irregular sand extraction are some of the main environmental problems found in the course of the main river. In recent years, the Tocantins river basin has undergone droughts and significant reductions in the level of the main river and its tributaries, compromising the basin’s sustainability, multiple uses of water and also the production of energy, as the basin houses the hydroelectric power plant of Estreito, which has an installed rated capacity of 1,087 MW.

Concerning groundwater, particularly in the metropolitan region of São Luís, there are reports of wells with high levels of salinization. In 2013, there were a total of 462 records of deep wells, distributed by several municipalities of the State (Cunha et al., 2013). Currently, there are more than 11,000 wells drilled (CPRM, 2018), which may indicate a scenario of overexploitation of the aquifers in Maranhão.

Conclusion

This study presents the application of a methodology that uses river basin sustainability indicators, bringing a contribution to the related literature, in particular to water resources management in terms of diagnosis of sustainability and support to planning and decision making. Sustainability indicators were used in the context of river basins, analyzing surface and underground waters when possible, since water resources are part of an integrated whole that has great relevance in the constitution of life and in a balanced ecosystem.

The application of the methodology to the basins of the state of Maranhão, located in Northeastern Brazil, determined sustainability with an overall intermediate performance in the river basins assessed in this research. These basins require priority measures in the hydrological dimension, especially for underground waters; attention to basic sanitation, including with regard to public supply; reduction of demands and waste; in addition to proper sewage collection and treatment. The results also highlighted the need to improve the state water management system, with actions oriented to the implementation and consolidation of the water resources policy instruments and promotion and support to river basin committees.

The spatialization of the indicators enables a clearer view of the water sustainability of the basins in Maranhão, so that all concepts regarding the performance of the water management structure, efficient use of available water as well as potentialities, availability and water demands could be unequivocally considered.

The application of this methodology may be a model for the assessment of other river basins, especially with conditions similar to the ones evaluated in this study. This tool, provided it is regularly applied, can provide an appropriate description of the evolution of basin conditions in terms of sustainability, assisting different stakeholders and water managers in the planning, decision-making and implementation of local strategies for sustainable development. Authors such as Corrêa and Teixeira (2006) recommend that sustainability indicators are annually applied to observe their evolution and to evaluate the effectiveness of the actions proposed from the surveys of the previous period.

Carvalho et al. (2011) have shown that the adoption of an average value as a measure to build the sustainability indice may be an area of weakness, which may represent a fragility in this study. Another difficulty found when conducting this research was the scarce availability of data in most of the state river basins, a fact that prevented the inclusion of other basins in this analysis and limited the assessment of more indicators.

However, we may conclude that these results were satisfactory and are applicable in regions with scarce data. Consideration was given to variables that fulfill the main components of sustainability, with social, economic, hydro-environmental and institutional characteristics, despite their incommensurability and the complexity of grouping information from indicators of such diverse nature, in particular in places where data availability is scarce. In addition, the aspects of the indicators used here allowed the joint and separate analysis of each dimension. This allows a unique look at the most critical issues of each basin, so as to act correctively and/or preventively to solve the main negative items.

The bottlenecks and limitations identified in this study represent a window of opportunity to improve the current situation in the basins, but it requires a more efficient coordination between the different institutions involved in water resources management.

In this specific application in river basins in the Northeastern part of Brazil, the results are particularly relevant, since there is a considerable gap in the literature on the integrated management of water resources for this region, and especially in the state of Maranhão, which can support more sustainable management actions.

As for future studies, it is recommended that researches shall be carried out on the potentialities, availabilities as well as superficial and underground water demands, by river basin, portraying the current reality of water resources in the State. It is also recommended that other studies shall be carried out to address the issue of water sustainability indicators at the site, including a greater number of indicators that consider other water sustainability criteria.

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APPENDIX

Annex 1. *Data used for the calculation of WUEI indices by municipality of River basin Mearim, State of Maranhão*

Municipality	Total number of households	% households supplied by wells	% households supplied by a water supply system	% households with a sewage network or a septic tank	% households with sewage treatment	% households with waste collection	Average percentage of physical losses water in the network
Açailândia	27,473	20.28	76.55	95.95	43.02	86.98	60.19
Altamira do Maranhão	2,632	30.01	51.13	76.75	0	17.04	51.23
Alto Alegre do Pindaré	7,282	23.05	76.79	78.97	28.44	25.39	68.7
Amarante do Maranhão	9,267	39.05	57.45	58.25	18.31	33.19	55.02
Anajatuba	6,503	46.71	42.77	59.16	19.15	14.01	55.71
Araguanã	3,051	62.3	37.05	81.02	0	46.39	64.51
Arame	6,961	58.44	38.37	51.02	15.49	41.02	7.6
Arari	6,915	25.76	66.8	71	17.41	45.2	11
Bacabal	26,215	15.11	81.15	76.37	68.98	65.71	48.64
Bacabeira	3,660	19.2	72.54	69.8	69.55	39.93	79.04
Barra do Corda	21,597	31.76	58.53	65.45	45.11	50.71	68.34

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Municipality	Total number of households	% households supplied by wells	% households supplied by a water supply system	% households with a sewage network or a septic tank	% households with sewage treatment	% households with waste collection	Average percentage of physical losses water in the network
Bela Vista do Maranhão	2,979	29.31	69.39	84.69	41	22.58	0
Bernardo do Mearim	1,496	59.93	38.24	66.01	6.98	40.59	86.88
Bom Jardim	9,610	36.46	58.79	75.82	51.12	27.14	74.14
Bom Jesus das Selvas	6,220	52.74	43.19	53.33	35.8	24.1	67.86
Bom Lugar	3,514	67.71	29.05	59.51	43.09	14.89	0
Brejo de Areia	1,276	55.66	41.24	52.26	11.22	3.44	0
Buriticupu	14,968	19.03	76.75	82.54	0	27.72	86.01
Buritirana	3,778	16.82	82.77	87.59	7.12	34.29	2.86
Cajapió	2,594	73.65	25.31	67.07	8.97	0.76	82
Cajari	4,306	75.22	16.85	52.14	21.1	11.67	91.17
Capinzal do Norte	2,884	31.61	66.72	59.54	44.63	24.49	0
Centro Novo do Maranhão	3,951	79.42	10.45	66.02	22.43	8.08	0
Conceição do Lago Açú	3,336	32.04	61.52	41.04	11.05	20.96	61.91
Dom Pedro	6,208	16.8	76.59	75.44	0	69.06	62.4
Esperantinópolis	4,806	25.34	70.39	67.81	15.18	53.26	69.81
Fernando Falcão	1,883	68.16	15.53	30.61	33.13	3.88	0
Formosa da Serra Negra	3,884	60.21	34.59	38.32	18.02	2598	0
Governador Newton Bello	2,831	63.91	34.84	65.48	11.3	22.22	56.18
Grajaú	14,913	23.06	72.3	63.49	39.01	58.59	9.46
Igarapé do Meio	3,022	29.91	64.5	49.65	14.32	3.01	0
Igarapé Grande	2,853	38.84	60.73	62.1	10.37	62.2	66.68
Itaipava do Grajaú	3,293	20.75	70.41	55.46	0	24.33	0
Jenipapo dos Vieiras	3,636	61.8	31.59	51.81	0	6.16	20.73
João Lisboa	5,407	15.27	84.13	92.42	31.7	53.58	72.45
Joselândia	3,913	19.08	77.34	58.98	12.39	17.81	53.43
Lago da Pedra	11,463	77.38	18.62	84.37	5.4	73.25	62.69
Lago do Junco	2,594	86.84	11.59	50.85	12.35	17.85	79.38
Lago dos Rodrigues	2,060	69.21	28.88	74.59	0	43.54	0
Lago Verde	3,684	46.78	47.5	48.19	9.33	18.75	78.55
Lagoa Grande do Maranhão	2,407	33.09	52.36	65.03	3.47	37.85	0
Lima Campos	3,185	34.61	61.72	67.67	7.06	53.55	65.23
Marajá do Sena	1,759	90.47	7.28	20.62	2	7.45	0
Matinha	5,579	62.68	37.15	88.9	13.9	27.22	74.5
Matões do Norte	2,519	23.82	45.32	33.35	10.7	16.99	81.44
Miranda do Norte	5,196	17.73	76.85	51.83	5.44	55.57	61.74
Monção	7,470	48.44	37.4	55.25	15.76	1.62	88.59
Montes Altos	2,381	37.69	61.42	59.23	6.32	48.48	48.91
Olho D'água das Cunhãs	4,857	55.91	39.19	64.31	31.4	18.07	91.14
Olinda Nova do Maranhão	3,264	44.54	52.9	48.2	8.22	26.26	0
Paulo Ramos	4,746	66.76	27.89	65.16	5.69	38.41	89.77
Pedreiras	10,630	16.83	78.79	59.18	9.13	66.3	72.43
Pedro do Rosário	5,294	94.78	3.71	75.69	5.06	13.5	0
Penalva	7,889	57.44	38.14	71.56	14.65	8.47	69.63
Peritoró	5,593	47.32	51.55	40.94	4.31	17.66	73.42
Pindaré-Mirim	7,750	6.25	92.38	76.66	6.38	53.35	61.53
Pio XII	5,399	26.09	60.02	63.4	31.49	34.9	50
Poção de Pedras	5,309	72.49	26.76	64.2	7.22	38.07	73.61
Presidente Dutra	11,923	2.17	95.99	83.81	13.11	59.37	58.84
Santa Filomena do Maranhão	1,753	12.26	82.3	15.18	0	0.97	73.17
Santa Inês	20,264	4.52	93.9	78.75	58.77	77.33	65.09
Santa Luzia	17,466	48.62	44.21	50.36	44.36	9.67	69.53

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Municipality	Total number of households	% households supplied by wells	% households supplied by a water supply system	% households with a sewage network or a septic tank	% households with sewage treatment	% households with waste collection	Average percentage of physical losses water in the network
Santa Rita	7.887	46.53	51.45	62.85	22.47	17.37	69.37
Santo Antônio dos Lopes	3,708	44.49	51.05	52.87	32.25	38.24	73.1
São Francisco do Brejão	2,672	24.97	73.93	81.69	11.78	63.08	18.28
São João Batista	5,069	50.51	20.91	71.92	0	7.46	98.06
São João do Carú	2,615	54.19	39.85	71.96	0	24.76	0
São José dos Basílios	1,870	35.92	59.66	46.49	3.09	13.34	79.57
São Luís Gonzaga do Maranhão	5,236	54.74	34.57	47.08	17.03	13.43	96.4
São Mateus do Maranhão	9,818	41.44	55.56	78.08	12.28	60.14	67.82
São Raimundo do Doca Bezerra	1,397	26.82	46.35	59.35	2.75	42.18	0
São Roberto	1,317	39.3	60.14	46.73	0	41.37	0
São Vicente Férrer	5,131	76.29	20.65	54.49	17.03	12.06	74.34
Satubinha	2,491	33.33	65.27	53.44	21	13.95	82.49
Senador La Rocque	4,530	9.14	90.4	74.2	12.86	41.02	76.62
Sítio Novo	4,097	49.76	41.02	45.08	8.9	41.49	0
Trizidela do Vale	5,101	13.55	80.29	70.08	6.97	68.6	73.6
Tufilândia	1,373	38.99	59	50.03	33	1.34	8.46
Tuntum	10,440	16.01	77.24	74.13	11.78	60.43	73.4
Viana	12,347	58.28	31.78	78.97	13.2	34.13	14.23
Vitória do Mearim	7,547	15.59	81.56	89.32	10.77	35.83	93.92
Vitorino Freire	8,222	57.92	40.64	80.24	8.05	40.64	80.73
Zé Doca	11,887	48.96	48.48	71.96	9.56	48.76	71.39

Annex 2. *Data used for the calculation of WUEI indices by municipality of River basin Itapecuru, State of Maranhão*

Municipality	Total number of households	% households supplied by wells	% households supplied by a water supply system	% households with a sewage network or a septic tank	% households with sewage treatment	% households with waste collection	Average percentage of physical losses water in the network
Aldeias Altas	5,795	67.64	24.74	35.63	4.7	19.2	83.2
Alto Alegre do Maranhão	7,282	26.6	72.08	77.64	33.21	47.82	75.51
Arari	6,915	25.76	66.8	71	17.41	45.2	20
Axixá	2,542	57.3	38.45	53.8	12	11.02	54.63
Bacabal	26,215	15.11	81.15	76.37	68.98	65.71	48.64
Bacabeira	3,660	19.2	72.54	69.8	69.55	39.93	79.04
Buriti Bravo	5,702	28.86	61.81	51.66	0	16.46	73.34
Cantanhede	4,713	25.96	67.63	56.21	10.03	31.63	38.75
Capinzal do Norte	2,884	31.61	66.72	59.54	44.63	24.49	0
Caxias	40,172	21.18	75.41	65.12	47.21	55.41	60.28
Codó	29,594	28.89	69.8	60.89	34.76	64.78	33.05
Colinas	9,855	42.27	51.01	60.43	31.27	30.56	82.89
Coroatá	15,930	23.62	72.4	57.89	7.2	50.36	73.6
Dom Pedro	6,208	16.8	76.59	75.44	0	69.06	62.4
Fernando Falcão	1,883	68.16	15.53	30.61	33.13	3.88	0
Formosa da Serra Negra	3,884	60.21	34.59	38.32	18.02	25.98	0
Fortuna	3,926	10.46	68.69	84.55	4.34	42.11	72.69
Gonçalves Dias	4,558	34.34	61.9	52.12	12.11	37.13	66.08
Governador Archer	2,582	15.02	84.31	70.88	8.32	55.68	30.34
Governador Eugênio Barros	4,093	18.22	79.73	57.3	0	29.16	91
Governador Luiz Rocha	1,891	6.37	91.65	56.3	6	4.3	81.4

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Graça Aranha	1,696	2.24	96.99	83.11	17.34	44.47	79.56
Itapecuru Mirim	15,710	37.56	58.52	63.79	15.24	34.1	58.43
Jatobá	2,139	8.71	89.45	99.61	0	3.68	81.56
Lagoa do Mato	2,687	26.04	65.9	50.72	1.98	15	5.29
Lima Campos	3,185	34.61	61.72	67.67	7.06	53.55	65.23
Loreto	2,669	33.14	61.47	66.57	3.44	45.18	73.79
Matões	7,598	50.42	46.62	55.4	4.47	13.12	0
Matões do Norte	2,519	23.82	45.32	33.35	10.7	16.99	81.44
Mirador	4,894	8.05	86.67	74.84	11	55.02	59.97
Miranda do Norte	5,196	17.73	76.85	51.83	5.44	55.57	61.74
Paraibano	5,291	12.25	82.67	69.25	5.91	61.72	65.63
Parnarama	8,654	42.91	53.17	51.89	0	22.42	20
Passagem Franca	4,562	12.83	85.08	52.95	7.54	32.3	0
Pastos Bons	4,694	20.48	76.59	68.26	14.65	50.91	18.94
Peritoró	5,593	47.32	51.55	40.94	4.31	17.66	73.42
Pirapemas	4,157	38.08	54.68	56.76	9.3	22.84	80.92
Presidente Juscelino	2,495	70.87	23.94	43.18	4.87	8.09	83.01
Rosário	9,448	28.22	64.07	71.15	19.07	49.23	56.65
Sambaíba	1,370	55.31	40.79	48.05	12.37	3.14	88.52
Santa Rita	7,887	46.53	51.45	62.85	22.47	17.37	69.37
Santo Antônio dos Lopes	3,708	44.49	51.05	52.87	32.25	38.24	73.1
São Domingos do Azeitão	1,679	38.58	57.33	79.04	9.44	49.84	46.6
São Domingos do Maranhão	8,853	26.53	65.06	74.81	13.2	32.9	79.46
São Félix de Balsas	1,213	85.31	10.16	46.46	5.87	10.93	91
São Francisco do Maranhão	3,289	51.58	39.24	22.62	0.87	15.62	43.81
São João do Soter	4,268	46.48	50.14	37.15	1.23	1.94	15.38
São João dos Patos	7,007	12.95	84.42	77.28	7.26	56.35	65.36
São Luís Gonzaga do Maranhão	5,236	54.74	34.57	47.08	17.03	13.43	96.4
São Mateus do Maranhão	9,818	41.44	55.56	78.08	12.28	60.14	67.82
São Raimundo das Mangabeiras	4,443	32.37	61.87	47.11	14.09	50.06	49.24
Senador Alexandre Costa	2,573	91.26	6.65	62.48	13.54	23.33	0
Sucupira do Norte	2,696	24.07	62.91	63.69	0	19.08	92
Timbiras	6,549	44.28	41.02	47.73	8.51	13.11	58.76
Timon	40,477	11.76	85.55	80.01	48.93	71.1	43.98
Tuntum	10,440	16.01	77.24	74.13	11.78	60.43	73.4
Turiaçu	7,784	83.09	12.93	65.81	5.87	15.1	15.8
Vargem Grande	11,100	48.39	37.43	41.96	14	14.7	69.13

Annex 3. *Data used for the calculation of WUEI indices by municipality of River basin Parnaíba, State of Maranhão*

Municipality	Total number of households	% households supplied by wells	% households supplied by a water supply system	% households with a sewage network or a septic tank	% households with sewage treatment	% households with waste collection	Average percentage of physical losses water in the network
Água Doce do Maranhão	2,730	71.45	26.72	60.47	3.97	16.11	0
Alto Parnaíba	2,647	37.9	59.16	31.69	12.22	33.2	29.09
Anapurus	3,328	37.32	57.69	50.31	5.88	12.9	68.19
Araioses	10,241	82.89	12.47	58.98	7.39	11.49	81.31
Balsas	21,310	32.57	65.47	75.37	43.2	73.95	28.69
Barão de Grajaú	4,735	22.75	66.38	46.79	0	41.51	79.9
Barreirinhas	12,162	72.16	21.27	77.99	15.43	19.17	71.43
Benedito Leite	1,424	15.4	70.01	51.19	1.54	41.67	74.21
Brejo	7,953	51.7	40.73	26.18	0	3.47	91.67

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Municipality	Total number of households	% households supplied by wells	% households supplied by a water supply system	% households with a sewage network or a septic tank	% households with sewage treatment	% households with waste collection	Average percentage of physical losses water in the network
Buriti	6,044	66.7	22.84	26.24	13.74	8.38	80.64
Caxias	40,172	21.18	75.41	65.12	47.21	55.41	60.28
Coelho Neto	11,110	20	72.15	79.03	4.21	48.13	85.52
Duque Bacelar	2,387	53.57	45.72	39.12	11.65	12.23	73.51
Feira Nova do Maranhão	1,988	80.2	18.62	46.7	0	27.64	0
Fortaleza dos Nogueiras	2,851	48.29	44.98	49.57	9.11	46.6	53.78
Lagoa do Mato	2,687	26.04	65.9	50.72	1.98	15	5.29
Loreto	2,669	33.14	61.47	66.57	3.44	45.18	73.79
Magalhães de Almeida	4,033	16.3	77.21	66.76	18.12	41.29	74.89
Matões	7,598	50.42	46.62	55.4	4.47	13.12	0
Milagres do Maranhão	1,800	51.97	40.79	32.76	10.54	10.75	0
Nova Colinas	1,186	40.49	50.08	50.79	12.2	29.08	13
Nova Iorque	1,202	40.56	55.44	46.26	0	26.68	67.05
Parnarama	8,654	42.91	53.17	51.89	0	22.42	20
Passagem Franca	4,562	12.83	85.08	52.95	7.54	32.3	0
Pastos Bons	4,694	20.48	76.59	68.26	14.65	50.91	18.94
Riachão	5,277	46.77	51.98	55.05	16	43.46	68.3
Sambaíba	1,370	55.31	40.79	48.05	12.37	3.14	88.52
Santa Quitéria do Maranhão	6,364	57.57	41.17	45.71	6.73	26.92	71.9
Santana do Maranhão	2,500	9.85	89.71	56.26	23.4	5.94	0
São Bernardo	6,289	53.19	37.31	57.56	12.66	25.69	49.07
São Domingos do Azeitão	1,679	38.58	57.33	79.04	9.44	49.84	46.6
São Félix de Balsas	1,213	85.31	10.16	46.46	5.87	10.93	91
São Francisco do Maranhão	3,289	51.58	39.24	22.62	0.87	15.62	43.81
São João dos Patos	7,007	12.95	84.42	77.28	7.26	56.35	65.36
São Raimundo das Mangabeiras	4,443	32.37	61.87	47.11	14.09	50.06	49.24
Sucupira do Riachão	1,213	22.97	72.83	56.26	9.45	0.07	42.18
Tasso Fragoso	1,935	35.77	62.19	55.13	13.04	55.13	59.97
Timon	40,477	11.76	85.55	80.01	71.1	71.1	43.98
Tutóia	11,344	66.54	27.64	53.86	20.65	14.92	27.7

Annex 4. *Data used for the calculation of WUEI indices by municipality of River basin Tocantins, State of Maranhão*

Municipality	Total number of households	% households supplied by wells	% households supplied by a water supply system	% households with a sewage network or a septic tank	% households with sewage treatment	% households with waste collection	Average percentage of physical losses water in the network
Açailândia	27,473	20.28	76.55	95.95	43.02	86.98	60.19
Buritirana	3,778	16.82	82.77	87.59	7.12	34.29	2.86
Campestre do Maranhão	3,529	8.13	91.44	89.4	6.31	68.43	0
Carolina	6,284	24.2	68.31	55.83	16.98	53.43	51
Cidelândia	3,515	19.2	80.19	84.62	0	45.89	53.6
Davinópolis	3,326	38.85	60.6	69.83	0	27.99	75.66
Estreito	9,117	22.52	76.17	61.4	11.6	70.77	0
Feira Nova do Maranhão	1,988	80.2	18.62	46.7	0	27.64	0
Governador Edison Lobão	4,243	18.67	80.12	80.07	17.09	67.67	0
Igarapé Grande	2,853	38.84	60.73	62.1	10.37	62.2	66.68
Imperatriz	68,537	6.32	93.29	67.83	56.34	89.22	71.59
João Lisboa	5,407	15.27	84.13	92.42	31.7	53.58	72.45

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Municipality	Total number of households	% households supplied by wells	% households supplied by a water supply system	% households with a sewage network or a septic tank	% households with sewage treatment	% households with waste collection	Average percentage of physical losses water in the network
Lajeado Novo	1,825	36.12	56.77	48.79	6.74	44.77	0
Montes Altos	2,381	37.69	61.42	59.23	6.32	48.48	48.91
Porto Franco	5,638	20.23	79.71	90.11	13.21	78.49	27.26
Riachão	5,277	46.77	51.98	55.05	16	43.46	68.3
Ribamar Fiquene	1,899	39.28	59.13	73.32	12	59.28	0
São Francisco do Brejão	2,672	24.97	73.93	81.69	11.78	63.08	18.28
São João do Paraíso	2,876	37.42	58.11	56.57	14.71	42.88	9.09
São Pedro da Água Branca	3,017	24.47	72.84	87.38	9.43	57.46	81.37
São Pedro dos Crentes	1,105	45.65	54.17	55.84	7.52	50.31	0
Senador La Rocque	4,530	9.14	90.4	74.2	12.86	41.02	76.62
Serrano do Maranhão	2,734	53.35	31.98	75.84	7	1.07	0
Sítio Novo	4.097	49.76	41.02	45.08	3.65	41.49	0
Vila Nova dos Martírios	2.728	17.76	75.3	90.7	0	71.51	0

Annex 5. *Data used for the calculation of WUEI indices by municipality of River basin Munim, State of Maranhão*

Municipality	Total number of households	% households supplied by wells	% households supplied by a water supply system	% households with a sewage network or a septic tank	% households with sewage treatment	% households with waste collection	Average percentage of physical losses water in the network
Afonso Cunha	1,301	51.53	42.04	46.18	11.53	50.25	83.2
Aldeias Altas	5,795	67.64	24.74	35.63	4.7	19.2	83.2
Anapurus	3,328	37.32	57.69	50.31	5.88	12.9	68.19
Axixá	2,542	57.3	38.45	53.8	12	11.02	54.63
Belágua	1,263	40.71	41.64	58.67	0	0.12	25
Brejo	7,953	51.7	40.73	26.18	0	3.47	91.67
Buriti	6,044	66.7	22.84	26.24	13.74	8.38	80.64
Cachoeira Grande	1,763	78.87	10.75	40.11	5.67	5.96	60
Caxias	40,172	21.18	75.41	65.12	47.21	55.41	60.28
Chapadinha	17,658	45.74	40.14	71.77	16.87	30.52	52.35
Codó	29,594	28.89	69.8	60.89	34.76	64.78	33.05
Coelho Neto	11,110	20	72.15	79.03	4.21	48.13	85.52
Duque Bacelar	2,387	53.57	45.72	39.12	11.65	12.23	73.51
Icatu	5,782	81.46	14.68	51.66	17	11.6	70.58
Itapecuru Mirim	15,710	37.56	58.52	63.79	15.24	34.1	58.43
Mata Roma	3,537	19.81	76.12	65.96	12.23	14.94	71.56
Milagres do Maranhão	1,800	51.97	40.79	32.76	10.54	10.75	0
Morros	3,774	46.94	33.42	44.58	7.32	19.79	78.75
Nina Rodrigues	2,541	75	22.56	75.56	13.17	13.43	73.9
Presidente Juscelino	2,495	70.87	23.94	43.18	4.87	8.09	83.01
Presidente Vargas	2,514	85.8	6.47	31.57	0	4.2	97
Santa Quitéria do Maranhão	6,364	57.57	41.17	45.71	6.73	26.92	71.9
Santa Rita	7,887	46.53	51.45	62.85	22.47	17.37	69.37
São Benedito do Rio Preto	3,918	62.17	31.1	77.57	0	3.68	97
Timbiras	6,549	44.28	41.02	47.73	8.51	13.11	58.76
Urbano Santos	5,324	55.72	26.8	59.12	6	13.49	74.35
Vargem Grande	11,100	48.39	37.43	41.96	14	14.7	69.13

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Annex 6. *Data used for the calculation of WUEI indices by municipality of River basin Gurupi, State of Maranhão*

Municipality	Total number of households	% households supplied by wells	% households supplied by a water supply system	% households with a sewage network or a septic tank	% households with sewage treatment	% households with waste collection	Average percentage of physical losses water in the network
Açailândia	27,473	20.28	76.55	95.95	43.02	86.98	60.19
Amapá do Maranhão	1,504	19.73	75.05	90.62	10.12	22.06	0
Boa Vista do Gurupi	1,812	96.05	2.83	74.5	4.57	40.89	0
Carutapera	5,078	61.14	33.76	32.69	20.3	21.8	60.07
Centro do Guilherme	2,593	72.19	26.17	65.65	2.33	32.15	0
Centro Novo do Maranhão	3,951	79.42	10.45	66.02	22.43	8.08	0
Cidelândia	3,515	19.2	80.19	84.62	0	45.89	53.6
Itinga do Maranhão	6,601	18.93	78.14	89.5	13	68.9	49.57
João Lisboa	5,407	15.27	84.13	92.42	31.7	53.58	72.45
Junco do Maranhão	988	51.61	46.53	89.14	8.77	29.59	0
Maracaçumé	4,605	59.55	30.12	53.93	6.25	56.23	25.67
São Francisco do Brejão	2,672	24.97	73.93	81.69	11.78	63.08	18.28
São João do Carú	2,615	54.19	39.85	71.96	0	24.76	0

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EFFECTS OF SOIL CONDITIONERS ON LAWN GRASS GROWTH IN DIFFERENT YEAR SEASONS

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Abstract. The field experiment was conducted in Poland between 2013 and 2015. The research was carried out in the split plot design with three replications. The following grass species were used in the experiment (factor B): *Lolium perenne*, *Festuca rubra* and *Poa pratensis*. Another experimental factor tested in the research was soil conditioners (factor A) as Substral (S), Humus Active Papka (HAP), Eko-Użyźniacz (EU), UGmax (UG). In each year of the experiment, among other things, grass growth, i.e. its slow rate of growth, was assessed. It was based on measurements of the height of plants before harvest. A 9-point scale was used. This assessment was conducted in three seasons (spring, summer, autumn). In all seasons red fescue had the most desirable intensity of growth (the slowest) and permanent ryegrass the fastest, both determined as an average effect of all soil conditioners. Of the applied soil conditioners, lawns treated with Humus Active Papka, grew the most slowly, taken as an average for all grass species and for all seasons. Of all grass species tested in the experiment red fescue had the smallest variation of growth intensity during the research.
Keywords: *lawns, regrowth, soil condition, grasses, Lolium perenne, Festuca rubra, Poa pratensis*

Introduction

In recent years a popular conviction has developed that presence of lawns in the vicinity of residential houses and in areas adjacent to various companies and establishments builds a positive image, demonstrating a high standard of living of their owners, or high performance in business (Czeluściński et al., 2017; Jankowski et al., 2017). In a situation when people are increasingly preoccupied with their professional life, a limited time they have to spend on treatment and maintaining lawns in a suitable condition has become a problem (Jankowski et al., 2012a,b,d,e). According to Rutkowska and Dębska-Kalinowska (2000) widely relevant species of grass planted on lawns used extensively include: red fescue (*Festuca rubra*), smooth-stalked meadow-grass (*Poa pratensis*), and perennial ryegrass (*Lolium perenne*).

According to many authors (Domański and Andrzejewska, 2011; Domański, 1998; Harkot and Czarnecki, 1998; Jankowski et al., 1999a,b; Prończuk and Prończuk, 2006), with a limited time devoted to lawn maintenance, a required feature of grass is its slow pace of growth. Therefore, it is important that species of grass selected for lawns should grow slowly. The slower growth of a species of grass, the more it is used as a component of a lawn mixture (Knot et al., 2017; Pooya et al., 2013; Salehi and Khosh-

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Khui, 2004). Slow growth of lawn grass considerably reduces mowing frequency, which directly translates into smaller costs of maintaining a lawn (Prończuk and Prończuk, 2008).

To make lawn maintenance simple, new solutions aiming at limiting amount of work, or at making it simpler, need to be worked out. It can be done by a proper selection of grass species, but also by use of special nutrients and growth regulators (Jankowski et al., 2012b,c,d). One of such solutions is application of organic products called soil conditioners, for a longer time positively affecting release of soil minerals and making it possible for plants to survive stressful conditions (Chen et al., 2004; Gąbka and Wolski, 2008). Use of such soil conditioners can be beneficial from a financial point of view, limiting the amount of money spent on grass lawn maintenance (Prończuk and Prończuk, 2008). Such products reduce mowing frequency, saving time and petrol, and with them applied, grass does not need as much fertiliser.

In the literature on the use of lawns there has been little information on possible use of soil additives to improve lawn maintenance. That is why, the aim of this paper is to investigate the impact of such products on grass quality, assessed by determining the rate of growth.

Material and methods

Characteristics of study species

Set up in 2012 the field experiment was conducted in the experimental facility of the Siedlce University of Natural Sciences and Humanities in Poland (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.

Characteristics of fertilizers

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 Active 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}.

Experimental design

In each year of the experiment, among other things, grass growth, i.e. its slow rate of growth, was assessed. It was based on measurements of the height of plants before harvest. That assessment was made according to the methodology developed by Domański (1998). A 9-point scale was used, in which 9 meant the highest value, with the following description: 1-very high; 3-large; 5-average; 7-small; 9-very small. This assessment was conducted in three seasons (spring, summer, autumn). In each year of the research spring assessment was made around 20 May, summer assessment around 20 August, and autumn assessment around 10 October.

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.

Characteristics of 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).

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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).

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.

Statistical analysis

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 interaction. Based on grass growth ratings, standard deviation and coefficient of variation were calculated for separate seasons, years, and soil conditioners.

Results and discussion

Spring grass regrowth

There was diversity of grass growth rate, depending on the species of grass, soil conditioner applied, and season of the year (*Tables 3-5*). In springtime this rate was varied in relation to all experimental factors (*Table 3*). Throughout the experiment during spring seasons, the slowest growth rate, as an average for all grass species, was in result of Humus Active Papka application (5.29) and the most intensive after using UGmax (4.71^{o}) or Substral (4.74^{o}). According to Prończuk and Prończuk (2006), in

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Denmark and in the Netherlands no mineral fertilisers are used on municipal lawns, which slows down the growth of grass and reduces the need for frequent mowing. In the USA, in an extensive system, most lawns (80%) are maintained without the use of mineral fertilisers as well (Cook, 2005).

Table 3. *Grass growth rates on a 9-point scale in spring seasons between 2013 and 2015*

Year (C)	Species (B)	Fertiliser (A) (S)	(EU)	(HAP)	(UG)	$\bar{x}$
2013	Smooth-stalked meadow-grass	6.8	5.2	5.0	7.2	6.05
	Perennial ryegrass	7.4	7.2	6.8	6.9	7.08
	Red fescue	8.1	8.3	7.8	7.9	8.03
2014	Smooth-stalked meadow-grass	1.0	7.0	6.9	2.0	4.23
	Perennial ryegrass	1.4	2.9	3.2	2.3	2.45
	Red fescue	2.3	2.0	1.8	2.2	2.08
2015	Smooth-stalked meadow-grass	5.0	2.2	5.1	1.9	3.55
	Perennial ryegrass	2.0	2.2	2.0	3.1	2.33
	Red fescue	8.7	9.0	9.0	8.9	8.9
		Mean effect of species				
Smooth-stalked meadow-grass		4.27	4.8	5.66	3.7	4.61
Perennial ryegrass		3.6	4.1	4.0	4.1	3.95
Red fescue		6.37	6.43	6.2	6.33	6.33
		Mean effect of fertiliser				
		4.74	5.11	5.29	4.71	4.96
		Mean effect of year				
2013		7.43	6.9	6.53	7.33	7.05
2014		1.57	3.97	3.97	2.17	2.92
2015		5.23	4.47	5.37	4.63	4.92

$LSD_{0.05}$
A=NS, B=2.12, C=2.12
A/B=NS, B/A=NS, A/C=NS
C/A=NS, B/C=2.69, C/B=2.69

In spring seasons, the fastest growth was observed on lawns with perennial ryegrass (3.95°) and the slowest for red fescue (6.33°), with the differences being statistically significant. Considering the effect of soil conditioners on different grass species, it was noted that perennial ryegrass treated with Substral and smooth-stalked meadow-grass treated with UGmax grew the fastest (3.6° and 3.7°, respectively). In turn, the slowest growth was on lawns with red fescue, with its responses to different soil conditioners ranging from 6.2° to 6. 43°.

In assessing grass growth in spring throughout the research, it was found that the most intensive growth was in 2014 (the average of 2.92°) and the least in 2013 (7.05°), with those differences between the effects of spring seasons being statistically significant. In springtime the type of applied soil conditioner had a greater impact on grass growth level than weather conditions. As regards the values of precipitation and air temperature (*Table 2*), the worst weather conditions occurred in the spring of 2015, when the intensity of grass growth was moderate (the average rate of 4.92°). In the spring of 2013 weather conditions were very conducive to grass growth (the average air temperature of 7.5°C in April, 15.3°C in May, and 17.7°C in June, and the precipitation of 57.6, 145.8, 111.9 mm, respectively), while the intensity of grass growth was the weakest (7.05°), comparing with the other spring seasons of the research. According to

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Martiniello and D'Andrea (2006) in warm and humid habitats, growth of green mass is faster and lusher. However, the excessive increase in air temperature, together with strong evaporation, can undermine the balance between energy spent on respiration and on accumulation of photosynthetic products necessary for growth.

Summer grass regrowth

In summer seasons, intensity of grass growth was also varied (*Table 4*), and, like in spring, perennial ryegrass grew the fastest (4.28) and red fescue the most slowly (6.54). Studying the effects of soil conditioners on different species it was found that, like in spring, the most intensive growth was on plots with perennial ryegrass treated with Substral (2.67^{o}) and on plots with smooth-stalked meadow-grass treated with UGmax (2.6^{o}). In turn, smooth-stalked meadow-grass and red fescue had the slowest growth when treated with Humus Active Papka (7.6^{o} and 7.43^{o}).

Table 4. *Grass growth rates on a 9-point scale in summer seasons between 2013 and 2015*

Year (C)	Species (B)	Fertiliser (A) (S)	(EU)	(HAP)	(UG)	$\bar{x}$
2013	Smooth-stalked meadow-grass	2.1	8.9	8.6	2.9	5.63
	Perennial ryegrass	2.9	7.8	8.4	4.1	5.8
	Red fescue	4.8	7.7	8.2	5.3	6.5
2014	Smooth-stalked meadow-grass	3.2	9.0	8.9	2.9	6.0
	Perennial ryegrass	2.9	6.9	7.2	3.2	5.05
	Red fescue	5.2	4.9	5.1	4.7	4.98
2015	Smooth-stalked meadow-grass	4.9	2.4	5.3	2.0	3.65
	Perennial ryegrass	2.2	1.9	2.0	1.8	1.98
	Red fescue	8.9	6.9	9.0	7.8	8.15
		Mean effect of species				
Smooth-stalked meadow-grass		3.4	6.77	7.6	2.6	5.09
Perennial ryegrass		2.67	5.53	5.87	3.03	4.28
Red fescue		6.3	6.5	7.43	5.93	6.54
		Mean effect of fertiliser				
		4.12	6.27	6.97	3.86	5.31
		Mean effect of year				
2013		3.27	8.13	8.4	4.1	5.98
2014		3.77	6.93	7.07	3.6	5.34
2015		5.33	3.73	5.43	3.87	4.59

$LSD_{0.05}$
A=2.55, B=NS, C=NS
A/B=2.05, B/A=1.84, A/C=2.05
C/A=1.84, B/C=1.59, C/B=1.59

The slowest growth in summer, taken as an average for all grass species, was caused by Humus Active Papka (6.97^{o}) and the fastest by UGmax (3.86^{o}). These results confirmed the justification of the use of those soil conditioners on lawns. According to Hamza and Suggars (2001) bio products are based on natural substances like plant extracts, phyto hormones, or humic substances. Therefore, they have positive impact on plant metabolism, stimulating life processes to overcome adverse environmental conditions.

Throughout the experiment grass growth varied in summer seasons. The worst weather conditions for the development of grass occurred in 2015 (the air temperature

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of 18.7 in July and 21.0 in August and precipitation of 62.6 and 11.9 mm, respectively), which was not reflected in slow growth. In 2015 the average value of this parameter for all species was 4.59, which was the fastest growth of all three summer seasons, suggesting that like in the spring, it was more dependent on the type of applied soil conditioners than on weather conditions. Of all summer seasons throughout the entire research grass had the slowest growth (best) in 2013, when treated with Humus Active Papka (8.4^{o}) or Eko-Użyźniacz (8.13^{o}). According to Calvo et al. (2014) this type of bio products facilitates assimilation of nutrients, which in turn affects metabolism of plants and at the same time quality of a lawn, like grass density or growth rate. In the present experiment grass treated with Substral had the worst, from the point of view of the user, growth rate in 2013 (3.27^{o}). Differences in the intensity of growth between applied soil conditioners in various years of research were statistically significant.

In other studies (Jankowski et al., 2011; Salehi and Khosh-Khui, 2004) it was found that the regrowth of lawn grass in monoculture was mainly dependent on the species of grass selected to be planted, as well as on the year of the studies, which was associated with weather conditions.

Autumn grass regrowth

In the autumn the intensity of grass growth was also varied (*Table 5*). Among the species of grass, perennial ryegrass had the most intensive growth (3.62^{o}), similar to previous seasons (spring, summer), while red fescue had the slowest growth (5.85^{o}). Differences in the intensity of growth between these species were statistically significant. Looking for best species and varieties of grass to be used on extensive lawns, Prończuk and Prończuk (2006) found that red fescue tolerated lack of nutrients in the soil better than other species. It is obvious that mineral fertilizers, mainly nitrogen, stimulate the growth and growth rate of grass.

Grass regrowth in regard to soil conditioners, species and study year

Throughout the three-year period red fescue treated with UGmax had the slowest (the best) growth (6.1^{o}) and perennial ryegrass with UGmax the most intensive (the worst) (2.97^{o}). Of the applied soil conditioners Humus Active Papka had the biggest effect on slowing grass growth (5.08^{o}). Throughout the research there were statistically significant differences in the intensity of growth in autumn, in which season lawn grass grew the most quickly in 2014 (3.83^{o}), while in 2013 the growth was the slowest (5.53^{o}). In various years of research the differences in the intensity of grass growth with different soil conditioners applied were statistically significant. In other studies Jankowski et al. (2012b,e) found that due to different weather conditions in various years of research there were also important significant differences in grass growth. Trinexapak ethyl used in the experiment conducted by the above authors showed favourable interaction between grass and mineral soil conditioners in terms of growth rate.

In assessing the intensity of grass growth (*Table 6*) as an average of all experimental years it was found that red fescue had the slowest growth (the average of 6.24^{o}), and perennial ryegrass the fastest (the average of 3.95^{o}). These differences were statistically significant. Of all species, red fescue treated with Humus Active Papka had the slowest growth of 6.44^{o}, as the three-year average. Comparing the effects of soil conditioners it was found that Humus Papka had the best ability to slow the intensity of grass growth, with the growth rate of 5.78^{o}, determined as an average for all species.

***Table 5.** Grass growth rates on a 9-point scale in autumn seasons between 2013 and 2015*

Year (C)	Species (B)	Fertiliser (A)				$\bar{x}$
		(S)	(EU)	(HAP)	(UG)	
2013	Smooth-stalked meadow-grass	6.9	4.8	5.1	7.4	6.05
	Perennial ryegrass	4.8	5.1	7.2	4.9	5.5
	Red fescue	4.9	4.8	5.2	5.3	5.05
2014	Smooth-stalked meadow-grass	5.0	4.8	4.9	3.8	4.63
	Perennial ryegrass	3.1	4.1	4.0	1.9	3.28
	Red fescue	4.2	3.0	2.9	4.3	3.6
2015	Smooth-stalked meadow-grass	4.6	5.2	5.4	5.0	5.05
	Perennial ryegrass	1.9	2.3	2.0	2.1	2.08
	Red fescue	9.0	8.9	9.0	8.7	8.9
		Mean effect of species				
Smooth-stalked meadow-grass		5.5	4.93	5.13	5.4	5.24
Perennial ryegrass		3.27	3.83	4.4	2.97	3.62
Red fescue		6.03	5.57	5.7	6.1	5.85
		Mean effect of fertiliser				
		4.93	4.78	5.08	4.82	4.9
		Mean effect of year				
2013		5.53	4.9	5.83	5.87	5.53
2014		4.1	3.97	3.93	3.33	3.83
2015		5.17	5.47	5.47	5.27	5.35

$LSD_{0.05}$
A=NS, B=1.73, C=1.73
A/B=NS, B/A=NS, A/C=NS
C/A=NS, B/C=1.54, C/B=1.54

***Table 6.** The effect of soil conditioners and species on annual grass growth rates on a 9-point scale between 2013 and 2015*

Species (B)	Fertiliser (A)				$\bar{x}$
	(S)	(EU)	(HAP)	(UG)	
Smooth-stalked meadow-grass	4.39	5.5	6.13	3.9	4.98
Perennial ryegrass	3.18	4.49	4.76	3.37	3.95
Red fescue	6.23	6.17	6.44	6.12	6.24
	Mean effect of fertiliser				
	4.6	5.39	5.78	4.46	5.06
$LSD_{0.05}$ dla: A=1.40, B=1.08					
	Mean effect of year				
2013	5.41	6.64	6.92	5.77	6.19
2014	3.16	4.96	4.99	3.03	4.04
2015	5.24	4.56	5.42	4.59	4.95
$LSD_{0.05}$ dla: A=NS, C=1.28					
	Season (D)				
Spring	4.74	5.11	4.22	4.71	4.7
Summer	4.12	6.27	6.97	3.86	5.31
Autumn	4.93	4.78	5.08	4.82	4.9
$LSD_{0.05}$ dla: A=NS, D=NS					

These results were confirmed by studies of other authors (Pooya et al., 2013; Jankowski et al., 2012a), who found that growth of lawn grass is an unstable feature, depending on the species, habitat conditions, content of macro-and micronutrients in the soil, and on the way the lawn is used.

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Similarly, throughout the years of research there was a varied intensity of grass growth. The slowest growth was achieved in 2013 as a result of treatment with Humus Active Papka (6.92^o). Similarly, in the following years of research on the same experimental units the growth was slow. According to many authors (Jankowski et al., 2011; Prończuk and Prończuk, 2008; Knot et al., 2017) lawn grass species, in addition to ensuring good appearance of a lawn, should be characterized by slow growth after mowing.

Determined as an average of all separate seasons (spring, summer, autumn) growth rate was the slowest in summer seasons (5.31^o). According to Martiniello and D'Andrea (2006) a decrease in growth and a smaller weight gain of grass during summer months is a natural phenomenon and to a large extent depends on temperature. Air temperature above 30^{o}C can completely stop weight gain of grass by stepping up the process of breathing. Among the applied soil conditioners in the summer, lawn treated with Humus Active had the slowest growth rate (6.79^o). In the autumn grass in the same experimental unit had the least intensive growth, too.

Grass regrowth variation

The calculated coefficient of variation of grass growth (*Table 7*) indicated that during the experiment the smallest variation of this parameter was for red fescue (39.1), with the slowest average growth (6.24), a feature most awaited by the user of the lawn.

Table 7. *Standard deviation and coefficient of variation of grass growth rate*

Species	Min.	Max.	Mean	Standard deviation	variation coefficient
Smooth-stalked meadow-grass	1	9	4.98	2.17	43.57
Perennial ryegrass	1.4	8.4	3.95	2.18	55.19
Red fescue	1.8	9	6.24	2.44	39.10

variation coefficient: 0 – 20% small variation, 20 – 40 % average variation,
40 – 60% large variation, > 60% very large variation

Conclusions

1. Growth rate was varied in relation to both the species of grasses (smooth-stalked meadow grass, perennial ryegrass, red fescue), the applied soil conditioner, and seasons and years of research.
2. In all seasons red fescue had the most desirable intensity of growth (the slowest) and permanent ryegrass the fastest, both determined as an average effect of all soil conditioners.
3. Of the applied soil conditioners, lawns treated with Humus Active Papka, containing in their composition not only a large amount of minerals but also permanent active humus with useful microorganisms, grew the most slowly, taken as an average for all grass species and for all seasons (spring, summer, autumn).
4. Among the species of grass, it was found that in the spring red fescue treated with Eko-Użyźniacz had the slowest growth of grass. In summer both smooth-stalked meadow-grass and red fescue treated with Humus Active Papka and in autumn red fescue treated with UGmax grew the most slowly. This proves the

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equivocal effect of soil conditioners on the level of growth rate of various grass species at different times of the year.

5. Of all seasons (spring, summer, autumn) it was found that, as an average for all species, grass grew the most slowly in summer and most intensely in springtime. This could be related to physiological development of grass as well as to the effects of the weather. During summer seasons grass treated with Humus Active Papka had the slowest growth, but it was the fastest as a result of the use of UGmax or Substral.
6. Of all grass species tested in the experiment red fescue had the smallest variation of growth intensity during the research, which, from a practical point of view, is a feature most appreciated by the user, as such grass does not require frequent mowing. In addition, Humus Active Papka deserves further attention as regards its potential for reducing grass growth.

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EFFECT OF THE AQUEOUS EXTRACT OF *EUPHORBIA GUYONIANA* (EUPHORBIACEAE) ON PATHOGENIC BACTERIA FROM LAND-BASED SOURCES

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Abstract. *Euphorbia guyoniana* is a medicinal plant endemic to Algeria. It is used by local populations for its medicinal properties. This study was to determine the antibacterial effect of the aqueous extract from *Euphorbia guyoniana* on pathogenic bacteria of telluric origin. The aqueous extract was obtained by confrontation with organic solvents method with a yield of 1.7%. The total content of the flavonoïd extract was evaluated by the method of Aluminum Trichloride and was found to be 0.31 mg EQ/gE. The extract obtained was characterized by Infrared Spectroscopy and revealed a richness of phenol, aldehyde and ester then analyzed by High-Performance Liquid Chromatography, which allowed us to identify 27 flavonoid compounds. The extract was tested by the diffusion method on agar, on 12 bacterial strains isolated from a henhouse and identified by VITEK. These strains seem to be sensitive to the flavonoids of *Euphorbia guyoniana* with MICs varying from 1.47 to 61.78 mg/ml. The order of sensitivity of the bacterial strains to the extract is represented in the following order: *Staphylococcus aureus* > *Streptococcus faecalis* > *Escherichia coli*. Thus, the flavonoids of *Euphorbia guyoniana* may be an alternative to chemical control of certain pathogenic microorganisms.
Keywords: *Euphorbia guyoniana, flavonoids, antibacterial activity, HPLC, VITEK*

Introduction

Within microbial communities in the soil, there may be some microorganisms that are pathogens for plants, animals and humans (Raaijmakers, 2009). Soil is a natural habitat that can contain some primary and opportunistic pathogenic bacteria. The rhizosphere may contain certain opportunistic bacteria such as *Burkholderia spp., Ochrobactrum spp.* and *Stenotrophomonas spp.* (Berg et al., 2005). *Pseudomonas aeruginosa* is a highly encountered bacterium in the soil (Colinon et al., 2013). Some primary pathogenic bacteria are natural soil inhabitants, such as *Bacillus cereus* and *Bacillus anthracis*, which can cause serious diseases in humans (food poisoning and pneumonia) (Ticknor et al., 2001; Reis et al., 2014). Other bacteria are highly pathogenic to humans such as *Clostridium botulinum* and *Clostridium tetani* (Smith, 1978, 1979). There are certain categories of bacteria with a saprophyte life in soil such as *Listeria monocytogenes* (Freitag et al., 2009). The importance of these pathogens from land-based sources is growing with the increasing practice of monoculture, mainly in the Mediterranean regions (Tramier, 1986).

Control of pathogens from land-based sources has always been difficult, however the use of biocidal soil disinfection products such as chloropicrin and methyl bromide has been found to be very dangerous for humans and useful organisms' cultures. Fortunately, these products have been definitely banned, which does not solve the problem of the control of diseases of land-based origin. Vector control during epidemics

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is achieved through chemical insecticides, but their use continues to give rise to a high level of contamination and ecological imbalance due to the appearance of resistance. This is why the World Health Organization (WHO) insists on the search for new methods of control that are basically biological.

Euphorbiaceae contains several families of chemical compounds such as alkaloids (De Nazare et al., 2005), flavonoids, Cyanogenetic compounds (Hunsa et al., 1995), ellagic acid (Mavar et al., 2004) Saponins (Tripathi and Tiwari, 1980) and terpenes (Mazoir et al., 2008). Among the species endemic to Algeria, *Euphorbia guyoniana* had a particular importance in the pharmacopoeia. According to Bellakhdar (1997), it is used by many Saharan populations against poisonous bites and stings and various infections. The latex of the plant is used to attack warts and to extirpate thorns.

This study attempts to establish for the first time the effect of the aqueous extract of a medicinal plant (*Euphorbia guyoniana*) on the human bacterial pathogen in the telluric environment for the purpose of biological control.

Materials and methods

Plant material

Experiments were carried out on the aerial and underground parts of *Euphorbia guyoniana*, collected from the Ghardaïa region (South Algeria) in February 2016. The botanical identification of the species was carried out at the botanical laboratory of the Higher National School of Agronomy (ENSA) in El-Harrach (Algeria). The whole plant (stems, flowers, leaves and roots) was used for the preparation of the extract. The plant material was ground after drying at ambient temperature in dark place in order to preserve the integrity of the molecules. The obtained ground product was stored in a hermetically sealed flask (*Photo 1*).

Photo 1. *Aerial part of Euphorbia guyoniana*

Extraction procedure

The extraction of flavonoids was carried out according to the Bruneton protocol (1999). The principle of this technique is based on the treatment of the plant material

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with various solvents. It is based on the degree of solubility of flavonoids in organic solvents. The recovered aqueous extract was stored in the dark in hermetically sealed vials and subjected to chemical and biological analysis.

Colorimetric determination of the flavonoïc extract

The content of *Euphorbia guyoniana* in flavonoids was determined by the method of Aluminum Trichloride ($AlCl_3$) cited by Bahorun et al. (1996); Djeridane et al. (2006) and Ayoola et al. (2008). This method is based on the formation of an aluminum flavonoid-ion complex having a maximum absorbance at 430 nm. The concentration of flavonoids was calculated from the calibration curve established with Quercetin and expressed in equivalent milligrams of Quercetin per gram of extract weight (mg EQ/gE).

Infrared spectroscopy analysis of the extract

The infrared spectrum of the aqueous extract, for a frequency range between 400 and 4000 cm^{-1}, was obtained by a NICOLET 560 type spectrometer.

High performance liquid chromatography (HPLC)

Qualitative analysis of the aqueous extract was realized using HPLC. The apparatus consisted of a Young Line YL9100 liquid phase chromatograph, equipped with a YL 9101 quaternary pump with integrated degasifier YL 9101, a UV/Visible detector YL 9120 and a YL 9131 oven. The column used was Agilent eclips XDB C 18 (5 µm) with a length of 25 cm and an internal diameter of 4.6 mm. The mobile phase was a mixture of ultrapure water / acetonitrile / acetic acid (50:47:2.5) in an isocratic system with a flow rate of 1 ml/min. The volume of extract and standards injected was 20 µl. The detection of the compounds was done with a UV detector at a wavelength of 280-320 nm.

Isolation and identification of pathogenic bacteria

The bacteria were isolated from the soil of a henhouse in the Bouira region (Algeria). Soil sampling was carried out by the suspension-dilution method described by Vidhyasekaran et al. (1997). The identification of bacteria was made by fresh macroscopic observation based on morphological criteria of the colony, microscopic observation including methylene blue staining and Gram staining, biochemical galleries and confirmed by VITEK.

Several bacterial strains have been identified in this soil, such as *E. coli* which is a commensal bacterium of the human digestive tract as well as of many animals. At a rate of 10^7 to 10^9 Colony Forming Unit (CFU) per gram of faeces, it accounts for 80-90% of the most dominant species of the aerobic bacterial flora of the human intestine (Tenaillon et al., 2010). These strains are both responsible for intestinal and extra-intestinal infections (urinary infections, bacteremias, meningitis ...) (Locatelli., 2013). It is mainly via the natural excretion of fecal matter by animals that these pathogenic bacteria have been introduced into aquifers, rivers and soil (Solo-Gabriele et al., 2000; An et al., 2002; Byappanahalli et al., 2006).

Enterococcus faecalis is also a commensal bacterium of the intestines of humans and warm-blooded animals. It is a species found in human excreta at concentrations ranging

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from 10^5 to 10^7 per gram of faeces (Noble, 1978; Leclerc et al., 1996). It is also found in faeces of animals such as cattle, poultry, pigs and sheep, but to a lesser extent (Leclerc et al., 1996; Franz et al., 1999; Wheeler et al., 2001). This species is an opportunistic pathogen, affecting only individuals with weakened immune systems, particularly in hospitals (Morrison et al., 1997). It may cause endocarditis, bacteremia, meningitis, urinary tract infections, intraabdominal infections and surgical wound infections (Chenoweth and Schaberg., 1990; Jett et al., 1994). Because of its natural presence in the intestines and fecal matter of humans and animals, this bacterium is frequently found in soil and on plants (Mundt., 1961; Fujioka et al., 1998; Byappanahalli et al., 2012; Valenzuela et al., 2012; Ran et al., 2013).

Staphylococcus aureus is a ubiquitous bacterium that is found specifically on mucous membranes, the nasopharyngeal sphere and skin of warm-blooded animals and humans (Ostyn et al., 2012). Staphylococci producing coagulases are essentially represented by the species *Staphylococcus aureus*. In addition to food poisoning and nosocomial infections in humans, this species may cause clinical and subclinical mastitis in ruminants, particularly cows, which is a common reason for milk contamination (Ostyn et al., 2012).

Evaluation of the resistance of isolated bacteria to antibiotics

The resistance of the isolated bacteria was tested by synthetic antibiotics, using the Muller Hinton agar diffusion method. The antibiotics used are Erythromycin (E_{15}) (15 mcg) Ciproflaxacin (Cip_5) (5 mcg) Clindamycin (Cl_{25}) (25 mcg) Nalidixic Acid (Na_{30})(30 mcg) and Carbenicillin (Cb_{100}) (100 mcg). Areas of inhibition were determined according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 2006) and bacteria were classified as resistant or susceptible to antibiotics.

Evaluation of the antibacterial activity

The evaluation of the antibacterial activity of the aqueous extract of *Euphorbia guyoniana* was carried out by the diffusion method in agar medium recommended by several authors (Belaiche, 1979; Garbonnelle et al., 1987; Joffin and Leyral, 2014; Koba et al., 2004). Petri dishes containing the Muller Hinton agar were inoculated with a quantity of bacterial suspension (0.5 McFarland), according to the recommendations of the NCCLS. Sterilized paper discs of 6 mm of diameter, impregnated with 10 μL of extract were placed on the surface of agar. The plates were kept for 2 h at 4 °C and then incubated overnight at 37 ° C. The sensitivity of the strain to the extract is manifested by the size of the diameter of the bacterial-free zone surrounding the disc. The antimicrobial activity was determined by measuring the Minimum Inhibitory Concentrations (MICs). Three replicates are performed for each bacterium.

Statistical analysis

The results were expressed as mean ± Standard Error of Mean (M ± ESM). The statistical analysis was performed using the Statistica software® (version 6, Genistat Conseils Inc., Montreal). After the analysis of the variance, the comparison of the averages is performed by the student's test for matched samples. The test is considered statistically significant when the value of $p \leq 0.05$, for a confidence interval of 95%.

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Results

Extraction yield on flavonoids

Aqueous extract containing flavonoids was obtained with a yield of flavonoids of 1.7%. The reason of using the aqueous extract for the study of the antibacterial activity in spite of its low rate is its richness in very polar flavonoids.

Colorimetric determination of flavonoids

The content in flavonoïds is reported in equivalent mg of quercetin/g of the plant. The concentration of the flavonoid in the aqueous extract is 0.31 mg EQ/gE.

Infrared spectroscopy analysis of flavonoic extracts

The results of the infrared characterization of the aqueous extract are shown in the *Table 1*.

Table 1. *Infrared analysis of the flavonoïc extract*

Wave lengh (cm^{-1})	Bonds	Nature of the bond	Function
3406.58	-OH free	Broad band	Phenol
1609.47	C=O	Mean band	Aldehyde
1079.29	C-O	Weak band	Ester

In the aqueous extract of *Euphorbia guyoniana,* and referring to the work of Mabry et al. (1970), the broad band around 3406.58 cm^{-1} is associated with the elongation vibration of the OH bond (phenol function). The mean band at 1609.47 cm^{-1} corresponds to the elongation vibration of the C=O bond (aldehyde function). Finally, a weak band of 1079.29 cm^{-1} is associated with the elongation vibration of the C-O bond (ester function).

HPLC

The HPLC analysis revealed the presence of 41 compounds, of which 27 could be identified in the flavonoid extract of *Euphorbia guyoniana* (*Fig. 1, Table 2*).

Table 2. *HPLC of the flavonoid extract of Euphorbia guyoniana*

Number	R_T (Min)	%	Compound name
01	34.203	0.4	P-coumaric acid
02	46.703	1.0	Rosmarinic acid
03	62.753	2.0	Quercetin
04	17.237	9.5	Gallic acid
05	27.320	0.4	caffeic acid
06	41.137	0.3	Rutin
07	50.403	19.1	Ellagic acid
08	56.103	6.6	Myricetin
09	27.320	0.4	Syringic acid
10	37.903	0.2	Ferulic acid

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Number	R_T (Min)	%	Compound name
11	64.670	4.0	Kaempferol
12	23.520	0.3	Proanthocyanidin dimer
13	41.137	0.3	Myricetin 3-O-glucoside
14	61.787	1.5	Amentoflavone
15	45.320	1.6	Isoferulic acid
16	47.953	2.5	Quercetin-3-β-Ogalactoside
17	49.470	1.5	Luteolin-7-β-Oglucoside
18	56.103	6.6	Quercetin-3-O-α-rhamnoside
19	73.337	1.5	Luteolin
20	10.187	4.4	Hydroxytyrosol
21	58.370	3.0	Apigenin-7-Oglucoside
22	54.737	4.7	Apigenin-7-Orutinoside
23	49.470	1.5	Luteolin-7-Oglucoside
24	23.520	0.3	Salicyclic acid
25	43.053	0.3	Benzoic Acid
26	47.953	2.5	M-coumaric acid
27	58.370	3.0	O-coumaric acid

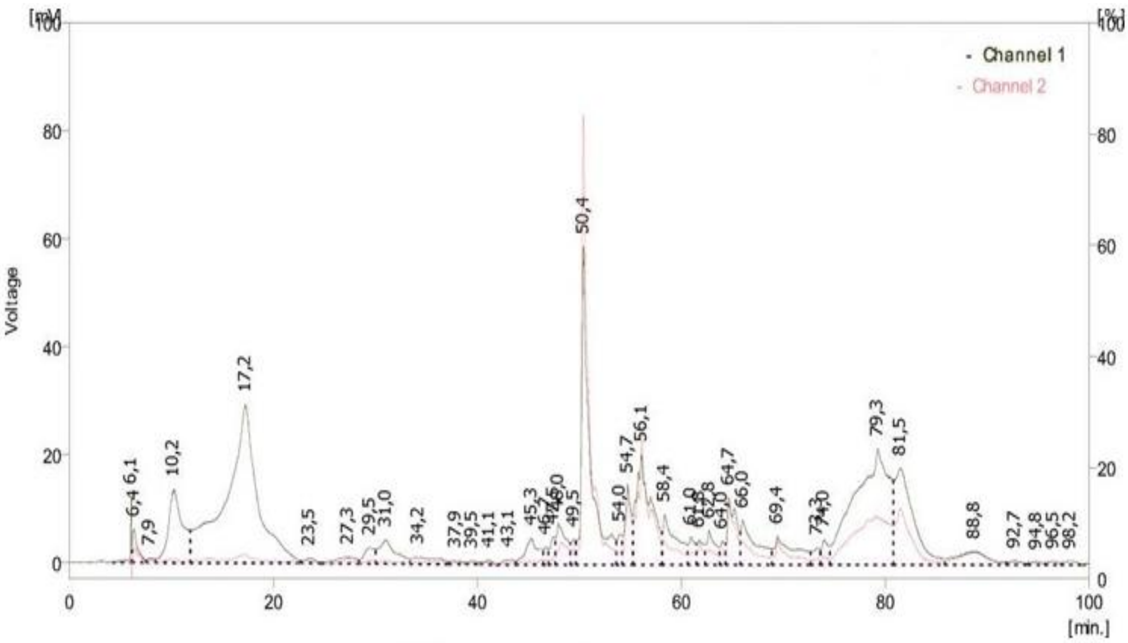

Figure 1. *Chromatogram of HPLC applied to flavonoids*

The analysis showed that the aqueous extract of *Euphorbia guyoniana* is rich in molecules with antibacterial activity. The major compounds are ellagic acid (19.1%), gallic acid (9.5%), Myricetine and Quercetin-3-O-α-rhamnoside (6.6%), Apigenin-7-Orutinoside (4.7%), Hydroxytyrosol (4.4%) and Kaempferol (4.0%), The other identified molecules are present at rates of less than 3%.

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Identification of isolated bacteria

The results of the identification of isolated bacteria are shown in *Tables 3* and *4*.

In total, twelve bacterial strains are isolated and identified from a henhouse soil belonging to three species: *Escherichia coli* (Gram negative), *Staphylococcus aureus* (Gram positive) and *Enterococcus faecalis* (Gram positive).

Table 3. *Identification of bacterial strains*

Sample	Macroscopic appearance		Microscopic appearance		Culturing		Biochemical tests	
	Form	Size	Fresh state	Gram staining/ Grouping mode	Culture media	Conditions	Oxidase	Catalase
01	Very small colony Pink	1 mm	Cocci immobile	G + Bunch of grapes	CHAPMAN	24 h at 37 °C	-	+
02	Large colony Yellowish	3 mm	Bacillus mobile	G- Isolated	HEKTOEN	24 h at 37 °C	-	+
03	Large colony Orange	4 mm	Bacillus mobile	G- Diplocoque	HEKTOEN	24 h at 37 °C	-	+
04	Small colony Transparent	1.5 mm	Cocci immobile	G+ Chain	BEA	24 h at 37 °C	-	-
05	Large colony Salmon	3 mm	Bacillus mobile	G- Diplocoque	HEKTOEN	24 h at 37 °C	-	+
06	Large colony Yellowish	4 mm	Bacillus immobile	G- Clusters	HEKTOEN	24 h at 37 °C	-	+
07	Small colony Golden	1.5 mm	Cocci immobile	G+ Bunch of grapes	CHAPMAN	24 h at 37 °C	-	+
08	Very small colony Yellowish	0.5 mm	Cocci immobile	G+ Bunch of grapes Isolated	CHAPMAN	24 h at 37 °C	-	+
09	Small colony Whitish	1.5 mm	Cocci immobile	G+ Chain Diplocoque	BEA	24 h at 37 °C	-	-
10	small colony Yellowish	1.5 mm	Cocci immobile	G+ Bunch of grapes	CHAPMAN	24 h at 37 °C	-	+
11	Small colony Transparent Surrounded by a black halo	1.5 mm	Cocci immobile	G+ Chain Isolated	BEA	24 h at 37°C	-	-
12	Small colony transparent	0.5 mm	Cocci immobile	G+ Chain	BEA	24 h at 37°C	-	-

+: presence; -: absence; G+: Gram positive; G-: Gram negative

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***Table 4.** Biochemical galleries*

Sample	Biochemical galleries													Name of the germ
	NR	Mob	Gaz	Glu	Suc	Lac	H2S	Urease	Indol	CIT	LDC	ODC	ADH	
01	+	-	-	+	+	+	-	+	-	+	/	/	/	*Staphylococcus aureus* (Staphylococcaceaes)
02	+	+	+	+	+	+	-	-	+	-	+	+	-	*Escherichia coli* (Enterobacteriaceae)
03	+	+	+	+	+	+	-	-	+	-	+	-	-	*Escherichia coli* (Enterobacteriaceae)
04	+	-	-	+	+	+	-	-	-	-	-	+	+	*Enterococcus faecalis* (Streptococcaceae)
05	+	+	+	+	+	+	-	-	+	-	+	+	-	*Escherichia coli* (Enterobacteriaceae)
06	+	+	+	+	+	+	-	-	+	-	+	-	-	*Escherichia coli* (Enterobacteriaceae)
07	+	-	-	+	+	+	-	+	-	+	/	/	/	*Staphylococcus aureus* (Staphylococcaceaes)
08	+	-	-	+	+	+	-	+	-	+	/	/	/	*Staphylococcus aureus* (Staphylococcaceaes)
09	+	-	-	+	+	+	-	-	-	-	-	+	+	*Enterococcus faecalis* (Streptococcaceae)
10	+	-	-	+	+	+	-	+	-	+	/	/	/	*Staphylococcus aureus* (Staphylococcaceaes)
11	+	-	-	+	+	+	-	-	-	-	+	+	+	*Enterococcus faecalis* (Streptococcaceae)
12	+	-	-	+	+	+	-	-	-	-	+	+	+	*Enterococcus faecalis* (Streptococcaceae)

+: presence; -: absence; /: unaccomplished

Antibacterial activity

Isolated strains used to evaluate antimicrobial activity showed an important resistance to the extract, *Tables 5* and *6* show the results.

The results showed that the antibacterial activity of the aqueous extract of the plant differs from one strain to another. An important antibacterial activity of the extract is observed for *Staphylococcus aureus* with zones of inhibition ranging from 21 (± 1) to 31.33 (± 1.5) mm, and positive controls for Erythromycin, Ciproflaxacin And clindamycin which showed respectively clear inhibition zones of 6 (± 0) to 34 (± 1) mm, 6 (± 0.5) to 36 (± 0.5) mm and 6 (± 0) to 20 (± 0.5) mm according to the strain (*Fig. 2*). A high sensitivity of *enterococcus faecalis* to the extract was noted with zones of inhibition ranging from 22 (± 2) to 28.33 (± 1.2) mm, and also positive controls for Erythromycin, Ciproflaxacin, clindamycin, And nalidixic acid with inhibition zones of 6 (± 0.5) to 20 (± 2.6) mm, 6 (± 0) to 34 (± 0) mm, 6 18 (± 0) mm and 6 (± 0.5) to 15 (± 0) mm respectively (*Fig. 3*). Finally, a lower sensitivity was observed in *Escherichia coli* for the extract with an inhibition zone between 18.33 (± 1.5) and 28.66 (± 1.5) mm,

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and positive controls for Ciproflaxacin, Clindamycin and carbenicillin with inhibition zones of 40 (± 0) to 42 (± 1) mm, 12 (± 1) to 25 (± 0) mm, and 8 (± 0) to 32 (± 1) mm respectively (*Fig. 4*).

Table 5. *Antibiotic and extract susceptibility tests*

Strain		**Areas of Antibiotic Inhibition**					**Areas of extract inhibition**
		E_{15}	**Cl_{25}**	**Cip_5**	**Cb_{100}**	**NA_{30}**	
Escherichia coli	01	/	16 (S)	41 (S)	13 (S)	/	28 (S)
	02	/	12 (S)	42 (S)	32 (S)	/	19 (S)
	03	/	16 (S)	42 (S)	24 (S)	/	20 (S)
	04	/	25 (S)	40 (S)	8 (S)	/	18 (S)
Staphylococcus aureus	01	6 (R)	6 (R)	6 (R)	/	/	21 (S)
	02	34 (S)	8 (S)	34 (S)	/	/	28 (S)
	03	24 (S)	18 (S)	36 (S)	/	/	27 (S)
	04	34 (S)	20 (S)	36 (S)	/	/	32 (S)
Enterococcus faecalis	01	6 (R)	6 (R)	6 (R)	/	6 (R)	28 (S)
	02	18 (S)	12 (S)	16 (S)	/	15 (S)	22 (S)
	03	20 (S)	18 (S)	34 (S)	/	6 (R)	28 (S)
	04	6 (R)	6 (R)	6 (R)	/	6 (R)	26 (S)

S: sensitive; R: resistant; /: unaccomplished

Table 6. *Values of minimum inhibitory concentrations*

Strain		**MIC_S (mg/ml)**
Escherichia coli	01	61.78
	02	55.12
	03	55.12
	04	50.23
Staphylococcus aureus	01	1.47
	02	8.88
	03	3.25
	04	4.75
Enterococcus faecalis	01	23.23
	02	25.87
	03	19.87
	04	17.05

From the results obtained above, it is clear that the aqueous extract of *Euphorbia guyoniana* is much more active against the different microbial strains than the synthetic antibiotics by exhibiting larger zones of inhibition. These results are confirmed by significant tests with $p < 0.05$. Except for Ciproflaxacin, which exhibits slightly larger inhibition zones on the Escherichia coli strains compared to the extract. This important antibacterial activity is due to the richness of the extract in flavonoïds, known to be effective antibacterial substances.

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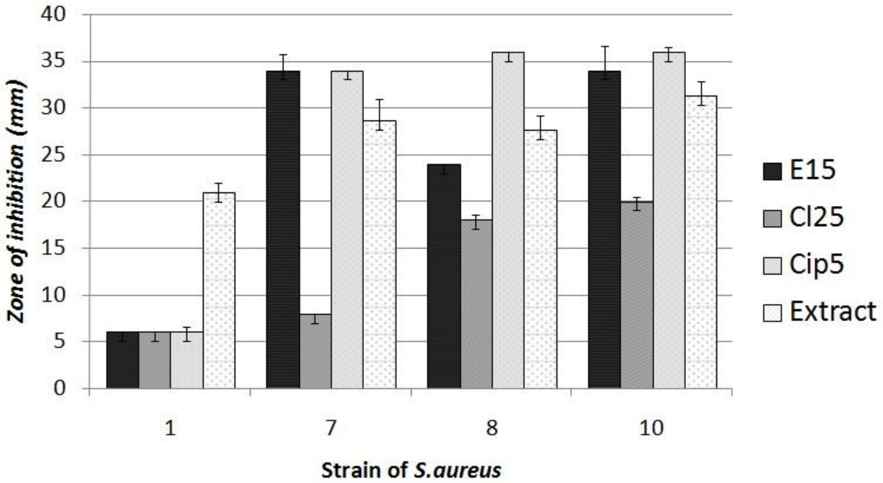

Figure 2. *Sensitivity of S. aureus strains of the extract and antibiotics*

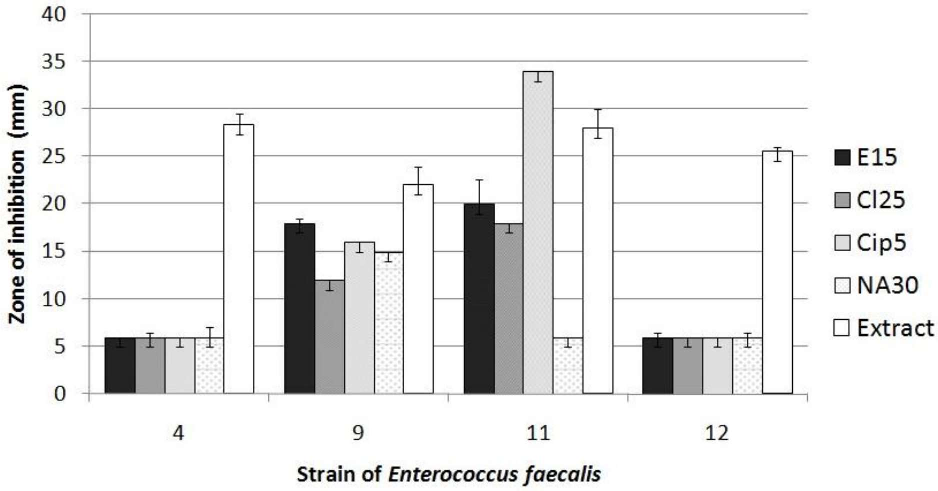

Figure 3. *Sensitivity of the strains Enterococcus faecalis to the extract and antibiotics*

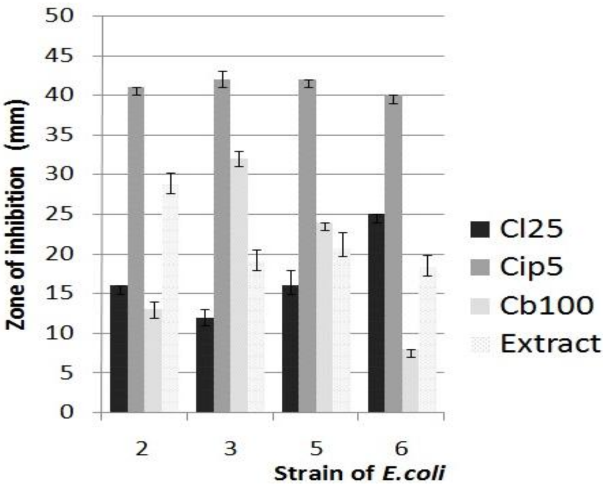

Figure 4. *Sensitivity of E. coli to the extract and antibiotics*

Discussion

The value of the flavonoid yield of the *Euphorbia guyoniana* plant found in our study (1.7%) was higher than that obtained by Kemassi (2014) for the aqueous extract of the same plant harvested from the Ghardaïa region which was obtained by Maceration with acetone, ie 0.082%. In addition, higher yields were noted by Herouini et al. (2015) for the flavonoid extract of the roots (6.3%) and the aerial part (4.3%) of *Euphorbia guyoniana* harvested in Oued sebseb (Algerian Sahara) obtained by reflux. According to Haba et al. (2008), *Euphorbia guyoniana* is a plant rich in secondary metabolites including diterpenes, triterpenes, steroids and aromatic compounds. The yield of flavonoids appears to depend on the nature of the biotope and the extraction method, knowing that the number of washes carried out in the extraction protocol could lead to substantial losses of the aglycones, hence the disadvantages of the method of extraction by solvent confrontation. Similar works have reported the existing of variability in the yield values extraction of secondary metabolite depending on the procedure of extraction (Moreira et al., 2005; Sagdic and Ozcan, 2003; Celiktas et al., 2007; Turkmen et al., 2007).

Concerning the concentration of aqueous extract in flavonoids; Andrianarisoa and Tsirinirindravo (2009) found a concentration of approximately 63.39 µg/µl for the aqueous extract of the leaves of *Dalechampia clematidifolia* (*Euphorbiaceae)* harvested in Madagascar, which represents a significantly lower value to that found for the species *Euphorbia guyoniana*.

HPLC analysis by Smara et al. (2014) on the aerial parts of the same plant (*Euphorbia guyoniana*) harvested from the Oued Souf region (Algerian Sahara) revealed the presence of a hydrolyzable tannin, a single coumarin and two flavonoids (flavonol) namely Quercetin-3O-β-D-glucuronide and kaempferol-3O-β-D-glucuronide. In our study, Quercetin is detected as a molecule, and associated with galactoside, glucoside or rhamnoside. As for kaempferol, this compound is also identified in the free form.

Very little study concerning the antibacterial activity of the aqueous extract of *Euphorbia guyoniana* was carried out. Herouni et al. (2015) used separately the flavonoid aqueous extracts of the aerial and subterranean parts of *Euphorbia guyoniana* on *Staphylococcus aureus* and *Escherichia coli* isolated from several infections, and noted a less marked activity than that observed in our study. These authors obtained zones of inhibition comprised between 7 and 8 mm.

This difference in the inhibitory effect of the bacterial growth observed may be related to the richness of the extract tested in flavonoid compounds known for their antibacterial activity. In the case of this study, the important antibacterial effect observed can be attributed either to the richness of the extract of flavonoid compounds (ellagic acid, gallic acid, Myricetine, Quercetin-3-O-α-rhamnoside, Apigenin-Orutinoside, Hydroxytyrosol and Kaempferol) or to the virulence of the bacterial strain.

Moreover, the efficiency of an extract of a plant also depends on the extraction method (Moreira et al., 2005; Sagdic and Ozcan, 2003; Celiktas et al., 2007; Turkmen et al., 2007), the part of the plant used (Yeo Sounta, 2014; Natarajan et al., 2005) and the harvest season.

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Conclusion

The evaluation of the antibacterial activity of the aqueous flavonoid extract of the medicinal plant *Euphorbia guyoniana* showed a remarkable inhibitory effect on pathogenic bacteria of telluric origin. In perspective other studies are necessary for the development of formulations for pharmaceutical use based on this extract in order to fight against these pathogens.

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EFFECT OF MYCORRHIZAL INOCULATION AND METHANOL SPRAYING ON SOME PHOTOSYNTHETIC CHARACTERISTICS AND YIELD IN WHEAT CULTIVARS UNDER END-SEASON DROUGHT STRESS

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Abstract. A two-year study was carried out with split-plot factorial arrangement based on randomized complete block design in three replications in Khorramabad, Iran, during 2012-2014. The studied factors in the main-plots included end season drought stress at three levels (normal irrigation, mild stress and severe stress with irrigation based on 40%, 60% and 80% moisture depletion of soil water available to plants) and mycorrhizal biofertilizer factors and methanol spraying at four levels (without mycorrhiza inoculation + distilled water spraying, control; mycorrhiza inoculation + distilled water spraying; without mycorrhiza inoculation + methanol spraying; and mycorrhiza inoculation + methanol spraying). Moreover, the three irrigated wheat cultivars Aflak, Dena and Alvand with factorial arrangement were in sub-plots. Results demonstrated that drought stress decreased stomatal conductance, leaf transpiration, photosynthesis rate and grain yield. Crop plants under mild stress conditions experienced lower carbon dioxide in sub-stomatal chamber, whereas, its accumulation in those plants that were under severe stress conditions increased significantly. The studied cultivars responded to drought stress differently. Yield reduction in Alvand was more severe than in Aflak and Dena under mild and severe drought stress conditions. The results suggested that mycorrhizal inoculation and methanol spraying play an important role in enhancing drought tolerance in susceptible wheat cultivars to drought stress. Moreover, they can be used in irrigated wheat farming to reduce damages caused by end season drought stress.
Keywords: *irrigation, photosynthesis, severe stress, transpiration, methanol spraying, wheat*

Introduction

As the most important cereal in many parts of the world, wheat is the main food for most people (Rauf et al., 2007; Shewry, 2009). Wheat is one of the most important cereal crop and considered as a staple food of the vast majority of the human population including urban and rural societies and it is also a major source of straw for animal feeding (Sharma et al., 2012).

Drought is one of the critical environmental adversities affecting the growth, development and final yield of crop species (Geng et al., 2016; Daryanto et al., 2017), and the frequency and severity of drought stress events are expecting to increase due to global climate change (Cook et al., 2014; Zhao and Dai, 2015; Joshi et al., 2016). Drought stress perturbs a broad range of plant physiological and biochemical processes, including decreased plant water status, inhibited photosynthetic processes, induced oxidative stress damage and so on, which ultimately lead to growth retardation and the

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reduction of crop yield (Perdomo et al., 2015; Saeidi and Abdoli, 2015; Daryanto et al., 2017). Drought stress is one of the major problems in successful crop production throughout the world (Auge, 2001) as well as in Iran. Drought stress is a key environmental phenomenon affecting cereal yields, often occurs over the wheat grain filling period, and eventually reduces crop yield in most cultivated areas in the world (Altenbach et al., 2003). In Iran, a significant portion of the 2.4 million hectares of irrigated wheat is damaged due to drought stress during flowering and grain filling phases (Jalal Kamali et al., 2012).

Crop growth is reduced under drought stress conditions due to limited photosynthetic rate. The damage caused by drought is primarily attributed to the inhibition and disruption of photosynthesis, which is the main mechanism of plant growth and maintenance of natural environments, and it threats the growth and yields of plants (Shao et al., 2016). Water deficit is often complemented by high temperatures that increase evapotranspiration and affect photosynthesis and ultimately decrease yield (Mir et al., 2012). Water deficit affects photosynthesis negatively by changing inner structure of the chloroplast, mitochondria and chlorophyll content and minerals (Huseynova et al., 2016). Allahverdiyev and Huseynova (2017) reported that stomatal conductance, net photosynthesic rate and transpiration rate decreased significantly in flag leaves of wheat genotypes in response to drought stress at anthesis. The intercellular CO_2 concentration in flag leaf of most genotypes increased under drought condition. Mild drought stress reduces photosynthesis through reversible stomatal factors. Under more severe or prolonged stress conditions, non-stomatal factors aggravate unfavorable conditions and the effects of stress generally become irreversible (Ahmadi and Baker, 2000). Blum et al. (1981) suggested that those genotypes are suitable for dry regions that are capable of retaining more water without closing their stomata.

Since crops production and, therefore, food security depend on the management of limiting factors, it is necessary to develop efficient strategies that allow the improvement of crop yield under water deficit stress (FAO, 2012). Water is undisputedly the major factor for the declining food production in many parts of the world, particularly in the arid and semiarid regions. Consequently, the world is now being challenged to produce “more crop per drop” of water. Therefore, in recent years there are increasing numbers of studies to understand the mechanism by which plants alleviate drought stress, and arbuscular mycorrhizal (AM) fungi seems to be an excellent alternative to serve the purpose. However, the mechanism by which AM fungi promotes drought tolerance is still to be understood well (Dar et al., 2018). Recently, it has been observed that the symbiotic interaction of plants with AMF, in addition to being important from the agricultural and ecological point of view (Yang et al., 2008), could be a sustainable mitigation practice for water deficit stress (Aroca, 2012). AM fungi allow host plants to grow more efficiently under biotic and abiotic stress conditions (Gholamhoseini et al., 2013). A recent finding indicates that AM fungus have a key role in modifications of root hairs consequently helping the plants to overcome the drought (Li et al., 2014). In association with symbiotic AM fungi, plants could explore larger volumes of soil to absorb water and nutrients thereby impart stress tolerance to the plants (Smith et al., 2009). Arbuscular mycorrhizal (AM) symbiosis often modifies gas exchange of the host plant (Koide, 1993; Smith and Read, 2008; Ruiz-Lozano and Aroca, 2010; Auge et al., 2014). Mycorrhizal association has been shown to increase the carbon fixation abilities of the plants. In a number of systems, higher photosynthetic rates have been reported when the plants are in association with

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AM fungi. For instance, in black locust, Yang et al. (2014) observed high stomatal conductance, high transpiration rates and high photosynthetic rates with reduced internal CO_2 concentration in fungal colonized plants than the non-colonized plants. Such higher photosynthetic rate as a consequence of fungal association has also been reported by Zhu et al. (2012). They observed high photosynthetic and transpiration rates in AM fungi colonized plants of maize than in non-colonized plants both under control and drought stress conditions. Arbuscular mycorrhiza stimulation of carbon exchange rate, stomatal conductance, and transpiration rate has been significantly associated with mycorrhizal stimulation of shoot dry weight, leaf phosphorus, leaf nitrogen:phosphorus ratio, and percent root colonization. (Auge et al., 2016). This relationship between the stomatal opening and the relative increase in the photosynthetic activity have been described in corn (Estrada et al., 2013),

Studies over recent years revealed that growth and yield of C_3 plants improved by methanol spraying, and methanol can be considered a carbon source for such plants. In general, the main contribution of these substances is to reduce the effects that stresses induced on crops have in performing photorespiration (Downie et al., 2004). Since nearly 90% of plant dry matter results from carbon dioxide assimilation through photosynthesis, an increase in photosynthesis rate can boost the crop production capacity (Makhdum et al., 2002). Methanol is a carbon source therefore increases CO_2 concentrations in plants and enhances growth and yield because the most important factor that affects dry weight of plants is CO_2 assimilation through photosynthesis (Mirakhori et al., 2009). Higher photosynthetic capacity can be achieved by using compounds including methanol, ethanol, propanol, and butanol as well as amino acids including glycine, glutamate and aspartate (Ramberg et al., 2002). In plants facing drought stress, methanol spraying prevents reduction of biomass (Rajala et al., 1998).

This study attempted to determine the effect of end season drought stress on yield and on photosynthesis and leaf gas exchange in three wheat cultivars so as to identify cultivars that are susceptible or tolerant to end season drought stress. Moreover, this study intended to analyze and clarify the effect of mycorrhiza inoculation and methanol spraying on wheat yield and leaf gas exchanges in an effort to mitigate and moderate the adverse effects of drought stress.

Materials and methods

Experimental site and treatments

A two-year experiment was carried out with split-plot factorial arrangement based on randomized complete block design in three replications in Khorramabad (33°20′N 48°21′E, altitude = 1171 m), Iran, during 2012-2014. The main factor included end season drought stress at three levels (normal irrigation, mild stress and severe stress with irrigation based on 40%, 60% and 80% moisture depletion of soil water available to plants) and factorial combination of mycorrhizal inoculation and methanol spraying (non-inoculation + non-praying as control; mycorrhizal inoculation; methanol spraying; mycorrhizal inoculation + methanol spraying) with wheat cultivars of Aflak, Dena and Alvand considered as sub factors. The climatic parameters (annual and long-term) of the experimental site are presented in *Table 1* during cropping years 2012-2013 and 2013-2014.

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Table 1. *The climatic parameters of the experimental site during cropping years 2012-2013 and 2013-2014 and average of 30 years (long-term)*

Month	2012-2013 cropping year					2012-2013 cropping year					Average of 30 years (long-term)				
	Precipitation (mm)	Relative moisture (%)	Temperature (°C)			Precipitation (mm)	Relative moisture (%)	Temperature (°C)			Precipitation (mm)	Relative moisture (%)	Temperature (°C)		
			Minimum (°C)	Maximum	Average			Minimum	Maximum	Average			Minimum	Maximum	Average
October	2.6	26.3	6.2	34.8	21.4	0	26	1.8	33.4	18.9	10.2	34.7	0	32	20.7
November	59	59.8	1.8	27.4	14.6	69.6	60	1.2	27.2	13	53.4	51.6	-7.8	25	14.4
December	30	69.9	-3.6	18.4	7.8	71.6	65	-4.6	20.8	8.3	77.6	62.6	-8	9.6	8.6
January	72.9	61.5	-6.2	17.2	5.3	70.8	63	-7	16.6	4.1	77.6	66.6	-13.6	17.6	5.9
February	68.4	59.8	-4.6	20.6	8.3	40.8	61	-7.6	18.8	5.9	83.5	66.1	-14.6	18	6.3
March	28.3	54.7	-3	25.6	10.6	68.4	61	-2	22.4	10.6	81.3	58.7	-11	24	9.9
April	29.8	47.5	0.6	27.4	14.9	86.9	57	-1.4	29.8	13	78.5	56.8	-7	33	13.7
May	72.4	52.8	3.2	30.6	17.2	22.8	48	5.8	33.6	19.5	56.8	50.6	-1.8	37	18.4
June	0.2	24.9	10.6	41.2	24.2	2	35	9.6	36.6	23.4	5.1	33.4	5	43	24.2
Sum	363.6					432.9					525.6				

Two bread wheat (*Triticum aestivum* L.) cultivars of Aflak and Alvand are spring and intermediate types, respectively, and Dena, as durum wheat (*T. durum* L.) cultivar, has a spring growth habit (Saeidi et al., 2005). The studied cultivars have been introduced by the Agricultural Research, Education and Extension Organization (AREEO) in the past few years, and are cultivated in temperate, tropical and subtropical regions of Iran. The mycorrhiza bio-fertilizer was a combination of two mycorrhiza fungi species called *Funnelifomis mosseae* and *Rhizophagus intaradices* and contained 15 spores g^{-1} and 930 hyphae of mycorrhiza cm^{-3}. Before sowing, Mycorrhiza (40 kg ha^{-1}) was poured uniformly into the sowing lines at the depth of 6-7 cm. Sowing density was 450 seeds m^{-2}. Each sub-plot consisted of 6 sowing lines with a spacing of 20 cm. The area of each plot was 7.2 m^2.

Prior to sowing, the soil was sampled to determine its physical and chemical characteristics (*Table 2*). Based on the soil analysis results, the chemical fertilizers of urea, triple super phosphate and potassium chloride were applied at 200, 100 and 50 kg ha^{-1}, respectively. Given the conventional practices in the region and the amount of regional rainfall, irrigation operations were carried out if necessary in all the experimental plots uniformly prior to beginning stage of stress treatments. At the wheat spike emergence phase, the normal irrigation and stress treatments were applied. Irrigation in normal irrigation and stress treatments were based on moisture depletion of soil in the range of field capacity to permanent wilting point. Soil moisture depletion percentage was measured by a Time Domain Reflectometer (TDR), which determines the volumetric percentage of soil moisture at the desired depth. Irrigation water volume for normal irrigation, mild stress and severe stress was 185, 132.5 and 80 mm, respectively in cropping year 2012-2013, and this amount was 147.5, 90 and 42.5 mm, respectively in cropping year 2013-2014. Industrial methanol (98%) at the concentration of 20% v/v was sprayed at spike emergence three times at 10-days intervals. Moreover, to each liter of the methanol solution, 1 g of the amino acid glycine and 1 mg of tetrahydrofolate were added.

Table 2. *Soil physical and chemical characteristics at the experimental site*

Year	Soil texture	Soil particles			pH	EC (dS/m)	O.C. (%)	N (%)	P (mg/kg)	K (mg/kg)	Zn (mg/kg)	B (mg/kg)	Cu (mg/kg)	Fe (mg/kg)
		Sand	Silt	Clay										
2012	Loam clay	20	57	23	7.7	0.77	1.02	0.098	8.8	332	0.33	0.2	1.6	4.8
2013	Loam clay	20	57	23	7.8	0.81	1.08	0.101	8.6	335	0.31	0.2	1.6	4.6

Leaf gas exchange measurement

The photosynthesis and leaf gas exchange measurements on the field were made by using a portable gas analyzer (ADC, Hoddeston UK, model LCA4) to measure the photosynthesis rate per leaf area unit (μmol CO_2 m^{-2} s^{-1}), stomatal conductance (mmol m^{-2} s^{-1}), transpiration rate (mmol H_2O m^{-2} s^{-1}) and internal CO_2 concentration (mmol). Measurements were conducted at 11 AM, at 26-27 °C and at the light intensity of 1100-1200 micromole photon m^{-2} s^{-1}. From each plot, five plants were randomly selected. Then, the flag leaves of the selected plants were placed inside a glass container of LCA-4 for 40 s and the related value was read and recorded (*Fig. 1*). Grain yield was measured at full maturity in the harvesting area of 3 m^2 from the middle four rows of each plot. Then, the seeds were weighed for grain yield calculation.

Data analysis

Combined analysis of the data obtained from two experimental years was performed using the expected value of mean square treatment. Moreover, the variance tests were also performed based on expected value of variance for the sources of variations. ANOVA operations and statistical calculations, comparison of the means, and calculation of simple correlation coefficients of traits were carried out using SAS, SPSS and MSTATC. The diagrams were drawn using Excel, and comparison of the means of the traits was performed employing Duncan's multiple range test (DMRT) at the 5% probability level.

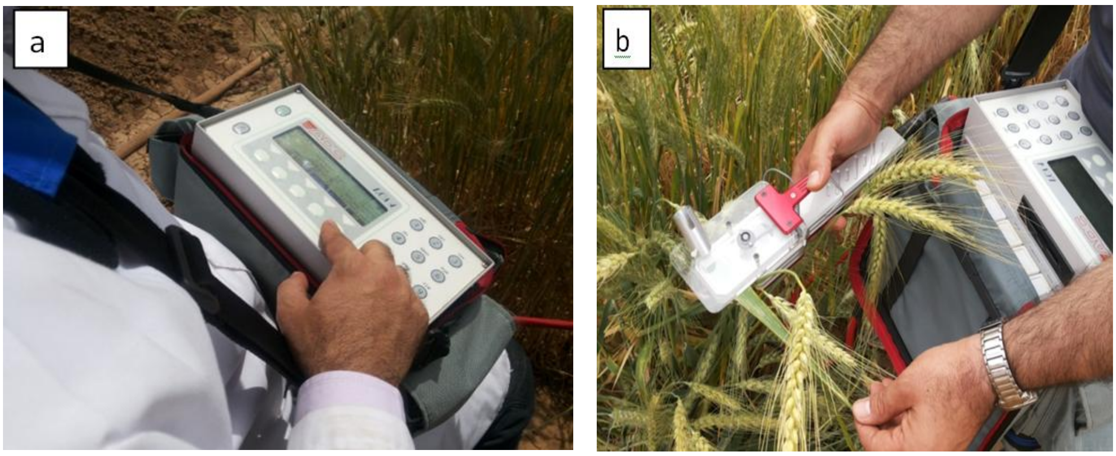

Figure 1. *Photos about the portable gas analyzer device (ADC, Hoddeston UK, model LCA4) (a). Measuring flag leaf photosynthesis and gas exchange of wheat plants in experimental plots (b)*

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Results

Leaf gas exchange

Effect of year on stomatal conductance, leaf transpiration, internal CO_2 concentration and photosynthetic rate was significant (*Table 3*). Stomatal conductance and photosynthetic rate in the second year improved and leaf transpiration and internal CO_2 concentration were higher in the first year than in the second year (*Table 4*) which could be due to variations in climatic conditions among experimental years.

Table 3. *Combined analysis of variance (mean squares) on the effect of water deficit stress, mycorrhiza-methanol spraying and cultivar on grain yield, photosynthesis and gas exchanges of wheat flag leaf (two-year analysis)*

S.O.V.	D.F.	Leaf transpiration	Stomatal conductance	Internal CO_2 concentration	Photosynthesis rate	Grain yield
Year (Y)	1	50.07**	654.52*	3918.52*	103.19*	17351561.9ns
Replication (Year)	4	0.567	13.643	150.245	3.110	2420430.2
Water deficit stress (S)	2	262.86**	298304.03**	162214.35**	867.46**	56711491.26**
S×Y	2	0.06ns	4.95ns	569.45ns	0.022ns	282271717ns
Error (a)	8	2.226	167.130	2127.55	3.36	8946809
Mycorrhiza-methanol (M)	3	26.46**	13424.46**	1267.59**	84.02**	5024713.9**
Y×M	3	0.07ns	996.59ns	43.81ns	0.20ns	466122.42ns
S×M	6	2.97**	14.42**	3172.06**	6.44**	783005.6**
Y×S×M	6	0.023ns	18432.94ns	140.74ns	0.146ns	323121.19ns
Cultivar (V)	2	23.74**	9.75ns	23.722ns	95.99**	43692271.45**
Y×V	2	0.06ns	2059.82ns	40.80ns	0.49ns	545193.2ns
S×V	4	3.43**	3.02**	1963.86**	11.29**	3800573.81**
Y×S×V	4	0.005ns	227.15ns	72.56ns	0.04ns	111023.44ns
M×V	6	0.56ns	4.32ns	80.27ns	0.86ns	596028.2*
Y×M×V	6	0.023ns	147.61ns	77.22ns	0.113ns	142153.6ns
S×M×V	12	0.27ns	36.38ns	170.09ns	1.72ns	395060.4ns
Y×S×M×V	12	0.014ns	3.98ns	41.60ns	0.069ns	306104.5ns
Error (bc)	132	0.508	119.36	131.17	1.21	225166.6
C.V.		10.7	10.6	6.4	8.9	8.6

ns, *, and ** are non-significant, significant at 5% and 1% probability levels, respectively.

Effect of water deficit stress on leaf gas exchange was significant (*Table 3*). Mild and severe water deficit stress decreased leaf stomatal conductance by 55.5% and 69.2%, leaf transpiration by 18.6% and 45.1% and photosynthetic rate by 15.6% and 69.2%, respectively, compared to the normal irrigation. Mild stress resulted in 26.6% decrease in internal CO_2 concentration while severe stress resulted in 26.9% increase in internal CO_2 concentration, compared to the normal irrigation (*Table 4*).

Increasing depletion of plant available water under normal irrigation to 60% and 80% of moisture depletion and increasing stress intensity, stomatal conductance and leaf transpiration decreased in all treatments and the highest decreasing observed at

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80% depletion of soil available water. The highest decreasing trend was devoted to the control and the lowest was for combined treatment. Under normal irrigation, mild and severe stresses, stomatal conductance and leaf transpiration in mycorrhizal inoculation, methanol spraying and combined treatment were more than control (*Figs. 2a* and *3a*).

Table 4. *Mean comparison results of main effects of year, water deficit stress, mycorrhiza-methanol spraying and cultivar on grain yield, photosynthesis and gas exchanges of wheat flag leaf (mean of two years)*

Treatments	Leaf transpiration (mol H_2O $m^{-2}s^{-1}$)	Stomatal conductance (mmol $m^{-2}s^{-1}$)	Internal CO_2 concentration (mmol CO_2)	Photosynthesis rate (µmol CO_2 $m^{-2}s^{-1}$)	Grain yield (kg ha^{-1})
First year	7.1b	101b	182.2a	11.7b	5234.9a
Second year	6.1b	104.5a	173.7b	13.1a	5801.7a
Water deficit stress (S)					
Normal irrigation	8.4a	175.8a	177.7b	15.5a	6373.3a
Mild stress	6.8b	78.2b	130.5c	13b	5580.1b
Severe stress	4.6c	54.2c	225.5a	8.6c	4601.5c
Mycorrhiza-methanol (M)					
Control	5.7c	82.7d	171.7c	10.6c	5233.1c
Mycorrhiza inoculation (AM)	6.9b	110.2	176.8b	12.7b	5574.3b
Methanol spraying (ME)	6.6b	98.7c	180.4ab	12.7b	5342.7c
AM+ ME	7.3a	119.3a	182.8a	13.5a	59230.a
Cultivar (V)					
Aflak	6.8a	103.3b	178a	12.8b	5869.4b
Dena	7.1a	118.4a	178.4a	13.4a	6059.9a
Alvand	6b	86.5c	177.3a	11.1c	4625.5c

Means having similar letters have no significant difference at 5% probability level through Duncan multiple range test

By increasing the depletion of soil available water content internal CO_2 concentration decreased in all treatments, so that it was at the lowest level under mild stress condition (60% soil moisture depletion). But this decrease value in mycorrhizal inoculation, methanol application and combined treatment was less than control (*Fig. 4a*). By increasing the stress intensity and increasing the percentage of soil moisture depletion to values higher than 60%, the amount of carbon dioxide covered by the intestinal space gradually increased in all treatments and increased to extreme stress (80% soil moisture depletion). However, the internal CO_2 concentration in the case of mycorrhizal inoculation and methanol spraying was less than control (*Fig. 4a*). Under normal irrigation conditions, stomatal conductance of leaves in Dena cultivar was more than Aflak and Alvand cultivars (*Fig. 2b*). Leaf transpiration rate in Aflak cultivar was more than Alvand cultivar, while transpiration rate in Dena and Alvand cultivars and Aflak and Dena cultivars showed no significant difference (*Fig. 3b*). Internal CO_2 concentration under normal irrigation showed no significant difference. Under mild and

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severe stress conditions, stomatal conductance in Aflak and Dena cultivars was more than in Alvand cultivar (*Fig. 2b*). Under severe stress conditions, internal CO_2 concentration increased for all cultivars compared to normal irrigation, but CO_2 concentration in Aflak and Dena cultivars was less than in Alvand cultivar (*Fig. 4b*).

Under mild stress conditions, transpiration in Aflak and Dena cultivars was more than in Alvand. Under severe stress, there was no significant difference among three cultivars and Dena and Alvand cultivars had the highest and the lowest transpiration rate, respectively (*Fig. 3b*). Mild stress decreased internal CO_2 concentration for three studied cultivars compared to normal irrigation. However, decreasing value in Aflak and Dena cultivars was less than in Alvand which could be attributed to higher drought resistance of cultivars. Severe stress resulted in increasing CO_2 (*Fig. 4b*).

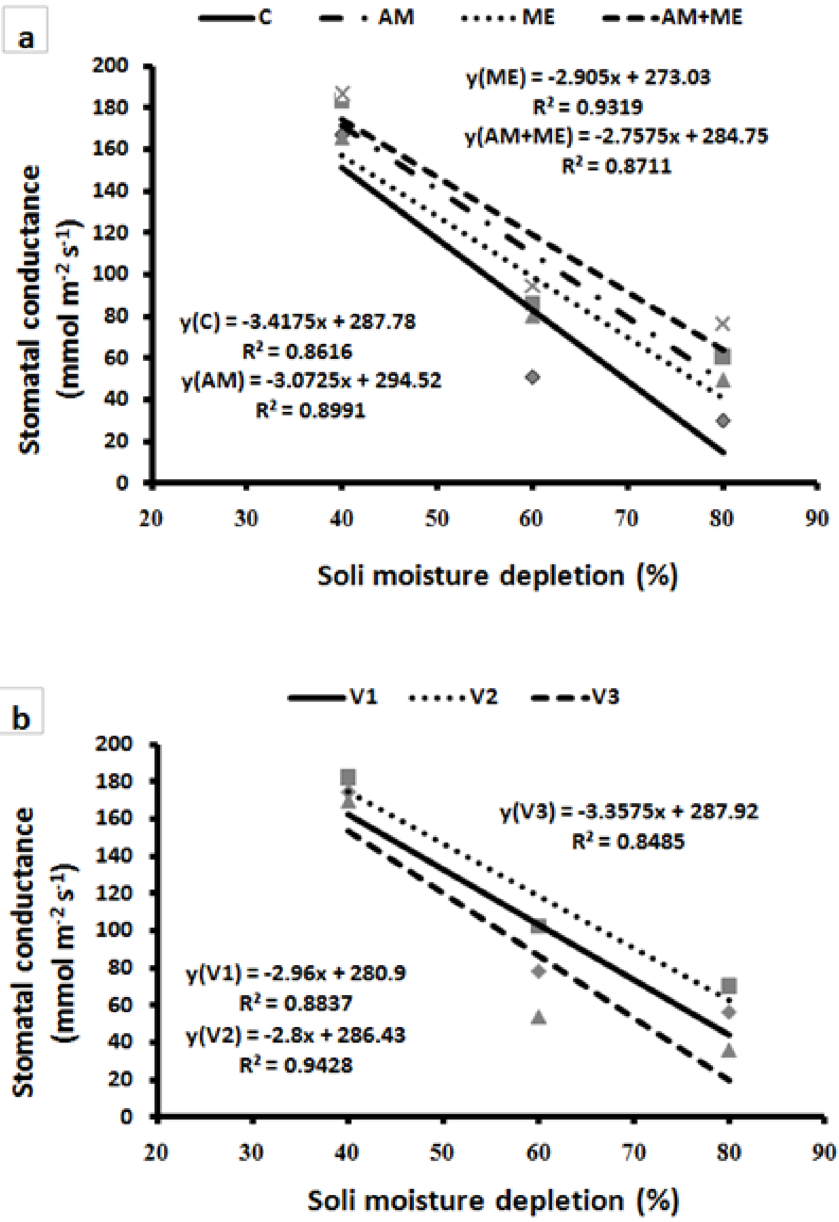

Figure 2. *Regression curve for interaction of irrigation levels × mycorrhiza- methanol (a), irrigation levels × cultivar (b) on leaf stomatal conductance. (C: control, AM: mycorrhizal inoculation, ME: methanol spraying, V_1: Aflak, V_2: Dena, V_3: Alvand)*

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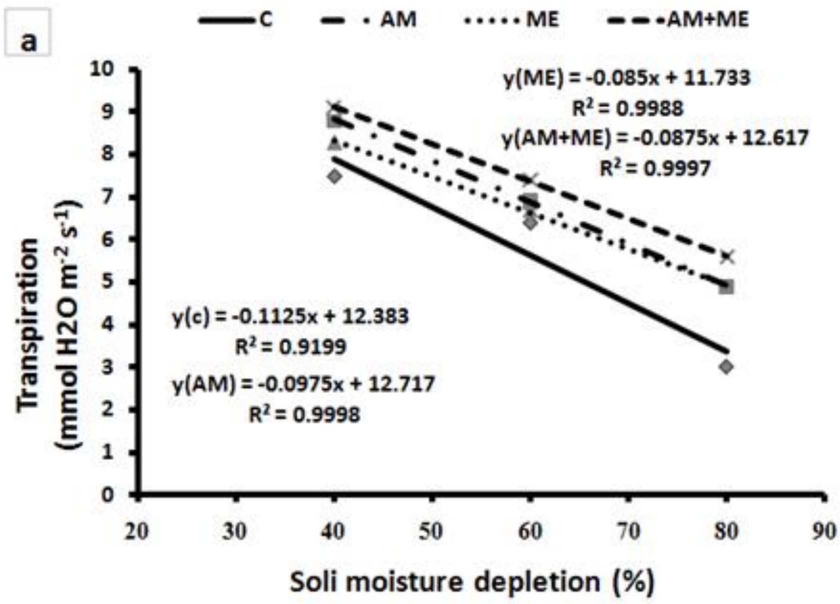

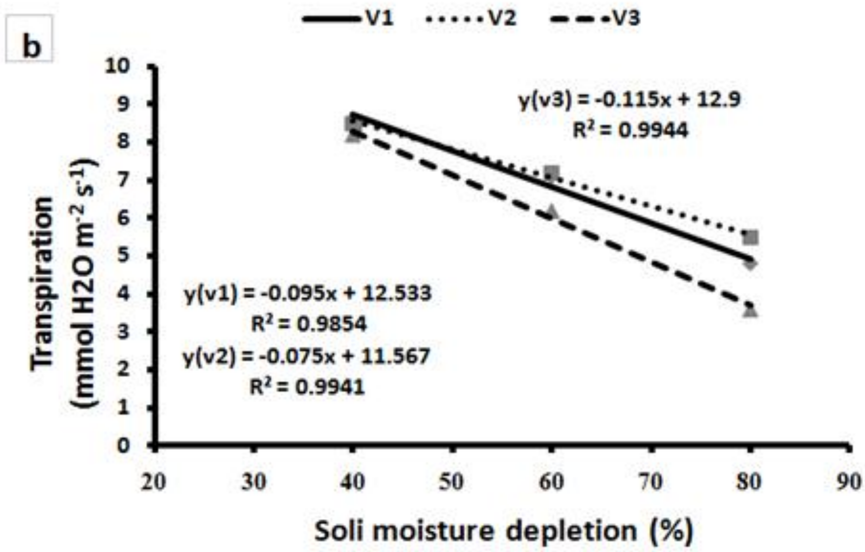

Figure 3. *Regression curve for interaction of irrigation levels × mycorrhiza- methanol (a), irrigation levels × cultivar (b) on leaf transpiration. (C: control, AM: mycorrhizal inoculation, ME: methanol spraying, V_1: Aflak, V_2: Dena, V_3: Alvand)*

Photosynthetic rate

Increasing depletion of plant available water under normal irrigation up to 60% and 80% and increasing stress level resulted in the decrease of photosynthestic rate in all treatments which the highest decrease observed in 80% moisture depletion. The highest deceasing trend devoted to control and the lowest was for methanol+mycorrhiza application. At all irrigation levels, photosynthetic rate in mycorrhizal inoculation, methanol spraying and combined application treatment was more than in the control (*Fig. 5a*).

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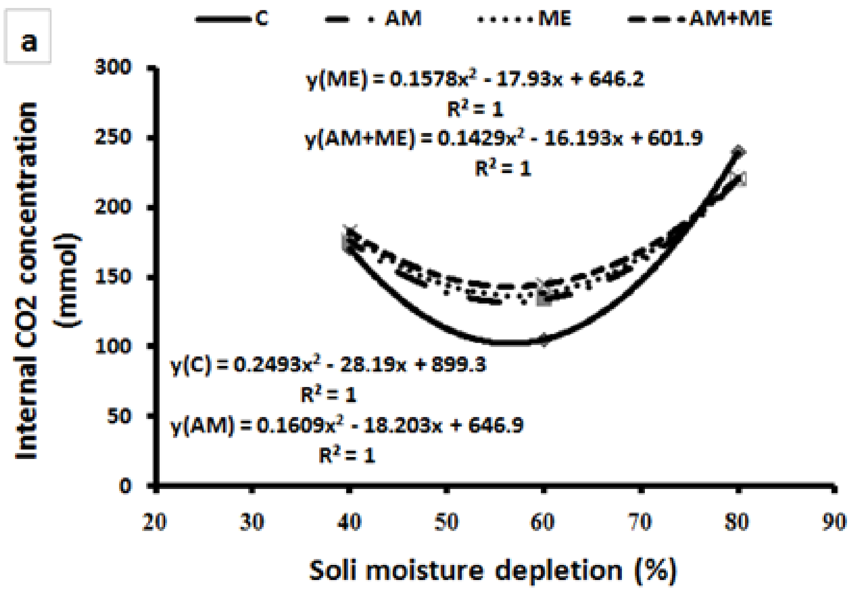

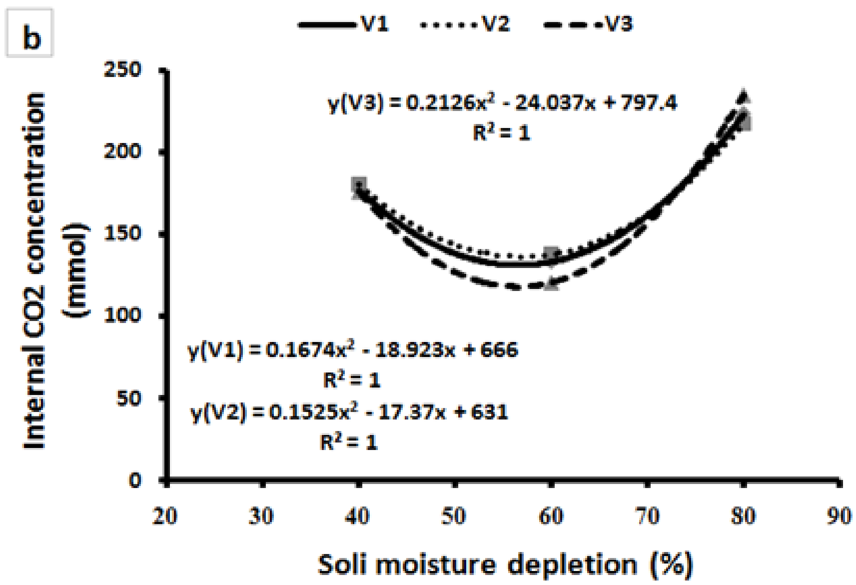

Figure 4. *Regression curve for interaction of irrigation levels × mycorrhiza- methanol (a), irrigation levels × cultivar (b) on Internal CO_2 concentration. (C: control, AM: mycorrhizal inoculation, ME: methanol spraying, V_1: Aflak, V_2: Dena, V_3: Alvand)*

According to our results, effect of water deficit stress on stomatal conductance was significant (*Table 3*). Mild and severe water deficit stresses resulted in the decrease of stomatal conductance compared to normal irrigation (*Table 4*); therefore, one of the effective factors on declining photosynthetic rate in the current study could be stated as decrease in stomatal conductance at both mild and severe stress conditions. The positive and significant correlation between photosynthesis and stomatal conductance (*Table 5*) confirms that decrease in stomatal conductance causes decrease in photosynthetic rate.

Increasing depletion of plant available water under normal irrigation up to 60% and 80% and increasing stress level resulted in the decrease of photosynthetic rate and the

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highest decrease was at 80% moisture depletion (severe stress), however decrease in Aflak and Dena cultivars was less than in Alvand. The highest decreasing trend was observed in Alvand and the lowest in Dena (*Fig. 5b*).

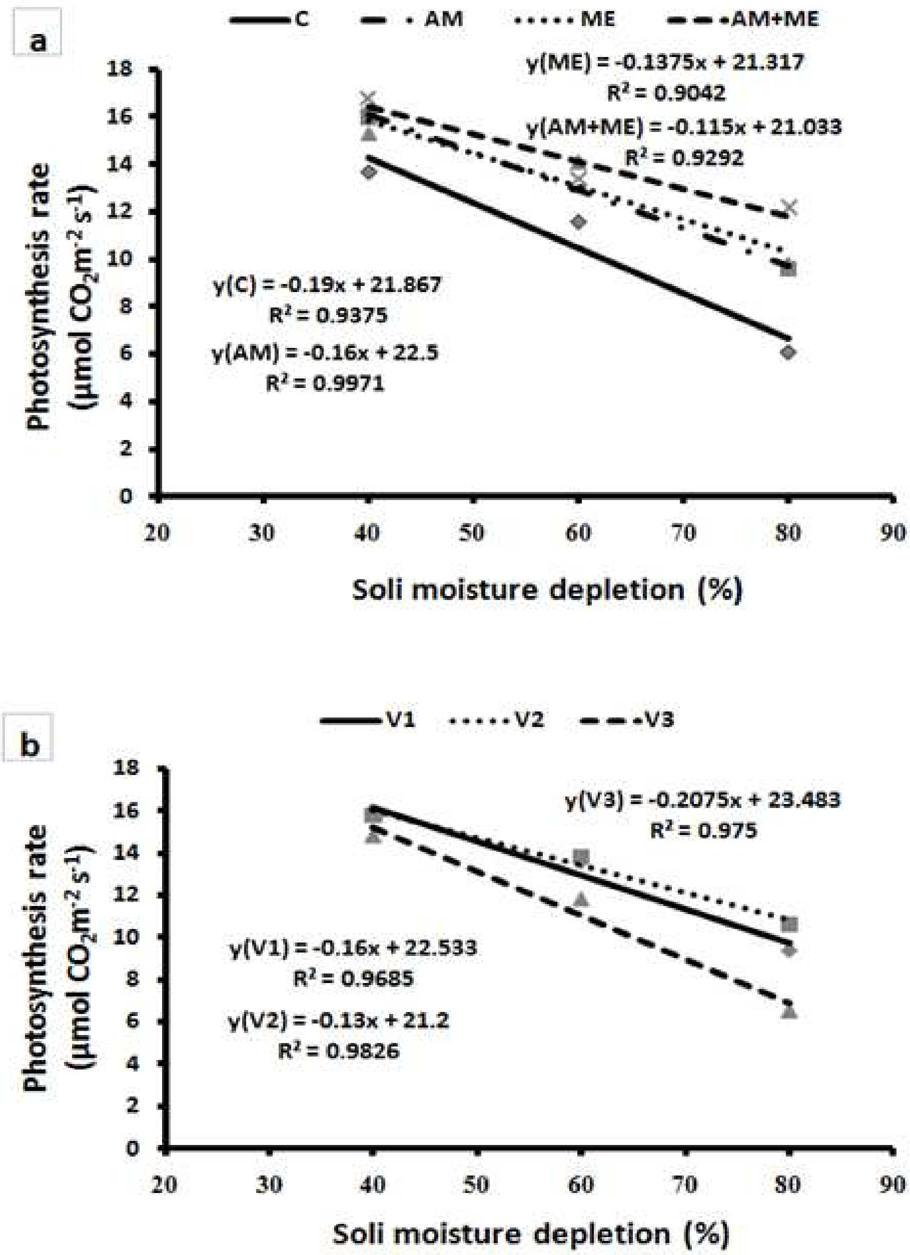

Figure 5. *Regression curve for interaction of irrigation levels × mycorrhiza- methanol (a), irrigation levels × cultivar (b) on leaf photosynthetic rate. (C: control, AM: mycorrhiza inoculation, ME: methanol spraying, V1: Aflak, V2: Dena, V3: Alvand)*

Grain yield

Interaction of water deficit stress×mycorrhiza-methanol and water deficit stress×cultivar on grain yield were significant (*Table 3*). Grain yield under mild and severe stress conditions decreased by 12.6% and 30% compared to normal irrigation,

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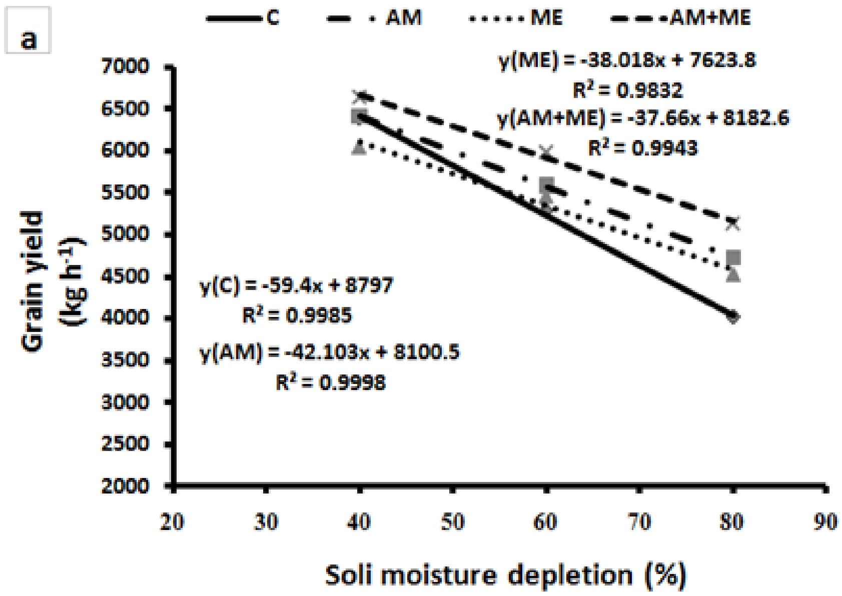
respectively (*Table 4*). Increasing depletion of plant available water under normal irrigation up to 60% and 80% and increasing stress level resulted in yield reduction for all studied treatments and highest decrease was at 80% moisture depletion. The greatest decreasing trend observed in control and the lowest in combined treatment application. At all irrigation levels grain yield in mycorrhizal inoculation, methanol spraying and combined treatment was more than in the control (*Fig. 6a*).



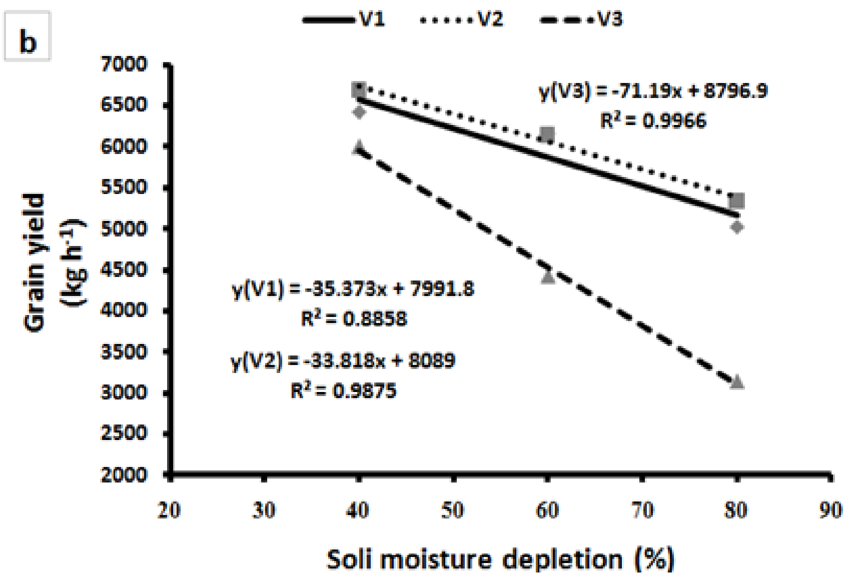


Figure 6. *Regression curve for interaction of irrigation levels × mycorrhiza- methanol (a), irrigation levels × cultivar (b) on leaf photosynthetic rate. (C: control, AM: mycorrhiza inoculation, ME: methanol spraying, V1: Aflak, V2: Dena, V3: Alvand)*

Increasing depletion of plant available water under normal irrigation up to 60% and 80% and increasing stress intensity resulted in the decrease of grain yield and the

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highest decrease was at 80% moisture depletion; however, decrease in Aflak and Dena cultivars were less than in Alvand. The highest decreasing trend was in Alvand cultivar and the lowest in Dena cultivar (*Fig. 6b*).

Mean comparisons showed that in control treatment, all studied cultivars had significant difference in terms of grain yield and the highest and the lowest yield associated to Dena and Alvand, respectively. Aflak and Dena cultivars reacted well to mycorrhizal inoculation, so that their yield increased by 12.8% and 7% compared to control, while there was no significant difference in yield of Alvand between control and mycorrhizal inoculation treatments. Studied cultivars had different response to methanol application. Aflak and Dena had no significant difference in terms of grain yield in methanol spraying, but Aflak showed 7.1% increase compared to the control (*Fig. 7*).

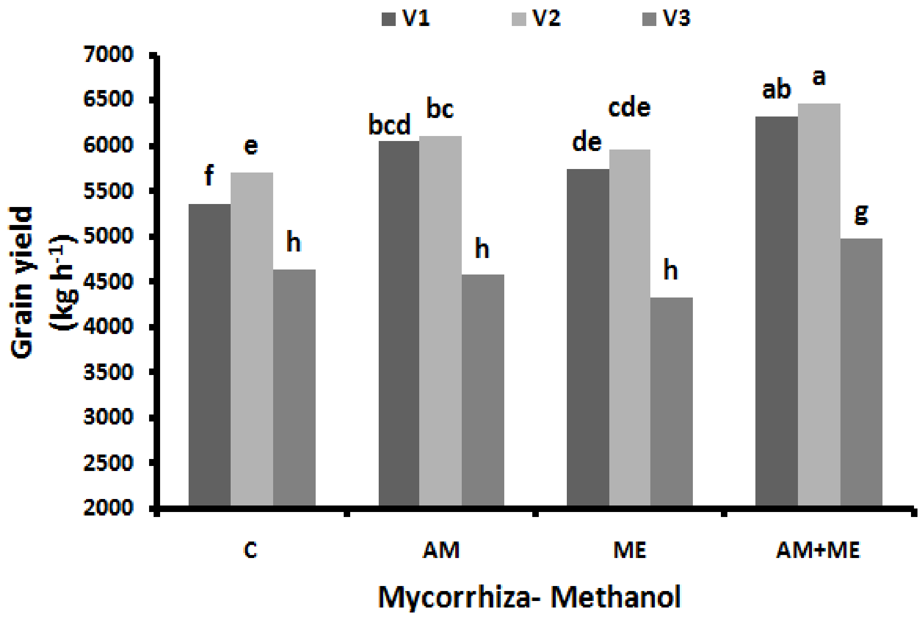

Figure 7. *Mean comparisons for interaction of mycorrhiza- methanol × cultivar on grain yield (e). Means having similar letters have no significant difference at 5% probability level through Duncan multiple range test. (C: control, AM: mycorrhiza inoculation, ME: methanol spraying, V1: Aflak, V2: Dena, V3: Alvand)*

Discussion

Leaf gas exchange

Siddique et al. (1999) found that wheat plants under drought stress conditions had considerable decrease in photosynthetic rate and stomatal conductance. Allahverdiyev and Huseynova (2017) report stomatal conductance, net photosynthetic rate and transpiration rate decreased significantly in flag leaves of wheat genotypes in response to drought stress at anthesis. Possibly increase in stomatal conductance in methanol spraying under water deficit conditions could be attributed to methanol contribution in enhancing stomata cells turgor and decrease in stomata closure. Nonomura and Benson (1992) concluded that methanol spraying in plant aerial parts increased plant turgor and prevented leaves subjected to direct sunlight from wilting, especially in hot regions. Zheng et al. (2008) reported that methanol spraying caused an increase in stomatal

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conductance and wheat plant photosynthesis. In a study conducted on cotton plants in Pakistan dry areas, methanol spraying resulted in the increase of stomatal conductance in treated plants (Makhdum et al., 2002). Zheng et al. (2008) found that methanol spraying increased wheat plant transpiration. Mycorrhizal symbiosis caused to delay in declining water content in leaves during drought stress and therefore stomata could be open for a longer period (Simpson and Duff, 1990). In another study on bean plants conducted by Zlatov and Yordanov (2004) resulted that intracellular CO_2 concentration decreases under drought stress conditions, which closes the leaf stomata and prevents the entry of CO_2 into the leaves. Arbuscular mycorrhiza stimulation of carbon exchange rate, stomatal conductance, and transpiration rate has been significant (Auge et al., 2016). Arbuscular mycorrhizal (AM) symbiosis often modifies gas exchange of the host plant (Koide, 1993; Smith and Read, 2008; Ruiz-Lozano and Aroca, 2010; Auge et al., 2014).

It was found that higher drought stress resistance in wheat cultivars is associated to higher stomatal conductance and partially to mesophilic conductance (Siosehmardeh et al., 2004). Difference in transpiration rate could be due to difference in sensitivity and tolerance rate of these cultivars to drought stress and stomatal reactions to water deficiency. Aflak and Dena cultivars kept their stomata less closed due to drought stress tolerance and had higher stomatal conductance and consequently had greater transpiration. Resistant wheat genotypes to drought having higher transpiration rate under drought stress conditions are able to uptake more water from soil and have higher leaf water content. Consequently, these genotypes have higher stomatal conductance and transpiration rate in comparison with drought sensitive cultivars. Roohi and Siosemardeh (2008) reported that leaf stomatal conductance and transpiration in resistant wheat genotypes was higher than in sensitive genotypes under water deficit conditions by 27% and 24%, respectively. The intercellular CO_2 concentration in flag leaf of most genotypes increased under drought condition (Allahverdiyev and Huseynova, 2017). It appears that Aflak and Dena had stomata openness ability due to drought stress resistance and therefore decrease in internal CO_2 concentration under mild stress condition was lower than in Alvand which had lower drought stress resistance. Also, these cultivars had lower destructive impact of severe stress conditions on biochemical processes and owing to higher mesophilic conductance, internal CO_2 concentration was lower than in Alvand. Mild drought stress reduces photosynthesis through reversible stomatal factors. Under more severe or prolonged stress conditions, non-stomatal factors aggravate unfavorable conditions and the effects of stress generally become irreversible (Ahmadi and Baker, 2000). Decrease in photosynthesis under higher drought levels is due to destructive biochemical processes (Johnston and Fowler, 2002). Photosynthesis limiting factors divided into stomatal factors which cause decrease in CO_2 diffusion into intercellular space due to decrease in stomatal conductance and non-stomatal factors which limit photosynthetic rate through direct impact of water deficit on carbon processing biochemical processes (Ahmadi and Baker, 2000).

Photosynthetic rate

The damage caused by drought to plants is primarily attributed to the inhibition and disruption of photosynthesis, which is the main mechanism of plant growth and maintenance of natural environments, and it threats to the growth and yields of plants (Shao et al., 2016). Siddique et al. (1999) concluded that wheat plants under drought stress had considerable decrease in stomatal conductance and photosynthetic rate. It appears that methanol foliar application resulted in the increase of CO_2 concentration in leaf cells and largely compensated CO_2 deficit and prevented further decrease in photosynthesis under drought stress conditions. Methanol, in comparison with CO_2, is a smaller molecule which could be easily utilized by C_3 plants for increasing photosynthetic rate (Li et al., 1995; Kotzabasis et al., 1999). Increase in photosynthetic rate through methanol foliar application has been reported by many authors (Setua et al., 2009; David et al., 2003). Zheng et al. (2008) studied the effect of methanol foliar application at various concentrations on winter wheat and reported that methanol increased stomata conductance, transpiration rate, internal CO_2 concentration and photosynthesis rate. It appears that methanol impact on C_3 plants is due to decrease in their photorespiration, because under filed conditions when air temperature, light intensity and consequently photorespiration were high, methanol foliar application increased plant growth (Nonomura and Benson, 1992; Fall and Benson, 1996). Photosynthesis rate in mycorrhizal plants was more than non-mycorrhizal plants which could be resulted from the effect of mycorrhiza on stomata opening. It is known that plants subjected to water deficit stress decrease photosynthesis because of an accumulation of ROS that damages the photosynthetic apparatus (Abbaspour et al., 2012), which can limit the NADPH and ATP supply of the Calvin cycle. As a consequence of AMF symbiosis, plants improve the water status, which increases photosynthesis by increasing stomatal conductance and, therefore, CO_2 fixation (Boldt et al., 2011). Relationship between the stomatal opening and the relative increase in the photosynthetic activity have been described in corn (Estrada et al., 2013). Mycorrhizal association has been shown to increase the carbon fixation abilities of the plants. In a number of systems, higher photosynthetic rates have been reported when the plants are in association with AM fungi. For instance, in black locust, Yang et al. (2014) observed high stomatal conductance, high transpiration rates and high photosynthetic rates with reduced internal CO2 concentration in fungal colonized plants than the non-colonized plants.

Grain yield

Drought is one of the critical environmental adversities affecting the growth, development and final yield of crop species (Geng et al., 2016; Daryanto et al., 2017), and the frequency and severity of drought stress events are expecting to increase due to global climate change (Cook et al., 2014; Zhao and Dai, 2015; Joshi et al., 2016). Drought stress perturbs a broad range of plant physiological and biochemical processes, including decreased plant water status, inhibited photosynthetic processes, induced oxidative stress damage and so on, which ultimately lead to growth retardation and the reduction of crop yield (Perdomo et al., 2015; Saeidi and Abdoli, 2015; Daryanto et al., 2017). It can be inferred that mycorrhizal inoculation and methanol application under mild and severe drought stress conditions inhibited from decrease in grain yield and lead to decreased and mediated damages due to water deficit stress in wheat crop. Under water stress conditions, mycorrhizal inoculated wheat plants produced better yield than control. It can be concluded that under moisture stress, mycorrhiza had effective contribution and increased water and nutrient uptake from soil through developing hypha and fungi mycelium and prevented from decrease in growth and grain yield under drought stress conditions. Mycorrhiza treatment improved all of wheat growth traits and drought resistance (Abo-ghalia and Khalafallah., 2008). Mycorrhiza fungi establish

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symbiotic association with roots of many plants cause increase in nutrient and water uptake, alleviating negative impacts of environmental stresses and improvement in plant growth and yield in sustainable agriculture systems (Sharma, 2002). Al-Karaki et al. (2004) concluded that under normal irrigation and drought stress conditions, mycorrhiza improved wheat grain yield and biomass and had great contribution in alleviating impacts of drought stress in field conditions. Some authors reported that methanol foliar application had no significant difference on most of studied traits in wheat (Milton et al., 1995; Ekiz et al., 1996). Contrarily, some authors reported that methanol spraying improved wheat growth and yield (Nonomura and Benson, 1992; Zheng et al., 2008). Other studies have shown that methanol application in crop plants under water deficit increased their biomass, while treating water-supplied plants with methanol, decreased their biomass (Nonomura and Benson, 1992; Ramirez et al., 2006).

Results of the present study revealed positive and significant correlation among grain yield with photosynthetic rate (r = 0.84), stomatal conductance (r = 0.86) and transpiration rate (r = 85) (*Table 5*). Grain yield under moisture stress decreased photosynthetic rate and stomatal conductance increased, while in mycorrhizal inoculation, methanol spraying and combined treatments, grain yield decreased less compared to control due to higher photosynthetic rate and stomatal conductance (*Figs. 6a, 1a* and *5a*).

Table 5. *Correlation of grain yield with photosynthetic rate and leaf gas exchanges under mild and severe water deficit stress conditions*

Traits	GY	SC	ICC	PR	LT
Grain yield (GY)	1				
Stomatal conductance (SC)	0.86**	1			
Internal CO_2 concentration (ICC)	-0.46*	-0.36ns	1		
Photosynthesis rate (PR)	0.84**	0.83**	-0.77**	1	
Leaf transpiration (LT)	0.85**	0.82**	-0.76**	0.97**	1

ns, *, and ** are non-significant, significant at 5% and 1% probability levels, respectively.

Under mild and severe stress conditions, yield for all studied cultivars decreased compared to normal irrigation, but decreasing value in Alvand (26.1% and 42.5%, respectively) was more than in Aflak (4.2% and 22% respectively) and in Dena (8.1% and 20.2%, respectively) cultivars (*Table 4*), which could be due to the fact that Alvand is a late maturing cultivar and the observed decrease represents more sensitivity of Alvand to late season water deficit stress. Karimzadeh Shurshjani et al. (2012) reported that Durum wheat cultivars, being early mature had less yield decrease compared to bread wheat cultivars under late season drought and yield decrease was higher in drought sensitive cultivars. Other authors have reported significant reductions in wheat yield due to late season drought stress (Dastfal et al., 2009; Gonzalez et al., 2010). The reason for decreasing grain yield for Alvand cultivar compared two other ones is that stomatal conductance, leaf tranpiration and photosynthetic rate decreased more under mild and severe drought stress conditions (*Figs. 1b, 2b* and *5b*). These results show that Alvand cultivar is more vulnerable to late season drought stress than other cultivars. Siosemardeh et al. (2005) found that decrease in stomatal conductance and mesophyll conductance and consequently decrease in photosynthetic rate are the main factors

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affecting yield of various wheat cultivars under drought stress conditions and resistant cultivars have higher stomatal conductance and mesophyll conductance as well as photosynthetic rate than sensitive cultivars.

Ekiz et al. (1996) understood that yield of irrigated wheat cultivars had no significant difference in methanol spraying treatments. In combined treatments, there was no significant difference between Aflak and Dena, while Alvand had lower yield than other two cultivars. Yield of cultivars studied increased compared to control in combined treatment. Results of other studies represent various responses of wheat cultivars to mycorrhizal inoculation (Vierheilig and Ocampo, 1991).

Conclusion

According to the results obtained, it can be concluded that late season drought stress caused decrease in stomatal conductance, leaf transpiration, photosynthetic rate and increase in internal CO_2 concentration and consequently decreased grain yield of wheat, while mycorrhizal inoculation and methanol spraying alleviated negative impacts of water stress and improved photosynthetic rate and leaf gas exchanges compared to control under drought stress conditions and consequently increased grain yield. Also, the differential response of cultivars to imposed water stress condition indicates the drought tolerance ability of wheat cultivars. Results showed that cultivar and vulnerability rate and their tolerance are effective in wheat response to late season drought stress. Decrease in photosynthetic rate, gas exchanges and yield in Alvand cultivar under mild and severe drought stress was more than in Aflak and Dena cultivars; that represent sensitivity of Alvand cultivar to late season drought stress conditions. In total, it can be concluded that use of drought-tolerant wheat cultivars cause decrease in destructive impacts of terminal season drought stress. Also, mycorrhizal application and methanol spraying improve photosynthesis, leaf gas exchanges and grain yield and could be considered as cropping management strategies for decreasing damages of late season drought stress in irrigated wheat crop systems. In the future studies, it is recommended for evaluation of effect of milder levels of water deficit stress on the growth and yield of wheat, because severe stress have a lot of negative impact on leaf gas exchange and growth, and the sharp decline in wheat yield.

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COBALT AND ARSENIC CONCENTRATION IN HERBS GROWING IN FIELD POND AREAS IN POLAND

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Abstract. This paper deals with cobalt and arsenic accumulation in above-ground parts of herbs growing in intermittent field ponds with no outlets in Poland. Those ponds were located in different surroundings. In the experiment described here the following herbs were analysed: *Potentilla anserina* L., *Mentha arvensis*, *Achillea millefolium* L., *Comarum palustre* L., *Lysimachia vulgaris* L. and *Lycopus europaeus* L. Both plant and soil samples were collected from three moisture sections: wet, periodically wet and dry. Cobalt and arsenic content in plants and soil was determined with inductively coupled plasma-atomic emission spectrometry, or the ICP–AES method, after dry mineralization of the material. Cobalt and arsenic content was significantly dependent on the herb species. The highest content of cobalt was found in *Mentha arvensis*, and arsenic in *Lysimachia vulgaris* L. and *Comarum palustre* L., with *Achillea millefolium* having the lowest content of arsenic. The highest content of those metals was in the bottom sediment and in the soil from the depression with arable land around. Arsenic had a higher accumulation coefficient in herbs than cobalt. Cobalt content in herb biomass did not exceed the limits, but in the case of arsenic it was too high in all herbs apart from *Achillea millefolium* L.
Keywords: *selected heavy metals, plants, soil, in-field ponds*

Introduction

Arsenic and cobalt belong to elements very common in the environment, and in higher levels they are poisonous to humans and animals (Bowell, 1994; Hamilton, 1994; Kabata-Pendias and Pendias, 1999). Arsenic compounds are used as herbicides and defoliants in agriculture and forestry mainly. The amount of arsenic in soil depends on its content in parent rock, the type of soil formation and on anthropogenic factors. Anthropogenic pollution is mainly of industrial origin but there are other sources like using communal and industrial waste or plant protecting products in agriculture (Krysiak and Karczewska, 2007). In soil, arsenic is adsorbed by iron oxides, mainly by iron hydroxides of different degrees of crystallisation (Bowell, 1994; Voigt et al., 1996; Wenzel et al., 2001). Because of that this metal is not very mobile and not easily available to plants. A change of soil pH or soil aeration can cause a partial destruction of iron oxides, and adsorbed arsenic is released (Masscheleyn et al., 1991; Marin et al., 1993).

Gál et al. (2008) indicate that there are different kinds of cobalt ions in soil depending on soil pH, which, together with reduction potential, determines bioavailability of the metal. Suttle et al. (2003) and Bakkaus et al. (2008) provide

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different degrees of cobalt bioavailability, from 3 to 13% and from 1.5 to 37%, respectively. In agricultural areas free from industrial pollution, cobalt can get into the soil together with phosphorus fertilisers.

Of course, its presence, like presence of other heavy metals in the soil-plant environment, can be harmful to people and animals. Plants growing in natural habitats, even if not directly exposed to pollution, can contain amounts of heavy metals which are too high (Ražic et al., 2008; Malinowska and Jankowski, 2016, 2017; Bolan et al., 2017).

The aim of the experiment was to determine cobalt and arsenic content in herbs used in the pharmaceutical, cosmetic and food industries. They were sampled from different moisture sections of three in-field ponds.

Materials and methods

The plant material was sampled in 2014, from mid June till the end of July. Six herb species growing in the areas of three field ponds on the Siedlce Plateau were sampled: *Potentilla anserina* L., *Mentha arvensis*, *Achillea millefolium* L., *Comarum palustre* L., *Lysimachia vulgaris* L., *Lycopus europaeus* L. Three experimental areas with three ponds were marked with capital letters: A, B and C (*Fig. 1).*

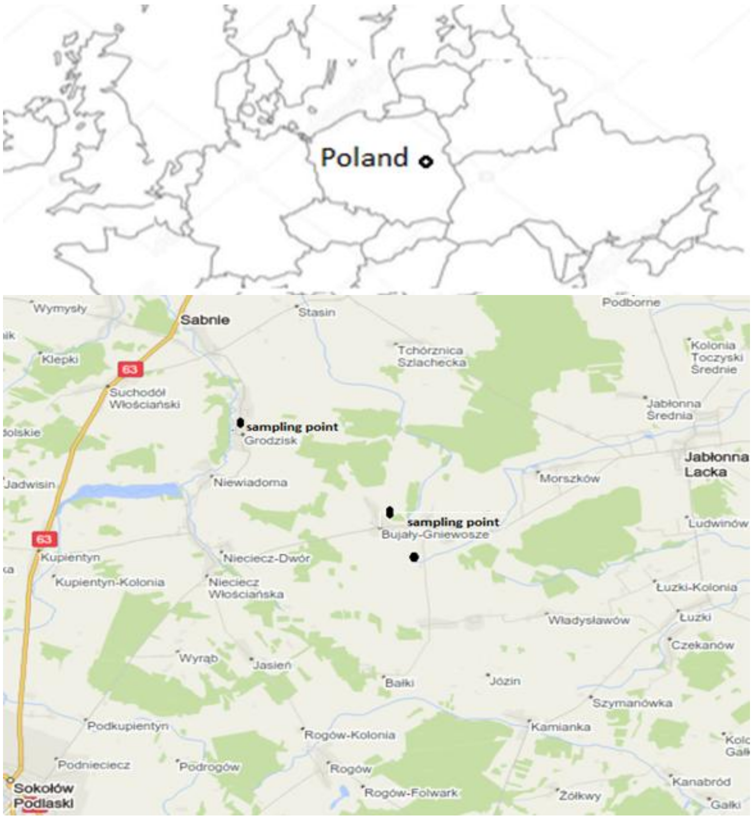

***Figure 1.** A map of the sampling site in an urban area, Poland*

Pond A was surrounded by arable land, B by permanent grassland and pond C was overgrown with bushes, with cultivated fields 100 m away from it. What decided about the choice of the ponds was the fact that they were surrounded by fields, but also the

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diversity of vegetation and the pond sizes, ranging from 15000 m^2 to 850 m^2. According to the administrative division they were located in the Sokołów Podlaski County, the eastern part of the Mazovian Voivodeship, with luvisols and rusty soil mostly. Each experimental area was divided into three transects, with the length varying from 18 to 80 m, stretching from the edge of the pond to the edge of the field. Around each pond in every transect three concentric moisture sections were marked: I – wet, with water stagnating in early spring after snow has melted or after heavy rainfall; II – periodically wet, with water stagnating only in early spring after snow has melted; III – dry, without any water stagnating there (Franczak and Franczak, 2015). The number of plant and soil samples taken from the transects varied from 85 to 100 in each experimental area. Additionally, bottom sediment samples were taken from each pond. 5-7 plants of each species were collected from each transect.

Plant material (from above-ground parts, without roots) was ground to particles of 0.25 mm in diameter, and 1 g of it was weighed and put into a porcelain crucible. Then the crucible was placed in a muffle furnace for 15 h to oxidise organic matter at the temperature of 450 °C. Next 10 ml of hydrochloric acid solution (1:1) was added and the mixture was put into a sand bath to evaporate, to decompose carbonates and to remove silica. After adding 5 ml of 10% hydrochloric acid solution, the contents of the crucible were filtered through a hard filter into a 100 ml volumetric flask and water was added up to the mark. Cobalt and arsenic concentration was determined using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). An internal quality control procedure was used to verify the accuracy of the methods. It was assumed that the average recovery for each spike concentration should be between 85 and 115% of true value. Two measurements were taken for each series of samples with the recovery being within the 85÷115% range. The limit of detection for arsenic and cobalt was 0.01 $mg \cdot kg^{-1}$. Soil pH, the value of which ranged from 5.45 to 6.50, was measured with the potentiometric method.

All of the data were statistically analysed and differences between means were assessed using analysis of variance (The Statistica programme, Version 10.0 StatSoft, was applied). Tukey's test was used to determine $LSD_{0.05}$ for means that were significantly different (StatSoft, Inc. 2011). The intensity of cobalt and arsenic accumulation by the herbs was determined with the accumulation coefficient (AC).

$$AC = c_p / c_s$$

where c_p is the metal content in the plant and c_s is the metal content in the soil (Wesołowski and Radecka, 2003). The results were interpreted as follows:
AC < 0.01 - no accumulation;
AC < 0.1 - slight accumulation;
AC - 1 - medium accumulation;
AC > 1 - high accumulation.

Additionally, the correlation coefficient between the content of each metal in the soil and the same content in the plants was calculated.

Results and discussion

Cobalt concentration in the biomass of herbs used in the experiment significantly varied, depending on the plant species and on the moisture section, and ranged from

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0.011 to 0.931 mg·kg^{-1} DM (*Table 1*). There was no significant variation in this concentration between moisture sections in the area with arable land. Out of all experimental areas and all moisture sections the highest average concentration of this metal was in *Mentha arvensis* (0.600 mg·kg^{-1}), and the lowest, over 16 times lower, in *Achillea millefolium* L. (0.037 mg·kg^{-1}). The average cobalt concentration in herbs sampled from different moisture sections was the highest in the periodically wet sections (0.261 mg·kg^{-1}), while the lowest was in the dry sections (0.189 mg·kg^{-1}).

Table 1. *Cobalt concentrations (mg·kg^{-1}) in the biomass of some herbs*

Species	A				B				C				Mean			
	I	II	III	Mean	I	II	III	Mean	I	II	III	Mean	I	II	III	Mean
Potentilla anserina L.	0.123	0.102	0.140	0.122	0.136	0.123	0.145	0.135	0.133	0.112	0.103	0.116	0.131	0.112	0.129	0.124
Mentha arvensis	0.623	0.741	0.563	0.642	0.745	0.931	0.684	0.787	0.541	0.342	0.232	0.372	0.636	0.671	0.493	0.600
Achillea millefolium L.	0.036	0.058	0.039	0.044	0.023	0.054	0.063	0.047	0.023	0.026	0.011	0.020	0.027	0.046	0.038	0.037
Comarum palustre L.	0.236	0.203	0.298	0.246	0.148	0.201	0.198	0.182	0.303	0.318	0.287	0.303	0.229	0.241	0.261	0.244
Lysimachia vulgaris L.	0.056	0.032	0.030	0.039	0.099	0.820	0.094	0.338	0.060	0.036	0.050	0.049	0.072	0.296	0.058	0.142
Lycopus europaeus L.	0.120	0.165	0.199	0.161	0.124	0.137	0.148	0.136	0.203	0.208	0.105	0.131	0.149	0.200	0.152	0.167
Mean	0.199	0.217	0.212	0.209	0.213	0.378	0.222	0.271	0.211	0.174	0.131	0.172	0.207	0.261	0.189	0.219

LSD$_{0.05}$ for:	A	B	C	Mean
S-species	S = 0.041	S = 0.013	S = 0.054	S = 0.034
M-moisture section	M = n.s.	M = 0.007	M = 0.031	M = 0.019
S/M; M/S interaction	S/M = 0.071	S/M = 0.018	S/M = 0.075	S/M = 0.047
	M/S = 0.057	M/S = 0.022	M/S = 0.093	M/S = 0.058

A - area with the cultivated field; B - area with permanent grasslands; C - area with bushes; I - wet section; II - periodically wet section; III - dry section; n.s. - not significant difference

Among different experimental areas, the highest average concentration of cobalt was in herbs growing around the pond with permanent grasslands (0.271 mg·kg^{-1}), while the lowest was in herbs from the area with bushes (0.172 mg·kg^{-1}). Bakkaus et al. (2005) say that dicotyledonous plants can contain much more cobalt than monocotyledonous plants. Like iron, cobalt in vascular plants is transported to tissues in an active and passive way (Palit et al., 1994). Because of the similarity of the uptake process there might be an antagonistic relationship between cobalt and other metals, in particular iron and manganese. According to Kabata-Pendias and Pendias (1999) cobalt concentration in plants should range from 0.08 to 0.1 mg·kg^{-1}, while the toxic concentration of this metal varies between 30 and 40 mg·kg^{-1} DM. In the present experiment the average cobalt concentration in the plants was 0.219 mg·kg^{-1}, which is lower than the limit. Symanowicz et al. (2014) found a similar cobalt concentration in *Galega orientalis* Lam., with mineral fertilisers applied.

Arsenic concentration in the analysed herbs varied considerably depending on the plant species and the moisture section (*Table 2*). Concentration of this metal ranged from 0.301 to 1.36 mg·kg^{-1} DM. The highest concentration of arsenic was found in *Lysimachia vulgaris* L. (0.848 mg·kg^{-1}) and in *Comarum palustre* L. (0.757 mg·kg^{-1}), with the lowest in *Achillea millefolium* L. (0.448 mg·kg^{-1}). The average concentration of this metal in all the herbs was 0.645 mg·kg^{-1}. The natural barrier for the uptake of

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arsenic by the above-ground parts of plants is the root system. Das et al. (2004) say that the concentration of arsenic in plant roots is ten times higher than in the above-ground parts. According to the Ministry of Health Regulation (2003) arsenic concentration in medicinal herbs and dry culinary herbs should not be higher than 0.5 mg·kg^{-1} DM. In the experiment described here, out of six analysed herbs, only *Achillea millefolium* L. had arsenic concentration below the limit. In all the other herbs the concentration was much higher that the limit set by the Regulation of 2003. Thus, in *Lysimachia vulgaris* L. the concentration was 70% higher, and in *Comarum palustre* L. it was 50% higher.

Table 2. *Arsenic concentrations (mg·kg^{-1}) in the biomass of some herbs*

Species	A				B				C				Mean			
	I	II	III	Mean	I	II	III	Mean	I	II	III	Mean	I	II	III	Mean
Potentilla anserina L.	0.871	0.645	0.620	0.712	0.541	0.954	0.530	0.675	0.501	0.451	0.403	0.452	0.638	0.683	0.518	0.613
Mentha arvensis	0.641	0.784	0.502	0.642	0.870	0.987	0.623	0.827	0.417	0.514	0.498	0.476	0.643	0.773	0.541	0.652
Achillea millefolium L.	0.501	0.412	0.436	0.450	0.512	0.578	0.509	0.533	0.411	0.368	0.301	0.360	0.475	0.453	0.415	0.448
Comarum palustre L.	0.899	0.785	0.623	0.769	0.880	1.020	0.877	0.926	0.544	0.688	0.501	0.578	0.774	0.831	0.667	0.757
Lysimachia vulgaris L.	0.991	0.836	0.521	0.783	0.841	1.360	0.801	1.00	0.754	0.801	0.723	0.759	0.862	0.999	0.682	0.848
Lycopus europaeus L.	0.421	0.499	0.413	0.444	0.647	0.921	0.578	0.715	0.412	0.566	0.509	0.496	0.493	0.662	0.500	0.552
Mean	0.721	0.661	0.519	0.633	0.716	0.970	0.653	0.779	0.507	0.565	0.489	0.520	0.648	0.734	0.554	0.645

$LSD_{0.05}$ for:	A	B	C	Mean
S-species	S = 0.010	S = 0.025	S = 0.023	S = 0.035
M-moisture section	M = 0.006	M = 0.014	M = 0.013	M = 0.020
S/M; M/S interaction	S/M = 0.014	S/M = 0.043	S/M = 0.033	S/M = 0.060
	M/S = 0.017	M/S = 0.035	M/S = 0.041	M/S = 0.049

A - area with the cultivated field; B - area with permanent grasslands; C - area with bushes; I - wet section; II - periodically wet section; III - dry section

When it comes to moisture sections the highest average concentration of arsenic was in herbs from periodically wet sections (0.734 mg·kg^{-1}), it was lower in wet sections and the lowest in dry sections (0.554 mg·kg^{-1}). This diversity might have been caused by leaching of plant protection products into groundwater. The average arsenic concentration was also different in different experimental areas. The most arsenic was in plants growing around the pond with permanent grasslands and the lowest in the area with bushes, on average 0.779 mg·kg^{-1} and 0.520 mg·kg^{-1}, respectively.

Cobalt and arsenic concentration in bottom sediment of the three different ponds varied (*Table 3*). The highest concentration of cobalt (3.12 mg·kg^{-1}) and arsenic (4.38 mg·kg^{-1}) was found in the bottom sediment sampled from the pond surrounded by arable land. In the sediment from the pond with bushes the concentration was much lower, with 2.03 mg Co·kg^{-1} and 2.87 mg As·kg^{-1}. Many publications (Dhar et al., 1997; Kondo et al., 1999; Karim, 2000; Anawar et al., 2003), report that arsenic can get into groundwater and accumulate in bottom sediment. Reduction processes, taking place in permanently flooded areas, can be the main source of contamination of groundwater by arsenic in south eastern Asia, in countries like Bangladesh, India and Vietnam (Burton et al., 2008).

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Table 3. *Cobalt and arsenic concentration in bottom sediment (mg·kg^{-1} DM)*

Metal	A	B	C	Mean
Co	3.12	2.63	2.03	2.59
As	4.38	3.54	2.87	3.60

A - area with the cultivated field; B - area with permanent grasslands; C - area with bushes

The amount of cobalt and arsenic in the soil was also related to the moisture section and to the experimental area (*Table 4*). On average the highest concentration of both metals was in the soil of the depression with arable land. Out of all three moisture sections the highest concentration of cobalt and arsenic was in the wet section (1.74 mg·kg^{-1} and 2.06 mg·kg^{-1}, respectively). The amount of those metals in the soil was much lower than their amount in non-contaminated soil reported by Kabata-Pendias and Pendias (1999). The release of arsenic into the soil is a complicated process, chemical and physicochemical, but first of all it involves a biochemical reduction of the components, both solid and liquid parts (Anawar et al., 2003; Solaiman et al., 2009; Postma et al., 2010). One of the main factors affecting this process is humus content in the soil.

Table 4. *Cobalt and arsenic concentration in soil (mg·kg^{-1} DM)*

Area	Co				As			
	I	II	III	Mean	I	II	III	Mean
A	1.94	2.29	1.03	1.75	2.94	3.05	2.40	2.80
B	1.75	1.23	1.48	1.49	1.85	1.69	0.989	1.51
C	1.52	1.36	0.984	1.29	1.39	1.26	1.32	1.32
Mean	1.74	1.63	1.17	1.51	2.06	2.00	1.57	1.88
$LSD_{0.05}$ for:								
M-moisture section	M = 0.288				M = 0.166			
Ar-area	Ar = 0.288				Ar = 0.166			
M/B; B/M-interaction	M/Ar = 0.499	Ar/M = 0.499			M/Ar = 0.288	Ar/M = 0.288		

A - area with the cultivated field; B - area with permanent grasslands; C - area with bushes; I - wet section; II - periodically wet section; III - dry section

There was a wide range of accumulation coefficient values of the soil-plant system (*Figs. 2* and *3)*. The accumulation coefficient for arsenic was higher than for cobalt but for both heavy metals it was lower than one, on average 0.111 for cobalt and 0.353 for arsenic. When the value of accumulation coefficient is higher than one, then the accumulation of the metal in plants is high (Kloke et al., 1984). According to the above publication both cobalt and arsenic have the lowest accumulation coefficient of all heavy metals, ranging from 0.01 to 0.1. The values obtained in this experiment are within the same range. The accumulation coefficient was closely related to the herb species, with the highest value for cobalt in *Mentha arvensis*, and arsenic in *Lysimachia vulgaris* L.. *Achillea millefolium* L. had the lowest value of the accumulation coefficient both for cobalt and arsenic.

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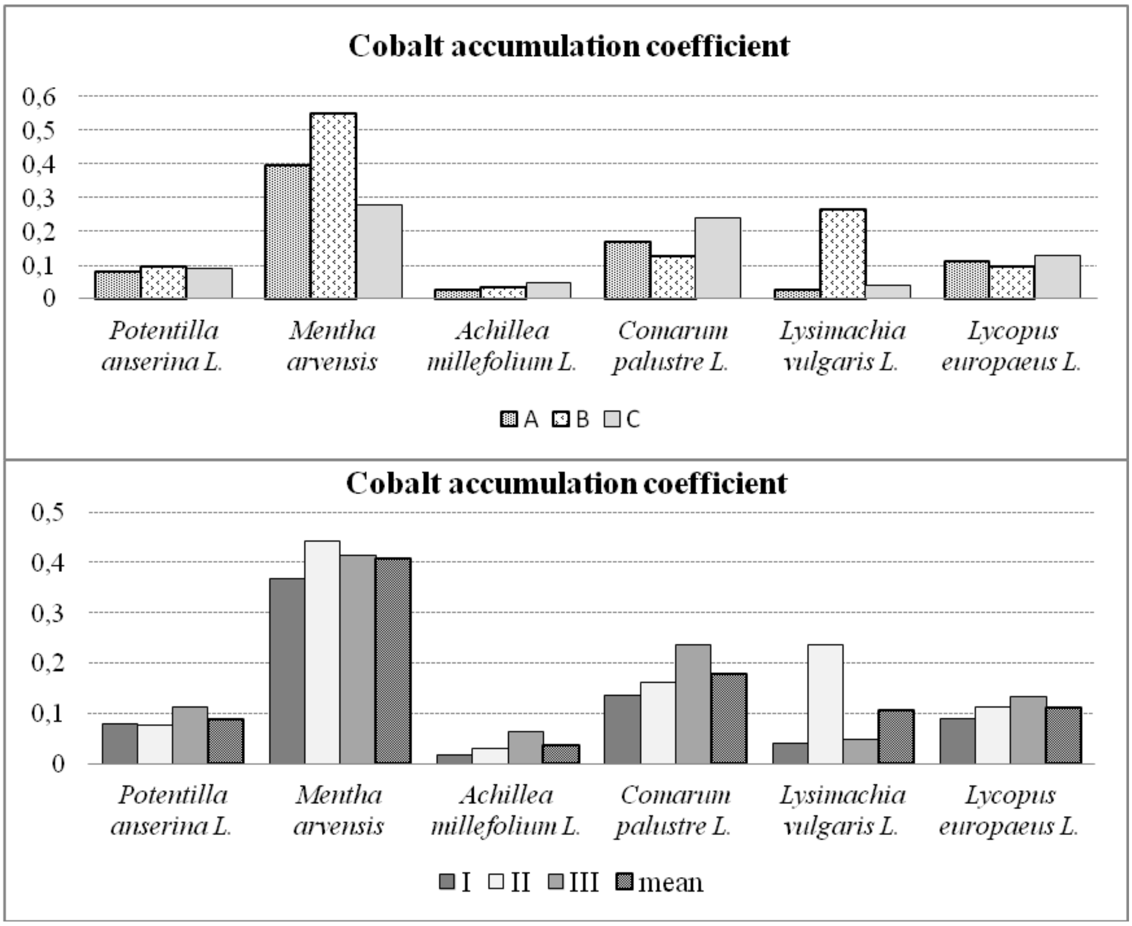
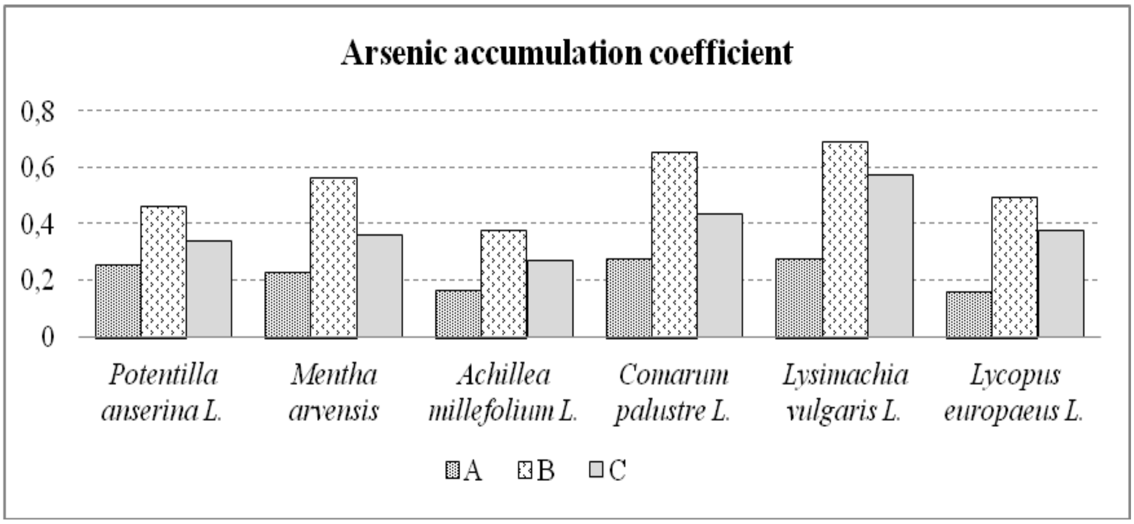


Figure 2. *Cobalt accumulation coefficient in plants growing in different experimental areas and different moisture sections. (A - area with the cultivated field; B - area with permanent grasslands; C - area with bushes; I - wet section; II - periodically wet section; III - dry section; S - species; Ar - area)*



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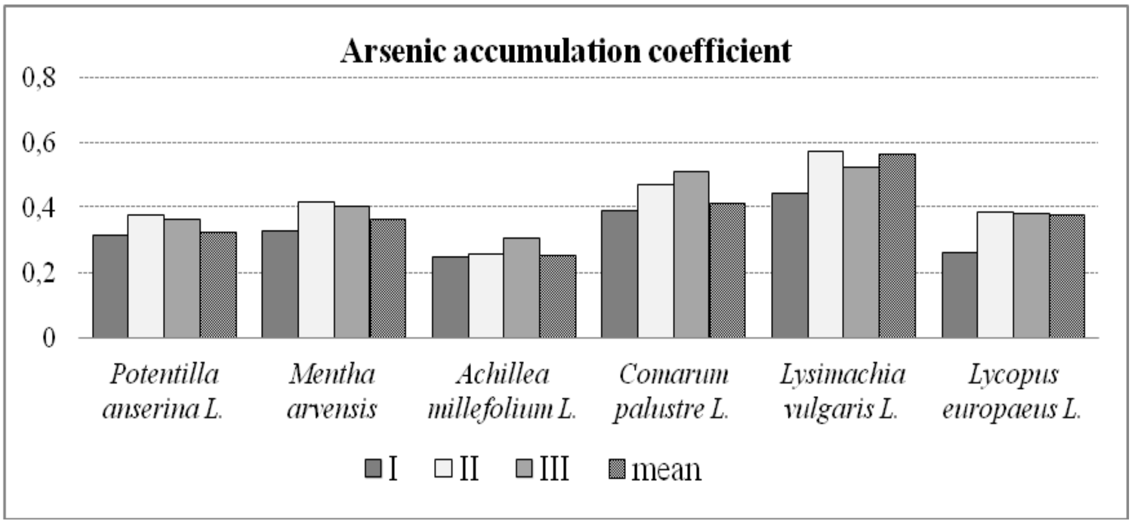

Figure 3. *Arsenic accumulation coefficient in plants growing in different experimental areas and different moisture sections. (A - area with the cultivated field; B - area with permanent grasslands; C - area with bushes; I - wet section; II - periodically wet section; III - dry section; S - species; M - moisture section)*

In plants growing in periodically wet and dry sections accumulation coefficient was much higher than in plants growing in wet sections where the concentration of both metals was the highest. There was also a relationship between cobalt and arsenic accumulation and the experimental area. The accumulation coefficient was much higher in plants from the area with permanent grassland than in the area with arable land. Accumulation of soil heavy metals by plants depends on many factors, first of all on pH but also on soil moisture and organic matter content. According to Kabata-Pendias and Pendias (1999) plants can alter chemical mobility and, at the same time, availability of metals in the rhizosphere. Additionally, the correlation coefficient between cobalt and arsenic content in the soil and in the plants was calculated but there was no significant relationship between those values (*Table 5*).

Table 5. *Correlation coefficient between cobalt and arsenic concentration in the soil and in the biomass of herbs*

Species	Co	As
Potentilla anserina L.	0.149	0.533
Mentha arvensis	0.385	0.264
Achillea millefolium L.	0.094	0.150
Comarum palustre L.	-0.596	0.227
Lysimachia vulgaris L.	-0.217	0.042
Lycopus europaeus L.	-0.215	-0.295

$p \leq 0.05$; critical value for $r = 0.632$; $N = 9$

Conclusions

There were significant differences between cobalt and arsenic content in the biomass of different species. Out of all moisture sections and experimental areas the highest

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concentration of cobalt was in *Mentha arvensis*, for arsenic it was the highest in *Lysimachia vulgaris* L. and Comarum *palustre* L., and the lowest in *Achillea millefolium* L.

The highest content of cobalt and arsenic was found in bottom sediment and in the soil of the area with arable land, which might have been a result of plant protection products application and intensive fertilization of cereals.

In the soil-plant system the accumulation coefficient was higher for arsenic than for cobalt. For both of the metals the coefficient was lower than one, which means that the accumulation was low.

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DEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS USING BACTERIAL ISOLATE FROM THE CONTAMINATED SOIL AND WHITE ROT FUNGUS *PLEUROTUS OSTREATUS*

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Abstract. The use of microbial consortia composed of bacteria and fungi presents higher rates of polycyclic aromatic hydrocarbon (PAH) degradation and is, therefore, essential for the remediation of contaminated soils. A microbial consortium compound comprising *Ochrobactrum intermedium* and white rot fungus *Pleurotus ostreatus* was found to be capable of degrading PAHs of crude oil in soil. This research studied the degradation capacity of a consortium composed of *O. intermedium* and *P. ostreatus* and the degradation capacity of both the bacteria and the fungus individually. The soil was artificially contaminated using two crude oil concentrations corresponding to 86,000 and 172,000 mg kg^{-1}. The concentrations of PAHs extracted from the two concentrations of crude oil were 138.16 and 268.03 mg kg^{-1}, respectively, with respective extraction resins of 78.3 and 73.5%. In total, the biodegradation of 10 PAHs were studied. Fluoranthene, indene[1,2,3-cd]pyrene and benzo[g,h,i]perylene were completely removed by the consortium after 50, 80 and 50 d of incubation, respectively. The rate at which anthracene, pyrene, chrysene and benzo[a]anthracene biodegraded in the presence of the microbial consortium were 96%, 86%, 98% and 98%, respectively, after 110 d of incubation. In this study, higher rates of degradation were obtained with the consortium than when the components were used individually, showing that consortia composed of fungi and bacteria are an efficient technology for degrading xenobiotics, as is the case with PAHs.
Keywords: *PAHs, soil microcosm, microbial consortium, biodegradation, white-rot fungi*

Introduction

The most resistant components of petroleum, polycyclic aromatic hydrocarbons (PAHs) are environmentally problematic due to their toxic, mutagenic and carcinogenic properties (Sahoo et al., 2012; Sinha et al., 2012). Low molecular weight (LMW) PAHs exhibit highly toxic characteristics and affect reproduction rates and mortality in aquatic biota, while high molecular weight (HMW) PAHs have higher mutagenic and carcinogenic effects in living beings (Boonchan et al., 2000). There are different transformation pathways for PAHs in nature, such as volatilization, adsorption, photooxidation, chemical oxidation, bioaccumulation and microbial degradation (Yuan et al., 2002). Soil is one of the abiotic resources most affected by PAH pollution. Physical-chemical technologies are generally used to reduce PAH soil contamination, although they have drawbacks, among which are their high cost, difficulty of operation and generation of secondary contaminants (Mamma et al., 2004). Bioremediation is an

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economical and efficient method for both eliminating PAHs from the soil and reducing the toxicity of the medium (Frenzel et al., 2010).

The bioremediation technology is based on the use of the catabolic ability of microorganisms using xenobiotics as energy sources. Successful bioremediation requires microorganisms with the capacity to degrade the contaminant (Venosa and Zhu, 2003). The application of isolated pure microorganisms or microbial consortia that have the ability to degrade PAHs and which have been taken from contaminated sites represents a possible alternative for the bioremediation of contaminated sites with similar environmental characteristics if the soil lacks the microbial flora capable of degrading the contaminant (Liu et al., 2011). Numerous studies have focused on degrading PAHs to deal with environmental pollution, reporting the use of a large number of species, with the most studied genera being *pseudomonas*, *mycobacterium*, *alcanivorax*, *sphingomonas*, *microbulbifer*, *shingomonas*, *micrococcus*, *cellulomonas*, *gordonia*, and *penicillium*, among others (Brito et al., 2006; Vila et al., 2010). Fungi genera such as *Chrysosporium*, *Bjerkandera*, *Irpex*, *Agrocybe*, *Lentinus*, *Pleurotus* and *Trametes* are among those that are reported to have been used (Valentín et al., 2006; Chupungars et al., 2009).

Although various studies have been able to degrade PAHs using pure microorganisms, these results can be improved through the use of microbial consortia (Bacosa et al., 2010; Colombo et al., 2011) and bacteria-fungus consortia (Boonchan et al., 2000; Li et al., 2008). One of the advantages of using microbial consortia is their multiple metabolic capacities, which increase the efficiency of the degradation process (Ghazali et al., 2004), the degradation of PAHs with consortia formed by using bacteria-fungus is a very promising technique because of the beneficial characteristics of both kingdoms. Thus, the aim of this study was to evaluate the capacity of the consortium formed by *Ochrobactrum intermedium* and *Pleurotus ostreatus* to degrade PAHs in a microcosm of soil contaminated with oil, with the objective of achieving superior remediation results than those achieved with individual microorganisms.

Materials and methods

Chemicals

The crude oil used in this study was obtained from the Lázaro Cardenas refinery in Minatitlán, Veracruz, Mexico. The chemicals used in the extraction and cleaning of the PAHs were of analytical grade, while the chemicals used in the quantification of the PAHs were of an HPLC grade, with both procured from Sigma-Aldrich (USA).

Microorganisms and culture medium

The bacterium was isolated from sediments in a hydrocarbon contaminated creek in Poza Rica, Veracruz, Mexico, at a depth of 40 cm from a site constantly polluted by oil, located at coordinates 20°32’28.77”N and 97°28’02.60”O. Bushnell Haas medium (BHM) was used as the culture medium in this study, with the following composition: (g l^{-1}): 2 NH_4NO_3; 2 KH_2PO_4; 2 K_2HPO_4; 1 NaCl; and, 0.2 $MgSO_4.7H_2O$. The micronutrients used were (mg l^{-1}): 1 $CaCl_2.2H_2O$; 1 $MnSO_4.H_2O$; 1 $FeCl_3$; 0.5 $ZnSO_4.7H_2O$; and, 2 $CuSO_4$. The pH was adjusted to 7.0 using 10.5 M NaOH. The BHM was sterilized by autoclaving at 121 °C for 15 min. *P. ostreatus* (ATCC38540) was grown in darkness on a potato dextrose agar (PDA) plate at 27 °C for 3 d.

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Enrichment and isolation of the PAH-degrading bacterium

The pre-enrichment medium used to isolate the PAH-degrading bacterium was BHM with dextrose (1 g) as a carbon source. The inoculated flasks were incubated in darkness for seven days at 30 °C at 150 rpm, after which 10 ml of said culture was transferred to a flask containing 90 ml of BHM supplemented with 1 g of dextrose and 1 g of oil as carbon sources. The flasks were then incubated under the same conditions described above, with the procedure then undertaken five further times using 1 g of petroleum as a unique carbon source. The PAH-degrading bacterium was isolated via enrichment, using Maya crude oil, while the isolation of the strain was carried out on nutrient agar plates by means of conventional spread-plate techniques, with the plates incubated at 30 °C for 48 h. The isolated strain was stored at -20 °C in liquid culture containing 20% glycerol (v/v). The PAH-degradation capacity of the isolated strain was determined by means of a qualitative evaluation. Petri dishes with BHM agar and oil as a carbon source were inoculated with 1 ml of isolated strain culture and incubated in darkness at 30 °C for 72 h. A Petri dish without inoculate served as a control to determine the loss of hydrocarbons due to abiotic factors (Bacosa et al., 2013). The emulsification capacity was evaluated by adding 10 ml of the bacterial culture after 48 h of incubation to an Erlenmeyer flask containing 90 ml of BHM supplemented with 2 ml of oil and 1 ml of Tween 80. An uninoculated control flask was also used. All flasks were incubated in darkness at 30 °C in an orbital shaker set to 150 rpm. Each assay was performed in triplicate.

The 16S rRNA sequence analysis of isolated strain

The cells of the strain were grown in 50 ml Luria Bertani medium at 30 °C in darkness for one night. DNA was extracted according to Sambrook and Roussell (2001). To amplify the 16S rDNA gene, a polymerase chain reaction (PCR) was performed using two universal primers, the forward primer Eu 530F (5'-TGA CTG ACT GAG TGC CAG GAC CCG CGG-3') and the reverse primer Eu 1449R (5'-TGA CTG ACT GAG GCT ACC TTG TTA CGA CTT-3'), described by Borneman et al. (1996). Each 50 µl reaction contained the following: 3 µl of DNA template; 0.25 µl of Eu 530F; 0.25 µl of Eu 1449R (final concentration 20 pmol); 25 µl of master mix (Vivantis); 0.5 µl of $MgCl_2$ (final concentration 2.0 mM/L); and, 21 µl of water. The PCR mixtures were preheated in Eppendorf Mastercycler EP Gradient thermal cycler at 95 °C for 5 min prior to running the following cycles: 95 °C - 1 min; 55 °C - 1 min; and, 72 °C – 1 min. The reactions were conducted for 30 cycles in a DNA thermal cycle. After the last cycle had been completed, a chain-elongation step was carried out at 72 °C for 5 min. The amplified DNA was sequenced by the Macrogen Company (Korea). The sequence was subjected to a BLAST similarity search on the NCBI-USA website (http://www.ncbi.nlm.nih.gov) and deposited into GenBank. Many relevant 16S rDNA gene sequences with authenticated and published names were selected as references from Gen-Bank, while the phylogenetic tree was constructed using the MEGA 6.0 software.

Experimental design

The degradation of PAH in a soil microcosm by the microbial consortium consisting of *P. ostreatus* and isolated bacteria was evaluated via the application of a 3 × 3 × 2 factorial design, using controls comprising contaminated soil and soil contaminated with substrate, both without inoculum, while each treatment was performed in triplicate. The bacterial degradation was determined using 50 g of sieved soil (2 mm) placed into

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glass bottles (150 ml), while the fungal and consortium degradation were assessed in 40 g of sieved soil and 10 g of substrate (corn stubble and coffee pulp 1: 1, w: w) to obtain a final weight of 50 g. The moisture content was adjusted to 70%, while the molar ratio C: N: P was 100: 10: 1. All soil samples were autoclaved for 60 minutes at 121 °C. 4.3 with 8.6 g of crude oil added dropwise to each bottle to achieve uniform distribution and final concentrations of 86,000 and 172,000 mg of total petroleum hydrocarbons kg^{-1} soil. 30 ml of acetone was then added, after which a homogeneous mixture of the soil was obtained. 30 ml of acetone was added to homogenize the soil with the oil of the soil, after which the acetone was completely evaporated from the glass bottles. Then, 3 ml of bacterial inoculum (3.2×10^8 CFU/ml) was added to a set of bottles containing 50 g of soil, while another set of bottles contained 40 g of soil and 10 g of substrate invaded with the consortium, and the third set of bottles contained only 40 g of soil and 10 g of substrate invaded with mycelium of *P. ostreatus*. All bottles were incubated at 30 °C in the dark, under three different incubation times (50, 80 and 110 d).

PAH extraction and analytical methods and data analysis

The extraction of PAHs was undertaken in accordance with Li et al. (2008), with the soil dried and passed through a 60-mesh sieve, and approximately 2 g of dry soil placed in a 50 mL test tube, to which 15 mL of acetone:dichloromethane (1:5) mixture was added. The PAHs were extracted via sonication for 40 min in an ultrasonic bath (Brand Branson, MH Series, MOD 1800), after which two extractions were taken from each sample and the supernatants then mixed. The extracts were concentrated in a rotary evaporator and resuspended in 2 ml of cyclohexane. For the cleaning, 2 ml of the sample resuspended in cyclohexane was eluted with 15 ml of a dichloromethane:hexane mixture (2:3) through a C-18 cartridge. The eluate was concentrated and resuspended in 2 ml of acetonitrile (Tian et al., 2008), while the characterization of residual PAH in the soil after the completed incubation times was performed using an Agilent 1260 HPLC System with a diode array and fluorescence detectors. The separation of the 16 PAHs was carried out on a ZORBAX Eclipse PAH (4.6 mm x 50 mm, 1.8 µm) column. The mobile phase comprised an acetonitrile:water mixture (60:40), a flow rate of 0.8 mL/min, a sample volume of 20 µl, and a run time of 16 min. The data was recorded as means ± standard deviation. One-way analysis of variance (ANOVA) was conducted to determine the significant differences in the degradation of the PAHs by microorganisms at different time periods. The differences between treatments were analyzed via a Tukey (HSD) test using the SAS 9.0 software. Differences between means at a level of 5% ($P < 0.05$) were considered significant.

Results and discussion

Degradation of PAHs by O. intermedium

Figure 1 shows the biodegradation of PAHs by *O. intermedium, P. ostreatus* and the consortium of both microorganisms. When the PAH concentration was 138 mg kg^{-1}, during the 80 d of incubation, a linear degradation was observed, while an increase in the rate of degradation was observed after 80 d of incubation. During the incubation of Control A, soil contaminated with oil without inoculum, a slight decrease of non-significant PAHs was observed. A trend of increasing degradation was observed at

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268 mg kg^{-1} PAH. As with Control A, Control B did not present a significant decrease of PAHs. This study found that *O. intermedium* has a higher rate of degradation when exposed to a concentration of 268 mg kg^{-1} of PAHs, and offers higher degradation potential the longer the incubation time, with the degradation trend presenting a constant drop along with falling PAH concentrations (Tirado-Torres et al., 2017). Cerqueira et al. (2011) observed that the most challenging method of PAH elimination is that which uses microorganisms.

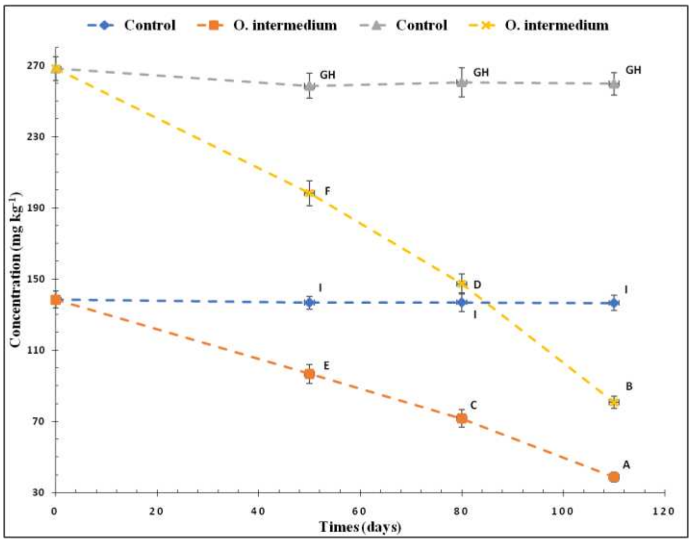

***Figure 1.** Changes in the concentrations of PAHs during biodegradation in crude oil PAH-contaminated soils. Control: Sterile soil without inocula. Letters represent significant differences among treatments (Tukey, P < 0.05)*

Limited by the low bioavailability of high molecular weight compounds, native microbial flora has a low capacity to remove PAHs from contaminated soils. Therefore, soil remediation processes are extremely complex in the case of hydrocarbon contamination (Janbandhu and Fukelar, 2011). The isolation of certain microorganisms with the ability to degrade PAHs has enabled significant progress in the development of bioaugmentation techniques and soil remediation (Mao et al., 2012). While few studies of *O. intermedium* have been conducted and especially research seeking to verify its potential to degrade PAHs, it is well known that microorganisms producing biosurfactants present a better option for the bioremediation of contaminants with hydrophobic characteristics, such as hydrocarbons, given that they increase their bioavailability (Wang et al., 2008).

In this study, *O. intermedium* was isolated and adapted to degrade oil PAHs in contaminated soils. Previous studies were reported to use *O. intermedium* isolated from the rhizosphere in soils contaminated by heavy metals and that it has the ability to reduce metal contaminants and subsist in contaminated soils (Sultan and Hasnain, 2007; Waranusantigul et al., 2011). Recently, a study carried out in a liquid medium proved that the species produces biosurfactants with a high level of thermal stability and tolerance to extreme levels of salinity and, thus, able to degrade up to 40% of PAHs in sludge contaminated with oil, while, in soil, the microcosms are able to degrade 70% of the most hydrophobic compounds in only three weeks (Bezza et al., 2015).

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Degradation of PAHs by P. ostreatus

Figure 2 shows the action of *P. ostreatus* on PAHs in soils contaminated with oil during incubation at 50, 80 and 110 d in this study. Compared to control assays, *P. ostreatus* presented significant degradation rates at concentrations of 138 mg kg^{-1}, at which a constant degradation between 80 and 110 d of incubation was observed. *P. ostreatus* was observed to significantly degrade PAHs at concentrations of 268 mg kg^{-1}. An adaptation stage was observed to occur during the first 50 d, while, at 80 and 110 d, a linear decreasing trend was observed, signifying a higher rate of degradation.

Initially, the study of the degradation potential of microorganisms was given on a laboratory scale and mainly comprised degradation tests on liquid culture. Earlier studies found that the *P. ostreatus* strain ATCC38540 is highly efficient in degrading PAHs in liquid culture (Tirado-Torres et al., 2016). Therefore, this study inoculated *P. ostreatus* in soil contaminated with petroleum to study its capacity to degrade the PAHs present in the crude oil.

P. ostreatus was able to degrade up to 115.2 mg kg^{-1} of a PAH concentration of 138.3 mg kg^{-1} and 186.2 of 268.2 mg kg^{-1} after 110 d of incubation compared to the respective control. During the first 50 d of remediation, the PAH elimination efficiency was attributed to the sorption of the contaminant in the co-substrate added to the treatment, plus the soil particles (Johnsen et al., 2005). At 80 and 110 d, the elimination of PAHs was attributed to the release of ligninolytic enzymes produced by *P. ostreatus* to break the cellulose of the co-substrate, since at those it was observe traces of co-substrate and an increase of the biomass of *P. ostreatus*. This suggests that the addition of the co-substrate enabled the fungus to execute an efficient cometabolic process (Bouchez et al., 1999).

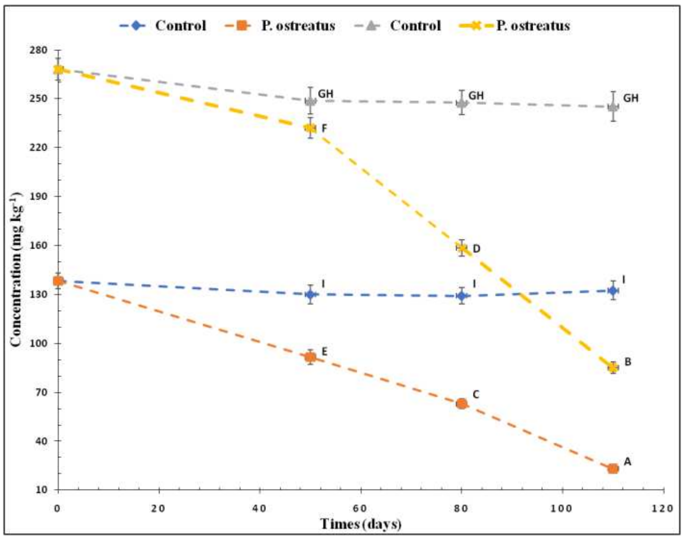

Figure 2. *Concentrations of PAHs during biodegradation in crude oil PAH-contaminated soils for fungus. Control: Sterile soil without inocula. Letters represent significant differences among treatments (Tukey, P < 0.05)*

Basidiomycetes act by means of extracellular enzymes in the co-metabolism process, during which these extracellular compounds act on the contaminant (Namhyun et al., 2000). White-rot fungi are basidiomycetes with the capacity to generate extracellular

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enzymes such as laccase, lignin peroxidase and manganese peroxidase, which are found to obtain good results in the degradation of xenobiotic agents (Hatakka, 1994). Acevedo et al. (2011), studied the efficacy of eliminating PAHs with a Chilean white-rot fungus (*Anthracophyllum discolor*) in contaminated soil, finding that *A. discolor* showed a high PAH removal capacity associated with the production of the extracellular enzyme MnP, results which are comparable with the ability of *P. ostreatus* to degrade PAHs as they are both white-rot fungi. In previous studies conducted in liquid culture, the presence of PAHs has been shown to stimulate MnP production in *P. ostreatus* (Bezalel et al., 1996). The production of ligninolytic enzymes by white-rot fungi, as well as other biochemical molecules, is known to facilitate the removal of xenobiotic agents such as PAHs (Baldrian et al., 2008).

Degradation of PAHs by O. intermedium-P. ostreatus consortium

This section presents the residual concentrations of PAHs in contaminated soils during the biodegradation induced by the consortium formed by *O. intermedium* and *P. ostreatus* (*Fig. 3*). As observed, after the inoculation of the consortium, the PAHs were efficiently degraded by 110 d, attaining a degradation efficiency of 86.7 and 75.5% for the PAH concentrations of 138 and 268 mg kg^{-1}, respectively.

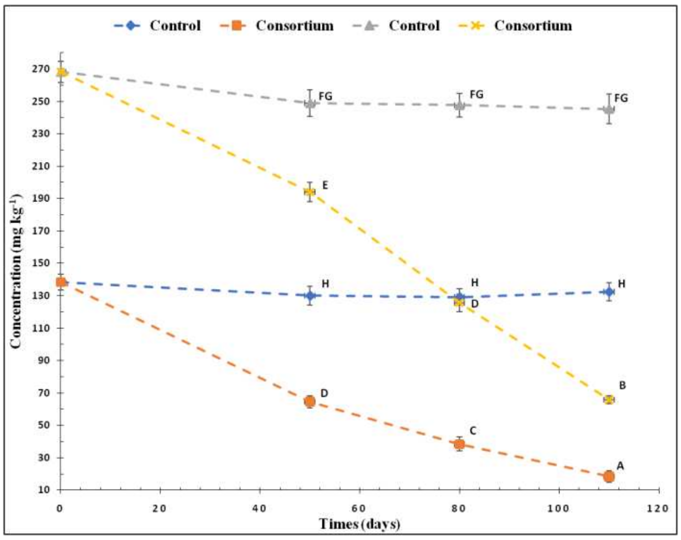

Figure 3. *Concentrations of PAHs during biodegradation in crude oil PAH-contaminated soils for consortium. Control: Sterile soil without inocula. Letters represent significant differences among treatments (Tukey, P < 0.05)*

While the consortium was observed to present a high rate of degradation at 138 mg kg^{-1} of PAHs after 50 d of incubation, a decrease in degradation efficiency occurs between 80 and 110 d. However, the consortium requires less time to adapt to the contaminated environment than the organisms used separately. At PAH concentrations of 268 mg kg^{-1}, the consortium presented similar behavior to that obtained at 50 d of incubation with 138 mg kg^{-1} of PAHs. An increase in the degradation response is observed between 50 and 80 d of incubation, while a decrease in the rate of degradation is observed between 80 and 110 d due to the decreasing concentration of PAHs. These results demonstrate that the consortium presents a greater degradation capacity in the

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first 80 d of remediation than the microorganisms in their isolated form. This part of the study reveals that *O. intermedium* and *P. ostreatus* act in synergy to efficiently metabolize PAHs from contaminated soil.

In this study, the consortium proved to have a greater capacity to degrade soil PAHs due to the synergism between the two microorganisms, which facilitates the degradation of PAHs. The consortium removed 119.9 and 202.4 mg kg^{-1} of PAHs at respective concentrations of 138.3 and 268.2 mg kg^{-1} in soil at 110 d of incubation. This is attributed to the enzymatic activity of the fungus, which formed a habitat that was less toxic to the bacteria (Chagas-Spinelli et al., 2012). Basidiomycetes normally synthesize enzymes that, to an extent, degrade complex PAHs (Sack et al., 1997; Aranda et al., 2010). Bacteria that generate enzymes such as laccase can contribute to an improved degradation of PAHs (Zeng et al., 2010). At 110 d of incubation at the two concentrations handled in this study, it was observed that the consortium achieved the highest level of PAH removal, while the other two treatments achieved levels close to the degradation rate of the consortium. This can be attributed to the limitation of nutrients necessary for the consortium (Hollender et al., 2003), while limiting the source of energy can also affect the efficiency of microorganisms (Kim et al., 2009). The consortium's efficiency in degrading PAHs is due to the qualities of the co-metabolism generated by *P. ostreatus*, as initiated by the addition of co-substrate, which, when decomposed by the extracellular enzymes expelled by the fungus, releases nutrients outside the cell that are available both for their products and for the bacteria (Šnajdr et al., 2011).

In addition, bacteria are able to form biofilms around fungal hyphae in order to bridge pores and penetrate soil aggregates (Kohlmeier et al., 2005). Fungi can also actively take and transfer PAHs through their hyphae via the cytoplasmic stream (Furuno et al., 2012). The primary degradation strategy carried out by fungi and bacteria comprises the release of biosurfactants in order to obtain greater contaminant bioavailability (Johnnsen and Karlson, 2004; Bento et al., 2005). The strong competitive capacity of a particular fungal species is considered a premise of successful soil colonization (Baldrian, 2008). Although, in this study, *P. ostreatus* has been shown to coexist with *O. intermedium*, other studies have reported decreasing *P. aeruginosa* biomass when forming a consortium with *P. ostreatus* (Tornberg et al., 2003). In contrast, Federici et al. (2007) observed an increase in bacterial counts of *mycobacterium, nocardia* and *rhodococcus* when used in consortium with *P. ostreatus* (DeBruyn et al., 2007). It should be noted that interactions between fungi and bacteria can positively affect biodegradation applied metabolically in conjunction with a fungal co-metabolism (Mueller et al., 1989), as well as negatively affecting the degradation of PAHs in soil (Borras et al., 2010). In some cases, the use of consortia has not obtained results showing the potential to degrade xenobiotics (Tornberg et al., 2003), while, in other cases, the positive cohabitation of consortium has been obtained with high rates of PAH degradation (Federici et al., 2007).

In this study, the consortium degraded 75.5 to 86.7% of PAHs, while Gallego et al. (2007) observed that a microbial consortium degraded from 31 to 55% of PAHs. It should be reiterated that, as Toledo et al. (2006) state, the comparison of results varies greatly due to the concentrations of hydrocarbons and the incubation times obtained. In another successful study of consortia, Díaz-Ramirez et al. (2008) formed a microbial consortium of six strains which, after 18 d of incubation, degraded 31% of PAHs. Ruberto et al. (2006) found that a combination of bioaugmentation and biostimulation in

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a microbial consortium caused a 46.6% elimination of 1,744 mg kg^{-1} of phenanthrene in soil after 56 days of incubation in Antarctic soil. The results obtained in this study show that fungal and bacterial consortia can be an efficient technology for degrading xenobiotics, as is the case for PAHs.

Bioremediation treatments: individual PAH removal

Previous studies have reported that microbial consortia obtain higher degradation efficiencies in liquid culture with the addition of surfactants in order to increase the bioavailability of xenobiotics (Bautista et al., 2009; Molina et al., 2009; González et al., 2011). The present study was carried out in soil without the addition of surfactants in order to observe the capacity of the consortium without needing to apply biostimulation. Sixteen PAHs were used in this study, of which six were not detected in the initial treatment period: acenaphthene (ACE); Acenaphthylene (ACY); benzo [b] fluoranthene (BBF); benzo [k] fluoranthene (BKF); Benzo [a] pyrene (BaP); and, dibenzo [a, h] anthracene (DahA). *Table 1* shows the 10 compounds detected in the chromatographic analysis and the degradation efficiencies obtained by the treatments at 110 days of incubation under a constant temperature of 30 °C. It can be seen that the compounds which were completely removed by both the consortium and *P. ostreatus* are FLT, IND and BgP. The compounds that showed significant degradation are ANT and CHY, with removal efficiencies ranging from 96 to 98%.

Table 1. *Removal of individual PAH compounds in soil contaminated by crude oil after the 110-day incubation period for the three different treatments and two controls*

PAH	Rings	Initial concentration Mg.kg^{-1}	PAHs removed from treatments (%)				
			C-1	C-2	O.I	P.O	C.O
Naphthalene (NAP)	2	74.16 ± 2.3	1.53 ± 0.8$_d$	3.06 ± 0.8$_d$	76.24 ± 2.8$_c$	84.52 ± 0.8$_b$	88.77 ± 0.1$_a$
Fluorene (FLU)	2	27.83 ± 0.1	1.70 ± 0.8$_d$	5.68 ± 1.7$_c$	41.59 ± 1.3$_b$	69.77 ± 0.6$_a$	69.77 ± 0.9$_a$
Fenanthrene (FEN)	3	4.06 ± 0.5	1.31 ± 0.9$_d$	0.28 ± 0.1$_d$	69.74 ± 0.6$_c$	76.27 ± 1.6$_b$	85.23 ± 1.7$_a$
Anthracene (ANT)	3	7.43 ± 0.1	1.89 ± 0.3$_d$	4.02 ± 1.0$_c$	93.50 ± 0.4$_b$	92.50 ± 1.4$_b$	96.27 ± 0.6$_a$
Total LMW	**2 y 3**	**113.48 ± 1.8**	**1.60 ± 1.1$_d$**	**3.71 ± 0.9$_d$**	**68.64 ± 0.5$_c$**	**81.13 ± 1.3$_b$**	**84.47 ± 0.6$_a$**
Fluoranthene (FLT)	4	6.75 ± 0.1	5.04 ± 0.5$_c$	5.03 ± 0.3$_c$	88.72 ± 0.5$_b$	100 ± 0.0$_a$	100 ± 0.0$_a$
Pyrene (PYR)	4	4.77 ± 0.4	2.47 ± 0.8$_e$	6.69 ± 0.9$_d$	68.03 ± 0.8$_c$	78.57 ± 0.8$_b$	86.51 ± 1.7$_a$
Chrysene (CHY)	4	6.19 ± 0.6	2.83 ± 0.8$_d$	1.87 ± 0.5$_d$	94.63 ± 0.6$_b$	91.60 ± 1.4$_c$	98.23 ± 0.9$_a$
Benzo[a]anthracene (BaA)	4	3.37 ± 0.2	5.14 ± 0.7$_b$	8.59 v 0.8$_b$	98.08 ± 0.8$_a$	95.67 ± 1.3$_a$	98.18 ± 0.5$_a$
Indene[123-cd]pyrene (IND)	5	1.44 ± 0.1	5.86 ± 0.8$_d$	13. 04 ± 0.9$_c$	75.74 ± 2.8$_b$	100 ± 0.0$_a$	100 ± 0.0$_a$
Benzo[g,h,i]perylene (BgP)	6	2.35 ± 0.1	4.69 ± 0.5$_c$	11.99 ± 0.5$_b$	97.59 ± 1.5$_a$	100 ± 0.0$_a$	100 ± 0.0$_a$
Total HMW	**4 a 6**	**24.87 ± 0.4**	**4.80 ± 0.9$_c$**	**7.04 ± 0.7$_c$**	**87.31 ± 1.8$_b$**	**93.21 ± 0.8$_a$**	**96.73 ± 0.4$_a$**
Total PAHs		**138.35 ± 5.42**	**1.33 ± 0.9$_d$**	**4.30 ± 0.53$_d$**	**72.04 ± 0.5$_c$**	**83.30 ± 0.51$_b$**	**86.69 ± 0.21$_a$**

Data are presented as mean ± SD. Means in the same row with a letter in common are not significantly different among treatments ($P < 0.05$). C-1: Soil without inoculum C-2: Soil and cosubstrate without inoculum O.I: *O. intermedium* P.O: *P. ostreatus* C.O: *O. intermedium-P. ostreatus*

These results can be used for future studies of those compounds which have been totally eliminated. *Figure 4* shows the degradation kinetics generated by the three treatments for each PAH. FLT was completely degraded after 80 d of treatment with both *P. ostreatus* and the consortium. IND was completely degraded after 50 d of treatment with the consortium, whereas treatment with *P. ostreatus* achieved complete degradation after 80 d. BgP, the aromatic compound used in this study with the greatest number of rings, was the PAH that was completely eliminated in less than 50 d of

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treatment with the consortium, whereas it was totally degraded after the 50 d of *P. ostreatus* treatment. The time required to obtain total degradation was shortest for BgP, followed by IND and, finally, FLT. Several studies have concluded that petroleum components have different susceptibilities to degradation by microorganisms (Greenwood et al., 2008; Bacosa et al., 2010).

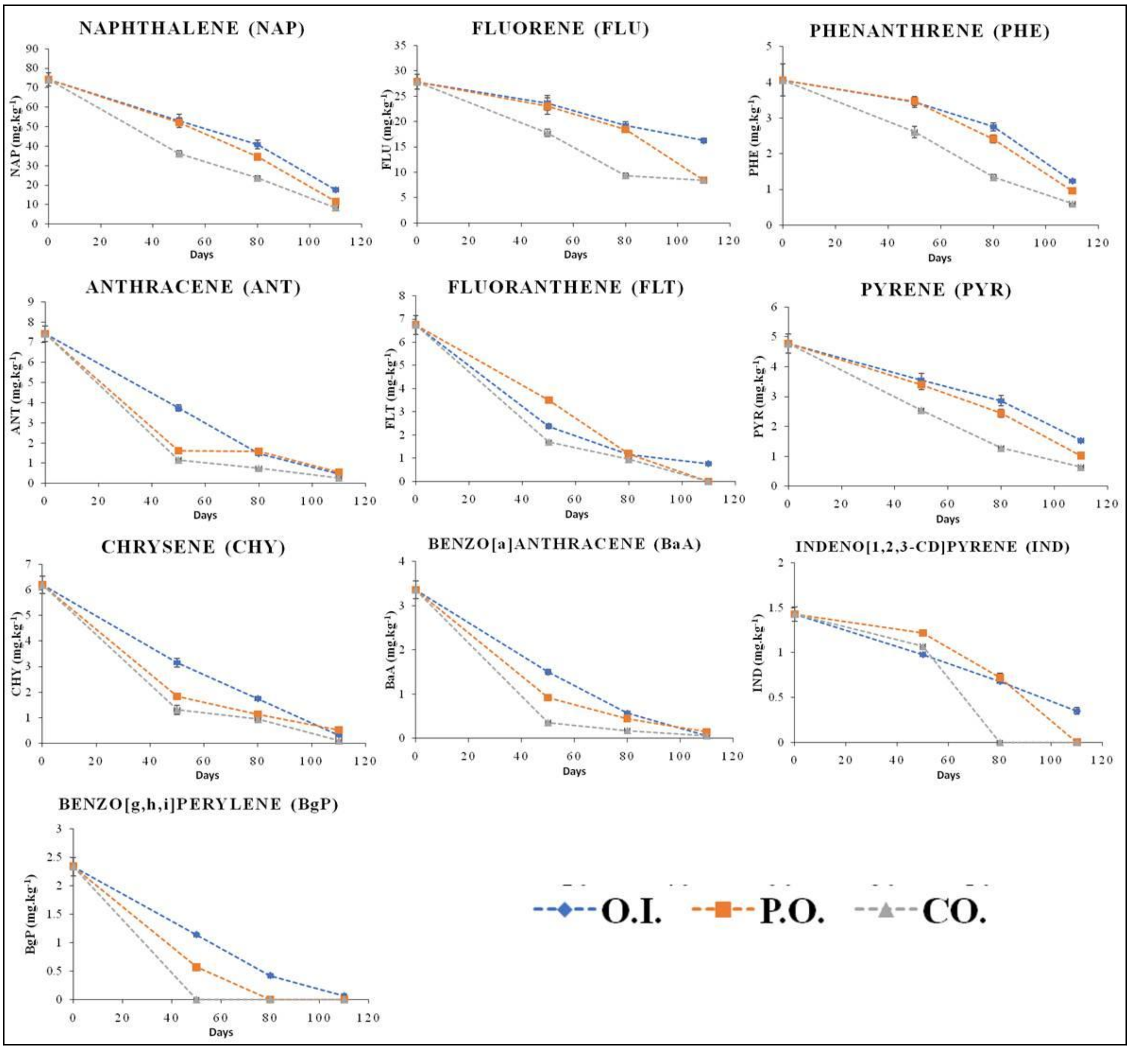

***Figure 4.** Concentration of individual PAH compounds during the 110-day experimental period for O.I. (O. intermedium), P.O. (P. ostreatus) and CO. (consortium) treatments*

Degradation efficiencies of 69.7 to 85.2% PHE were obtained, proving that this compound can be degraded by actions associated with the microorganisms used in this study. Other studies using microorganisms have reported the biodegradation of PHE by such microorganisms as *Sphingobacterium* sp., *Achromobacter* sp., and *Bacillus* sp. (Janbandhu and Fulekar, 2011), while, in consortia, they have been able to degrade PHE at rapid rates without any additional carbon source (Arulazhagan and Vasudevan, 2009). Chagas-Spinelli et al. (2012), studied the degradation of PAHs via three treatments, including BSBA consortium, obtaining, after 126 d of incubation, degradation efficiencies similar to those found in this study. PAHs comprising two aromatic rings,

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such as NAP and FLU, achieved high degradation efficiencies, of 100 and 98.3%, at concentrations of 2.1 and 12.3 mg kg^{-1}, respectively. Although the concentrations used in the present study were much higher, the high degradation can be attributed to the fact that LMW-PAHs degrade more easily due to their lower hydrophobicity than those of HMW-PAHs (Lors et al., 2012).

The total degradation of FLT and IND was found to be possible in this research, results which are superior to those reported by Chagas-Spinelli et al. (2012), who obtained 86.6 and 93.9% degradation in their most effective treatment, with the FLT concentration 2.4 times lower and the IND concentration 4.4 higher than the concentrations used in this study. The efficiency of *O. intermedium* was found to be similar to that obtained by the research undertaken with the BSBA microbial consortium, whereas the consortium used in this study managed to eliminate both FLT and IND completely, meaning that the use of consortia formed by fungi and bacteria is more efficient than the microbial consortia. This is attributed to the ability of *P. ostreatus* to both generate extracellular enzymes and obtain greater xenobiotic bioavailability for microorganisms (Namhyun et al., 2000). As PYR is classified as an HMW, its degradation process is more difficult than that of LMWs. This compound has been previously studied in liquid culture using a microbial consortium, in which it was degraded by up to 80%, although the increased PAH concentration decreased the degradation capacity of the consortium (Arulazhagan and Vasudevan, 2009). It is known that HMW PAHs, due to their high hydrophobicity, require longer degradation time and sometimes different microorganisms to degrade them (Luo et al., 2009; Boonchan et al., 2000). In this study, the concentrations of PYR were low so that its high degradation efficiencies had to be obtained by all three treatments. These results show that consortia composed of a bacteria-fungus mixture can more efficiently degrade existing LMW and HMW PAHs in soil (Li et al., 2008).

Conclusions

The consortium formed by certified *P. ostreatus* strain and *O. intermedium* strain isolated from soil contaminated with hydrocarbons were able to degrade PAHs in soil. The highest rate of degradation occurred using the consortium during the first 80 days of incubation, suggesting synergistic effects. In particular, the total degradation of HWM indene [1,2,3-cd] pyrene and benzo [g, h, i] perylene Haps was achieved using the consortium. The results of this study show that the consortium is a promising technology for the elimination of PAHs from soils contaminated with hydrocarbons; however, further research on the consortium must be undertaken through a field study as well as toxicity testing of the treated soils.

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DETERMINATION OF METALS AND SELENIUM CONCENTRATIONS IN FEATHER OF ARMENIAN GULL (*LARUS ARMENICUS*) LIVING IN VAN LAKE BASIN, TURKEY

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Abstract. In this study, the heavy metals magnesium (Mg), zinc (Zn), copper (Cu), manganese (Mn), lithium (Li), tin (Sn), cobalt (Co) and selenium (Se) levels were determined in the feathers of Armenian Gull (*Larus armenicus*), which is a species classified as near threatened (NT) according to IUCN categories. The Gull lives on the islands of Turkey's largest lake, Lake Van. Different levels were detected due to the urban and rural location of the study areas. The highest levels of Mg, Zn, Mn, chromium (Cr), and Li were determined in the Sıhke pond near the city's dumpsite. The lowest levels were determined on Adır island, with the least human factors. While the levels of Co, Se, and Sn were high in the samples taken from Akdamar Island, the lowest levels were determined on Çarpanak Island and in Sıhke pond.
Keywords: *toxicology, bird, Akdamar Island, Çarpanak Island, Adır Island, Sıhke Pond*

Introduction

The increase in human population, and developing industry and technology have resulted in more waste disposal into nature. Human-derived wastes affect other species that are an indispensable part of the ecosystem in the wild. Although heavy metals are found at certain concentrations in nature and in living organisms, they have a durable and bio-accumulative nature, and therefore pose great risk to the food chain and wildlife with the effect of anthropogenic factors (Zolfaghari et al., 2007; Costa et al., 2013; Nriagu and Pacyna, 1988; Metcheva et al., 2010). Birds, in the higher trophic level of the food chain, which are most rapidly affected by degrading environmental conditions and respond to this change, are referred to as bioindicators (Burger, 1993). Hence, a suitable method for detecting metal accumulation in nature is to determine the amount of it in bird feathers. Heavy metals in the feathers can be due to external (direct contact) and internal (via blood) factors (Movalli, 2000). Heavy metals enter a birds' body via feeding and accumulate in the calamus area, which is connected to blood vessels (Dauwe et al., 2000, 2003). For heavy metal analysis, the use of feathers removed from the body without harming the organism is more convenient than using other organs (Burger, 1996; Movalli, 2000; Battaglia et al., 2005). Samples taken from living things are particularly good for species that are in danger or whose numbers are decreasing (Movalli, 2000). The Armenian Gull lives in limited areas located in Turkey, Israel, Egypt, and Iran (Anonymous, 2017). Unique to Turkey's eastern Anatolia and spread throughout the region, these birds feed on the endemic Lake Van fish of Van Lake in the summer while living on garbage in the city center in winter (Adızel et al., 2010; Nergiz and Durmuş, 2017). The number of these species, listed as near threatened (NT), by the International Union for Conservation of Nature (IUCN), is declining day-by-day

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in Turkey because of habitat degradation and the lack of healthy diet (Anonymous, 2017).

The aim of this study was to (a) determine the concentrations of heavy metals in the feathers of Armenian gulls, which live in different locations of the Van Lake Basin and are found on the higher trophic level of the food chain, (b) explain the differences in the concentrations between the areas ecologically, and (c) compare the results with similar studies.

Materials and methods

Feather samples of the Armenian gull were collected from dead gulls in Akdamar (328345 X; 4245530 Y), Çarpanak (333264 X; 4275058 Y), and Adır Islands (357054 X; 3203307 Y) in Van Lake and in Sıhke Pond (362132 X; 4266574 Y) near the city's dumpsite between May and June 2017 when reproduction largely takes place (*Fig. 1*).

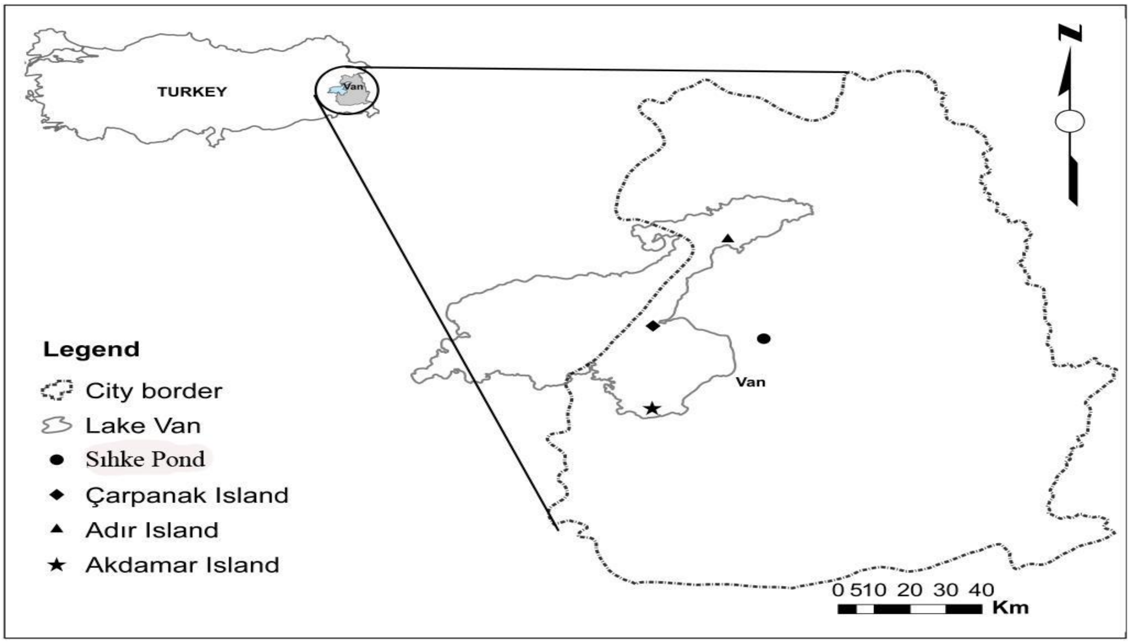

Figure 1. *Islands from which feather samples were taken in the Van Lake Basin*

Water samples were also taken from different regions of the lake for comparison. The 3 islands in Van Lake are the areas where these birds reproduce, mostly in the spring. During the breeding season, there is intensive activity to feed the offspring and deaths occur in adults and young as a result of this rush. In each area, the wing feathers were taken from 10 dead adult gulls. The feathers were put into plastic bags and taken to the Van Yuzuncu Yil University Scientific Research Centre for analysis.

Analysis of the samples

The feather samples brought to the laboratory were washed 3 times with distilled water and acetone in order to remove environmental pollutants such as soil and feces (Goede and Bruin, 1984). Next, the samples were placed in glass containers and dried in the drying oven at 70 °C for 24 h. The dried feathers were cut with steel scissors and at

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least 2 g (dry weight) from each sample was weighed on a precision scale and placed in glass containers. The prepared feather samples were burned using the tissue sample program in a microwave heating unit (Ethos Easy Advanced Microwave Digestion System, Milestone, 230/50 Hz, Italy) by adding 8 mL of 65% HNO_3 and 2 mL of H_2O_2 (Blust et al., 1988; Janssens et al., 2001). A 2 mL solution from the incinerated samples was diluted with distilled water to 10 mL and analyzed on the ICP-AAS (Thermo Scientific ICAP 600 series) instrument.

Calibration of the instrument was done with a certified solution (Multi element ICP QC standard solution-QCS-01-23E, Belgium). All of the metals were compared to the National Institute of Standards and Technology (NIST) and Standard Reference Material (SRM) standards. Accepted recoveries ranged from 95% to 105%.

Statistical analysis

The statistical analysis was done using SPSS software v23. Means and standard deviations (SD) are given in *Table 1*. The Kruskal-Wallis and Tukey post-hoc comparison tests were used to evaluate the effects of the heavy metal concentrations according to the localities.

Table 1. *Heavy metal concentrations (mg/kg dry weight) in the feathers of Larus armenicus*

Metal	n	Akdamar Island Mean ± SD (min–max)	Adır Island Mean ± SD (min–max)	Çarpanak Island Mean ± SD (min–max)	Sıhke Pond Mean ± SD (min–max)	Water samples Mean ± SD (min–max)	Fr	*p*
Co	10	0.089 ± 0.004 (0.002–0.04)	0.003 ± 0.001 (0.0004–0.16)	0.002 ± 0.0004 (0.0001–0.004)	0.054 ± 0.019 (0.004–0.164)	0.0005 ± 0.0001 (0.0001–0.001)	7.30	0.001
Cr	10	0.133 ± 0.039 (0.052–0.36)	0.128 ± 0.022 (0.056–0.29)	0.154 ± 0.058 (0.044–0.52)	0.410 ± 0.143 (0.07–1.44)	0.0005 ± 0.0001 (0.0001–0.001)	3.96	0.007
Li	10	0.029 ± 0.011 (0.01–0.12)	0.012 ± 0.002 (0.004–0.03)	0.018 ± 0.004 (0.004–0.04)	0.038 ± 0.014 (0.004–0.14)	0.0005 ± 0.0002 (0.0001–0.001)	3.33	0.018
Mg	10	13.24 ± 2.95 (3.22–14.94)	8.56 ± 1.14 (3.22–14.94)	11.04 ± 1.31 (4.76–17.58)	48.27 ± 14.55 (7.06–131.12)	0.0005 ± 0.0001 (0.0001–0.001)	7.24	0.001
Mn	10	0.232 ± 0.051 (0.048–0.54)	0.079 ± 0.011 (0.03–0.16)	0.088 ± 0.009 (0.05–0.13)	0.877 ± 0.291 (0.092–2.84)	0.0006 ± 0.0001 (0.0001–0.001)	7.00	0.001
Se	10	0.043 ± 0.004 (0.02–0.06)	0.040 ± 0.004 (0.004–0.56)	0.037 ± 0.003 (0.02–0.05)	0.0271 ± 0.004 (0–0.048)	0.0005 ± 0.0001 (0.0001–0.001)	14.66	0.001
Sn	10	0.032 ± 0.013 (0.004–0.14)	0.013 ± 0.001 (0.004–0.02)	0.012 ± 0.002 (0.004–0.024)	0.012 ± 0.002 (0.002–0.02)	0.0006 ± 0.0002 (0.0001–0.001)	4.64	0.003
Zn	10	2.62 ± 0.19 (1.52–3.17)	2.35 ± 0.08 (1.80–2.86)	2.42 ± 0.088 (2.06–3.84)	2.83 ± 0.198 (1.71–3.84)	0.0006 ± 0.0001 (0.0001–0.001)	75.81	0.001

The Kruskal-Wallis test followed by Tukey's comparison test between islands and water concentrations of metals. Statically significance was accepted at $P < 0.005$

Results and discussion

Different metal concentrations were determined in all of the feather and water samples taken from the study areas ($P < 0.005$). The mean metal concentrations in the water and feather samples from the 3 islands and a pond are shown in *Table 1*. In all of the areas, it was determined that the highest concentrations were magnesium (Mg) and zinc (Zn). The levels of cobalt (Co), chromium (Cr), lithium (Li), Mg, manganese (Mn), selenium (Se), tin (Sn), and Zn in all of the areas were different and statistically significant. The samples taken from Akdamar Island turned out to have concentration

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densities of Mg> Zn> Mn> Cr> Co> Se> Sn> Li (P < 0.005). The rank of concentrations from Adir Island was Mg> Zn> Cr> Mn> Se> Sn> Li> Co, on Çarpanak island it was Mg> Zn> Cr> Mn> Se> Li> Sn> Co, whereas in Sıhke Pond it was Mg> Zn> Mn> Cr> Co> Li> Se> Sn (P < 0.005).

In all of the studied areas, it was determined that the highest Mg (48.27 mg/kg), Zn (2.83 mg/kg), Mn (0.877 mg/kg), Cr (0.41 mg/kg), and Li (0.038 mg/kg) levels were in Sıhke Pond, which is in the vicinity of the city's dumpsite. The lowest levels of these same metals were found on Adır Island, where human activities are the least. The Co (0.089 mg/kg), Se (0.043 mg/kg), and Sn (0.032 mg/kg) levels were the highest in samples taken from Akdamar Island. While the lowest Co (0.002 mg/kg) and Sn (0.012 mg/kg) levels were determined on Çarpanak Island, and the lowest Se (0.027 mg/kg) concentration was detected in Sıhke Pond (*Table 1*).

Many studies have been carried out to determine the levels of heavy metals in bird feathers. Burger (1995, 1996), in his study of Herring gull feathers showed that metals accumulating in the feathers change depending on sex, age, and nutrition. It was also indicated that the level of Se in the samples taken from the dead gulls was higher than that in the living ones. In their study, it was shown that the level of Se was lower in adults than in juveniles. In our study, it was observed that the level of Se in adult gulls was lower than those of other metals and differed based on the feeding areas. From the data obtained in this study, it can be said that the concentrations were not at levels lethal enough to kill gulls but were sufficient to restrict locomotor activity and facilitate death to same extent (Burger, 1995).

Se levels in gull and tern feathers ranged from 1.200 to 18.700 ppb, with a median of 3.900 ppb (Burger, 1994). In this study, the Se concentration in the feathers as dry weight (mg/kg) (0.027 mg/kg–0.043 mg/L) exceeded the average value in the literature.

The accumulation of metals in the birds varies depending on the concentration and variety of food available (Zolfaghari et al., 2007). The highest heavy metal levels were found in the studies of predatory avian feathers at the top of the food chain (Braune and Gaskin, 1987). The Armenian gull is located in the higher trophic levels of the food chain in terms of being carnivorous, although it is not literally a predator. The concentration levels varied depending on the region and density of the fish that they feed on. In this study, it was observed that the levels in the gulls at Sıhke Pond near the city garbage dump were higher than the levels in other areas.

Se, Cr, and Mn levels have been determined in studies concerning the metal levels of ocean birds (Burger and Gochfeld, 2001). The levels determined in this study were higher than those reported in other studies. In the literature, an average of 11.8 ppm Mn was determined for marine birds (Burger, 1993). This level is much higher than thhat found in the present study, which is thought to be due to the density of the food.

Zn, Mn, Mg, Co, and Cr were analyzed in the feathers of cattle egret that are feeding with fish such as gull, during their reproduction period (Malik and Zeb, 2009). According to the results obtained, the Zn, Mn, and Mg levels were parallel but the Co and Cr levels were lower than those we determined.

When the Mn level obtained from this study was compared with studies conducted on fish-fed aquatic birds, the level (0.232 ± 0.051) found on Akdamar Island overlaped with that (0.021 mg/kg) which Abdullah et al. (2015) found in the feathers of *Bubulcus ibis*, while it was found that the concentrations in *Ardea cinerea* (0.099 ± 0.036) and *Egretta garzetta* (0.094 ± 0.027) found by Kim and Koo (2008) were similar to those on the islands of Adır (0.079 ± 0.011) and Çarpanak (0.088 ± 0.009).

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Pearl mullet (*Alburnus tarichi* Güldenstädt, 1814), an endemic species of fish, is the Armenian gulls' basic food during their breeding season. Pearl mullet, an anadromous fish, goes into fresh water to lay eggs in spring and is much hunted by the seagulls during this period. Species feeding on larger fish have been shown to accumulate higher levels of metals than those feeding on smaller fish (Burger, 2002). However, the size of the fish caught by the gulls is average and does not vary by much.

The levels of metals in the species of birds feeding on plant seeds, leaves, and fruit were found to be lower than those feeding on meat (Zolfaghari et al., 2007; Behrooz et al., 2009). However, the species we were studying is a carnivorous species and does not feed on plants. The levels of Mn and Cr detected in the studies of the phytophagous great tit (*Parus major*) were lower than those in our study, but the Se levels were higher than our findings.

As for in the vicinity of the industrial and residential areas, the concentrations of metals appear to increase and pose risks (Dauwe et al., 2000; Frantz et al., 2012). In India, the heavy metal concentrations in the feathers of 11 species of birds living in both urban and rural areas were examined. It was stated that among the reasons for the high levels of heavy metals are the vehicle traffic in urban areas, metal manufacturing industries, and human-derived factors (Manjula et al., 2015), which seems parallel with our results. The Mg, Zn, Mn, Cr, and Li levels were significantly higher in the populations of gulls around Sıhke Pond, where the pollution was most intense. Statistical differences between the areas were significant ($P < 0.005$).

Conclusion

Wetlands in Turkey are increasingly affected by contamination as a consequence of agricultural, industrial, and anthropogenic sources. Therefore, it is inevitable that these effects on wildlife, especially birds, be investigated. However, previous studies on contamination in Turkey were limited to a few studies of contaminants in water birds (Ayas and Kolankaya, 1996; Ayas et al., 1997; Ayaş, 2007).

The aim of this study was to determine the concentrations of heavy metals (Mg, Zn, Cu, Mn, Li, Sn, Co) and Selenium in gull feathers, indigenous to the eastern Anatolian region of Turkey and breeding on the islands in Lake Van. During the feathering period, the levels of some metals in internal tissues drop as they are stored in their feathers. The metal levels in the first emerging feathers show a combination of the stored state of the organic mercury that accumulates at the end of the feather, as well as the current diet intake (Burger, 1995; Zolfaghari et al., 2007). Feather samples from the dead gulls were taken from 3 islands in the lake and from the coasts of a pond (Sıhke) close to the city dumpsite. In all the feather samples taken, heavy metals were detected at different concentrations. Levels of Mg and Zn were determined as high in all of the areas. It was determined that the levels of Mg, Zn, Mn, Cr, and Li were higher in Sıhke Pond than in the other fields when the samples were analyzed. The absence of contaminating factors and contamination in the other 3 sites was the main reason for this disparity. In parallel with the increase of pollutant factors in natural ecosystems, the biological accumulation in living things is also increasing (Burger, 1993). Therefore, as it is in this study, the use of natural and contaminated areas is very useful for comparing metal concentrations in living organisms.

Although there are many studies on heavy metals in various species of seagulls in the literature (Burger, 1995; 1996), none has been done on the Armenian gull. Moreover,

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this is the first study that considers correlations between the element levels of *Larus armenicus* feathers. Our results are meaningful due to an adequate sample size from the relative areas and the number of detected elements.

Depending on environmental pollution, increased accumulation amounts, and harmful effects should be observed in all of the study localities. Additional studies are required to determine whether the levels measured in the individual feathers were toxic.

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STUDY ON THE ECOLOGICAL HEALTH EVALUATION OF A GEOPARK BASED ON DPSIR CONCEPTUAL MODEL – ILLUSTRATED BY THE QIANJIANG XIAONANHAI NATIONAL GEOPARK OF CHINA

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Abstract. With the rapid development of science tourism, geoparks are brought into focus of many geologists and tourists because there are of high scientific values for experts and we can disseminate science to the public. In recent years, there have been many problems in the construction of geoparks in China. The quick demand growth of geoparks conflicts with the ecological health of the area prominently. It is of great significance for ameliorating the planning and improving the development potential of a geopark and evaluate the ecological health of a geopark by combining qualitative and quantitative indicators. Illustrated by the example of Xiaonanhai National Geopark in Qianjiang, Chongqing and based on the theory of ecological health, this paper constructs the eco-health evaluation system for geological parks through DPSIR conceptual model. We use entropy and multi-level fuzzy synthesis evaluation method to determine the factor's proportion and calculate Geopark Ecological Health Index. The result shows that the highest score of Qianjiang Xiaonanhai Geopark is 0.6643, and the lowest score is 0.6120. Though the score reveals a general trend of increase, the gap between scores of indicators is large. Based on the conclusion, some countermeasures and suggestions to improve the eco-construction and management planning of Qiannan XiaonanHai National Geopark are put forward.
Keywords: *Qiannan Xiaonanhai National Geopark, health degree, index evaluation system, DPSIR conceptual model, fuzzy comprehensive evaluation*

Introduction

Geopark is a new name created by UNESCO in the study on the feasibility of the Geoparks Project. According to the definition of geoparks by the Ministry of Land and Resources of the People's Republic of China No. 77 (2000), geoparks should be relic resources with special scientific value, popular science education value, and aesthetic value at the national and even international levels. It is necessary to provide geological records that can present important geological evidence for a certain geological event or stage in a certain area, and have special geological features, fossils, or geological relics with typical geomorphological meaning.

In other countries, some research studies have already been conducted on geoparks in the past. In 1999, UNESCO officially launched the World Geopark Program. Italian studies expounded the scientific and educational importance about geological remains of soil profiles, tourism and recreational value, and demonstrated that the protection of geological sites could be better achieved through the implementation of geographic databases (Edoardo and Costantini, 1986). Researchers studied two cities and towns in

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the United States that carried out geological tourism and elaborated on its unique practices including the key points of geological tourism cooperation, social promotion, education innovation, and field discipline (Wandersee and Clary, 2005). The concept of geoscience tourism is likened to the three dimensions of a box, metaphorically referring to shapes, processes and tourism.

However, the research on geoparks in China started late, but in recent years, more research has been done on the geoparks. Characteristics of geological tourism were analyzed in the United States National Park, focusing on the study of the protection of geological landscapes, eco-system, resource and environment in the United States National Park while conducting tourism activities and thinking that it is of siginificant reference for China (Xie and Liu, 2003). The protection status of Wudalianchi Global Geopark was analyzed and the sustainable development model was studied. The proposed elements of the model were the followings: leading community residents to participate in tourism development, strengthening the legislative system, reforming the geopark management system, establishing an information base, creating a network of geological relics and training specialized management personnel (Qian and Zhao, 2006). Scholars at home and abroad mainly focused on the development, construction, and problems of geoparks, discussed and studied the types and protection of geological relics, geoparks, and tourism economy. In response to the initiative of UNESCO to establish the "Global Geopark Network System", the Ministry of Land and Natural Resources, which are in charge of the declaration, approval and construction of geoparks as well as the formulation of the relevant laws, regulations and policies of geoparks, officially launched the plan of China's national geopark construction in 2000. After more than 10 years of development, China has become a geopark power in quantity.

Geoparks not only provide a public place for scientific sightseeing, leisure, health care, public education, cultural and entertainment, but also bring great social and economic benefits because of its irreproducibility as geological landscape, cultural landscapes and ecological focus protected areas, which has great scientific research value.

Tourism industry used to be considered as a "smoke-free industry" and would not cause environmental problems (Li, 2012). However, with the continuous development of society and economy, the contradiction between tourism development and ecological protection has become increasingly prominent. The contradiction between the social and economic benefits and ecological and environmental benefits has become the major problem that geoparks face on the way of sustainable development. At present, the protection of the ecology and environment has become a hot topic in the field of academic research. The earliest health degree which was used to measure the health of the human body from multiple dimensions such as body, spirit, and society, gradually extended to other disciplines, which means a quantitative measurement of the health level of an industry or an experimental site. The research methods were mostly based on the characteristics of a certain industry and the index systems of evaluation were constructed based on the actual situation of the industry. Then the industry's health degree was evaluated, as an important indicator to measure socio-economic and ecological environmental benefits of the scenic area (Wang, 2016). Health degree is becoming more and more important in the field of ecological health.

Taijin was took as an example to establish an index system of urban ecosystem health assessment considering the aspects of vitality, organizational structure, resilience,

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ecosystem service function, and population health of the urban ecosystem (Zhao et al., 2013). The basic principles and methods of regional ecosystem health assessment were explored in the aspects of regional types, target units, model methods, selection of indicators, thresholds, and weight settings (Peng et al., 2007). The "pressure-state-response" (PSR) framework model was applied to establish an indicator system and constructed evaluation model for the ecosystem health assessment of river basins (Yan et al., 2008).

Nowadays, although some academic achievements have involved the evaluation of ecosystem health and various indicators of the tourism industry, the overall quantity is small and the comprehensiveness is inadequate. Moreover, index systems in the aspect of evaluation for the health of geoparks are rarely used. Constructing a health evaluation index system suitable for geological parks means a conceptual model for measuring and evaluating environmental and sustainable health development. It analyzes the interaction between human and environmental systems from a systemic perspective, and comprehensively analyzes and describes environmental problems and their common models for social development. It is divided into driving forces (D) indicators, pressure (P) indicators, status (S) indicators, impact (I) indicators and response (R) indicators (Qin and Lu, 2014). Evaluating the overall health of the geoparks is a new area that needs to be researched and developed urgently in the tourism industry and those research are of high innovativeness and practical value.

Based on the field investigation of the socio-economic development and eco-environmental protection of Qianjiang Xiaonanhai Geopark, this paper starts with the concept of health degree of tourism industry, and selects the factors that have an important influence on the eco-health of the geopark as evaluation indices. We construct the evaluation index system of eco-health of geoparks through DPSIR conceptual model, and use fuzzy comprehensive evaluation method to calculate each factor and overall health index of Qianjiang Xiaonanhai Geopark, in order to analyze the connotation of ecological health of Xiaonanhai Geopark and its significance provides a theoretical basis and references for its declaration of the world-class geopark, to promote its scientific planning, rational construction and sustainable development. At the same time, this paper tries to build a business-ecosystem assessment system suitable for geoparks, promote the development of standardized, rationalized, scientific and sustainable geoparks, and propose measures and advice for integrating internal and external resources and optimizing current development models in order to increase their efficiency, so as to make due contributions to local economic development.

Materials and Methods

The overview of the research area

Qianjiang Xiaonanhai National Geological Park is located in the Qianjiang District, Chongqing, the junction of Chongqing and Hubei province (east longitude 108°38'20"--108°49'48", north latitude 29°31'14"--29°43'27"). It is 32 kilometres north of the county seat, covers an area of about 30 square kilometres. On the June 10, 1856, a 6.25-magnitude earthquake with an intensity of 8° on the ground cut off rivers and blocked the lake, creating the magnificent landscape for the complete ancient earthquake ruins. Dakuayan and Xiaokuayan in the north, two precipices, and other relics remain clearly visible. The place is rich in multi-type geological resources. As a national geopark, Xiaonanhai is a non-renewable and non-replicable landscape with a high value of both

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science popularization for public and scientific research for experts and scholars. It can provide sightseeing, leisure and other tourist activities with scientific education. *Figure 1* shows the research site.

Figure 1. *The experimental site of Qianjiang Xiaonanhai National Geopark*

Qianjiang Xiao Nanhai scenic area health evaluation index content

Driving force indicators

According to the explanation of the driving force in DPSIR model, the "driving force" in the evaluation of the health of geoparks is the potential cause of environmental change. It can be divided into two categories: natural driving force and socio-economic driving force. The natural driving force mainly includes climate change and natural disasters. Their impact on the geoparks often take a long time to emerge. The landscape of Qianjiang Xiaonanhai Geopark is relatively stable, and the aim of evaluation is the current situation, so the driving force indicator here mainly refers to the socio-economic driving force. Socio-economic driving force includes population growth, tourism demand (number) growth, GDP growth and other factors. Population growth can easily lead to encroachment on landscape land. Economic growth has a direct impact on scenic spots development.

Pressure indicators

The pressure on geoparks is mainly caused by the contradiction between the ever-increasing demand for tourism and the irreproducibility and expansibility of geological landscape resources. Compared with other industries, tourism industry tend to be more vulnerable and often face the threat of being constantly weakened. The population density, regional development index, utilization of tourist space and environmental

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carrying capacity are chosen to reflect the pressure on natural environment of geoparks caused by human activities.

State indicators

Geopark health status is the result of the driving force and pressure. The status and dynamic changes of the geoparks eco-environment are the basis of the health degree analyses of geoparks. The eco-system can be mainly refelected by the vegetation, biology, air quality, surface water quality and landscape state. When selecting natural state indicators, forest coverage, biodiversity, negative oxygen ion content, landscape fragmentation, surface water quality, comprehensive air pollution index can be selected to describe the eco-system status of Qianjiang Xiaonanhai Geopark comprehensively. In terms of social and economic status, the level of tourists' consumption is chosen as the representative to reflect attractiveness.

Impact indicators

Impact indicators are related to human health and life closely. The changing health level in tourist area will in turn have an impact on many aspects of human life quality, health, socio-economic structure and so on. The impact indicators of the DPSIR model of Qianjiang Xiaonanhai Geopark is used to describe the final social and economic effect when the health degree of the geopark change. Its impact on society and economy is mainly reflected in the change of visibility of tourist areas abroad, the satisfaction of tourists, total tourism revenue, economic growth and people's living standards. Common indicators reflecting these changes include area visibility, tourist satisfaction, tourist period, GDP per capita and so on.

Response indicators

The pressure is caused by socio-economic and natural factors which shaped current status of geoparks. The current status, in turn, affects the scenic area development potential and direction in the future and affects the scenic economic belt. Therefore, a corresponding social response must be made. People tend to promote the sustainable development of geoparks by adopting active countermeasures and policies such as improving resource utilization efficiency, reducing pollution and increasing investment in environmental protection. Therefore, choosing the ratio of environmental protection input to GDP, infrastructure construction in tourist areas, improvement of laws and regulations reflects the human's guiding role in the process of sustainable development of the park.

Evaluation methods

The Qianjiang Xiaonanhai tourist spot is a complex ecosystem. There are many indicators involved in ecosystem health assessment and the ambiguities are difficult to determine. Therefore, the entropy method is adopted to get the weight of each evaluation indicators, and the multi-level fuzzy comprehensive evaluation method is adopted to calculate the geology park health index.

Evaluation system construction

The ecological health system of Qianjiang Xiaonanhai National Geological Park is typical and complex. It is a growing giant system with multilevel. For the giant

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multivariable system, we use the description of state variables to reflect its inherent laws. According to the principle of representativeness, the selection of indicators was based on a large number of the results of previous literature and studies (Qin et al., 2013). Based on the "Driving Force-Pressure-State-Influence-Response" (DPSIR) model, an assessment index system of ecosystem health in Qianjiang Xiaonanhai Geopark was constructed. According to the actual situation of Qianjiang Xiaonanhai Geopark, 22 evaluation indices were selected, including 3 driving force indicators, 4 stress indicators, 7 state indicators, 5 impact indicators and 3 response indicators (*Table 1*).

The source of evaluation indicators

The research data are mainly from "Qianjiang 2012-2016 National Economic and Social Development Bulletin", "Chongqing Province Statistical Yearbook", "Qianjiang Yearbook", public data of Qianjiang District Protection Bureau of Chongqing Municipality, field investigation of Qianjiang Xiaonanhai Geopark, and from the principal office of the park.

Determine the indicators weight

The weight of eco-health evaluation index in tourist area is to empower the evaluation index relative to the importance of eco-health in scenic area to distinguish its contribution size. Determining the weight of an evaluation indicator is a very important element that directly affects the outcome of the evaluation.

The method of determining the index weight can be divided into two major categories: subjective weighting and objective weighting. Among them, the subjective empowerment method mainly based on expert advice and experience, such as expert scoring method, analytic hierarchy process, etc., objective empowerment is calculated by certain mathematical methods, such as principal component analysis, mean square error, the coefficient of variation method (Zhu et al., 2012). In this paper, according to the actual situation of ecological indicators in Xiaonanhai Geopark, in order to minimize the limitation of weight calculation, entropy weight method was used to empower each evaluation index of Xiaonanhai Geopark. In a specific operation, entropy weight method was based on the degree of variation of each index, then the entropy of each index was calculated, and the weight of each index was corrected through the entropy so as to obtain more objective and scientific index weight. Based on the data of health degree of Qianjiang Xiaonanhai Geopark from 2012 to 2016, the objective weight is determined according to the basic idea of entropy weight method, the size of index variability. In the comprehensive evaluation, the smaller information entropy of an index indicates the greater difference between the index values, the more relevant information provided, the more important role an index plays in the comprehensive analysis and evaluation, and thus the greater weight it should have; and vice versa (Shi and Chen, 2015).

Before evaluating, the data needs to be dimensioned before the metrics are empowered. In order to get a more objective weight, in this study, the objective weight of the indicator is calculated by the entropy method, and the calculation principle of the entropy method are as follows (Zhu, 2014):

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1) Each indicator's dimensions and units are different and cannot be directly compared and calculated. Therefore, before each indicator's weight is calculated, it needs to be standardized:
   When the indicator is a positive indicator, the standardization formula is:

$$x_{ij}^{'} = \frac{x_{ij} - x_j^{\min}}{x_j^{\max} - x_j^{\min}} \tag{Eq.1}$$

   When the indicator is a negative indicator, the standardization formula is:

$$x_{ij}^{'} = \frac{x_j^{\max} - x_{ij}}{x_j^{\max} - x_j^{\min}} \tag{Eq.2}$$

2) In order to eliminate the negative value of the translation process, after some standard values are normalized, there may be small or negative values. For normalization and convenience of calculation, the normalized values are shifted to eliminate the above situation.

$$x_{ij}^{'} = H + x_{ij}^{'} \tag{Eq.3}$$

   where H is the magnitude of the index shift, generally takeing 1.
3) The dimensionless process of the original data:

$$y_{ij} = \frac{x_{ij}^{'}}{\sum_{i=1}^{n} x_{ij}^{'}} \tag{Eq.4}$$

4) The entropy of each index is calculated as follows:

$$e_j = -\frac{1}{\ln n} \sum_{i=1}^{n} y_{ij} \ln y_{ij} \tag{Eq.5}$$

5) The difference coefficient of the first index is calculated as follows:

$$g_j = 1 - e_j \tag{Eq.6}$$

   (Among them, j = 1 ，2 ，the index number is p).
6) The weight of the first indicator is calculated as follows:

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$$\omega_j = \frac{g_j}{\sum_{j=1}^{p} g_j} \qquad \text{(Eq.7)}$$

(Among them, j = 1 , 2 , the index number is p).

Table 1. shows the the weights of indicators.

Table 1. *Qianjiang Xiaonanhai Geopark ecosystem health*

Object Layer	Criterion Layer	Indicator Layer	Weights	Tendency
Qianjiang Xiaonanhai Geopark ecological health index system	Driving force (D)	Natural population growth rate（D1）	0.0391	-
		Tourist growth rate（D2）	0.0457	+
		GDP growth rate（D3）	0.0417	+
	Pressure (P)	Population density（P1）	0.0402	-
		Regional Development Index（P2）	0.0366	-
		Visitor space utilization concentration (P3)	0.0311	-
		Natural disaster days（P4）	0.0324	-
	Status (S)	Forest Coverage（S1）	0.0375	+
		Biodiversity（S2）	0.0387	+
		Tourism per capita consumption level（S3）	0.0487	+
		Negative oxygen ion content（S4）	0.0356	+
		Landscape fragmentation（S5）	0.0521	-
		Surface water quality（S6）	0.0684	+
		Air Pollution Index（S7）	0.0393	-
	influences (I)	Tourist area visibility（I1）	0.0414	+
		Tourist satisfaction（I2）	0.0818	+
		Suitable period（I3）	0.0345	+
		Total tourism revenue and GDP ratio（I4）	0.0485	+
		Per capita GDP（I5）	0.0358	+
	response (R)	The proportion of environmental protection in GDP（R1）	0.0466	+
		Tourism infrastructure intact rate（R2）	0.0424	+
		Improvement of laws and regulations（R3）	0.0818	+

"+" stands for positive indicators, "-" stands for negative indicators

According to the relationship between indicators and ecological health, we divided Qianjiang Xiao Nanhai scenic ecological health assessment indicators into two categories:

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1) Positive indicator. The index is positively correlated with the degree of ecological health (positive index).The higher the index is, the higher the degree of ecological health will be. These indicators are e.g.: GDP growth rate, biodiversity and forest coverage indicators.
2) Negative indicator. The index is negatively correlated with the degree of ecological health (negative index), the higher the index is, the lower the degree of ecological health will be. These indicators are e.g.: the natural population growth rate, air pollution index.

Evaluation criteria

After confirming indicators and evaluation methods of Xiaonanhai Scenic Spot, the determination of each indicator was essential, which was the key to health degree assessment. The standard value (or reference value) of eco-system health evaluation of geoparks is constantly changing. The evaluation criteria of this study are mainly referring to the international, national, industrial and local standards, and the relevant standards in previous literature. According to the background and target value of the local tourism area, the ecological system of Xiaonanhai Geopark is divided into five subtypes: morbid (0.0-0.2), generally morbid (0.2-0.4), sub-healthy (0.4-0.6), healthy (0.6- 0.8), very healthy (0.8 ~ 1.0). *Table 2* shows the health rating and content of geopark.

Table 2. *Geological park ecological health rating and content*

Rating level	Very healthy	Healthy	Sub - healthy	General morbid	Morbid
Normalized Value	0.8～1.0	0.6～0.8	0.4～0.6	0.2～0.4	0～0.2

The Evaluation of established model

Fuzzy comprehensive evaluation method combines qualitative and quantitative analysis, unifies accuracy and inaccuracy, and integrates multiple factors (Zhu et al., 2012). The mechanisms and factors that affect the eco-health of tourism areas are very complicated. There are many things and factors that have ambiguous properties (Li, 2012).The interaction between them and the influence of each factor on system function are hard to accurately measure in terms of quantity. Therefore, in this paper, fuzzy comprehensive evaluation method is used to calculate the health index of Qianjiang Xiaonanhai Geopark, so as to scientifically evaluate the health status of its ecosystem.

Establish evaluation set

It is assumed that the fuzzy evaluation result of the evaluation index of the geologic park health degree is V. The evaluation grades are divided into morbid, generally morbid, sub-healthy, healthy and very healthy. The five rating criteria are set to s1, s2, s3, s4, s5. The standard value of each indicator and standard source have been described above. Constructed fuzzy evaluation sets V is:

V = {v1, v2, v3, v4, v5} = {disease, general morbid, sub-healthy, healthy, very healthy}.

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Determine membership function

Membership function has various forms. The determination of the membership function was based on the nature of the evaluation index. General evaluation indices can be divided into two categories: positive (or inverse) and moderate indicators. For the positive (or inverse) index, the greater the value (or smaller), the better; for the moderate index, it is a better value when it is in the satisfactory range. The farther away from this interval, the worse its evaluation status will be (Yue and Liu, 2008):

1) The membership function of positive index
   According to the analysis of geology park health evaluation of the reality and evaluation purposes, ascending trapezoidal distribution function and linear triangular function can be used for the positive index membership function. The results are as follows:

$$y_{D1}(x)=\begin{cases} 0 & x \geq s_2 \\ \dfrac{s_2-x}{s_2-s_1} & s_1 < x < s_2 \\ 1 & x \leq s_1 \end{cases} \quad \text{(Eq.8)}$$

$$y_{D2}(x)=\begin{cases} 0 & x \leq s_1 or x \geq s_3 \\ \dfrac{x-s_1}{s_2-s_1} & s_1 < x \leq s_2 \\ \dfrac{s_3-x}{s_3-s_2} & s_2 < x < s_3 \end{cases} \quad \text{(Eq.9)}$$

$$y_{D3}(x)=\begin{cases} 0 & x \leq s_2 or x \geq s_4 \\ \dfrac{x-s_2}{s_3-s_2} & s_2 < x < s_3 \\ \dfrac{s_4-x}{s_4-s_3} & s_3 \leq x < s_4 \end{cases} \quad \text{(Eq.10)}$$

$$y_{D4}(x)=\begin{cases} 0 & x \leq s_3 or x \geq s_5 \\ \dfrac{x-s_3}{s_4-s_3} & s_3 < x < s_4 \\ \dfrac{s_5-x}{s_5-s_4} & s_4 \leq x < s_5 \end{cases} \quad \text{(Eq.11)}$$

$$y_{D5}(x)=\begin{cases} 0 & x \leq s_4 \\ \dfrac{x-s_4}{s_5-s_4} & s_4 < x < s_5 \\ 1 & x \geq s_5 \end{cases} \quad \text{(Eq.12)}$$

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2) The membership function of a reverse index
According to the analysis of geologic park's health status, the membership function of inverse indicators can adopt the half-trapezoidal distribution function and the linear triangular function as follows:

$$y_{D1}(x)=\begin{cases} 0 & x\le s_2 \\ \dfrac{x-s_2}{s_1-s_2} & s_2<x<s_1 \\ 1 & x\ge s_1 \end{cases} \tag{Eq.13}$$

$$y_{D2}(x)=\begin{cases} 0 & x\ge s_1 or x\le s_3 \\ \dfrac{s_1-x}{s_1-s_2} & s_2<x\le s_1 \\ \dfrac{x-s_3}{s_2-s_3} & s_3<x<s_2 \end{cases} \tag{Eq.14}$$

$$y_{D3}(x)=\begin{cases} 0 & x\le s_4 or x\ge s_2 \\ \dfrac{s_2-x}{s_2-s_3} & s_3<x\le s_2 \\ \dfrac{x-s_4}{s_3-s_4} & s_4<x<s_3 \end{cases} \tag{Eq.15}$$

$$y_{D4}(x)=\begin{cases} 0 & x\le s_5 or x\ge s_3 \\ \dfrac{s_3-x}{s_3-s_4} & s_4<x\le s_3 \\ \dfrac{x-s_5}{s_4-s_5} & s_5<x<s_4 \end{cases} \tag{Eq.16}$$

$$y_{D5}(x)=\begin{cases} 0 & x\ge s_4 \\ \dfrac{s_4-x}{s_4-s_5} & s_5<x<s_4 \\ 1 & x\le s_5 \end{cases} \tag{Eq.17}$$

where, x is the measured value of the evaluation index; s1 is the first level of the evaluation index (morbid) standard value; s2 is the second level of the evaluation index (general morbid) standard value; s3 is the third level of the evaluation index (Sub-healthy); s4 is the fourth level (healthy) of the evaluation index; s5 is the fifth level (very healthy) of the evaluation index.

The results of ecological health evaluation and empirical analysis

Membership matrix

Combined with the standard values of Table 2 and the intragroup weights of each indicator obtained through the normalization method, the data of 2012-2016 are

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calculated by MATLAB 2010b software, and the membership matrix of the driving force indicator is obtained as follows:

$$R_D^{2012} = \begin{bmatrix} 0 & 0.15 & 0.85 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix} \quad \text{(Eq.18)}$$

$$R_D^{2013} = \begin{bmatrix} 0 & 0 & 0.85 & 0.15 & 0 \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix} \quad \text{(Eq.19)}$$

$$R_D^{2014} = \begin{bmatrix} 0 & 0 & 0.95 & 0.05 & 0 \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix} \quad \text{(Eq.20)}$$

$$R_D^{2015} = \begin{bmatrix} 0 & 0.05 & 0.95 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix} \quad \text{(Eq.21)}$$

$$R_D^{2016} = \begin{bmatrix} 0 & 0 & 0.6 & 0.4 & 0 \\ 0 & 0.5867 & 0.4133 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix} \quad \text{(Eq.22)}$$

Fuzzy comprehensive evaluation

WD = (0.3089,0.3613,0.3298)

Thus, we can get the fuzzy evaluation result of driving force health degree from 2012 to 2016:

$$\begin{aligned} H_D^{2012} &= W_D \bullet R_D^{2012} \\ &= (0.3089, 0.3613, 0.3298) \bullet \begin{bmatrix} 0 & 0.15 & 0.85 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix} \\ &= (0, 0.0463, 0.2626, 0, 0.6911) \end{aligned} \quad \text{(Eq.23)}$$

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$$H_D^{2013} = W_D \bullet R_D^{2013}$$
$$= (0.3089, 0.3613, 0.3298) \bullet \begin{bmatrix} 0 & 0 & 0.85 & 0.15 & 0 \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix} \quad \text{(Eq.24)}$$
$$= (0, 0, 0.2626, 0.0463, 0.6911)$$

$$H_D^{2014} = W_D \bullet R_D^{2014}$$
$$= (0.3089, 0.3613, 0.3298) \bullet \begin{bmatrix} 0 & 0 & 0.95 & 0.05 & 0 \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix} \quad \text{(Eq.25)}$$
$$= (0, 0, 0.2935, 0.0154, 0.6911)$$

$$H_D^{2015} = W_D \bullet R_D^{2015}$$
$$= (0.3089, 0.3613, 0.3298) \bullet \begin{bmatrix} 0 & 0.05 & 0.95 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix} \quad \text{(Eq.26)}$$
$$= (0.3613, 0.0154, 0.2935, 0, 0.3298)$$

Health index

In this paper, we can get the result of comprehensive evaluation of driving force factor by using fuzzy centroid method, that is, the driving force factor health index is as follows (where cj takes the median of health score threshold, ie c1 = 0.1, c2 = 0.3, c3 = c5 = 0.9):

$$C_D^{2012} = \sum_{j=1}^{5} (h_j c_j) = 0.7672 \quad \text{(Eq.27)}$$

$$C_D^{2013} = \sum_{j=1}^{5} (h_j c_j) = 0.7857 \quad \text{(Eq.28)}$$

$$C_D^{2014} = \sum_{j=1}^{5} (h_j c_j) = 0.7795 \quad \text{(Eq.29)}$$

$$C_D^{2015} = \sum_{j=1}^{5} (h_j c_j) = 0.4843 \quad \text{(Eq.30)}$$

$$C_D^{2016} = \sum_{j=1}^{5} (h_j c_j) = 0.6143 \quad \text{(Eq.31)}$$

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By the same token, we can calculate the stress, status, influence, response factors and the health index integrated in 2012-2016.

The stress factor health index is calculated as follows:

$$C_P^{2012} = \sum_{j=1}^{5}(h_j c_j) = 0.4326 \quad \text{(Eq.32)}$$

$$C_P^{2013} = \sum_{j=1}^{5}(h_j c_j) = 0.4956 \quad \text{(Eq.33)}$$

$$C_P^{2014} = \sum_{j=1}^{5}(h_j c_j) = 0.5296 \quad \text{(Eq.34)}$$

$$C_P^{2015} = \sum_{j=1}^{5}(h_j c_j) = 0.5169 \quad \text{(Eq.35)}$$

$$C_P^{2016} = \sum_{j=1}^{5}(h_j c_j) = 0.5672 \quad \text{(Eq.36)}$$

The state factor health index is calculated as follows:

$$C_S^{2012} = \sum_{j=1}^{5}(h_j c_j) = 0.6270 \quad \text{(Eq.37)}$$

$$C_S^{2013} = \sum_{j=1}^{5}(h_j c_j) = 0.5930 \quad \text{(Eq.38)}$$

$$C_S^{2014} = \sum_{j=1}^{5}(h_j c_j) = 0.5324 \quad \text{(Eq.39)}$$

$$C_S^{2015} = \sum_{j=1}^{5}(h_j c_j) = 0.5152 \quad \text{(Eq.40)}$$

$$C_S^{2016} = \sum_{j=1}^{5}(h_j c_j) = 0.5967 \quad \text{(Eq.41)}$$

The impact factor health index is calculated as follows:

$$C_I^{2012} = \sum_{j=1}^{5}(h_j c_j) = 0.7293 \quad \text{(Eq.42)}$$

$$C_I^{2013} = \sum_{j=1}^{5}(h_j c_j) = 0.7635 \quad \text{(Eq.43)}$$

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$$C_I^{2014} = \sum_{j=1}^{5}(h_j c_j) = 0.7189 \quad \text{(Eq.44)}$$

$$C_I^{2015} = \sum_{j=1}^{5}(h_j c_j) = 0.7214 \quad \text{(Eq.45)}$$

$$C_I^{2016} = \sum_{j=1}^{5}(h_j c_j) = 0.7214 \quad \text{(Eq.46)}$$

The response factor health index is calculated as follows:

$$C_R^{2012} = \sum_{j=1}^{5}(h_j c_j) = 0.5546 \quad \text{(Eq.47)}$$

$$C_R^{2013} = \sum_{j=1}^{5}(h_j c_j) = 0.5958 \quad \text{(Eq.48)}$$

$$C_R^{2014} = \sum_{j=1}^{5}(h_j c_j) = 0.6097 \quad \text{(Eq.49)}$$

$$C_R^{2015} = \sum_{j=1}^{5}(h_j c_j) = 0.8109 \quad \text{(Eq.50)}$$

$$C_R^{2016} = \sum_{j=1}^{5}(h_j c_j) = 0.8273 \quad \text{(Eq.51)}$$

The general health index is calculated as follows:

$$C^{2012} = \sum_{j=1}^{5}(h_j c_j) = 0.6298 \quad \text{(Eq.52)}$$

$$C^{2013} = \sum_{j=1}^{5}(h_j c_j) = 0.6455 \quad \text{(Eq.53)}$$

$$C^{2014} = \sum_{j=1}^{5}(h_j c_j) = 0.6216 \quad \text{(Eq.54)}$$

$$C^{2015} = \sum_{j=1}^{5}(h_j c_j) = 0.6120 \quad \text{(Eq.55)}$$

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$$C^{2016} = \sum_{j=1}^{5}(h_j c_j) = 0.6643 \qquad \text{(Eq.56)}$$

Results

According to the results of the fuzzy comprehensive evaluation, the overall ecological health status of Xiaonanhai Geopark in 2012-2016 is "healthy", which is consistent with the reality of Xiao Nanhai scenic spot. The factors and indicators status analysis are as follows:

First, the "driving force" factors are all "healthy" except the "sub-healthy" state of 2015, but the score in 2015 and 2016 has dropped significantly. The economic benefits of Qianjiang Xiaonanhai Geopark generally show a downward trend. The natural growth rate of the local population is relatively high, which has a great burden on the environment. The growth rate of tourist numbers fluctuates from year to year shows even negative growth in 2015. This is also the main reason why the driving force declines in the score. The economic benefits brought by the geoparks are relatively limited. The driving force for economic growth and tourism development needs further improvement.

Second, the "stress" factor has been in a "sub-healthy" state (health degree: 0.4326-0.5672). However, due to the slowdown of the social and economic development in the "driving force" factor, the "stress" factor has a good development trend. The key indicators that have a negative effect on the "stress" factors are the "regional development index", "concentration of tourist space utilization" and "days of natural disasters". The method to balance the contradiction between the socio-economic benefits of the park and the pressure of the natural environment still needs to be further explored.

Third, the "state" factor is basically in a "sub-healthy" state except in "healthy" state in 2012. Due to the "stress" factor, the "state" factor tends to be "healthy" in 2016. However, the change of "pressure" factor is larger than that of "state" factor, which shows that the influence of "pressure" factor on the ecosystem health of Xiaonanhai scenic spot has some lag. According to the score, the overall state of the Xiaonanhai Geopark in Qianjiang is relatively stable, and the status of vegetation, biology and air quality are in a healthy state and above. However, the pollution status of surface water needs to be improved.

Fourth, affected by the "state" factor, the "impact" factor is basically in a "healthy" state (health: 0.7635-0.7214). Overall tourist satisfaction has brought positive publicity to the scenic spots. However, because of the limitations of such factors as the landscape quality, the geographical location of the park and the incompleteness of the surrounding recreational facilities, the popularity and the exposure of the media is relatively low. To a certain extent, all these limited the further development of the park.

Fifthly, due to the good development of "influence" factor, "health" has been maintained. In the face of "sub-health" state of "stress" factor and "state factor" (Health: 0.5546) The status gradually improved to become "very healthy" (Health: 0.8273). The state of infrastructure in the park is intact. With increasing investment in infrastructure and environmental protection in the park, the government has also formulated more complete laws and regulations for the tourist attractions and improved the internal governance framework of the park.

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Sixthly, although the "stress" factor and the "status" factor have the threat of being "sub-health", the overall health status of the park has shown a "healthy" state in recent years, and the overall development trend is good. However, due to the obvious difference between the indicators, the key factors affecting the health of Qianjiang Xiaonanhai Geopark are also the focus of sustainable development in the future. The main focus is on the growth of tourist population, regional development index, tourist space utilization, and tourist popularity of these indicators. In short, the protection should be taken into account to ensure the healthy development of regional ecosystems without exploiting.

Compared with previous studies, this paper adopts fuzzy comprehensive evaluation method instead of the commonly used AHP method, which can more comprehensively evaluate the health level of geoparks; Moreover, because the health assessment methods are relatively new, no one has yet addressed special evaluation of the health of geoparks. Therefore, this paper has a strong innovation in the establishment of the evaluation index system for geoparks and the calculation methods.

Figure 2 shows the 2012-2016 overall condition of factors and comprehensive score trends of the Qianjiang Xiaonanhai Geopark.

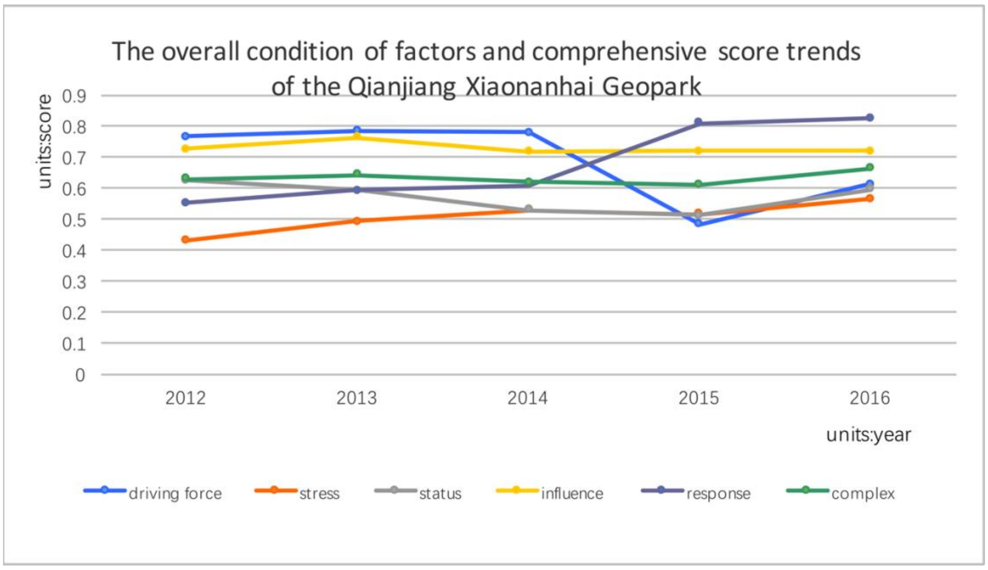

Figure 2. *The overall condition of factors and comprehensive score trends of the Qianjiang Xiaonanhai Geopark*

The protection and countermeasures of development

The Qianjiang Xiaonanhai Geopark has developed steadily in recent years, and the overall ecological health level is "healthy", but some indicators are too low, reflecting the obvious shortcomings in the construction and development of the geopark. Based on the results of empirical analysis and field investigation, we propose the following countermeasures and suggestions for Xiaonanhai Geopark:

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Improve the mechanism of talent introducing and the overall quality and management level of employees

The development of the Xiaonanhai Geopark needs leaders with long-term development strategic vision and a group of managers with the rich professional knowledge to undertake the important task of protecting geological relics. The duties of all staff positions should be cleared. The assessment system should be formulated, and all aspects should be introduced to professionals. They can provide professional guidance for the construction of geoparks based on the needs of the park.

Establish Qianjiang Xiaonanhai Geopark Museum, improve public facilities within the park, and further carry out popular science education, geological teaching and geological heritage tourism

At present, the science popularization facilities in Xiaonanhai Geopark are still in a backward state. We should further speed up the construction of popular science museums in the park, improve the design and the layout of geological knowledge explaining cards, equip the park with the corresponding geo-knowledge voice explaining equipment, give full play to the characteristics of science popularization in geological parks, set up a tourism network that takes science tourism as the leading role and build boutique tourism route.

Organize the implementation of funds, speed up the construction of key scenic spots and improve the surrounding facilities

Due to funding constraints, the process of Xiaonanhai Geopark internal construction is extremely slow. At present, the level of the key scenic spots construction in the Xiaonanhai Geopark is very low, and tourists have a very poor view. There are almost no matching facilities around the scenic spots and the tour process is extremely inconvenient. On the one hand, sources of funds can seek the support of national policies; on the other hand, the park can set up the Qianjiang Xiaonanhai Geopark Sustainable Development Fund, accepting donations and financing from all walks of life to provide the material guarantee for the internal construction of scenic spots.

Cooperate with scientific research organizations of universities, formulate scientific and feasible scientific research plans so that the sites can be scientifically protected and developed

The site of Xiaonanhai Geopark has great scientific value and potential for scientific research, and its future research should attract the attention of scientific research organizations in relevant disciplines. Xiaonanghai Geopark can cooperate with the organizations of universities and colleges in the relevant disciplines, regularly repair and reinforce the sites and formulate a series of scientific and feasible scientific research plans so as to protect better and scientifically utilize the precious relic resources.

Organize high-quality science popularization activities in geosciences, improve foreign media window and the public and media exposure and enhance the scenic spot popularity

The small amount of tourists in the Xiaonanhai geological park, to a large extent, is caused by the low popularity of the scenic spot. Geoparks can regularly organize activities of science popularization to increase the public interaction rate. At the same

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time, the park can establish and improve self-media platforms such as Weibo, official account of wechat and websites of scenic spots so as to improve the amout of information output and radiate influence and enhance the visibility of scenic spots.

Plan scenic spot development, improve space utilization efficiency and further improve the management system

At present, the interior planning of the Xiaonanhai Geopark is rather chaotic. There are 7 major programs in Xiaonanhai such as scenic spot planning, holiday area planning and nature reserve planning. The plans do not converge with each other. Therefore, the park planning and implementation are difficult. There should be a unified planning and layout designing of the Xiaoganhai Geopark, and a further improvement of the park's internal management system should be implemented, such as implementing the smooth management to avoid the restriction of various departments so as to enhance the efficiency of planning and implementation.

Based on the predecessors' ecological health theory, we compared most of the existing research index system evaluations to a large industry, such as tourism, and there is no specific evaluation index system for specific environments. There is very little research on a new concept, and we have constructed an ecological health assessment index system based on the DPSIR conceptual model specifically for geoparks, which makes up for the gap in this area to a certain degree, aiming at future generations to address the health of geoparks. The degree study provides reference. It has a high degree of innovation and uses Minjiang Xiaonanhai Park as a specific case. It proposes an evaluation method for the ecological health index of geopark tourist areas based on fuzzy mathematics and shows how to evaluate the ecology of the target area. The complete process of health puts forward scientific countermeasures and suggestions according to local conditions, which has high practical value and responds to the call of green tourism and is conducive to sustainable development.

Discussion

Based on the theory of ecological health, this paper takes Chongqing Xiaojianghai Xiaonanhai Geopark as an example. Based on the DPSIR conceptual model, an ecological health index evaluation system suitable for geoparks is constructed. The weights are confirmed by entropy weight method and multilevel fuzzy synthesis is used. The evaluation method calculates the ecological health degree of the geopark and concludes that the comprehensive health index of the park in the past five years is in a generally healthy state. The results show that the highest score of Qianjiang Xiaonanhai Geopark is 0.6643, and the lowest score is 0.6120. Based on local conditions, reasonable suggestions are proposed for the sustainable development of the Qianjiang Xiaonanhai Geopark.

Based on the predecessors' ecological health theory, compared with most of the existing research index system evaluations to a large industry, such as tourism, we found that there is no specific evaluation index system for specific environments. There is very little research on a health degree, and we have constructed an ecological health assessment index system based on the dpsir conceptual model specifically for geoparks, which makes up for the gap in this area to a certain degree, aiming at future generations to address the health of geoparks. The degree study provides future reference and has a high degree of innovation. It proposes an evaluation method for the ecological health

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index of geopark tourist areas based on fuzzy mathematics, and shows the complete process of how to evaluate the ecology of the health degree of a target area. Moreover, it can put forward scientific countermeasures and suggestions according to local conditions, which have high practical value. This research responds to the call of green tourism, which is conducive to sustainable development.

Conclusion

Ecological health and its evaluation are a very new and constantly updating field and ecological health of tourism areas is a new research direction. The development and protection of geopark tourism areas is a hot topic in current tourism industry. This article introduces the concept of a comprehensive assessment of health, and it is a groundbreaking and exploratory work in the geopark tourist area. However, due to time and energy and data acquisition reasons, there are still some fields worth deep study:

First, the size of sample on the study should be increased and a more detailed investigation and analysis should be done. More common conclusions could be drawn and further improvement of the construction of index evaluation system could be implemented.

Second, further follow-up research should be carried out, through longer-term data tracking and analysis, to understand the relevant factors and mechanisms of ecosystem health, and to select the same type of tourist areas at different stages of development for comparative research. Different tourism development stages should be compared and continuously study on the impact of ecological health.

Third, the concept of ecological health assessment could be applied to other areas and the research could be further expanded. The cross-contrast between different industries should be enhanced and the conceptual connotation of the research field should be enriched.

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IDENTIFICATION OF VEGETATION COVERAGE SEASONS IN IRAN USING ENHANCED VEGETATION INDEX (EVI)

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Abstract. Vegetation coverage seasons are affected by climatic elements and changes in climatic elements influence the vegetation growth period. The purpose of this research is to identify the vegetation coverage seasons of Iran, with the help of the Enhanced Vegetation Index (EVI). In this research, 16-day data of Iran's Enhanced Vegetation Index (EVI) was downloaded from MODIS Aqua website from July 14, 2002 to March 14, 2015. Then, on the basis of nearly 10 billion cells, the long-term mean of the 16-day EVI was calculated, and a time-location array was obtained at a dimension of 23*7541502. The results of cluster analysis on the Euclidean distances of this array were investigated by Ward method. This study shows that according to the local pattern of EVI in Iran, there are three different seasons, including a massive/dense season (from the April 14th to the June 1st) that the highest density is in the north and west of Iran, a transition season (from the June 17th to the October 7th) that the highest compression on the Caspian Sea coast, and less severity along the Alborz heights, and the season of thinning (from the October 23rd to the March 29th), the highest density of which is along the coast of the Caspian Sea, on the Khuzestan plain, and also in the southern half of the country.
Keywords: *vegetation, cluster analysis, EVI, MODIS, Iran*

Introduction

The MODIS sensor provides comprehensive information on the surface of the Earth, the Oceans, and the Atmosphere in a variety of different spectrums (Babu et al., 2016). By using the data of the MODIS, better maps of the vegetation coverage of the Earth can be provided. With these maps, estimates of the distribution of different types of vegetation coverage at ground level would be obtained (Mumtaz et al., 2017). Changes of vegetation coverage that occur during a year through different seasons, can be used as a factor for the interpretation of vegetation coverage maps (Di Vittorio and Georgakakos, 2018). In fact, the growing seasons show the growth conditions of different types of vegetation coverages throughout the year (Yang et al., 2017). The growing seasons are the most affected by the climate (Tsalyuk et al., 2017; Moriondo and Bindi, 2007) which determine the start and termination of the climate systems of the beginning and end of the growing seasons (Testa et al., 2018). In a study the relationship of vegetation coverage changes between the border of the desert of Africa and the West of Africa, which is known to have a minor effect on the monsoon cycle, was investigated (Zheng and Eltahir, 1998; Zhang et al., 2015); therefore, the coastal deforestation may be the cause of the disappearance of the monsoon, and have a great effect on the precipitation of the area (Ardo et al., 2018). According to research Ratana et al. (2005), the vegetation coverage index in Korado shows a high seasonal contradiction in the dry season between June and August, and the wet season between November and March; and also Muhammad et al. (2016) and Shi et al. (2017) have classified important products and vegetation coverage based on the Enhanced Vegetation Index (EVI) and images from the MODIS sensor.

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Erasmi et al. (2009) investigated the reduction of forage productivity, a 10-day combination index of the normalized vegetation coverage, that was used for imaging (and measuring) the 250-meter resolution of the MODIS sensor. In a study, using MODIS sensor data to rebuild the vegetation coverage dynamics, in response to climate patterns for the period 2001-2005 was used for North America. In a study by Cabello et al. (2012), the regional and spatial responses of the enhanced vegetation coverage profile are attributed to environmental factors, but in the dry regions of the Southeast of Spain on an annual scale is reviewed, and the effect of climate on this index has been revealed (Villani et al., 2011; Knezevic et al., 2017). The research results of Lorenz et al. (2013) show that vegetation coverage phenology has a limited effect on the mean of European summer climate (Hilker et al., 2015). The results of Jamali et al. (2011) showed that NDVI and EVI are highly correlated with soil moisture at all sites up to a 1 m depth implying strong relationships between vegetation growth and soil moisture at these depths, where most of the root biomass is located. Furthermore, EVI shows slightly higher correlations with soil moisture than NDVI at all sites during the growing season. In addition, EVI responds to soil moisture changes earlier than NDVI by about 0-4 days at the sub-Saharan sites where rainfall is a controlling factor. These slightly stronger relationships with EVI might occur because of improved corrections for variations in atmosphere, soil, and canopy background which make it sufficient for monitoring open canopy areas such as grasslands and savannas (Huete et al., 1997). Based on Peng et al. (2017) the effects of rainfall on vegetation coverage vary according to the rainfall time and the type of growing form, so that in urban areas, there is no significant relationship between the amount of precipitation and the vegetation coverage, in any time scale. In pasture lands, the strongest correlation is observed between the spring rainfall and the vegetation coverage changes, while the forest area shows the strongest correlation with annual precipitation (Pisek et al., 2015), and the strongest correlation with rainfall in spring and March is seen in the arable land (Allen et al., 2010), In the meadow areas, there is also a stronger correlation between rainfall and bushes (Son et al., 2014). Regarding the identification and classification of changes in the plant growth index (EVI) by MODIS sensor in Jiangxi province of China (Zhang et al., 2014; and Raghavendra and Aslam, 2017), in Karnatkay of India, in the southern part of Vietnam, and in Uttar Pradesh of India some research studies were done (Bansal et al., 2017). Since the vegetation coverage seasons show the condition of the vegetation coverage compression of an area throughout the year, and also its effect on the life of plant and animal ecosystems, the purpose of this study is to identify the seasons of Iran's vegetation coverage, based on the enhanced vegetation index (EVI) by using the MODIS Aqua sensor.

Data and Methods

In this research, the data of MODIS Aqua sensor were used for dividing the vegetation coverage into seasons in Iran. The MODIS Aqua sensor was launched on the June 4th, 2002 by NASA (Vermote et al., 2002). The time the satellite passed over the equator was 13:30 local time (Wang and Xie., 2009). To find the seasonal vegetation coverage of Iran, at first, the 16-day data of the Enhanced (Growth of) Vegetation Index (EVI) of the MODIS-Aqua with a local resolution of 500 meters in the time interval from July 14, 2002 to March 14, 2015were extracted from the MODIS website, which covered the territory of Iran in 6 tiles. The dimensions of each tile was 3600*2400 cells,

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and each square cell was 463.31 meters. Six tiles were said to be one mosaic, which means that there are 23 mosaics of 16 days throughout the year. Due to the fact that the used data also included the extraterritorial land of Iran, with the help of the in-polygon function of the MATLAB software, only the data that were covering the geographical intra-boundary of Iran were extracted. The long-term mean of vegetation coverage of Iran was calculated for every 16-days, that for each, an array of 7200*4800 cells was obtained. These estimates are representative of the local behavior of vegetation coverage in every 16-days of the year, with the arrangement of a time-local array of dimensions of 23*75841502. This array was the basis of our judgments for dividing the vegetation coverage into seasons in Iran.

EVI was developed to optimize the vegetation signal with improved sensitivity in high biomass regions and improved vegetation monitoring through a de-coupling of the canopy background signal and a reduction in atmosphere influences (Aulia et al., 2016; Ogilive et al., 2015).

$$\text{EVI} = G\,\frac{N - R}{N + C_1 R - C_2 B + L} \tag{Eq. 1}$$

where N, R, and B are atmospherically corrected or partially atmosphere-corrected (Rayleigh and ozone absorption) surface reflectances in near-infrared, red and blue bands respectively; G is a gain factor; C1, C2 are the coefficients of the aerosol resistance term, which uses the blue band to correct for aerosol influences in the red band, and L functions as the soil-adjustment factor, attributed to the interaction and feedbacks between the soil-adjustment factor and the aerosol resistance term (Liu and Huete, 1995; Jiang et al., 2008). The coefficients adopted in the MODIS EVI algorithm are, L = 1, C_1 = 6, C_2 = 7.5, and G = 2.5. EVI has been used recently in a wide variety of studies, including those on land cover/land cover change (Wardlow et al., 2007), estimation of vegetation biophysical parameters (Chen et al., 2004; Houborg et al., 2007), phenology (Ahl et al., 2006; Zhang et al., 2003; Bolton and Friedl, 2013), evapotranspiration (Nagler et al., 2005; Nouri et al., 2016), biodiversity (Waring et al., 2006; Sloan et al., 2014), and the estimation of gross primary production (GPP) (Rahman et al., 2005; Zhang et al., 2014).

Accordingly, at first by calculating the Euclidean distance of the vegetation coverage of the entire local points of the array k before all the mosaics, we measure the dissimulated degree of the sites (*Eq. 2*).

$$d_{rs}^2 = (k_r - k_s)(k_r + k_s)' \tag{Eq. 2}$$

d_{rs}^2 is the Euclidean distance of the point *r* with the coordinates of $(\varphi_r . \lambda_r)$ and the point *s* with the coordinates of $(\varphi_r . \lambda_r)$ or the Euclidean distance of the *r* group and the *s* groups;

k_r is the vegetation coverage of the *r* spot or group;

k_s is the vegetation coverage of the *s* spot or group;

After measuring the Euclidean intervals, a cluster analysis was performed by Ward method into an array of distances (M), and the 75841502 point of the *k* array was arranged together in accordance with the simulated degree (*Fig. 2*).

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In the method, the R and S groups are merged if the increase in the variance resulting from their integration is minimized relative to the integration of each of them with other groups (*Eq. 3*):

$$d(x.y) = \frac{n_r + n_s + n_{rs}^2}{(n_r + n_s)} \quad \text{(Eq. 3)}$$

Here n_{rs}^2 is the distance between the group *r* and the group *s*, that are obtained by the central transplant method. n_r is the number of members of the group *r*, and n_s is the number of members of the group *s*. Based on this analysis, Iran has three seasonal vegetation coverages (*Fig. 2*). For a better understanding of the phenomena, the Iranian Digital Elevation Model (DEM) is depicted in 500-m location with a sine-image system (*Fig. 3*). By distinguishing the vegetation coverage of each of the *k* array local spot, the map of seasonal vegetation coverage of Iran was drawn (*Figures 4, 5, 6*).

Results and Discussion

Figure 1 shows the Enhanced Vegetation Index (EVI). According to the definition of EVI the values between 1-0.8 represent a very dense vegetation cover, 0.8-0.6 represent dense cover, 0.6-0.4 stands for average cover, 0.4-0.2 correspond to sparse, 0.2-0 represent a poor and the background soil (Chakraborty et al., 2016). of course, a range of 0.1 indicates the background soil, and 0.2-0.1 can also indicate grasslands and bushes (Zhou et al., 2016), which are known as the soil in this research. The values of less than zero are also known as water resources, which may be lakes, wetlands, dams, snow, and even mountain ice (in the cold season). In general, in this study, the EVI values are considered to be more than 0.2 as vegetation covering.

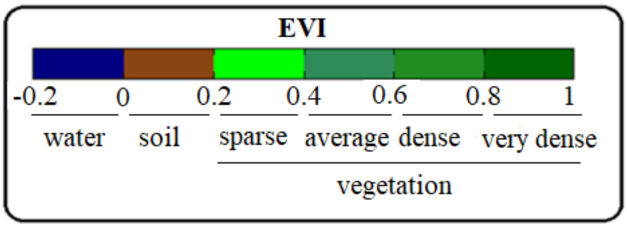

***Figure 1.** The Defined Values of EVI in this Research*

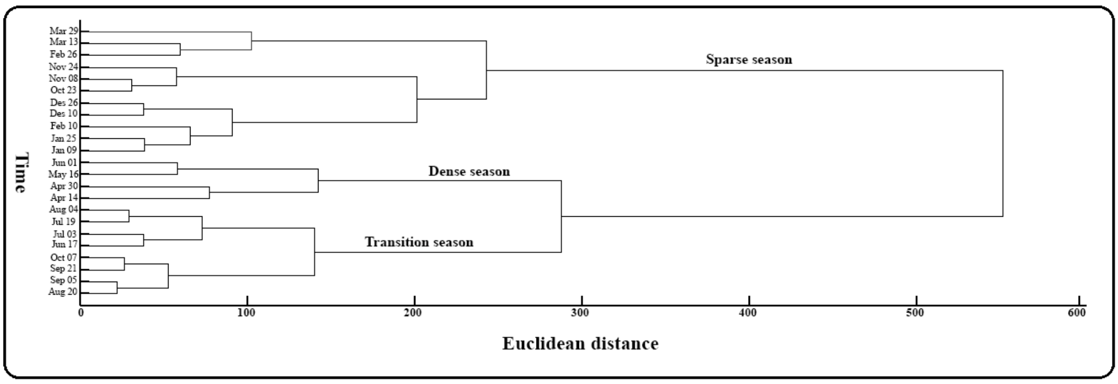

***Figure 2.** The Clustering of EVI by Ward method*

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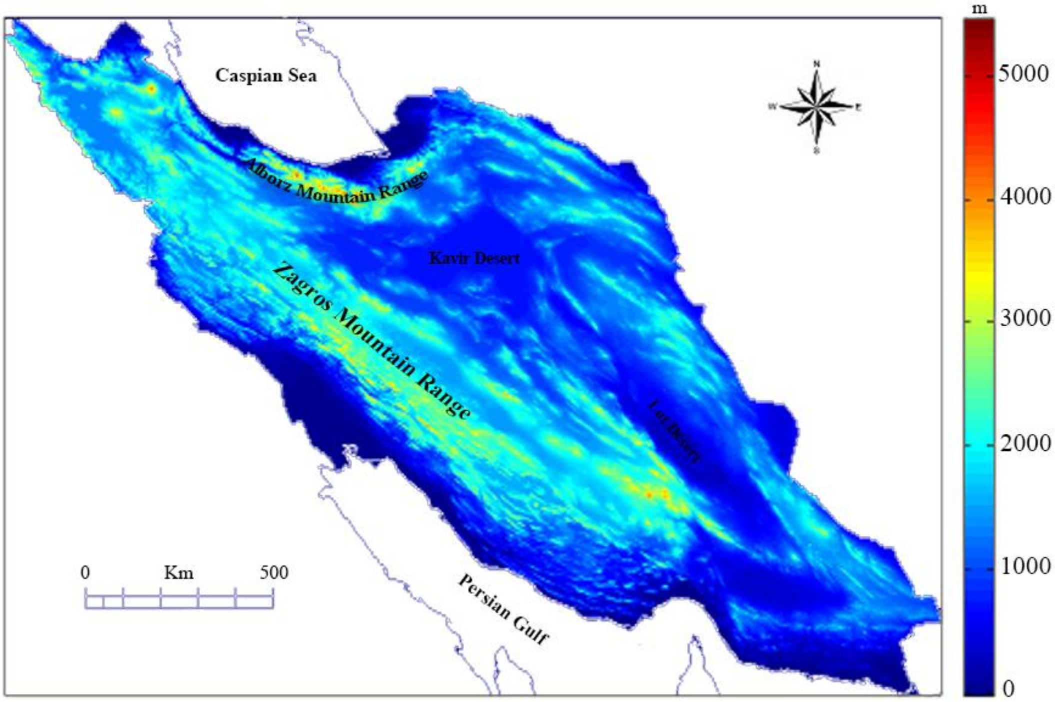

Figure 3. *Digital Elevation Model of Iran in 500 m spatial resolution with a sine image system*

By analyzing the clustering method, the country of Iran was divided into three dense, transition, and sparse seasons, regarding the vegetation coating, as shown in *Figure 2*.

The Sparse Season

Figure 4 shows the long-term mean of the vegetation coverage of the sparse season in the period from the October 23rd to the March 29th. During this time period, the vegetation coverage is insignificant, so that only 2.5 percent of the country's coverage is covered, and this amount is also sparse. The highest density of vegetation coverage in the Northern half of the country was only seen along the coasts of the Caspian Sea, and in the Southern half of the country in the plain of Khuzestan, Bushehr, West of Kohgiluyeh and Boyerahmad, South of Fars and Kerman, North and Northeast of Hormozgan. The main cause of the most densely populated vegetation cover in the mentioned areas is temperate temperature and winter rainfall, while in other parts of the country, there is no plant in this time period due to the low temperature, snow and ice cover. In addition to the mentioned areas, in the central regions of Southern Tehran, Qom and the East of Isfahan, in the Western regions of Western Kermanshah, East of Ilam and West of Lorestan, in the East of the country in the West and South of Xorasan Razavi and North of the South Xorasan, and finally in the North of the country, in the North of Ardabil Province, in the plain of Moghan very weak and dotted areas of the sparse vegetation coverage were observed. Mean, dense, and very dense vegetation is not seen in any part of the country. Because of the low temperature, snow and rain, the area of snow, ice cover and water is maximum amounts, that cover about 1.3 percent of the country's coverage. Through the broad surface, in the Northern parts of China (Mao et al., 2014), the coasts of Africa (Fetzel et al., 2015), the North of America (Potter et

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al., 2008), and the coastline of the country Mali (Spiekermann et al., 2015) seasonal variations of temperature changes the plant's vegetation coverage. The blue color in the map shows from one side, the snow cover on the Alborz and Zagros heights and the peaks of Sabalan and Sahand, and on the other side, the waters of Urmia Lake and the lakes of Fars Province. About 96 percent of the country's land is covered by poor vegetation/soil cover.

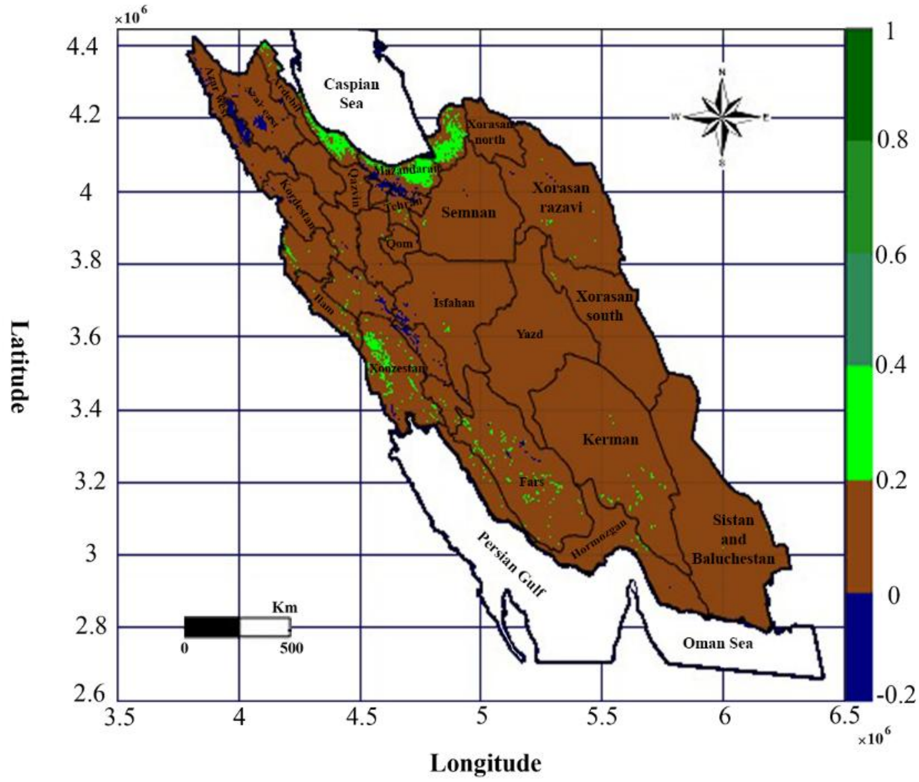

Figure 4. *The Long-term mean Sparse season from October 23rd to March 29th*

The Dense Season

Figure 5 shows the long-term mean of vegetation coverage of the dense season in the period from the April 14th to the June 1st when the vegetation coverage growth is at its peak. In this period, the sparse vegetation coverage is still very wide and covers about 12.5 percent of the country, its highest density was observed in the Western half of the country, along the Zagros mountains and throughout the Northern half of the country, and even the Northeast of the country. The mean vegetation coverage in this interval is at its maximum amount, and covers about 1.5 percent of the country. The highest density of the mean vegetation coverage was observed along the coasts of the Caspian Sea; in addition to this area, it was observed in the Northwest of the country in the provinces of West Azerbaijan, and also the Sahand slopes in Azar East and the Sabalan slopes in Ardabil and in the Western provinces of the country, the provinces of Kermanshah, Hamedan and Lorestan, also in the provinces of Chaharmahal and Bakhtiari and Kohgiluyeh and Boyer Ahmad in the Southern half of the country. Also, in the mountains of Southern Alborz slope and central regions of the provinces of Fars and Kerman, there is the mean vegetation coverage, spottedly. In this period of time,

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since it is at its peak of growing, there is also a dense vegetation covering about 1480 square kilometers of the country that only was observed by the Caspian Sea, especially by its Southern coast. The soil surface has declined due to the significant increase in vegetation coverage over this time period, and covers about 85.4 percent of the country. The coverage of snow, ice, and water involves about 0.4 percent of the country. The color blue on the map, in this period, shows the other water resources and rivers of the country, such as the lakes of Fars Province, Shadegan wetland in Khuzestan, Hirmand River in Sistan and Baluchestan, Zayandehrud in Isfahan, as well as rivers along the Alborz and Zagros heights. At this time, the extent of the snow and ice cover is very small, and can only be spotted in the Alborz and Zagros heights. In general, during the dense season 14.14 percent of the country is covered by vegetation including sparse, transition, and dense. Even in this season, where vegetation is maximized, there is no massive vegetation, and it shows the poor vegetation of Iran.

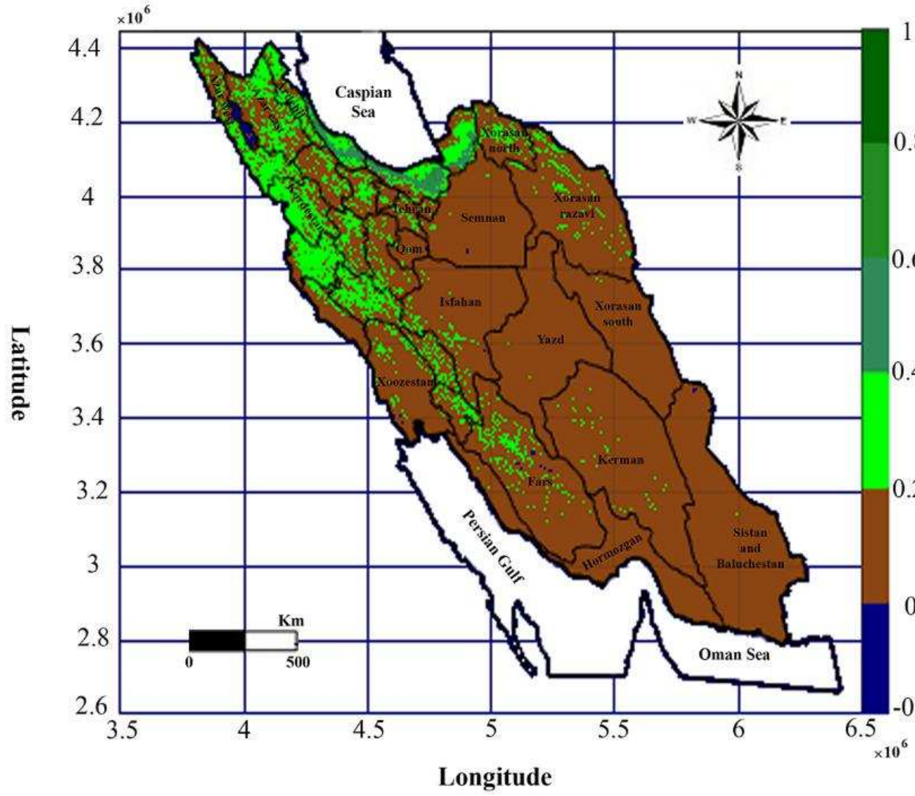

Figure 5. *The Long-term mean Dense season from April 14th to June 1st*

The Transition Season

Figure 6 indicates the long-term mean vegetation coverage of the transition season in the period from the June 17th to October 7th, which is exactly in the warm period of the year. During this period, the sparse vegetation coverage, which is sporadic in most parts of the country except the central and Eastern deserts of the Southeast, it covers about 3.8 percent of the country's territory. By looking at the map, it can be seen clearly that the sparse vegetation coverage, except the Caspian shores, in other parts of the country almost everywhere is consistent with the high altitudes of the country, so that in the Northern half of the country, this vegetation coating along the Alborz highlands, in the

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Western half of the corresponding of the Zagros heights and in the North-East of the country, which is in line with the altitudes of Xorasan. In addition to the mentioned areas, in the southern regions of the country, the sparse vegetation coverage in this interval is almost in line with the high altitudes, so that the existense of the vegetation coverage of Zagros, in the range of Kohgiluyeh and Boyer Ahmad provinces, Chaharmahal and Bakhtiari and Fars, as well as in the desert provinces of Kerman and Yazd, on the highlands of Laleh Zar and Shirkooh, are well known. The reason for the presence of denser vegetation coverage on the heights compared to the plain areas in this time interval on the one hand can be attributed to the presence of adequate amount of water, on the other hand to the proper temperature, which helps the growing of vegetation cover. In addition to the above altitudes, there is also the presence of sparse vegetation coverage along Zayandehrud and Khuzestan plain. The mean vegetation coverage covers about 1.2 percent of the country and its highest density is along the coasts of the Caspian Sea due to its favorable rainfall and temperate temperature in this area during this time period for vegetation cover. After the coasts of the Caspian Sea in the North and West of the country, the mean vegetation coverage is significant in the province of the East Azarbayjan, in the Sahand slopes along the Aras river in the North of this province, and in the Azar West Province, in the West of the Urmia Lake. In addition to the mentioned areas, the average vegetation cover is also found in limited areas in the southern Alborz and the central and northern heights of the Zagros Mountains. The dense and very dense vegetation coatings at this time, are not visible at any point in the country. The water content is also 0.34 percent. At this time of year, the amount of soil is significant and covers about 94.5 percent of the country's total area.

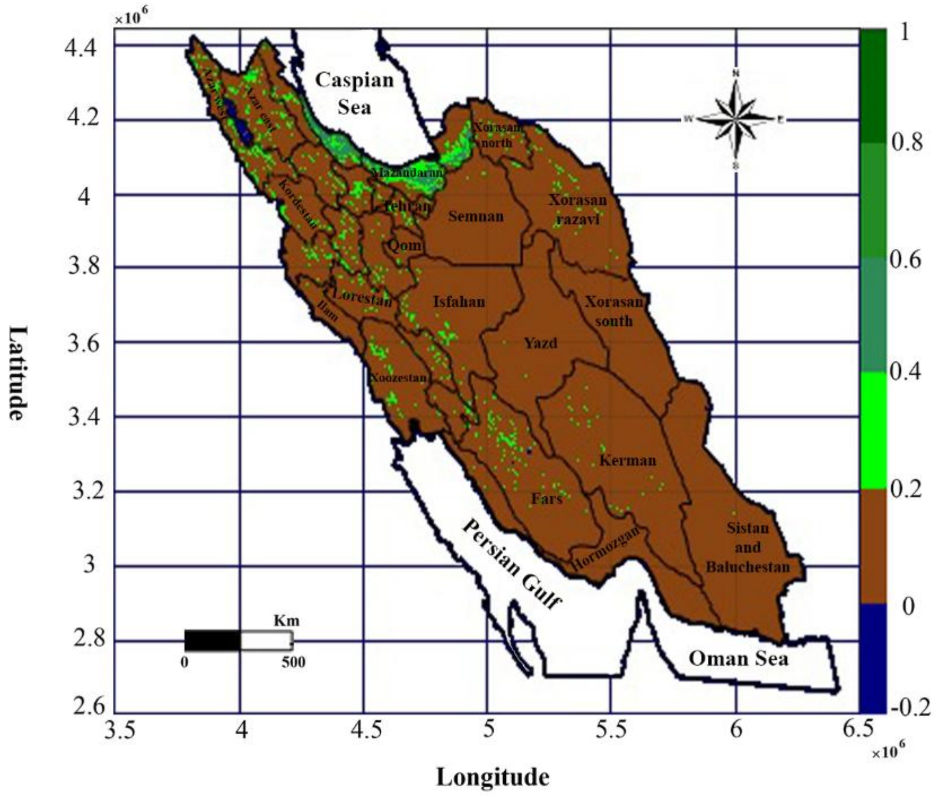

Figure 6. *The Long-term mean Transition season from June 17^{th} to October7^{th}*

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Conclusion

The vegetation coverage changes that occur during a year through different seasons, can be used as a factor in the interpretation of vegetation coverage maps. The purpose of vegetation coverage indices is to predict and evaluate some of the characteristics of the vegetation coating, such as the crown covering, biomass, leaf spread, or the percentage of vegetation coating. In this study, initially, from the total of 23 maps of the 13-years long-term mean period was taken, and in the next step a cluster analysis was carried out resulting in the creation of clusters of maps that are similar to each other. Then, it was classified into Iran's growing seasons by the Ward method; Iran has three vegetation (and growing) seasons over the year; the first season has a dense vegetation coating, and its period is from the April 14th to the June 1st. In this season, about 14 percent of Iran's area, due to the temperature and precipitation adjustments, is covered by plants. Its highest density is seen in the Zagros, Alborz, Northeastern highlands and along the coasts of the Caspian Sea. In the second season, the vegetation coverage is in the transitional stage, and covers the period from the June 17th to October 7th, and its highest density is observed along the coasts of the Caspian Sea and the highlands of Alborz and Zagros. The third season has a sparse vegetation covering, and its period is from the October 23rd to March 29th, and due to the coldness of the air in most parts of the country, only 2.5 percent of Iran's territory is covered. Its highest density is along the coasts of the Caspian Sea, Khuzestan plain, and Southern half of the country. For future studies, it is recommended to compare vegetation indices such as EVI and NDVI in the study area.

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THE EFFECTS OF DOSAGES OF WORM AND NITROGEN-PHOSPHORUS FERTILIZERS ON PLANT GROWTH OF *HYACINTHUS* SP. IN SİİRT PROVINCE, TURKEY

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Abstract. This research was carried out in order to determine the effects of worm fertilizer and nitrogen-phosphorus fertilizer dosage differences on flowering of hyacinth as a bulbous ornamental plant from 2016 to 2017 in Siirt University, Turkey. Solid worm fertilizer was applied at dosages of 25 g/bulb (V1), 50 g/bulb (V2), 75 g/bulb (V3) and 2 kg/decare (NP1), 4 kg/decare (NP2), 8 kg/decare (NP3) Nitrogen-Phosphorus (NP) were applied during the growth of hyacinth (*Hyacinthus orientalis* L. cv. "Purple Star"). According to the results, the lowest (the earliest time) mean values of the first flowering time, full flowering time, and harvest time were obtained as 135.25 days, 136.54 days, and 137.70 days in V1 treatment, respectively. The highest mean value on leaf number (5.71), leaf length (128.21 mm), stalk thickness (13.50 mm), and floret diameter (28.14 mm) were determined in V1. The highest mean values belong to flower diameter (66.96 mm) and length (116.33 mm) were evaluated in V2 and V3, respectively. Among NP fertilizers, in NP1 the highest mean value on floret number (37.69) while in NP2 the highest mean value on floret length (26.76 mm) were obtained. The highest mean values on leaf diameter and plant height were found as 28.86 mm and 179.54 mm in NP3 treatment. As a result, it has been determined that the hyacinth can be grown with worm fertilization without chemical fertilizer use under ecological conditions of Siirt province. Considering the adverse effects of chemical fertilizers on the environment, it is proved that there are no statistically significant differences between them and the worm fertilizer, which is a bio-fertilizer.
Keywords: *chemical fertilizer, flowering, hyacinth, ornamental plant, plant growth, worm fertilizer*

Introduction

Hyacinthus orientalis L. is a herbaceous flower belonging to the hyacinth branch of the Liliaceae family species (Hu et al., 2015). This species was originally cultivated in the Mediterranean region and in South Africa (van Sheepen, 1991). In 1768, more than 2000 varieties of this species were recorded (Darlington et al., 1951). According to Shen et al. (2004), nowadays the common hyacinth cultivars used for gardening are Holland and Rome species (Hu et al., 2015). The genus of hyacinths consists of 30 species among which only *H. orientalis* have horticultural importance due to their longevity as cut flowers. As well as grown hyacinths are generally between 10 and 30 cm tall and come in a wide range of colors. The swollen portion consists mostly of fleshy, food storing scales attached to a short flat stem. The plants start flowering in the first part of February (Smigielska et al., 2014). The plant prefers moist but light sandy to loamy soils. These soils could be acidic, neutral or alkaline. The hyacinth is widely cultivated in pots and is used as a cut flower worldwide (Hu et al., 2015). Its fragrance and graceful style have provided this flower with considerable ornamental value. Also, many distinctive ornamental plant groups live in Turkey due to its location that is the cross- section point of Mediterranean, Europe-Siberia and Iranian-Turanian phytogeographical regions and because different climatic conditions prevalent in its

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region, which cause natural floristic richness, biological difference and several habitat characteristics which are unique. Flower cultivation, which has a large natural potential in Turkey, is gradually growing importance and become a productive agricultural and commercial branch. Cut flower cultivation is a development of ornamental plant production having the largest part either in production or economic value. Cut flower production includes the processes, where fresh or dried flowers or their parts are used in clamps and arrangements in their original or colored forms.

This study was formulated to investigate the potential role of bio-fertilizer application for enhancing growth, and flowering of *Hyacinthus orientalis* in a sustainable agricultural production system in order to reduce the amount of excessive chemical material released to the environment. The experiment was conducted with varying dosage of nitrogen-phosphorus (NP) and worm fertilizer (V) on *Hyacinthus orientalis to* find out the effects on criteria of growth and flowering of plants.

Literature Review

Activities such as growing, choosing, procession, classification, storing and marketing of flowers are included in the cut flower production process. Nearly 50 countries produce cut flowers. Turkey is among the most important countries having the largest potential for cut flower growing with its climatic and topographical characteristics (Sönmez et al., 2013). Generally, farmers use chemical fertilizers to improve soil fertility and hence increase the yield of their crops. However, the use of chemical fertilizers causes a great impact on the soil quality and the surrounding environment. Vermicomposting is a non-thermopiles and simple biotechnological process of composting, in which certain species of earthworms and microorganisms are used for biological degradation of organic waste (Arancon and Edwards, 2005; Khan and Ishaq, 2011). Sangwan et al. (2010) who inferred that soil amended with 30% vermicompost produced more number of flowers in marigold (*Tagetes erecta* L.). The plants were grown in pot culture experiments and the largest flower diameter was produced in soil amended with 40% vermicompost. Further, the beneficial effect of vermicompost might be due to increased soil organic matter and improved physical properties of soil like bulk density and better aggregation. Edwards (1998) reported that vermicompost could promote early and vigorous growth of seedlings (Gopinathan and Prakash, 2014). Vermicompost has found to effectively enhance the root formation, elongation of stem and production of biomass, vegetables, ornamental plants etc. Also Chamani et al. (2008) studied the impact of vermicompost on the growth and flowering of *Petunia hybrida* 'Dream Neon Rose' in greenhouse conditions using 0, 20, 40 and 60% ratio of vermicompost and reported that vermicompost had positive, significant influence on the number of flower, leaf growth, and branch fresh and dry weights as compared to plants grown in vermicompost-lacking soil. Edwards and Burrows (1988) reported that vermicompost increased ornamental seedling emergence compared with those in control commercial plant growth media, using a wide range of test plants such as pea, lettuce, wheat, cabbage, tomato and radish however fertilizers are soil improvements applied to increase plant growth, the main nutrients added in fertilizer are nitrogen, phosphorus, potassium, other nutrients are added in small amounts. Fertilizers are required to bring out the best features of ornamental potted plants. For natural plants to grow and increase they need a number of chemical elements. Ashoorzadeh et al. (2016) found that vermicompost and NPK significantly improved the growth, yield and quality of rose flowers compared to control and that the improvement was greater when vermicompost and NPK fertilizers were mixed. Gangadharan and Gopinath (2000) reported that application of 10 t of vermicompost ha^{-1} + 80 percent recommended NPK dose per ha for obtaining higher yield and net return per ha in *Gladiolus* spp. was successful. Also Amarjeet et al. (1996) recorded an increase of flower stalk height and leaf length in tuberose with an application of a higher dose of NPK and increased flowering period and the largest panicle length was produced with 200:200:400 kg/ha NPK. In addition, they are eco-friendly, easily available and cost effective.

Materials and Methods

Experimental Site

The present experiment was carried out in the research and practice field of Siirt University Faculty of Agriculture, during the 2016-2017 vegetation periods (*Figure 1*). The research site is situated at the 37°58'12'' North latitude and 41°50'54'' East longitudes and at an altitude of 650 meters above sea level.

Figure 1. *Location of sampling site (Google maps, 2018)*

Siirt province has a semi-arid and subtropical climate with hot and dry summers, where maximum temperatures exceed 45°C, the winters are cold and the minimum temperatures can reach as low as 5°C in December and January months. Some climatic properties were presented in *Table 1.*

The soil is categorized as sandy loam with low aggregation. The chemical composition of the soil collected from 0-30 cm depth before planting of *Hyacinthus* bulbs. Some soil properties were determined as clay for texture type, neutral for pH, non-saline, medium lime, low for organic matter and phosphorus (P_2O_5) and sufficient for potassium (K_2O).

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Table 1. *Some climatic values of the year where the experiment was carried out (Anonymous, 2017)*

Parameter	November 2016	December 2016	January 2017	February 2017	March 2017	April 2017	May 2017	Average
Monthly avarege temperature (°C)	10.4	3.3	3.0	2.6	9.7	14.2	19.5	8.9
Monthly average of daily max. temperatures (°C)	16.45	7.2	7.4	7.9	14.8	20.0	25.1	14.1
Monthly average of daily min. temperatures (°C)	6	0.6	-0.2	-1.5	5.3	9.3	13.9	3.0
Average monthly 50 cm of soil temperature (°C)	15.6	8.6	6.6	5.9	10.1	14.5	19.4	11.5
Average monthly relative humidity (%)	49.7	73.1	65.9	64.9	63.5	59.3	51.7	61.1
Average monthly total precipitation (mm)	55.6	121.4	49.4	45.6	118.8	149.9	74.8	87.9

Experimental Details

This study was carried out with *Hyacinthus orientalis* L. cv. “Purple Star” as plant material. The experiment was laid out in a randomized block design with 7 treatments and three replicates including 12 bulbs per replicates. The plants were planted in November 2016, with solid worm fertilizer and 20:20:0 N:P:K fertilizer. The used solid worm fertilizer is obtained from *Eisenia foetida* culture worms known as red California worm produced by EkosolFarm producer company. Worm fertilizer dosages were used as 25 (V1), 50 (V2) and 75 (V3) g/plant on each bulb hole. 2 (NP1), 4 (NP2), and 8 (NP3) kg da^{-1} dosages calculated from N and P_2O_5 containing inorganic fertilizer were applied. The fertilizers were given at the time of planting.

Morphological Observations and Phenological Properties

Some of the observations and measurements performed are shown in *Figure 2*.

Figure 2. *Hyacinths in experimental site*

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First flowering time (day): The period taken for the opening of the floret was recorded from the date of spike initiation for the first florets opening.
Full flowering time (day): A number of days required for the full flowering time or the floret opening from the plantation moment to 50 percent sprouting of florets.
Harvest time (day): When the last 1-2 florets closed have been seen.
Leaf number/plant: A number of leaves per plant were counted at harvest time.
Leaf length (mm): Leaf length was measured with digital compass.
Leaf diameter (mm): Leaf diameter was measured with digital compass.
Plant height (mm): Plant height was measured from the soil surface to the top of the plant.
Flower height (mm): The height of the flower was measured from the bottom floret to the top floret.
Flower diameter (mm): The width of the flower was measured for each flower.
Floret length (mm): The height of the floret was measured at full flowering time.
Floret diameter (mm): The diameter of floret was measured at full flowering time.
Floret number/flower: The number of florets per one flower was counted at full flowering time.
Stalk thickness (mm): The stalk thickness was measured at full flowering time.

Statistical Calculations

Data were analyzed by the analysis of variance method for two-factor randomized block design, by SAS 9.1 Statistical Package Program. The LSD multiple comparison test was used to compare the averages. Tests were conducted at α= 0.05 significance level (Düzgüneş et al., 1987). Descriptive statistics, like mean and standard errors, were presented for the investigated features.

Results and Discussions

The results of the present investigation related to the impact of NP fertilizers and worm fertilizer treatments, mainly on growth and floral parameters are explained in this section. The effects of all fertilizer types on parameters were not found statistically significant. The data of the final observations of the various parameters during growth and flowering period were subjected to statistical analysis and the results have been presented through tables and suitable diagrams.

Table 2. *Effects of NP and worm fertilizers dosages on growth and floral parameters*

Treatment	first flowering time (day)	full flowering time (day)	harvest time (day)	leaf number (mm)	leaf length (mm)	leaf diameter (mm)	plant height (mm)	flower height (mm)	flower diameter (mm)	floret length (mm)	floret diameter (mm)	floret number	stalk thickness (mm)
Control	136.35	138.38	140.92	5.54	118.88	26.30	165.28	103.16	63.92	24.08	26.79	35.91	12.34
V1	135.25	136.54	137.70	5.71	128.28	27.63	174.38	115.83	64.63	25.33	28.14	32.14	13.50
V2	136.17	137.59	139.35	5.62	121.34	27.17	177.99	112.57	66.96	25.10	26.49	35.91	12.61
V3	135.67	137.11	138.67	5.41	126.03	26.52	178.36	116.33	60.72	26.25	25.69	33.80	12.79
NP1	135.90	137.38	139.55	5.38	127.52	28.31	175.02	115.17	64.48	25.21	26.48	37.69	13.35
NP2	135.34	136.61	138.33	5.54	127.18	26.46	176.09	114.24	66.94	26.76	26.90	36.64	12.89
NP3	136.15	137.73	138.55	5.66	123.27	28.86	179.54	111.39	61.80	25.42	26.17	34.37	12.62

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Phenological Observations

First flowering time (day)

The data regarding days to first flowering time are presented in *Table 2*. No significant difference was observed in the case of first floret opening of hyacinth. The first floret opening was the earliest (135.25 days) in V1 which was closely followed by V3 and NP2 (*Figure 3a*). The control plants took the maximum days (136.35) which was followed by other treatment for the first floret open. Sönmez et al. (2013) showed that the lowest number of days to primary candle formation were 116 days, the lowest number of days to mature flower formation were 117 days, the lowest number of days to harvest were 121 days, the lowest number of days to primary candle formation were 123 days, in the case of gladioli plant. Sharma et al. (2003) observed that on onion growth. In the study, flowering and bulb production attributes of tuberose were recorded that among the different nitrogen dosages minimum time for sprouting were 14.74 days, which was recorded with the application of 300 kg N/ha whereas maximum time for the sprouting of bulbs (21.57 days) was recorded in control. Early sprouting in tuberose with higher doses of nitrogen, phosphorus, and their interaction might be due to the stimulation of bulbs by comparatively high nutrient availability (N and P) and their respective absorption through bulbs surface and primary roots. Keisam et al. (2014) observed that first flowering time of gladioli was greatly influenced by different combinations of organic and inorganic fertilizers.

Full Flowering Time (day)

The data concerning the days taken to full flowering time, as influenced by different treatments were presented in *Table 2*. Statistically no significant difference was observed in the case of full flowering time (days) of hyacinth by different levels of NP and worm fertilizers. However, it can be revealed that days to the full flowering time was the earliest (136.54 days) in V1 which was closely followed NP2 and other treatments *(Figure 3b)*. Full flowering took the longest (138.38 days) for the control plant. However, Rajadurai and Beaulah (2000) also found that increasing levels of NPK fertilizers resulted in the full flowering of African marigold, which also brought about full flowering, contains essential plant nutrients like N, P, K, Ca, Fe, S, Mg, Zn, Mo, Cu, Mn, Co and B in a balanced amount in addition to organic fertilizers, which gave rise to full flowering.

Harvest time (day)

The data belonging to days taken to harvest time, as influenced by different treatments were presented in *Table 2* and *Figure 3c*. No significant difference was observed in the case of harvest time (day) of hyacinth by different levels of NP fertilizers and worm fertilizer. However, it can be revealed that days to the harvest time was the earliest (137.70 days) in V1 which was closely followed NP2 and other treatments. Harvest time took the longest time (140.92 days), for the control plants (*Figure 3c)*.

Similar results were also found by Chakkaborty et al. (2009). On the other hand, manure brought about the late flowering of marigold in 78.3 days i.e. 94.8 days later than in the case of worm fertilizer. The flowering period was also maximsed (up to 24.51 days) due to application of worm fertilizer. This could have arisen from differences in their

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source and nutrients composition as well as timing of nutrients availability to the flowering plants.

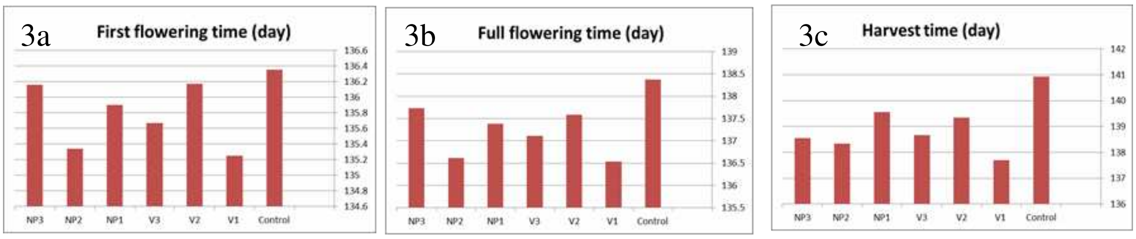

Figure 3. *Phenological properties a-First flowering, b-Full flowering, c-Harvest times (day)*

Morphological Properties

Leaf number

Leaf number was counted and no significant difference was found due to different levels of NP and worm fertilizer presented in *Table 2*. Leaf number varied from 5.41 to 5.71 per plant. The maximum number of leaf (5.71) was recorded with the V1 treatment. Results reveal *Figure 4a* that the increasing level of NP increasing the leaf number of hyacinth. And that is the increasing level of worm fertilizer decreased the leaves number of hyacinth. It was not a significant result because a maximum number of leaves was recorded in V1 and followed by other treatment. This result is in agreement with the findings of El-Desuki et al. (2006). They observed that nitrogen application had no significant effect on leaf number of onion. Also Sultana et al. (2015a) observed that number of leaf/plant of summer onion was found insignificant when the influence of integrated N from urea, cow dung, and worm fertilizer was compared. The highest number of leaves/plant (6.00) was observed after the treatment with 120 kg N/ha supplied from urea followed by other treatments. Alternatively, the minimum number of leaves (4.98) per plant was found in control plots, where no fertilizer was applied. Also Keisam et al. (2014) observed that leaf number of gladioli was greatly influenced by different combinations of organic and inorganic fertilizers. The highest number of leaves (5.60) was obtained from the mixture consisting of vermicompost (2.5 t ha^{-1}) + humic acid (0.2%) + Vesicular Arbuscular Mycorrhiza.

Leaf length (mm)

Leaf length of hyacinth varied from 118.88 mm to 128.28 mm over the treatments (*Table 2*). The leaf length (128.28) was recorded with V1 which was closely followed by NP1 and NP2 and V3. The minimum leaf length (118.88 mm) was produced from the control treatment. Results reveal (*Figure 4b*) that the increasing level of NP decreased the leaf height of hyacinth. However, the best performance was recorded in the leaf height with V1. On the other hand, Sönmez et al. (2013) observed that the highest leaf length (47.9 cm), the highest plant length (70.6 cm), and the highest leaf length 30.78 mm) was obtained in waste mushroom compost application, in the case of gladioli plant. Sultana et al. (2015b) observed that the results showed that the rate of effects within NPK fertilizers and worm fertilizer treatments were not found significant on leaf length of (*Zinnia elegans*) flowers. However, Keisam et al. (2014) observed that leaf length of gladioli was greatly influenced by different combinations of organic and inorganic fertilizers. The

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highest leaf length (50.03 cm) was obtained from the mixture consisting of vermicompost (2.5 t ha-1) + humic acid (0.2%) + Vesicular Arbuscular Mycorrhiza.

Leaf diameter (mm)

Leaf diameter of hyacinth varied from 26.30 mm to 28.86 mm over the treatments (*Table 2*). The widest leaf (28.86 mm) was recorded with NP3 which was followed by and statistically similar with NP1 and V1 and V2 *(Figure 4c)*. The narrowest leaf (26,30 mm) was produced from the control closely followed by other treatments. There was no significant difference. Sönmez et al. (2013) observed that the highest leaf length (47.9 cm), the highest plant length (70.6 cm). While the highest leaf diameter (30.78 mm) was obtained in waste mushroom compost application on gladioli plant. Keisam et al. (2014) observed that leaf width of gladioli was greatly influenced by different combinations of organic and inorganic fertilizers. The highest leaf width (8.62 cm) was determined on the mixture consisting of vermicompost (2.5 t ha-1) + humic acid (0.2%) + Vesicular Arbuscular Mycorrhiza.

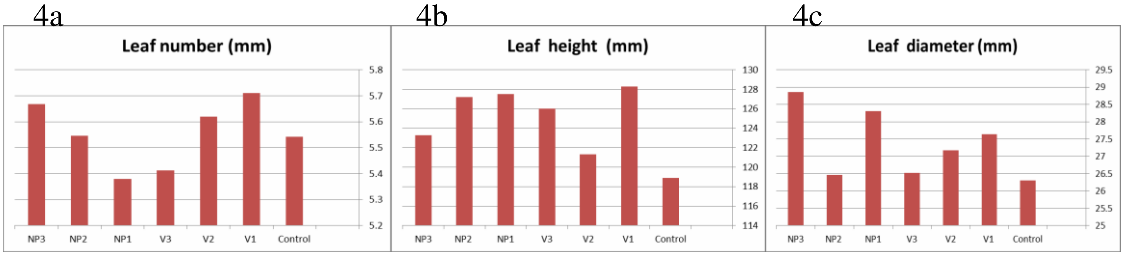

Figure 4. *Under treatments a-Leaf number, b-Leaf length, c-Leaf diameter (mm)*

Plant height (mm)

Plant height of hyacinth was measured on flowering. It was observed that plant height was not significantly influenced by different levels of NP and worm fertilizer application (*Table 2*). The tallest plant (179.54 mm) was recorded in NP3, which was followed by and statistically similar to other treatment. The shortest plant (165.28 mm) was found in control. No significant difference was found proving that the increasing level of NP are increasing the plant height of *H. orientalis* and that the increasing level of worm fertilizer increases the plant height of hyacinth (*Figure 5a*). El-Desuki et al. (2006) stated that plant height of onion increased with increasing rates of N up to 125 kg/ha and decreased thereafter. Also, Sultana et al. (2015b) observed that the impact of NPK fertilizers on the shoot heights of *Zinnia* plant was found similar with that of control. Shoot heights of plant increased with the rates of vermicompost application but the values obtained in the 10% and 20% were not significantly different.

Flower height (mm)

A noticeable variation in the flower height was recorded due to application of different levels of NP and worm fertilizer presented in *Table 2*. The longest flower (116.33 mm) was measured with V3, which was statistically similar with that of NP1, V1, and V2 and other treatment. The shortest flower (103.16 mm) was produced with control. Results revealed (*Figure 5b)* that the increasing level of NP decreased the flower height of hyacinth and that the increasing level of worm fertilizer increased the flower height of hyacinth. A similar result was found that soil application of fertilizer increased

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flowering branch length as compared to its foliar application and no-fertilization on gladioli plant (Mohammadi et al., 2014). Turkoglu et al. (2008) observed that flower height of narcissi plants as 18.38 mm.

Flower diameter (mm)

Flower diameter was not significantly influenced by different levels of NP and worm fertilizer supplement (*Table 2*). The highest flower diameter of 66.96 mm was found in plants grown under V2 which was closely followed by NP2, V1, NP1 and other treatments. The lowest flower diameter (60.72 mm) was recorded with the V3 closely flowed by control (*Figure 5c).* On the other hand, Mohammadi et al. (2014) found that soil application of fertilizer increased flower diameter compared to its foliar application and no-fertilization on gladioli plant.

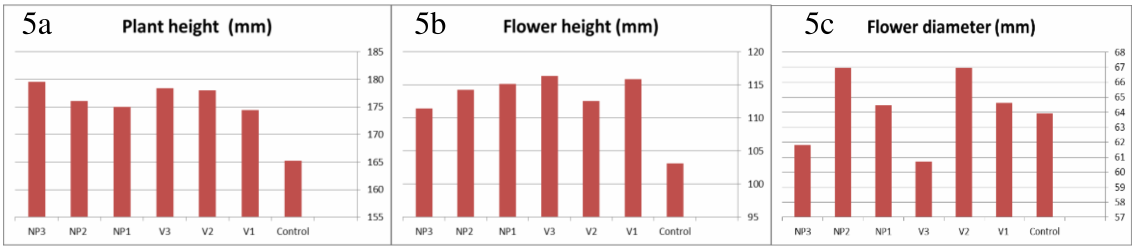

Figure 5. *Under treatments a-Plant height, b- Flower height, c- Flower diameter (mm)*

Floret length (mm)

Floret length of hyacinth was measured after full flowering time. It was observed that floret length was not significantly influenced by different levels of NP and worm fertilizer application (*Table 2*.). The highest floret length (26.76 mm) was recorded in NP2 which was followed by and statistically similar with other treatment without control, and the lowest floret length (24.08 mm) was found in control. Results revealed (*Figure 6a*) that the increasing level of NP2, increased the floret height but increasing level of NP3, decreased the floret length of *H. orientalis* and that the increasing level of V2, decreased the floret height but increasing level of V3, not significantly increased the floret height of hyacinth.

Floret diameter (mm)

Floret diameter was not significantly influenced by different levels of NP and worm fertilizer is presented in *Table 2*. The highest floret diameter (28.14 mm) was found in plants grown under V1, which was closely followed by other treatment except for V3. The lowest floret diameter (25.69 mm) was recorded with the V3. Results revealed (*Figure 6b)* that the increasing level of NP2, increased the floret diameter but increasing level of NP3, decreased floret diameter of hyacinth flower. And the increasing level of worm fertilizer do not increase the floret diameter of hyacinth.

Floret number/flower

Floret number was counted and no significant variation was found due to different levels of NP and worm fertilizer is presented in *Table 2*. Floret number varied from 32.14 to 37.69 per plant. The maximum number of floret (37.69) was recorded with the NP1. The lowest

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number of floret per plant was noted with the V1, (32.14) closely followed by NP3, V3 *(Figure 6c)*. Similar results were recorded by Sultana et al. (2015b). The rate of the effect of NP fertilizers was not found significant in the production of flowers of *Zinnia* plant. Also, Mohammadi et al. (2014) found that soil application of fertilizer increased flower number compared to its foliar application and no-fertilization in the case of gladioli. A similar result Senthilkumar et al. (2004) concluded that the appropriate ratio application of worm fertilizer resulted in higher bud number, flower number, shoot weight, root weight and plant height, which is in agreement with our study.

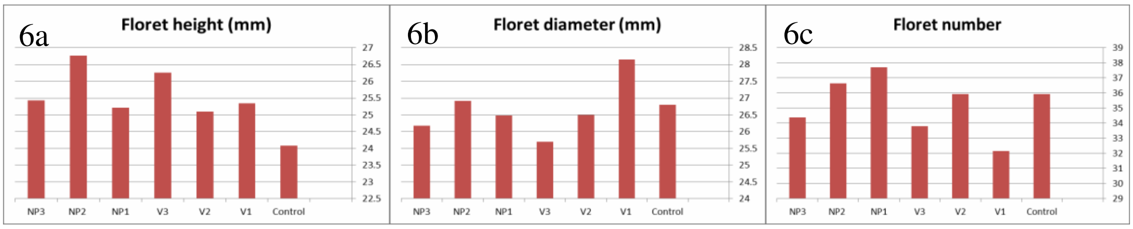

Figure 6. *Under treatments a-Floret height, b- Flower diameter (mm), c- Flower number*

Stalk thickness (mm)

Stalk thickness for hyacinth was not significantly influenced by different levels of NP and worm fertilizer supplement (*Table 2*). The highest stalk thickness of 13.50 mm was found in plants grown under V1. The lowest stalk thickness of 12.34 mm was recorded with the control and closely followed by other treatment except for V1, NP1. It is presented that the increasing level of NP decreased the stalk thickness of plants (*Figure 7*). Also, Turkoglu et al. (2008) observed 5.56 mm of stalk thickness in the case of narcissi plant. Padem and Alan (1995) observed that combined NPK applications may elevate the nitrogen, potassium and phosphorus contents and the increase in stalk length might be due to elevated levels of macronutrients which have a positive effect on floral characteristics of tomatoes.

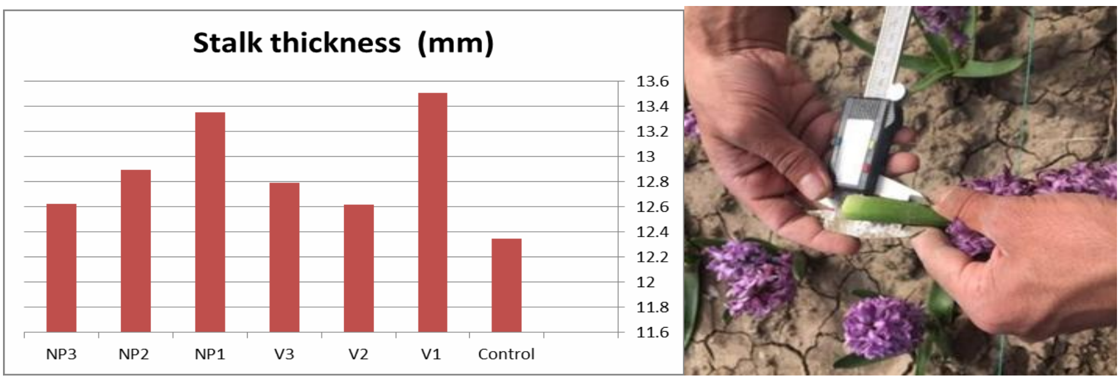

Figure 7. *Under treatments stalk thickness (mm)*

Conclusions

In this present study concerning the effects of dosages of worm fertilizer and nitrogen-phosphorus fertilizer on plant growth of hyacinth (*Hyacinthus orientalis* L. cv. “Purple Star”) in ecological conditions of Siirt was investigated. It is known that

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chemical fertilization has negative effects when it is used unconsciously and in excess, by mixing with soil and water. Since aesthetics are important in ornamental plants, it is considered that the best performance of the flower criteria is possible with fertilization and excessive chemical fertilization is carried out if necessary. However, the same effect can be made without harming the environment. In Siirt province there is no commercial production in terms of cut flowers, bulbous ornamental plants, indoor or outdoor ornamental plants. However, due to its climate structure, Siirt province is suitable for growing many ornamental plants. According to this study, worm fertilizer, which is new for Turkey not known or used in Siirt province yet, has positive effects on the flowering bulbous ornamental plants to encourage especially hyacinth growing. As a result, for some plant growing and flower criteria, worm fertilizer can be used instead of chemical fertilizer.

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BACILLUS SUBTILIS [EHRENBERG. (1835) COHN 1872] IMPROVES WATER USE EFFICIENCY IN MAIZE UNDER DROUGHT CONDITIONS AND TWO LIGHT INTENSITIES

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Abstract. The aim of this work was to evaluate the effects of *Bacillus subtilis* inoculation on the growth and the photosynthetic activity of maize plants under conditions of water stress in two regimes of light intensity. Plants were inoculated with two strains of *B. subtilis* directly onto the soil and maintained for 40 days in a growth chamber under controlled conditions. Two experiments were carried out under two conditions of light intensity (300 and 900 µmol m^{-2} s^{-1} photons). During the conduction of the plants, two water supply regimes based on evapotranspiration replacement were established. At 35 days of plant growth, the following physiological parameters were evaluated: photosynthetic rate, intercellular CO_2 concentration, stomatal conductance, transpiration and water use efficiency. Plants were collected (40 days) to evaluate the production of dry biomass. *B. subtilis* increased the water use efficiency in maize plants submitted to water stress under two light intensities. The maize gas exchange response to water stress and presence of rhizobacteria were differentiated for the two light conditions. The low light intensity condition provided lower stomatal conductance and CO_2 assimilation and greater efficiency in water use in plants inoculated with *Bacillus subtilis* (AP-3) under conditions of water deficit. The high intensity condition provided significant differences in the reduction of stomatal conductance in plants with stress compared to plants without stress when inoculated with *B. subtilis* PRBS-1.
Keywords: *rhizobacteria, gas exchange, Zea mays, abiotic stress, seed inoculation*

Introduction

The unpredictability of climate variability is one of the main sources of risk for agricultural activities (Hardaker et al., 1997). Most of the problems found in tropical crops are due to climatic causes: droughts, heavy rains and frost. For example, losses from drought reach up to 50% in some important tropical crops, such as bean, maize and soybean (Vivan et al., 2015).

Nowadays, plant growth-promoting rhizobacteria (PGPR) has been used as alternative for promoting plant resistance to environmental stresses, such as drought conditions. (Liu et al., 2013). Among the known PGPR, *Bacillus subtilis* [Ehrenberg. (1835) Cohn 1872] has received special attention because of its catabolic versatility and ability to colonize roots, as well as to induce the production of a large number of enzymes and metabolites that favor the growth of plants, under conditions of stresses (Mayaket al., 2004). Recently, Li et al. (2016) reported that *Bacillus subtilis* promoted an increase in the efficiency of water use under drought conditions by *Vicia faba*, and this effect was explained through the regulation of stomata during the stress period. Regarding this, Messina et al. (2015) reported that the impact of water deficit on maize yield can be mitigated by the management practices adopted and physiological characteristics of the hybrid used, reporting that the limitation of transpiration may also contribute to improving corn tolerance to drought.

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Also, it is known that *B. subtilis* produces phytohormones, such as auxins and gibberellins (Katz and Demain, 1977; Araujo et al., 2005) and these hormones could ameliorate the effect of stress in plants (Liu et al., 2013).

The metabolic alterations affecting plants under drought occur due to oxidative damage at the cellular level, which happens due to the unbalance between the formation of reactive oxygen species and their detoxification (Sandhya et al., 2010). There is a significant interaction between drought stress and the activity of antioxidant enzymes in plants. In this case, inoculation of rhizobacteria in plants could reduce the harmful effects by the activation of the antioxidant enzyme system (Han and Lee, 2005). Sandhya et al. (2010) found a decreased activity of the antioxidant enzymes in maize, under drought condition, inoculated with PGPR. In addition, C4 plants present high photosynthetic rate in response to optimal growth conditions. Although the factors limiting the photosynthesis have not yet been well known, Usuda et al. (1985) found that light intensity drives the increase on the production of enzymes related to photosynthesis.

When roots are sensitized by drought, they transmit chemical signals that regulate the stomatal mechanism and shoot growth (Davies and Zhang, 1991). These chemical signals are mainly composed of plant hormones, such as cytokinins, abscisic acid (ABA) and ethylene (Dodd, 2003). The production of indole-acetic acid and cytokinins induced by rhizobacteria would be an additional mechanism to attenuate the deleterious effects of water deficit in plants (Arkhipova et al., 2007). Specifically, inoculation of a cytokinin-producing strain of *B. subtilis* increased the growth of lettuce plants in water deficit soils (Arkhipova et al., 2007).

The hypothesis of this study is that *B. subtilis* could ameliorate the effect of drought on growth and photosynthesis in maize. Thus, this study evaluated the effects of *B. subtilis* inoculation on the growth and photosynthetic activity of maize under drought conditions and two regimes of light intensity.

Material and methods

Bacillus subtilis

Two strains of *Bacillus subtilis* (AP-3 and PRBS-2) were used in the experiments. They were isolated from soil under soybean cultivation and are characterized by Araujo et al. (2005). The strains were maintained in nutrient agar culture medium under refrigerated conditions.

Study site, treatments and experimental set-up

The study was conducted in laboratory of Unoeste, Presidente Prudente, São Paulo, Brazil. The experiments were carried out in a completely randomized arrangement, in a 3 × 2 factorial scheme (control and treatments with inoculation of two *B. subtilis* isolates in two irrigation regimes) and five replications, which totaled 30 plots. In the first experiment, plants were conducted under a light intensity regime of 900 µmol m^{-2} s^{-1} and in the second experiment plants were conducted at the intensity of 300 µmol m^{-2} s^{-1}. Both experiments were conducted for 40 days in a growth chamber and were maintained in the same photoperiod, temperature and humidity conditions.

The soil used in the experiments was collected from the 0-20 cm horizon and characterized as an acrisol (FAO, 1998), whose chemical characteristics showed the

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following values: pH ($CaCl_2$ 0.01 mol L^{-1}) 5.7; 3.9 mg dm^{-3} of P (Mehlich^{-1}); 2.2 mmolc dm^{-3} of K; 20.3 mmol dm^{-3} of Ca; 4.3 mmol dm^{-3} of Mg; 14.3 mmol dm^{-3} of H + Al; 151 mmole dm^{-3} of CEC and 65% of base saturation. Soil base saturation was increased to 80% (Raij et al., 1997) with application of dolomitic limestone. The soil was conditioned in plastic pots (2.5 kg), irrigated until field capacity and incubated for 60 days.

Corn seeds (*Zea mays* L. Hybrid SYN 7205) were inoculated with *B. subtilis* isolates at the time of sowing. The aqueous suspension containing each isolate at the concentration of 10^9 cells per mL was applied directly onto the seeds in the planting pit in the amount of 0.1 mL. After sowing and inoculation of the rhizobacteria, the pots were allocated into a plant growth chamber model FITOTRON® SGC 120 (Weiss Technik, UK) under temperature conditions of 28/18 °C day / night and cycle of 16/8 h day/night. Relative humidity was programmed to 60%. Initially, soil moisture was maintained at 100% field capacity. After 15 days of plant emergence, the water stress was induced in the pots by the replacement of only 30% of the evapotranspirated water, and the control plants received 100% replacement. The daily control of evapotranspiration was carried out using the gravimetric method (Catuchi, 2011). Soil moisture contents were monitored by humidity and temperature sensors (Prochek®, Decagon Devices).

CO_2 and H_2O gas-exchange

At 35 days of plant growth the physiological parameters related to photosynthesis were evaluated through an infrared gas analyzer (IRGA, model Li- 6400XTR, Li-Cor). Spot measurements were performed on 5 plants per treatment under photons saturating irradiance of 1,200 µmol m^{-2} s^{-1}. The following parameters were evaluated: liquid assimilation of CO_2 (A, mmol m^{-2} s^{-1}), stomatal conductance (gs, mmol m^{-2} s^{-1} H_2O), intercellular CO_2 concentration (Ci, ppm) and transpiration (E, mmol m^{-2} s^{-1} of H_2O). From these variables, the water use efficiency was calculated as A/E (WUE, mmol mol^{-1}).

Plant growth analysis

The plants were harvested 40 days after sowing, separating the root system and above-ground biomass to determine dry biomass. The shoot was separated from the root 2 cm above the soil level and washed in water containing mild detergent to remove soil. The quantification of biomass dry weight was performed in the laboratory after drying the material on forced ventilation oven (60-70 °C) to constant weight. The evaluation of the growth promotion was carried out by comparing the dry root and shoot masses of the inoculated plants and control, within each water replacement condition.

Data analysis

The data obtained as mean value of five replicates and significance was considered at the 95% confidence level. Normality test and ANOVA using SISVAR software (Ferreira, 2014) was performed to evaluate the effect of rhizobacteria at water stressed and non-stressed conditions and the Tukey test was used to compare the means.

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Results

ANOVA showed highly significant ($P \leq 0.05$) effects of treatments with *B. subtilis* in in most variables analyzed. It is also possible to emphasize the interactions found between aerial part and concentration of intercellular CO_2 in the two light intensity (*Table 1*).

Table 1. *ANOVA results of treatments into stress in H (high) and L (low) intensity with F values*

Sources	Shoot		Root		A		Ci		gs		E		WUE	
	H	L	H	L	H	L	H	L	H	L	H	L	H	L
Treatment	ns	*	*	*	ns	*	*	*	*	*	ns	*	*	*
Stress	*	*	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	*	ns
T x S	*	*	ns	ns	ns	ns	*	*	*	ns	ns	ns	*	ns

*P ≤ 0.05; ns: not significant

Plant growth analysis

The highest luminous intensity (900 µmol m^{-2} s^{-1}) promoted a reduction on shoot growth under drought condition, but did not alter root growth. Although rhizobacteria could ameliorate the drought stress, the inoculation of both strains of *B. subtilis* was not able to interfere significantly with the damages caused by drought in plant growth (*Table 2*). For root growth, *B. subtilis* (PRBS-1) reduced the root biomass in the non-stressed-plants, when compared to the non-inoculated control.

Table 2. *Shoot and root growth as a function of inoculation with B. subtilis in maize plants submitted to water stress under high light intensity*

Treatments	Shoot dry mass (g $plant^{-1}$)		Root dry mass (g $plant^{-1}$)	
	Without water stress	With water stress	Without water stress	With water stress
Non-inoculated	1.86±0.2 ^{aA}	1.48±0.2 ^{aB}	2.89±0.5^{a}	2.59±0.3 ^{a}
B. subtilis AP-3	1.99±0.3 ^{aA}	1.48±0.4 ^{aB}	2.27±0.5 ^{ab}	2.10±0.2 ^{a}
B. subtilis PRBS-1	2.04±0.3 ^{aA}	1.42±0.3 ^{aB}	1.86±0.3 ^{b}	2.50±0.4 ^{a}

Mean ± standard error. Values followed by distinct letters, lowercase in the columns and upper case in the lines, differ by Tukey test at 5%

In the second experiment under a lower luminous intensity (300 µmol m^{-2} s^{-1}), maize plants showed different responses regarding the presence of rhizobacteria than those found under the greater luminous intensity. It was found that plant inoculation with the *B. subtilis* (PRBS-1) resulted in increased shoot and root biomass production under drought condition (*Table 3*).

CO_2 and H_2O gas-exchange

Under high light intensity, maize did not show significant changes in photosynthetic parameters in non-inoculated plants under drought condition, but there was an increase in water use efficiency. Inoculation of maize with *B. subtilis* (PRBS-1) promoted a reduction in the rate of CO_2 assimilation and stomatal conductance when the plants

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underwent drought condition as compared to the non-stressed plants (*Table 4*). Under drought condition, plants inoculated with *B. subtilis* (AP-3) showed a significant reduction in intercellular CO_2 concentration. On the other hand, under normal condition, *B. subtilis* (AP-3) provided the highest rates of intercellular CO_2 than non-inoculated plants.

Maize plants inoculated with *B. subtilis* (AP-3) showed higher water use efficiency compared to non-inoculated plants, both under normal and drought condition (*Table 5*). However, the plant-water saving observed in the inoculated plants was not reflected in the improvement or maintenance of the physiological variables related to photosynthesis. It suggests that, under high luminous intensity, the economy of water may have been resulting of other effects promoted by the presence of PGPR.

Table 3. *Shoot and root growth as a function of inoculation with B. subtilis in maize plants submitted to water stress under low light intensity*

Treatments	Shoot dry mass (g plant^{-1})		Root dry mass (g plant^{-1})	
	Without water stress	With water stress	Without water stress	With water stress
Non-inoculated	2.21±0.09 ^{bA}	0.59±0.09 ^{bB}	0.93±0.18 ^{aA}	0.40±0.08 ^{bB}
B. subtilis AP-3	2.14±0.08 ^{bA}	0.70±0.06 ^{bB}	0.90±0.2 ^{aA}	0.47±0.1 ^{bB}
B. subtilis PRBS-1	2.37±0.06 ^{aA}	1.71±0.15 ^{aB}	0.98±0.25 ^{aA}	0.80±0.15 ^{aA}

Mean ± standard error. Values followed by distinct letters, lowercase in the columns and upper case in the lines, differ by Tukey test at 5%

Table 4. *Net assimilation of CO_2 (A), intercellular CO_2 concentration (Ci) and stomatal conductance (g_s), as a function of inoculation with B. subtilis in maize plants subjected to water stress under high light intensity*

Treatments	A (µmol CO_2 m^{-2} s^{-1})		Ci (ppm)		g_s (mmol H_2O m^{-2} s^{-1})	
	Without water stress	With water stress	Without water stress	With water stress	Without water stress	With water stress
Non-inoculated	11.7±1.8	13.1±1.7	207±22 ^{bA}	188±19 ^{aA}	82±8.9^{aA}	91±11^{aA}
B. subtilis AP-3	11.4±1.9	13.8±2.0	276±28 ^{aA}	214±25 ^{aB}	95±7.3^{aA}	85±9.0^{aA}
B. subtilis PRBS-1	15.0±2.3	10.6±1.8	225±27 ^{abA}	238±29 ^{aA}	103±12^{aA}	70±8.5^{aB}

Mean ± standard error. Values followed by distinct letters, lowercase in the columns and upper case in the lines, differ by Tukey test at 5%

Table 5. *Transpiration rate (E) and water use efficiency (WUE) as a function of inoculation with B. subtilis in maize plants submitted to water stress under high light intensity*

Treatments	E (mmol H_2O m^{-2} s^{-1})		WUE (mol CO_2 mol H_2O^{-1})	
	Without water stress	With water stress	Without water stress	With water stress
Non-inoculated	2.0±0.3	2.2±0.5	4.5±0.5 ^{cB}	5.9±0.3 ^{bA}
B. subtilis AP-3	2.1±0.4	2.0±0.6	5.5±0.4 ^{bB}	6.6±0.3 ^{aA}
B. subtilis PRBS-1	2.3±0.3	1.8±0.4	6.4±0.4 ^{aA}	5.6±0.2 ^{bA}

Mean ± standard error. Values followed by distinct letters, lowercase in the columns and upper case in the lines, differ by Tukey test at 5%

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With lower luminous intensity (300 µmol m^{-2} s^{-1}) the assimilation and intercellular concentration of CO_2 were reduced in the non-inoculated plants under drought condition. In the absence of drought, plants inoculated with *B. subtilis* (PRBS-1) showed a lower photosynthetic rate as compared to the non-inoculated plants; this condition could be explained by the decrease in stomatal conductance (*Table 6*). However, under drought condition, inoculation of plants with both *B. subtilis* (AP-3 and PRBS-1) did not result in photosynthesis alteration; although plants inoculated with the *B. subtilis* (PRBS-1) have shown higher intercellular CO_2 concentration and lesser stomatal conductance.

Inoculation with *B. subtilis* promoted a lesser transpiration in plants under drought condition. It means that inoculated plants presented lower water losses, as a direct consequence of the stomatal conductance reduction. However, this reduction of stomatal conductance in inoculated plants was not sufficient to decrease the photosynthetic activity. This fact may have contributed to the improvement of the inoculated plants' performance in terms of water use efficiency (*Table 7*), both under normal and drought conditions.

Table 6. *Net assimilation of CO_2 (A), intercellular CO_2 concentration (Ci) and stomatal conductance (g_s), as a function of inoculation with B. subtilis in maize plants subjected to water stress under low light intensity*

Treatments	**A (µmol CO_2 m^{-2} s^{-1})**		**Ci (ppm)**		**g_s (mmol H_2O m^{-1} s^{-1})**	
	Without water stress	**With water stress**	**Without water stress**	**With water stress**	**Without water stress**	**With water stress**
Non- inoculated	4.91±0.4^{a}	4.05±0.3 ^{a}	308±54 ^{aA}	171±35 ^{bB}	39±2.6 ^{a}	36± 2.1 ^{a}
B. subtilis AP-3	4.00±0.3 ^{ab}	3.76±0.4 ^{a}	284±32 ^{aA}	216±48 ^{abA}	30±2.1 ^{b}	28±3.0 ^{b}
B. subtilis PRBS-1	3.15±0.3 ^{b}	3.52±0.2 ^{a}	238±42 ^{aA}	258±42 ^{aA}	26±1.9 ^{b}	25±1.7 ^{b}

Mean ± standard error. Values followed by distinct letters, lowercase in the columns and upper case in the lines, differ by Tukey test at 5%

Table 7. *Transpiration rate (E) and water use efficiency (WUE) as a function of inoculation with B. subtilis in maize plants submitted to water stress under low light intensity*

Treatments	**E (mmol H_2O m^{-2} s^{-1})**		**WUE (mol CO_2 mol H_2O^{-1})**	
	Without water stress	**With water stress**	**Without water stress**	**With water stress**
Non-inoculated	0.40±0.09 ^{a}	0.47±0.11 ^{a}	8.2±1.1 ^{b}	10.1±0.9 ^{c}
B. subtilis AP-3	0.36±0.08 ^{ab}	0.30±0.05 ^{b}	13.9±1.9 ^{a}	12.6±1.2 ^{a}
B. subtilis PRBS-1	0.25±0.05 ^{b}	0.29±0.06 ^{b}	10.7±1.3 ^{a}	12.3±1.1 ^{b}

Mean ± standard error. Values followed by distinct letters differ by Tukey test at 5%

Comparing the behavior of the plants inoculated with *B. subtilis* (AP3) in relation to the non-inoculated plants, it was observed that in the condition of low light intensity there were larger changes in the photosynthetic parameters of the inoculated plants (*Fig. 1*).

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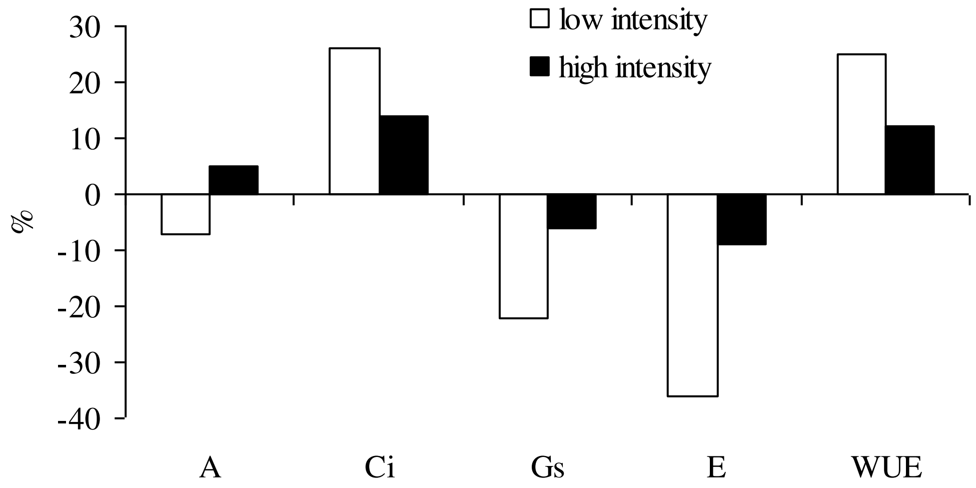

Figure 1. *Percentage variations found in photosynthetic parameters in corn (A = transpiration; Ci = intercellular CO_2 concentration; Gs = stomatal conductance; E = Net assimilation of CO_2 and WUE = water use efficiency) In the comparison of plants in the treatment with B. subtilis (AP-3) with those of the control treatment under water stress and two light intensities: low (300 µmol m^{-2} s^{-1}); high (900 µmol m^{-2} s^{-1})*

Discussion

Net CO_2 assimilation rate (A) increased with the increase of light intensity but did not correlate with increased conversion of CO_2 to carbohydrates (Sun et al., 2012). In our study it was verified that the plants under high luminous intensity assimilated more than twice CO_2 and presented greater stomatal conductance compared to the plants under low light intensity. However, the concentration of intercellular CO_2 (Ci) was similar in both conditions. One of the primary effects of drought condition in plants is related to the partial or total closure of the stomata (Lopes and Lima, 2015). However, other studies have shown that photosynthesis is not only regulated by the CO_2 diffusion through stomata, and that other non-stomatal factors can affect photosynthetic activity in response to stressful environments (Becker and Fock, 1986; Yuet et al., 2009). Becker and Fock (1986) reported the reduction of CO_2 assimilation, but did not observe changes in intercellular CO_2 (Ci), in plants under drought condition. However, these authors found a marked reduction in the activity of important enzymes in the photosynthetic process.

Confirming hypothesis, the results found in our study suggest that *B. subtilis* can ameliorate the deleterious effects of drought stress, by interfering with the gas flow related to photosynthesis, and water status of the plants. However, this activity was affected by light intensity, because in the low light intensity there were significant changes in few parameters evaluated, including plant growth. Based on the interactions found by ANOVA (*Table 1*), it was observed that the high light intensity condition provided stress interactions with the treatments in the stomatal conductance and water use efficiency parameters, which was absent in the low light intensity condition.

Li et al. (2016) concluded that leaf application of *B. subtilis* in beans (*Phaseolus vulgaris*) improved the efficiency of water use, mainly by the closure of the stomata. These same authors also reported that the restriction of photosynthesis can be attributed to the reduction of the atmospheric CO_2 input caused by the stomatal limitation induced by the presence of the rhizobacteria. *B. subtilis* (PRBS-1) inoculation in maize resulted

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in lower stomatal conductance when plants were subjected to water stress conditions. The partial reduction of stomatal conductance proportionally limits transpiration more than CO_2 entry into leaves (Chaves and Oliveira, 2004). Our results corroborate this findings, since in the condition of lower luminous intensity, the effects of rhizobacteria inoculation occurred by the reduction of transpiration under drought condition. However, the reduction of transpiration was not followed by a reduction in photosynthesis, which resulted in higher water use efficiency (WUE). The observed increase in intercellular CO_2 concentration as an effect of the PRBS-1 isolate under water stress conditions explain the absence of reduction in the photosynthetic activity of the inoculated plants. Previous studies also indicated that the accumulation of intercellular CO_2 acts as a defense mechanism against water deficit, since carbon and water retention in foliar tissue are observed with the increase of the WUE (Blum, 2005; Tardieu, 2012).

Liu et al. (2013) concluded that the increase in the growth of *Platycladus orientalis* plants when inoculated with *B. subtilis* under drought conditions, results from the effect of increased cytokinin concentration in the leaves. It is also noteworthy that the *B. subtilis* isolates used in this study have already been characterized as phytohormone producers (Araujo et al., 2005). However, it is well known that when plants are stimulated to close the stomata in stressful situations, there may be an improvement in their efficiency in the use of water (Davies et al., 2002). Our results showed higher values of water use efficiency in *B. subtilis* inoculated plants, besides increased plant growth when plants were submitted to water stress in lower light intensity conditions, when compared to non-inoculated plants. However, the interaction with water stress only occurred with the condition of high luminous intensity.

In the analysis of the parameters related to the gas exchange, for both water regimes it was verified that in the condition of lower light intensity, the plants were more responsive to the rhizobacteria inoculation, mainly in the condition of water stress. In *Figure 1* it can be verified that the isolate AP-3 of *B. subtilis* provided better photosynthetic performances in the plant when it was cultivated in the condition of lower luminosity. Under a lower luminous intensity, there was a reduction in stomatal conductance and consequently lower water loss due to transpiration as the effects of *B. subtilis* plant inoculation. The reduction of transpiration in plants inoculated by rhizobacteria has been reported by Bresson et al. (2013) as a mechanism supporting plant tolerance to drought. Messina et al. (2015) concluded that maize hybrids with limited transpiration characteristics would be more suitable for cultivation in regions with a higher prevalence of dry periods. In this respect, it was observed that *B. subtilis* (PRBS-1) reduced the transpiration of maize plants even in the absence of water deficit, and on the other hand, promoted a significant increase of plant dry mass (*Table 6*). However, when the plants were conducted under higher luminous intensity, the transpiration was not influenced by water stress or rhizobacteria inoculation.

Few studies compare the photosynthetic activity of corn in different conditions of light flux, under water stress. It is known that C4 plants have great potential for acclimatization to environmental changes and that too much light absorption can cause serious problems, which is why special mechanisms protect the photosynthetic system from excessive light (xanthophyll cycle). The multiple levels of photosynthesis control enable plants to successfully grow in a changing environment with different habitats (Taiz and Zeiger, 2004). In a study with maize growth in two light conditions under direct sunlight (2000 $\mu mol\ m^{-2}\ s^{-1}$) and in the growth chamber (400 $\mu mol\ m^{-2}\ s^{-1}$), plants

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grew 2.3 times more at the lowest luminous density (Usuda et al., 1985). In our study, the plants in conditions of low light density had lower photosynthetic rates and also had decreased shoot growth. Mojses and Kalapos (2008) in a study with luminous intensity variation in invasive and non-invasive C4 plants verified differences and the adaptation potential of these plants to semiarid conditions, represented by the lower luminous intensity, where there was a greater reduction of gas exchange by stomata and an increase in water use efficiency. The condition of lower light intensity in corn and inoculation of *B. subtilis* (AP-3) also resulted in greater closure of the stomata and increase in water use efficiency, comparing the non-inoculated plants (*Fig. 1*). In this sense, maize cultivation in tropical regions, at times of the year with less light radiation, such as winter, can provide better responses to inoculation of rhizobacteria in order to mitigate the effects of drought.

Several studies have already presented the potential of the *B. subtilis* rhizobacterium as a plant growth promoter, highlighting the production of antibiotics in the control of pathogens (Araujo et al., 2005); production of growth regulators (Molla et al., 2001) and nutrient solubilization (Kim et al., 1998), among others. In addition to these benefits previously presented in other studies, we can indicate that this species demonstrates potential to reduce the damages caused by water stress in agricultural crops, confirming what has already been reported by Li et al. (2016), who concluded that *B. subtilis* acts as a regulator of photosynthesis and promotes improvements in the efficiency of water use. However, light intensity can influence this regulation by factors not evaluated in this study. In this sense, our findings on the effects of the inoculation of *B. subtilis* on maize growth, in water deficit conditions, may foster new studies about this mechanism of action with a focus on field experiments.

Conclusion

In conclusion, the inoculation of *B. subtilis* increased the water use efficiency in maize plants subjected to water stress, under two light intensities. The maize gas exchange response to water stress and presence of rhizobacteria were differentiated for the two light conditions. The low light intensity condition provided lower stomatal conductance and greater efficiency in water use, in plants inoculated with *Bacillus subtilis* AP-3 under conditions of water deficit. The high intensity condition provided significant differences in the reduction of stomatal conductance in plants with stress compared to plants without stress when inoculated with *B. subtilis* PRBS-1.

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THE USAGE OF ARTIFICIAL NEURAL NETWORKS IN MICROBIAL WATER QUALITY MODELING: A CASE STUDY FROM THE LAKE İZNİK

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Abstract. The aim of this study was to develop faecal pollution model structures with artificial neural networks (ANNs) for cost-effective lake water quality management studies. In this study 5 artificial neural networks model structures were applied to predict the Faecal coliform concentrations for 4 different coast areas "Göllüce, İnciraltı, Darka, Orhangazi" and all data of the coasts in Lake İznik-Turkey. The Levenberg–Marquardt and backpropagation algorithm was proposed for feed-forward neural networks training. According to performance functions root mean squared error (RMSE), neural network model structures provided acceptable results. Correlation values (R) were found between 0.590 and 0.999. Increasing the number of hidden layer in the model structures was not raised the model efficiency in each trial. Type and number of input parameters were more effective for some model efficiency. Increasing the number of hidden layer and inputs in the model structures did not raise the model efficiency in each trial. Because depending on the numbers and chemical compositions of the substrates in the lake water microorganism's metabolism and their growth rates could be influenced differently and the larger error values of the modeling results determined in Göllüce and Orhangazi Coasts which influenced by pollution sources. Water quality modeling studies and increasing of monitoring would provide more productive results for protection and management of coastal.
Keywords: *faecal pollution, mathematical modeling, deep lake, water management, Turkey*

Introduction

One of the main elements that compose the rich biodiversity in the world and Turkey is wetlands. While wetlands of different qualifications increase the ecosystem diversity on one hand, the species using these wetlands raise the species' and genetic diversity on the other hand (Karadeniz et al., 2009). However, wetlands, especially lakes, are considered to be potential agriculture-residential-industrial areas in the development plans for years because of their high values. Wetlands are polluted by domestic, industrial and agricultural pollutants, and thus their biological diversities are destroyed (Oktem et al., 2012).

Contaminations of the environment with pathogen, hazardous and toxic chemicals are the major problems facing the industrialized nations and urbanized regions today. Microbial pollution in coastal and wetland areas is quite important for human and other species (Cordier et al., 2014). Particularly, pathogens, which include bacteria, viruses and protozoans, can come from a variety of point and nonpoint sources. For over a century, bacterial indicator organisms have been used in the USA to assess the presence of faecal contamination, and consequently pathogens, in drinking and bathing waters (NRC, 2004). Commonly used indicators are total coliform, faecal coliform, faecal streptococci, Escherichia coli and Enterococcus (Mas and Ahlfeld, 2009).

Artificial Neural Networks studies starting in 1940s are performed in hydrology, meteorology, ecology and water quality fields (Morid et al., 2007; Ranković et al.,

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2010; Kim and Valdes, 2003; Partal and Kişi, 2007; Reimer and Sodoudi, 2004; Mishra and Desai, 2006; Belayneh and Adamowski, 2012). Several lake water quality studies were related to especially phytoplankton production, algal blooms, dissolved oxygen and other spatial and temporal variability of limnological properties and eutrophication (Soyupak et al., 2003; Karul et al., 2000). Microbial contaminants are non-conservative, irregularly distributed and may even increase in concentration due to growth in the environment. The interrelationship and interactions between microbial communities in water creates additional modeling challenges that have been overcome by applications of ANNs to multi-parameter databases. Important and meaningful results were obtained with modeling of pathogens with ANN (Neelakantan et al., 2002; Brion and Lingireddy, 2003; Mas and Ahlfeld et al., 2007; Ogwueleka and Ogwueleka, 2010; WHO, 2010; Wu et al., 2014).

The purposes of this study were to design and develop ANN model structures for prediction of fecal coliform in order to evaluate microbial contamination and to predict the pollution impact on bathing, agricultural, aquaculture, recreation and tourism areas of Lake İznik as this has national and international importance, and also to observe the differences among the modeling results in the lake coasts occurring due to sewage inputs or nonpoint sources according to relevant environmental conditions. This study would be supportive of data management and could be the basis for basin and coastal management and the early warning system studies for public health. Also, this study was important with regards to international environmental scientific developments because the feed forward neural network (FNN) models could supply cost-effective environmental management tools for the investigation of any monitoring stations and pathogenic pollution.

Materials and methods

Study area and data

Lake İznik, the largest lake in the Marmara region and the fifth largest in Turkey, is located between the districts of İznik and Orhangazi in the Province of Bursa. It is located between 40°23′ and 40°30′N latitudes and 29°20′ and 29°42′E longitudes. The lake, which is located 85 m above sea level, has a length of 32 km and a maximum width of 12 km. It is about 15-16 km from the Bay of Gemlik. It has a surface area of 310 km^2 and a water volume of 12.2 billion m^3. The maximum depth of the lake (80 m) has been measured near Karacakaya (Ozturk et al., 2005; Yağcı and Ustaoğlu, 2012). It has 1 outlet, Karsak stream, and 5 inlets, Orhangazi, Kuru, Karasu, Ekinlik and Söloz streams. Karsak stream connects the lake to the Sea of Marmara but it has a number of natural and artificial barriers that marine fishes are unable to cross (Özuluğ, et., al., 2005).

Lake İznik, one of the 76 wetlands of international importance in Turkey, is a natural protected (NP) wetland according to the criteria of The Convention on Wetlands of International Importance (The Ramsar Convention) (RAMSAR, 2007). The ecosystem of Lake İznik was declared to be a natural protected area in 1990 and conforms to the international criteria with its characteristics (BGPEFD, 2008) and The Lake İznik and İznik basin had been an area of settlement throughout the history. The archeological findings in the tumulus in İznik depression suggest that human settlement started 7150 years ago. İznik town later became an important cultural center during the Roman, Byzantine and Ottoman empires (Ülgen et al., 2012).

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Moreover, Lake İznik is a significant bird watching area in Turkey; there are eight host and five stationary bird species, 13 in total, in this area (Akpınar et al., 2010). Lake İznik is a significant water resource not only with its water capacity but also in terms of agriculture, industry, water productions and recreational activities for the region (Başar et al., 2004). Therefore pollution monitoring and preservation of the Lake are important nationally and internationally.

Point and nonpoint (diffuse) pollution sources existed in the watershed, which discharge pollutants into the lake. The pollution loading sources of Lake İznik are agricultural, animal, sewage, forest, aerial (Akkoyunlu et al., 2011) and industrial (Oktem et al., 2012). Partially treated and untreated sewage and nonpoint pollution of fertilizers and pesticides from surrounding agricultural areas reached the lake (Akçaalan et al., 2014; Albay and Aykulu, 2002). Lake water is also used for irrigation and the lake is a popular place for recreation during summer months. Around the lake, especially in Orhangazi region in the west banks of the lake, the planless misuse of 1st class farming lands (open to settlement), which are appropriate for irrigation farming, puts the future of the Lake İznik in danger (Meşeli, 2010).

It is a well-known fact that the pollution in Lake İznik increases proportionately to the population growth. The common use of the septic tanks in Orhangazi and İznik around the lake causes the pollution of the lake to increase. Also there are 45 villages were around the lake and pollution loads of their domestic waste water affects the lake. But a certain amount of the used water (70-90% in general) is thought to return to the sewages (Oktem et al., 2012). Moreover, Lake İznik is polluted by the waste water of the Orhangazi Industrial Area, the tankages of İznik and Osmangazi, Marmara Birlik Olive Processing Plants in İznik, and by Ispak industrial plants in Orhangazi. Moreover, the lake was under threat because of the small olive oil plants around it and chemistry, automotive, metal and food industry sectors were present in the lake basin (WWF, 2011). Industries using the water of the lake in processing goods discharge their waste water to the lake without sufficient purification. Phosphorus in the industrial pollution was determined to be 3 times higher than that in the domestic waste water; and nitrogen in the industrial pollution was determined to be 1.5 times higher than that in the domestic waste water. In addition Istanbul-Bursa highway passes through Orhangazi on the west coasts of the Lake İznik and this heavy traffic caused air pollution (Oktem et al., 2012).

Several studies investigated the extent of anthropogenic pollution, such as nutrients, lead, and polycyclic aromatic hydrocarbons (PAH) in the sediment of the recent past and identified differences in the spatial distribution and sedimentation history (Franz et al., 2006; Ünlü et al., 2010; Viehberg et al., 2012). The spatial distribution of Pb indicating anthropogenic pollution sources coincides with elevated concentration in the delta area of the Sölöz stream which feeding of the Lake İznik. Interestingly, Ünlü et al. (2010) identified high concentrations of polycyclic aromatic hydrocarbons in the same area, thus supporting human-induced contamination (Viehberg et al., 2012).

Samples were taken from 4 different stations from 2010 to 2015. Sampling stations and regions were given in *Table 1* and features of the sampling stations were as follows:

Göllüce Village is located in the district limits of İznik sub-province. It is on the main road interconnecting with Gemlik and İznik sub-provinces. There are aggregated reeds at the entrance to the lakeside public beach. İnciraltı public beach is nearby the İznik sub-province. The Darka area is a public beach in front the Darka Holiday Village. Orhangazi Area is near the outlet of the Lake and Orhangazi sub-province. All

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sampling stations and regions are in the swimming areas. Location of Lake İznik and sampling stations were shown in *Figure 1*.

Table 1. *Sampling stations and region*

Station no.	Sampling regions	Number of data	Coordinates
1	Göllüce area	27	40°22'56.49"N, 29°35'36.73"E
2	İnciraltı area	27	40°25'46.64"N, 29°42'45.24"E
3	Darka area	27	40°24'37.25"N, 29°42'12.04"E
4	Orhangazi area	27	40°28'50.37"N, 29°20'48.76"E
	Total data	108	

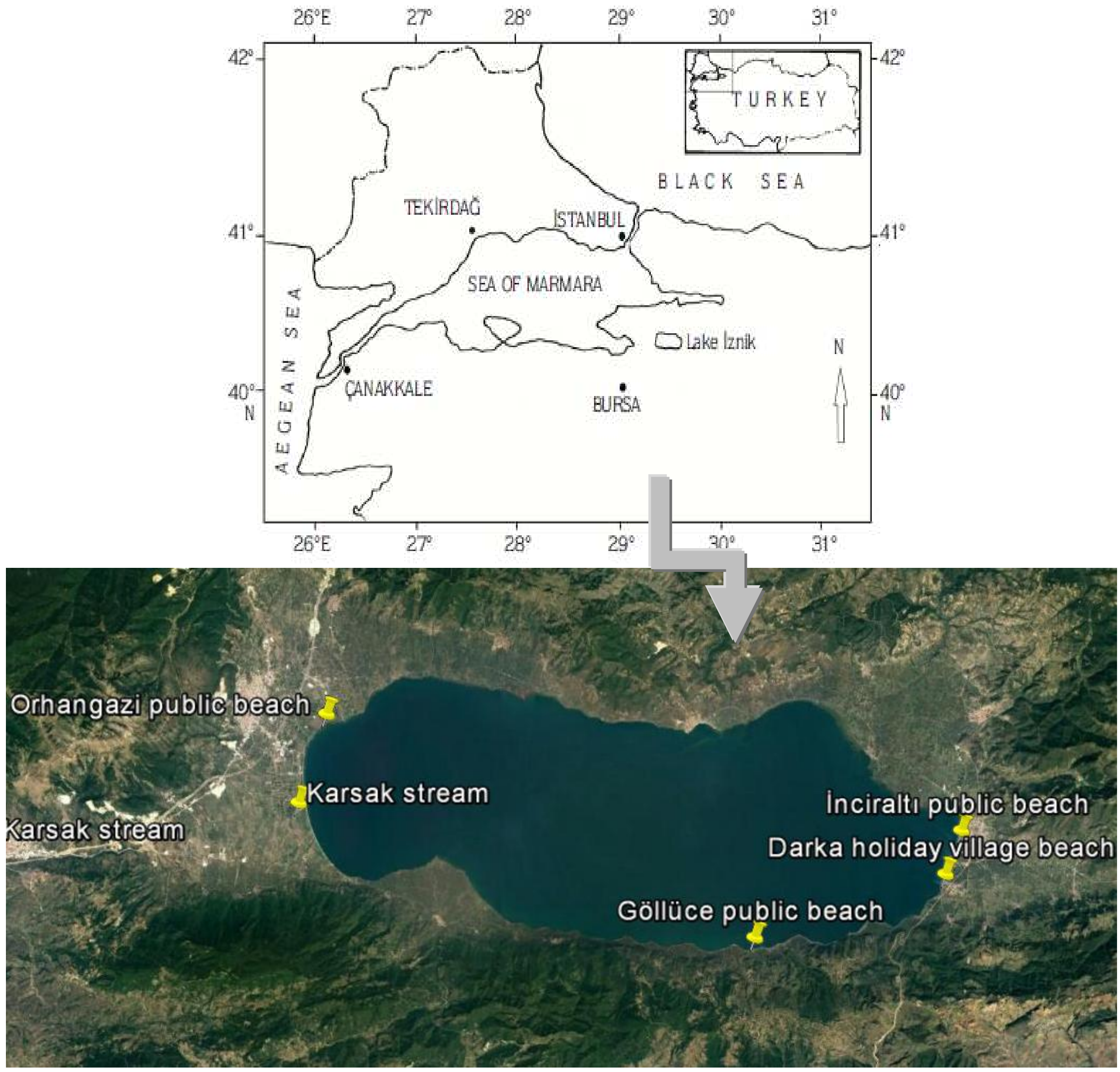

Figure 1. *Location of Lake İznik and sampling stations (Ozuluğ et al., 2005 and Google Earth, 2016)*

In this study, the microbiological pollution levels of the beaches in Lake İznik were examined with using the data obtained from the Bursa Provincial Directorate of Public

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Health. Total coliform, fecal coliform and fecal streptococci parameters were evaluated and modeled. The samples collected for measurements were taken from 30 cm below of the surface and put into the polyethylene (PE) single use bottles of 500 mL volume, kept in the thermo isolated boxes, having cooling bars at +4 °C, and brought to the lab in 24 h at the most. Bacterial count were performed by membrane filtration (MF) method and determined as CFU/100 mL (APHA, 1992; YSKY, 2006).

ANN model and training algorithm

Artificial neural networks are formed of a set of simple elements, the alleged artificial neurons. These elements are inspired by biological nervous systems. Models of neural networks are separated into two categories: feed forward neural networks and recurrent neural networks. Feed forward neural networks propagate data linearly from input to output and they are the most popular and most widely used models in many practical applications. (Hornik, 1991) Showed the feed forward neural network (FNN) with as few as a single hidden layer and arbitrary bounded and smooth activation functions can approximate a continuous nonlinear function (Şen, 2004). The multilayer FNN represented in *Figure 2* (Rankovic et al., 2010; Efe and Kaynak, 2002; Okkan and Mollamahmutoğlu, 2010).

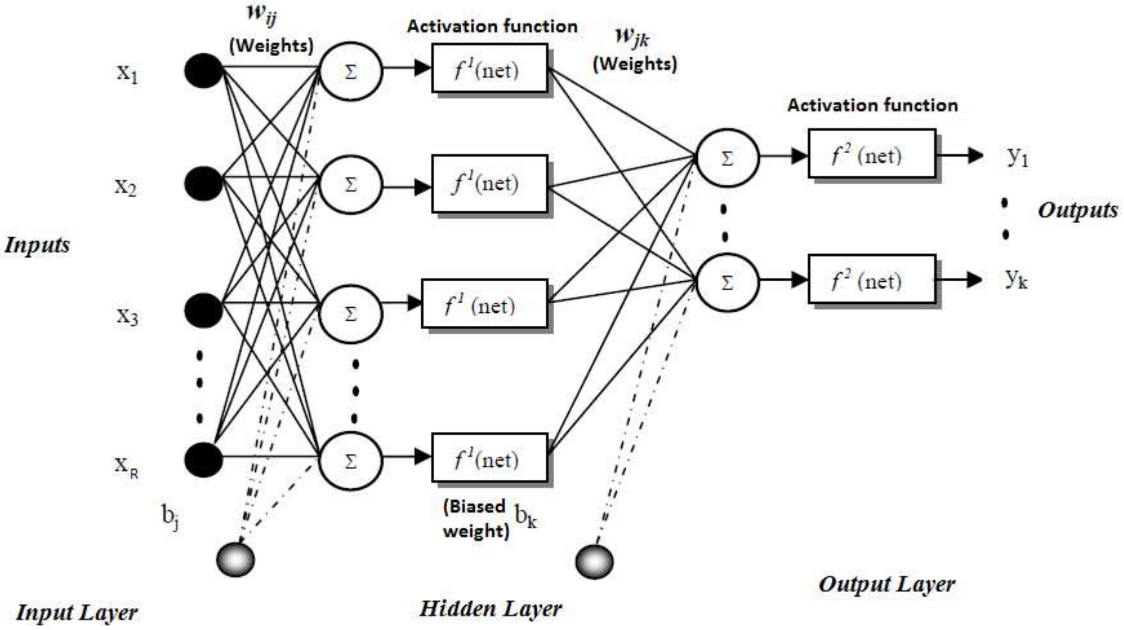

Figure 2. *Multi layer feed forward neural network*

As shown in *Figure 2,* the elements constituting ANN are input layer, hidden layers (number of hidden layers are one or might be more), output layer linkages between the layers and linkage weights.

The data of the problem is in the input and output layers. Productivity and importance of information in the input layer are provided with the weights. Net (basis) function expresses that productivity of input data to neuron. For an analytical study, the connection networks are mathematically represented by a basis function u (w, x). The

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net value is a linear combination of the inputs and the output of a neuron can be expressed as *Equation 1:*

$$out = f(n) \tag{Eq.1}$$

where (*Eq. 2*)

$$n = \sum_{j=1}^{R} w_j x_j + b \tag{Eq.2}$$

$x_1, x_2, \ldots, x_R$ are the input signals; $w_1, w_2, \ldots, w_R$ are the weights of neuron; b is bias value; and $f(\cdot)$ is the activation function.

Activation function is a function determining the neuron output with processing the net inputs obtained from net function. The linear and sigmoid are common used activation functions in the construction of artificial neural networks.

An example of the sigmoid is the logistic function, defined by *Equation 1:*

$$f(n) = \frac{1}{1+e^{-n}} \tag{Eq.3}$$

Also, as sigmoid function can be used hyperbolic tangent function and it gives the outputs range between [0-1] (*Eq. 2;* Haykin, 1999):

$$f(n) = \frac{1-e^{-n}}{1+e^{-n}} \tag{Eq.4}$$

The inputs $x_1, x_2, \ldots, x_R$ are multiplied by weights $w_{i,j(1)}$ and summed at each hidden neuron i. Then the summed signal (*Eq. 5*):

$$n_{i(1)} = \sum_{j=1}^{R} w_{i,j(1)} x_J + b_{i(1)} \tag{Eq.5}$$

The node activates a nonlinear function $f_{(n)}$. The output y at a linear output node can be calculated as *Equation 6:*

$$y = \sum_{i=1}^{z} w_{1,i(2)} \frac{1-e^{-\left(\sum_{i=1}^{R} x_j w_{i,j(1)} + b_{i(1)}\right)}}{1-e^{-\left(\sum_{i=1}^{R} x_j w_{i,j(1)} + b_{i(1)}\right)}} + b_{1(2)} \tag{Eq.6}$$

where R is the number of inputs, z is the number of hidden neurons, wi.j(1) is the first layer weight between the input j and the i^{th} hidden neuron, w1,i(2) is the second layer weight between the i^{th} hidden neuron and output neuron, bi(1) is a biased weight for the ith hidden neuron and b1(2) is a biased weight for the output neuron (Rankovic et al., 2010).

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The learning algorithms using in ANN are heuristics, partial Newton methods, matched gradient methods and Levenberg Marquardt methods. In this study feed forward neural network structure was used and Levenberg Marquardt training algorithm supervised training algorithms was preferred for training. Pupose Levenberg Marquardt algorithm is the least squares calculation method based on maximum neighborly idea (Hagan and Menhaj, 1994). The sum of squared errors of any one of the element in the training set (N number of elements) could be calculated as follows (*Eqs. 7* and *8*)*:*

$$E(w) = \frac{1}{2}\sum_{i=1}^{N}(y_{mi} - y_i)^2 = \frac{1}{2}(y_m - y)^T(y_m - y) \tag{Eq.7}$$

$$w = [w_{1,1(1)}, w_{1,2(1)}, \dots, w_{z,R(1)}, b_{1(1)}, b_{2(1)}, \dots, b_{z(1)}, w_{1,1(2)}, w_{1,2(2)}, \dots, w_{1,z(2)}, b_{1(2)}]^T \tag{Eq.8}$$

Two number of different hidden layer neuron for each input neurons was tested in this study. This was because; in the recent study were two different approaches used in determining the number of hidden layer neurons. The first approach for a network model which in the N number of neuron in the input layer: (N + 1) / 2 and the second approach: (2 * N + 1) were introduced (Hagan and Menhaj, 1994; Oğuztürk, 2010).

Performance and sensitivity analysis of ANN

The performance of the forecasts from the data-driven models was evaluated. The Pearson correlation coefficient is one of the most commonly used performance in selecting proper inputs for the ANN (Rankovic et al., 2010). Correlation coefficient is described as the degree of correlation between the empirical and modeled values (*Eq. 9*):

$$r = \frac{\sum_{i=1}^{N_0}(y_i - \bar{y})(y_{mi} - \bar{y}_m)}{\sqrt{\sum_{i=1}^{N_0}(y_i - \bar{y})^2 \sum_{i=1}^{N_0}(y_{mi} - \bar{y}_m)^2}} \tag{Eq.9}$$

where y_i and y_{mi} enounce the network output and measured value from the i^{th} element; $\bar{y}$ and $\bar{y}_m$ conceive their average respectively, and N_o describes the number of measurements.

Another performance measure is mean absolute error (MAE). The MAE is used to measure how close forecasted values are to the observed values. It is the average of the absolute errors. The smaller values of MAE and MSE (mean square error) provide the better performance (Belayneh and Adamowski, 2012). MAE and MSE are estimated as follows (*Eqs. 10* and *11*):

$$MAE = \frac{1}{N_0}\sum_{i=1}^{N_0}|y_{mi} - y_i|^2 \tag{Eq.10}$$

$$MSE = \frac{1}{N_0}\sum_{i=1}^{N_0}(y_{mi} - y_i)^2 \tag{Eq.11}$$

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ANN application

In this study, the total coliform, fecal coliform and fecal streptococci parameters, which were measured between the years of 2010 and 2015, were modeled. The total number of measurements used in modeling for each parameter was 108 and 27 number of data was evaluated for each of lake coasts and parameters.

The amount of data needed to train a neural network is very much problem-dependent. The quality of training data (i.e., how well the available training data represents the problem space) is as important as the quantity (i.e., the number of records, or examples of input-output pairs). The key is to use training data that generally span the problem data space. For relatively small datasets (fewer than 20 input variables, 100 to several thousand records) a minimum of 10 to 40 records (examples) per input variable is recommended for training (NNMP, 2016). The total data number used in ANN modeling studies, related to water quality, ranged from 60 and 442 (Brion and Lingireddy, 2003; Mas and Ahlfeld, 2007; Radojevic et al., 2013). For these reasons the total number of data used in this study is suitable for water quality studies.

In this study, all the ANN model structures were created with the MATLAB ANN toolbox (Matlab, R2015). The tangent sigmoid transfer function was the activation function for the hidden layer, while the activation function for the output layer was a linear function. All the ANN models in this study were trained using the Levenberg Marquardt (LM) back propagation algorithm. The LM back propagation algorithm was chosen because of its efficiency.

All the ANN models the cross validation technique was used to partition the data sets; 80% of the data was used to train the models, while the remaining 20% of the data was used to test and validate the models, with 10% used for testing and 10% used for validation (Principe et al., 1999; Keskin et al., 2011; Keskin et al., 2009; Belayneh and Adamowski, 2012). In microbiological modeling studies, between 60 and 80% of the total data were used in training process, between 10 and 20% were used in test and validation stage (Brion and Lingireddy, 2003; Mas and Ahlfeld, 2007; Radojevic et al., 2013).

The training set was used to compute the error gradient and to update the network weights and biases. The error from the validation set was used to monitor the training process. If the network overfits the data the error in the validation set will begin to rise. The testing data set is an independent data set and is used to verify the performance of the model.

The data based on monthly average values was used in this study and 4 model structure (a, b, c, d) were tested for each 5 model (Model 1-Total data of the Lake, Model 2-Göllüce area, Model 3-İnciraltı area, Model 4-Darka area and Model 5-Orhangazi area). The results in the model structures related to ANN modeling experiments with a different number of hidden layers were defined in a comparative way.

In the (a) and (b) model structures total coliform and fecal streptococci and in the (c) and (d) model structures total coliform were chosen as input parameters. In all of the model structures, fecal coliform was selected as output parameter.

Total coliform parameter does not always indicate fecal contamination or the presence of the pathogens in water. Some bacteria, which are non-fecal source, answer to the definition of coliform bacteria. Most of the fecal streptococci bacteria are fecal source and in practice, they are the most important indicator of contamination with human feces. However, certain types and their subtypes are also present in plant

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material. Fecal streptococci rarely grow in contaminated water and are more resistant than coliform bacteria. Fecal coliforms are a subgroup of the total coliform bacteria and are of fecal origin. While total coliforms may be given some permission since they are phytogenetic and soil born, fecal coliforms are allowed to a very limited number of permission (Casadevall and Pirofski, 2014; Alberts et al., 2002). For this reason, Fecal coliform was chosen as an output parameter and modeled with the aim of estimating the sewages containing human and animal waste and providing support to the prevention works afterwards.

Results and discussion

Water quality and basic statistic of the data set

According to the "Blue Flag Criteria" (TÜRÇEV, 2013), microbial contaminant levels for the last 4 years are required in order to determine the level of pollution of beaches. Therefore, the measurements performed at the Lake coasts during the swimming season, in other words in summer months, between the years of 2010 and 2015, were evaluated within the context of this study. The results of the measurements were evaluated according to "Table of Criteria for Water Quality Required for Swimming and Recreation in Swimming Water Quality Act" (*Table 2*).

Table 2. *Table of criteria for water quality required for swimming and recreation (YSKY, 2006)*

Parameters	Reference values	Mandatory values
Total coliform /100 ml	1000 (2006-2014 years) 500 (2015 year)	10000
Faecal coliform /100 ml	200 (2006-2014 years) 100 (2015 year)	2000
Faecal streptococci /100 ml	100	1000

According to this table; if the measured values are under the reference values, the water is in good quality, between the reference values and mandatory value is in medium quality and above the mandatory value, is in poor quality.

When the variation of microbial pollution in the coastal areas was examined the parameters were changed with good and medium quality. However, according to the values of 2015, faecal coliform and faecal streptococci parameters were found to be in good class and the pollution decreased in the all coasts. It was determined that total coliform and faecal streptococci concentrations were no statistical difference among the months but slight increase in these parameter's concentrations were observed in the each month until late spring to early fall. These parameters could be soil and plant origin. Therefore intensive agricultural activity and rain in these months may increase the concentrations (Akkoyunlu et al., 2011; WWF, 2011). According to the statistical calculations (ANOVA tables) for all of the Lake there was no difference among the months and monitoring stations of the coasts. But the differences among the years for faecal coliform and faecal streptococci parameters were found significant ($p \leq 0.05$) statistical. According to the ANOVA analysis results for the each station the monthly

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alterations of faecal streptococci in Darka and Orhangazi public beaches were found as significant ($p \leq 0.05$) statistically (Demirci and Can, 2015). Basic statistic of the data set for this water quality and modeling studies was given in *Table 3*.

Table 3. *Basic statistic of the data set*

Coasts	Parameters (CFU / 100 ml)	Mean	Std	Max	Min
Göllüce Public Beach	Total coliform	511.148	636.474	3210	5
	Faecal coliform	135.093	132.533	521.5	2
	Faecal streptococci	73.537	63.613	220	2
İnciraltı Public Beach	Total coliform	310.296	315.901	1500	28
	Faecal coliform	110.222	113.191	440	7.5
	Faecal streptococci	65.370	89.917	420	2
Darka Holiday Village	Total coliform	310.611	301.155	1200	15
	Faecal coliform	66.889	75.676	400	2.5
	Faecal streptococci	51.889	62.364	250	3
Orhangazi Public Beach	Total coliform	518.185	690.173	3300	5
	Faecal coliform	110.259	110.555	425	2
	Faecal streptococci	78.130	92.063	400	2
Lake	Total coliform	412.560	520.574	3300	5
	Faecal coliform	105.616	111.137	521.5	2
	Faecal streptococcus	67.231	77.799	420	2

Moreover, the correlation of the three parameters with each other was considered statistically significant ($p < 0.05$). In previous scientific studies, when the correlation between indicator bacteria has been examined, it has been determined that the numbers of indicator bacteria decreased or increased in parallel with each other (Demirci and Can, 2015; Gürün and Kımıran-Erdem, 2013).

The effect of neural network structure on performance function (RMSE) and model estimation power (R)

The effect of neural network structure by changing of inputs and number of hidden neurons (layer) on model performance was investigated. Root Mean Square Error (RMSE) has been adopted as a measure of performance (as performance function) for comparing the effectiveness of tested structures (Belayneh and Adamowski, 2012; Soyupak et al., 2006). The performance function (RMSE) values and correlation coefficients (R) obtained for training, validation, testing and whole data sets. The model structures and their performance functions (RMSE) values and correlation coefficients (R) of the data sets were presented in *Table 4*. In all model structures, Faecal coliform was chosen as output parameter.

In "Model 1" all data of Lake İznik a and b structures, total coliform and faecal streptococci parameters were chosen as inputs. These models were run quite efficiently. But RMSE values of model 1-b were smaller than model 1-a, R correlation numbers of model 1-b were bigger than model 1-a except verification values. Enhancing of hidden

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neuron numbers in Model 1-b was raised to whole data set of R correlation number. In model 1 –c and d total coliform parameter was selected as input. These models were run efficiently. In model 1-d, hidden neuron numbers were raised. For this reason RMSE values of training and testing stages decreased and these R values and R number of whole data increased. İznik sub-province had advanced biological treatment plant with membrane technology and its sewerage system had completed. But the first stage of the treatment plant was put into use just (BUSKİ, 2016). Even so the treatment plant was not run at full capacity the pollution in the lake decreased (Demirci and Can, 2015). Also the monthly variations of the parameter's concentration was not been considered as statistically significant (Demirci and Can, 2015). This situation could be attributed that the pollution load was not belong to a specific period and point pollution sources disturb the ecological balance of the environment because of their uninterrupted waste inputs and therefore change the competitive environment among microorganisms continuously (Gürün and Kımıran Erdem, 2013). As a consequence, it was estimated that there was some inaccuracy in the model results and R values were not too high. Bursa Metropolitan Municipality planned to start up the second stage of the biological treatment plants in İznik sub-province. So it was estimated that microbiological and chemical pollution in the Lake would decrease (BBB, 2016).

In Göllüce Coasts "model 2" a and b structures, total coliform and faecal streptococci parameters were inputs as model 1. These models were run efficiently. RMSE values of model 2-a and b training and testing stages were bigger than model 1-a and b but verification RMSE values were smaller than model 1. Also verification R values were bigger than model 1, others not. It was found that the performance of model 2 (Göllüce area) less than model 1 (All Data of the Lake). Livelihood of the village of Göllüce was fruit and vegetable farming, especially olive cultivation. Therefore pesticides and fertilizers polluted this coast as diffuse sources. Moreover entering of domestic wastewater was in this coast because of incomplete sewerage system (LGP, 2016).

Microbial growth on and utilization of environmental contaminants as substrates have been studied by many researchers. Most times, substrate utilization results in removal of chemical contaminant, increase in microbial biomass and subsequent biodegradation of the contaminant (Okpokwasili and Nkweke, 2005). Wastewaters from industrial, municipal and agricultural sources are characterized by presence of mixtures of chemicals. Pollutant mixtures may contain only organic chemicals or may also include inorganic substances such as heavy metals. Co-contamination of natural environments with mixtures of pollutants is an important problem. The removal of one component may be inhibited by other components in the mixture and different conditions may be required to degrade different compounds within the mixture. Strong interactions among components of a pollutant mixture have been reported (Egli, 1995; Klečka and Maier, 1988; Meyer et al., 1984; Saéz and Rittmann, 1993). The utilization pattern can change with different mixture compositions, depending on the chemical nature and concentration of the substrate, oxygen concentration and microbial growth rates. In addition to biodegradation stimulation due to increased growth at low substrate concentrations, stimulation of one compound by another in a mixture can be by induction of catabolic enzymes required for degradation of the second pollutant (Arvin et al., 1989). This mechanism produces simultaneous degradation of pollutants in mixtures and has been reported for pentachlorophenol and chlorinated aromatics, toluene and *p*-xylene (Okpokwasili and Nkweke, 2005).

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Table 4. *The model structures and their performance functions (RMSE) values and correlation coefficients (R) of the data sets*

Coast area	Model No.	Inputs	Outputs	ANN	RMSE			R			
					Training	**Validation**	**Testing**	**Training**	**Validation**	**Testing**	**Whole data set**
All data of the lake (Model 1)	Model 1a	Total coliform Faecal streptococci	Faecal coliform	2-2-1	74.859	40.435	64.555	0.755	0.866	0.944	0.77
	Model 1b	Total coliform Faecal streptococci	Faecal coliform	2-5-1	61.829	71.010	29.998	0.843	0.801	0.948	0.84
	Model 1c	Total coliform	Faecal coliform	1-1-1	80.205	74.946	53.999	0.701	0.758	0.791	0.71
	Model 1d	Total coliform		1-3-1	76.559	98.575	19.931	0.766	0.635	0.833	0.76
Göllüce Public Beach (Model 2)	Model 2a	Total coliform Faecal streptococci	Faecal coliform	2-2-1	101.040	9.751	112.817	0.678	0.995	0.939	0.72
	Model 2b	Total coliform Faecal streptococci	Faecal coliform	2-5-1	86.518	14.042	89.323	0.753	0.998	0.943	0.80
	Model 2c	Total coliform	Faecal coliform	1-1-1	74.568	206.662	88.096	0.679	0.990	0.971	0.69
	Model 2d	Total coliform		1-3-1	85.081	70.479	50.687	0.793	0.880	0.927	0.79
İnciraltı Public Beach (Model 3)	Model 3a	Total coliform Faecal streptococci	Faecal coliform	2-2-1	65.290	32.746	37.764	0.842	0.999	0.899	0.85
	Model 3b	Total coliform Faecal streptococci	Faecal coliform	2-5-1	55.068	33.122	104.545	0.817	0.985	0.998	0.86
	Model 3c	Total coliform	Faecal coliform	1-1-1	76.811	26.362	22.684	0.715	0.873	0.993	0.79
	Model 3d	Total coliform		1-3-1	61.428	24.584	132.438	0.785	0.883	1.000	0.78
Darka Holiday Village (Model 4)	Model 4a	Total coliform Faecal streptococci	Faecal coliform	2-2-1	27.567	16.169	174.660	0.659	0.883	0.998	0.59
	Model 4b	Total coliform Faecal streptococci	Faecal coliform	2-5-1	29.681	11.991	11.371	0.947	0.999	0.982	0.95
	Model 4c	Total coliform	Faecal coliform	1-1-1	62.617	50.989	13.630	0.645	0.694	0.930	0.63
	Model 4d	Total coliform		1-3-1	25.872	9.532	12.136	0.949	0.907	0.956	0.95
Orhangazi Public Beach (Model 5)	Model 5a	Total coliform Faecal streptococci	Faecal coliform	2-2-1	60.452	48.69257	115.34	0.769	0.968	0.978	0.79
	Model 5b	Total coliform Faecal streptococci	Faecal coliform	2-5-1	18.9066	31.68544	42.662	0.986	0.975	0.999	0.98
	Model 5c	Total coliform	Faecal coliform	1-1-1	66.6663	63.32148	73.752	0.756	0.990	0.976	0.80
	Model 5d	Total coliform		1-3-1	75.671	24.87342	85.111	0.742	0.937	0.991	0.75

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Model 1 (All of the Lake data) had more data than model 2. Therefore performance of model 2 might be fallen. Also, it was found that pollution concentrations of Göllüce coast higher than Lake Average. Because it was considered that pollution loads and varieties concentrated in Göllüce coast. For this reason metabolisms and growth rate of microorganisms were affected and model performance dropped.

In model 2-c and d total coliform parameter was selected as input. These models were run efficiently. In model 2-d, hidden neuron numbers were raised. For this reason RMSE values of validation and testing stages decreased and R values of validation and whole data were increased. The structure of b in model 2 like model 1 was the most efficient structure among the others. Increasing of the numbers of input parameters and neurons in hidden layer raised the model 2 performance.

In İnciraltı coasts "Model 3" a and b structures, total coliform and faecal streptococci parameters were inputs as model 1 and model 2. These model structures were run more efficiently and R values were bigger than model 1 and model 2. When model 3 (c and d) compared with model 1 and model 2 it was seen that model 3 c and d structures run better than model 1 c and d especially model 3 c. There was no difference between model 3 c and d. İnciraltı coast was cleaner than Göllüce coast as shown in *Table 3*. Also, there was cleaner than the lake average in terms of total coliform. Therefore microbial modeling was efficiently due to decrease interactions between pollutants. The raising of neuron numbers of hidden layers in the structures had same number of inputs applied in model 3 (İnciraltı coast) was not enhance model efficiency. The raising of neuron numbers of hidden layers in the structures had same number of inputs in the more contaminated coasts (model 1 and model 2) increased the model efficiency. The raising of input's numbers in the all structures in model 3 enhanced the model efficiency.

In Darka Holiday Village "Model 4" inputs and outputs parameters were like other models. In a and b structures RMSE values were less than other models excluding testing value of a structure. For this testing RMSE value R number of whole data set "a" less than other model structures even the smallest R value. Structure b was one of the most efficient running models. When c and d were compared structure d better than c and other d structures of models. Darka Holiday Village was the cleanest coast in the Lake according to *Table 3*. The pollution in Darka coast was caused mainly domestic waste water originating from holiday village not from agriculture (BBB, 2016; Municipality of İznik, 2016). Unlike the clean area of İnciraltı, increasing of neurons in hidden layers raised the performances of the structures in the Model 4 had same and different numbers of inputs.

According to comparison of Orhangazi coast "Model 5" with other models RMSE values of structure b smaller than other model's structures and it could be said that the most effective one. R values of all Model 5 structures were found 0.75 and over. When and b were compared rising of hidden layer number was increased the performance. According to comparison of c and d rising of hidden layer number was not increased the performance. In the Orhangazi area which was one of the most contaminated coasts enhancing of inputs and hidden layer numbers provided better model performance. Orhangazi was one of the places where agriculture and industry were the most intense in Bursa (BBB, 2016). In sites co-contaminated with metals and organic compounds, metal toxicity inhibits the activity of organic degrading microorganisms, impacting both their physiology and ecology, thus reducing the rate of biodegradation of the organic compounds (Said and Lewis, 1991; Roane et al., 2001; Maslin and Maier, 2000). Also, faecal contamination and growth of pathogens were associated with environmental parameters such as rainfall, temperature, wind, sunlight or different hydrometeorological and hydrodynamic variables (WHO, 2010).

Model structures experimented in this study had shown that the results of the statistical model might not always be in linear trend and gave results with a small inaccuracy. In addition, all these experiments had shown that to increase the number of neurons in hidden layer had not increased at every turn the model efficiency. Species and number of the input parameters might be more effective in the coasts where cleaner than the others with lower concentrations. However, when the whole models were examined the increasing of numbers of inputs with neurons in the hidden layers raised the model performances at every trial. Thus the structure of "b" was found as the most efficient structure in the all models.

As a result, it was found that all model structures of the 4 coasts and all data of the lake were successfully. R and RMSE values calculated were in the acceptable range (Soyupak et al., 2003; Brion and Lingireddy, 2003; Yonar and Kılıç, 2014; Mas and Ahlfeld, 2007; Ogwueleka and Ogwueleka, 2010; Radojevic et al., 2013). In water pollution and quality studies when number of data and tests were increased RMSE and MSE values were calculated smaller (Soyupak et al., 2003; Yonar and Kılıç, 2014). *Figure 3* displays the observed time series and forecasted values with the model structures. In addition, the corresponding scatter plots are also presented.

Conclusions

This study is important to support the modeling studies of microbiological parameters and to more effective monitoring can be done by measuring fewer parameters with the predictions made. Because there are limited number of studies in deep lakes this study is original in terms of artificial neural network modeling of coliform bacteria in deep lakes. The ANN structures were successfully used to forecast the faecal coliform concentrations in Göllüce, İnciraltı, Darka, Orhangazi Coasts-Lake İznik. Multilayer feed forward networks were used. This study indicated that a neural network could be used to predict microbial pollution in deep lakes.

In the regions, where point sources of pollution were continuous, the modeling study gave a result with a certain margin of error since they disturb the ecological balance of the environment and therefore change the competitive environment among microorganisms continuously. Besides, the error values (RMSE) of the modeling results of the coasts had more pollution load and pollutant diversity (Göllüce and Orhangazi areas) were found to be higher than the others coasts of Darka and İnciraltı, which are relatively cleaner, since the metabolism of microorganisms was effected by the number and chemical structure of substrates (pollutants), existing in the environment, and depending on this, their rates of growth were affected. *This study showed that the use of artificial neural networks to forecast of microbial pollution of lake coasts where wide variety of contaminants (industrial and domestic) was found might not provide accurate results.*

For this reason summer and winter data could be assessed separately and together, and repeat of the models by increasing the number of data would enhance the model performance. The effect of neural network structure by changing of inputs and neuron's number of hidden layer on model performance was investigated. *This study showed also that increasing the number of inputs together with the neurons in hidden layer raised the performance of each model structure.*

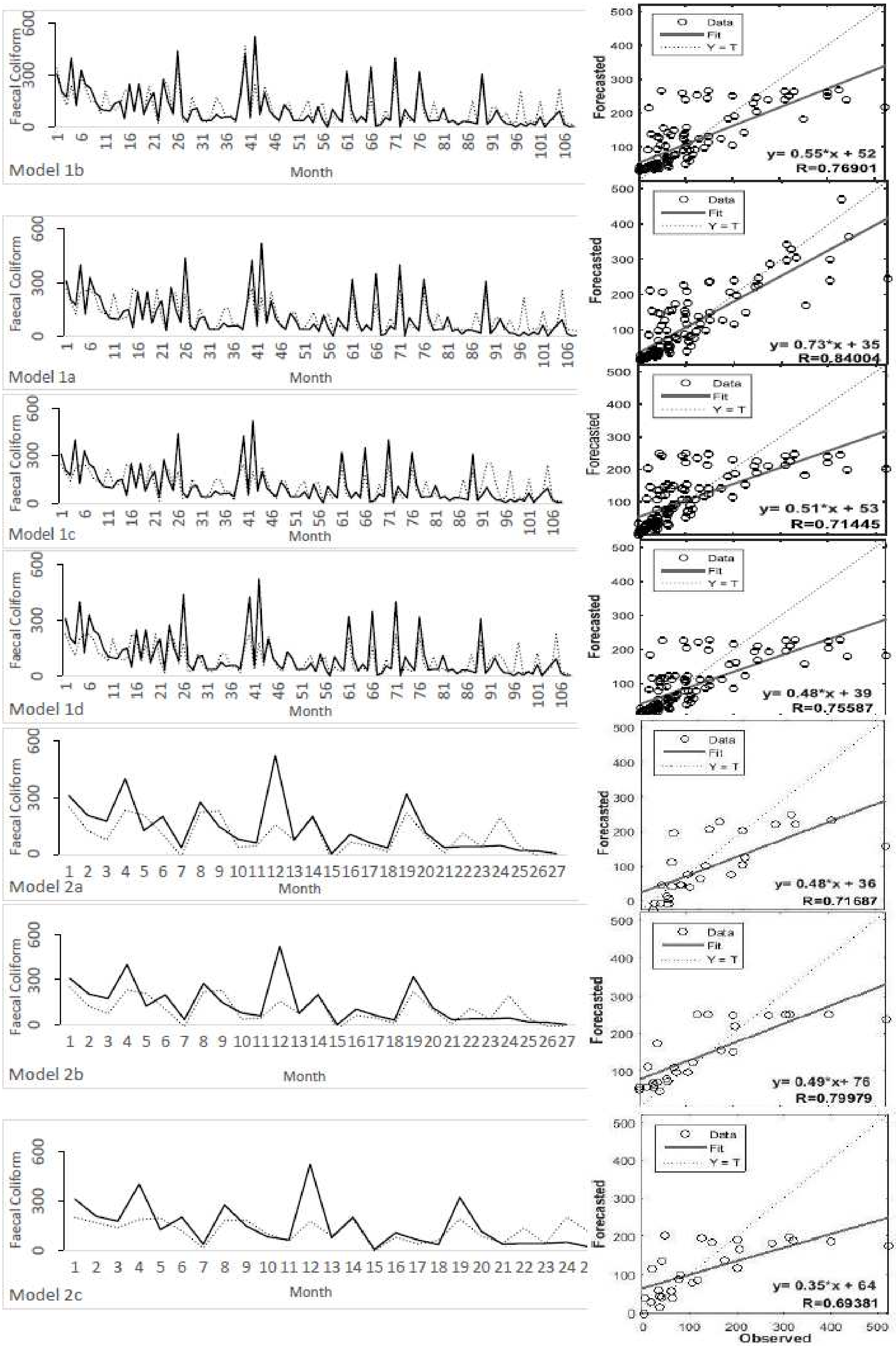

Figure 3. *The observed time series and forecasted values with the model structures*

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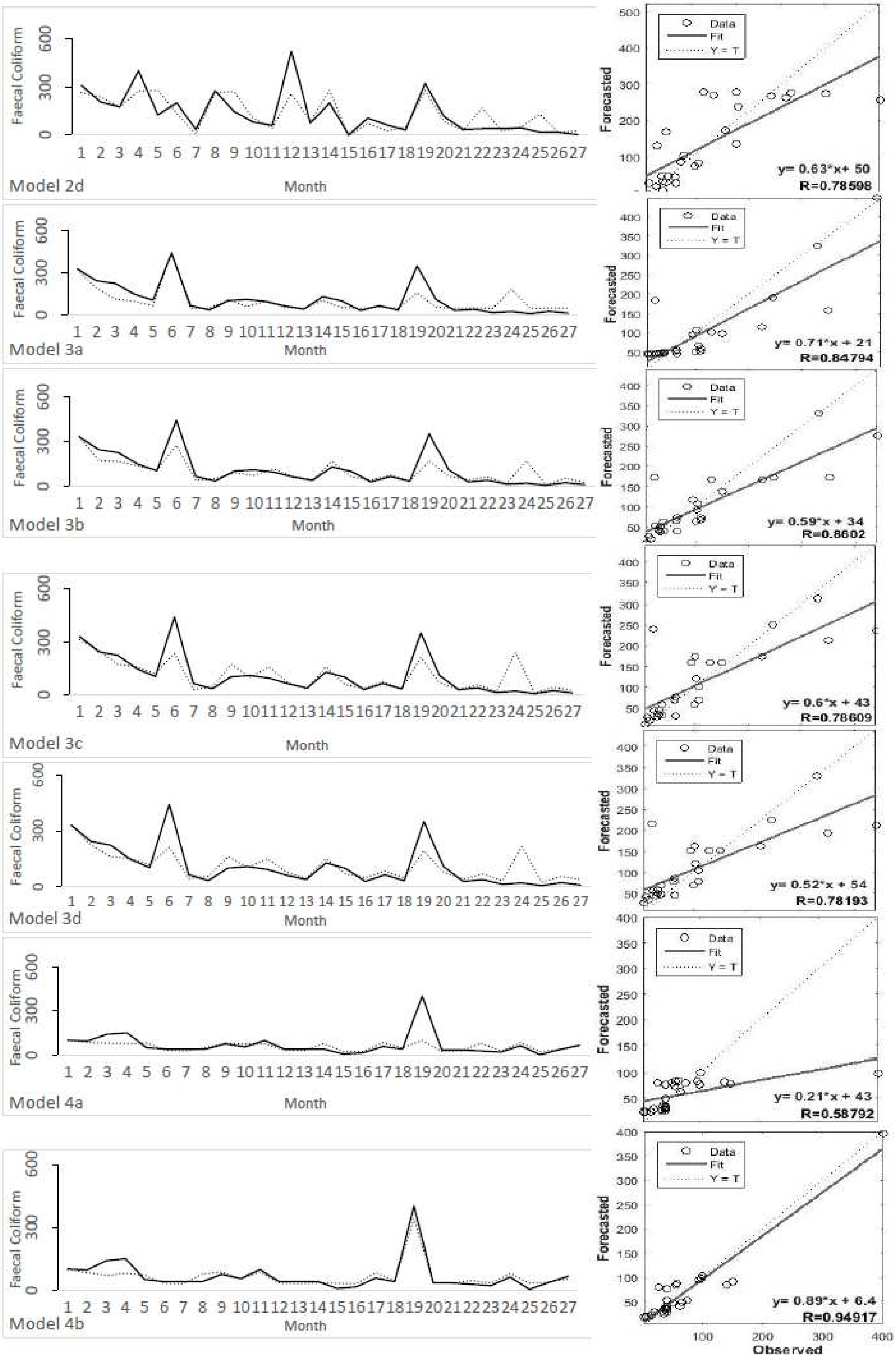

Figure 3. *(Continued) The observed time series and forecasted values with the model structures*

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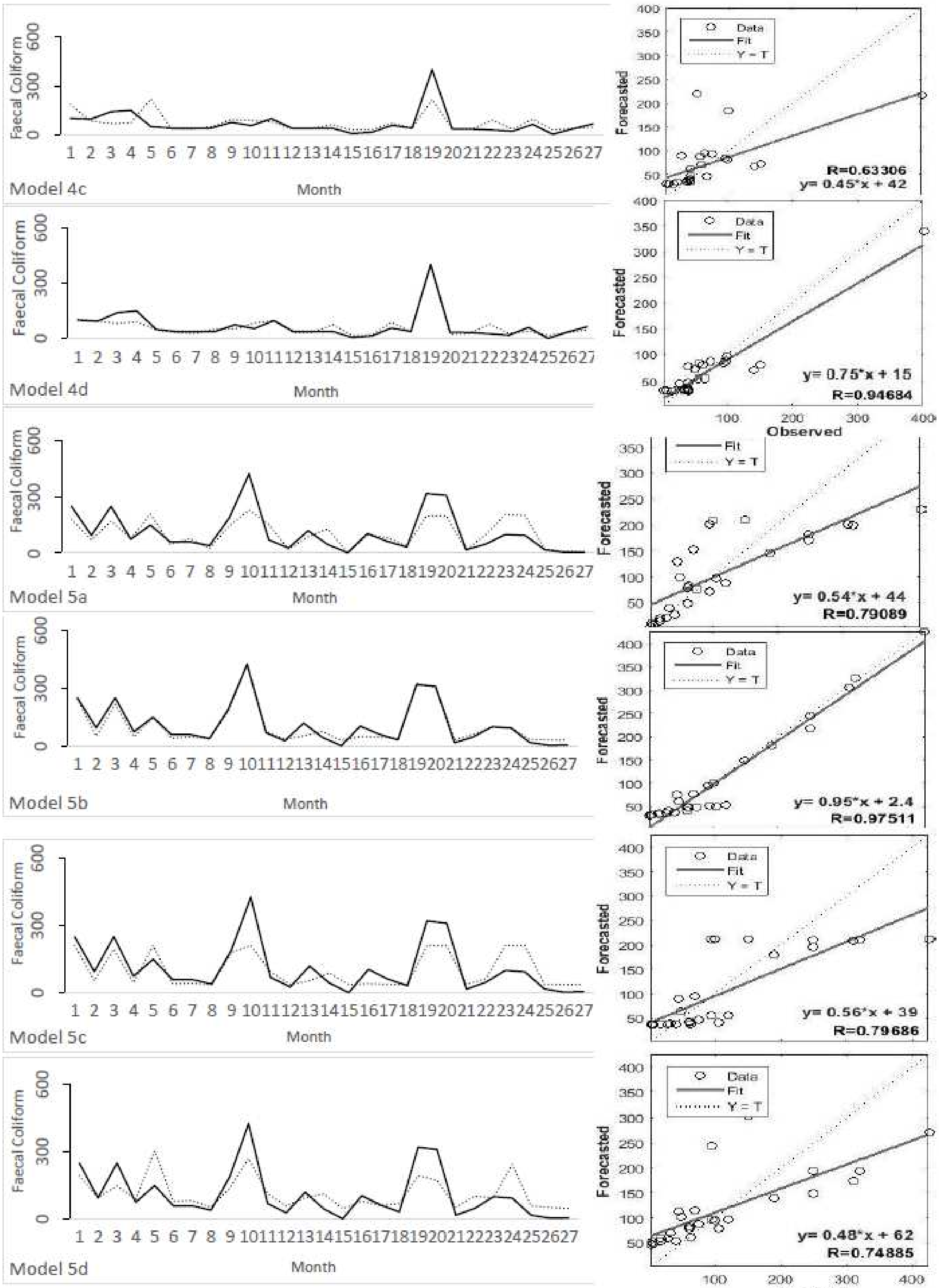

***Figure 3.** (Continued) The observed time series and forecasted values with the model structures*

The development and application of microbial source tracking methods in order to identify sources of faecal pollution could also provide useful additional data. For future studies and providing information to the more effective integrated coastal and basin

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management, it is necessary to simulate pathogen organisms through different statistical and process based dynamic water quality models, using environmental and hydrodynamic parameters in water and in the atmosphere, and compare the model results. Also, different artificial neural network model structures should be applied for different microbiological parameters, and model performances should be examined in the future. In addition to that it will be useful to establish early warning systems, which are very important for public health, on the examined coasts by using the results of the modeling studies. Investigations to improve the knowledge-base of the area would also be of greatest importance to validate coastal and lake models and identify, for example, the role of diffuse sources from animal and agricultural soil origins which could carry human pathogenic bacteria or viruses.

The fact that monitoring and modeling works to protect of Lake İznik has national and international ecological values of in respect to water birds, endemic flora and fauna, tourism, recreation, fishing and agricultural are vitally important for the conservation of natural equilibrium in the world. Consequently continuation and development of modeling studies will be important in the future to provide information to the protection and supervision mechanisms.

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DEVELOPMENT OF FIRE BRICKS FROM ORGANIC WASTE: AN ECO-FRIENDLY ENERGY SOLUTION

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Abstract. The growing population poses serious threats not only to forests but also to the overall environment due to fuel and wood consumption used for various purposes. However, converting organic waste into fire bricks could be a useful step towards alternative energy sources. In the present study, fire bricks were prepared using organic waste i.e., cardboard, rice husk, saw dust, cow dung and newspapers. Firstly, the brick manufacturing materials were soaked in water in various ratios and converted into paste with mortar and pestle to make bricks. Secondly, the fire bricks were analyzed for gas emissions, burning time period, flue gas temperature and net efficiency. The results reveal that gas emissions were below the National Environmental Quality Standards (NEQS). Moreover, the results were also compared with a commonly used fuel wood (*Vachellia karroo*) to calculate the statistical variations for various parameters. Thus, the net efficiency range of fire bricks was from 39.7 to 58.9%, significantly higher than that of the fuel wood. The flue gas temperature varied from 230 to 430 °C. Therefore, the bricks were found to have high heat intensity, to be easy to use, and to have needed small space for their storage. Additionally, fire bricks are cost-effective compared to fuel wood. It is concluded that high quality and durable fire bricks can be made while using a combination of rice husk and newspapers (RHNP), rice husk and cardboard (RHCB), sawdust and cardboard (SDCB), cow dung and cardboard (CDCB), cow dung and newspapers (CDNP), sawdust and cow dung (SDCD) in a ratio of 2:3. It is due to all these benefits, that fire bricks were considered eco-friendlier in efficiency than fuel wood.
Keywords: *fire bricks, alternative energy, fuel wood, biomass recycling, waste management*

Introduction

Energy is a crucial and significant factor for socioeconomic development in any country. However, the ever-growing population is putting more pressure on energy sources. It is said that both sufficient and cost effective energy resources are essential for the steady development around the globe. The energy demand is raised with urbanization and industrialization. Thus, fossil fuel is excessively used around the world for urbanization and modernization. Coal, oil and gas meet 80% of the energy requirements of the world, however; contribution of renewable energy resources and

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nuclear power in energy sector is 13.1% and 6.5%, respectively in the total energy demands (IEA, 2007).

Municipal solid waste is being generated in large amounts with low gas emissions threatening the environmental health. In order to collect, store, process, and dispose the municipal solid waste, usually unsuitable practices are being employed. These unfit techniques in turn harm the health of humans, plants and animals. Suitable and secure management of solid waste is deemed necessary, especially in an area which is denser for better environmental health. In developing countries, waste after its collection is generally discarded on land surface without any protection that causes water and air pollution (Dong et al., 2003; Mosler et al., 2006).

Recently, it has been witnessed that the fossil fuel scarcity is increasing with high energy requirements around the world. It is need of the hour to go for environment-friendly and sustainable alternate energy resources. The interest of switching towards alternate energy sources will not only substitute fossil fuels but it is also good for environmental health. Conventional fuel like firewood is rapidly declining which increases dependency on innovative sources of energy for household usage (Kavitha and Joseph, 2007).

Wood and charcoal had been considered as renewable energy sources and agricultural waste is available in sufficient amounts in rural areas. The residues like rice husk, sawdust, corn stover and cotton stalk are best options to be used for cooking purposes at household level. However, the use of these residues without processing is problematic due to their raw form which produces large amounts of smoke, leading to air pollution. Biomass resources when burned in their raw form generate less heat and have low mass to volume ratio. All these factors lead to poor efficiency to use them as fuel (Emerhi, 2011). Moreover, it is not convenient to use, transport and store these agricultural residues in their pure form.

Massive flammable materials are compacted to make them feasible for using as fuel. Thus, this technique is becoming popular around the globe. Fuel bricks have been produced to use not only for household purposes but also for industries. The reason behind the production of fire bricks is to meet the growing energy requirements and to protect the environment from the harms of traditional fuels. At the same time, this technique also manages crop remains in a very efficient way (Vongsaysana, 2009). Moreover, heat efficiency of these crop residues can also be intensified by converting them into fuel bricks (Wilaipon, 2007).

Calorific values of the agricultural and organic residues would increase in brick form due to compaction. The loose agricultural wastes have lower calorific value which is difficult to handle (Oladeji, 2010). For electricity production, industrial use and for cooking food, the use of fire bricks can be a good option (Styles et al., 2008). Therefore, the present study aims to switch traditional use of fuel wood to the high energy efficient and environment-friendly alternative energy sources through agricultural and other organic residue-based fire bricks.

Materials and methods

Study area description

The current study was carried out in the Punjab Province of Pakistan which is renowned for its agricultural based economy. Geographic location of the study area is

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30.183° latitude and 73.06° longitude. All the waste material used in this study was collected and processed for brick development in the district Pakpattan of the province.

Collection and preparation of samples

Cardboard and newspapers were collected from shops. Likewise, rice husk was collected from rice grinding mills and sawdust from carpenter shop. Fresh cow dung was collected from cow farm house. Similarly, rice husk and sawdust were cleaned to remove dirt and undesirable particles. Newspapers were torn into the smallest possible pieces. These pieces were placed in the bucket and soaked in water for 24, 48 and 72 h. *Table 2* shows the effects of soaking time on quality of fire bricks.

These samples were converted into paste while using grinder. The same procedure was repeated for cardboard sample preparation, and cardboard sample was also converted into paste while using crusher. This practice was adopted to check the most suitable combination and quality of sample materials to convert them into smooth bricks. The quality of sampling combinations in different ratios was ranked in three different categories such as good, average and best. Only the best quality rank was proceeded further for analysis. The quality (average, good and best) of the fire bricks was based on the measurement of porosity and dry bulk density of the bricks (*Table 1*). The porosity (n) of a material indicates the storage capacity of a porous medium. It is a fraction of the voids in total volume of the porous medium (*Eq. 1*). The dry bulk density (ρ_b) is calculated from the dry mass of the material and its total volume (*Eq. 2*) (Fetter, 2001).

$$n(\%) = \frac{V_v}{V_t} \times 100 = \frac{V_w}{V_t} \times 100 \qquad \text{(Eq.1)}$$

$$\rho_b (g / cm^3) = \frac{m_s}{V_t} \qquad \text{(Eq.2)}$$

where V_v is the volume of voids which is equal to volume of water V_w in saturated medium, V_t is total volume and m_s is dry mass of the material.

Table 1. *Criteria used for the quality of fire bricks*

Porosity (%)			**Dry bulk density (g/cm^3)**		
Average	Good	Best	Average	Good	Best
35-60	73-90	60-72	0.31-0.5	0.15-0.3	0.1-0.15

Vachellia karroo (formerly known as *Acacia karroo*) is a fast growing tree and is abundantly found in Pakistan, India, Nepal and Africa. It is commonly found along highways, canals, river sides, rangelands and among the major trees considered for agro-forestry in Pakistan. It produces high-density wood (800-890 kg/m^3) which is commonly used for fuel wood besides other beneficial uses. Fuel wood of *Vachelli karroo* was used as control in this study (*Fig. 1a*).

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Binder preparation and formation of bricks

Wheat flour was used to prepare binder. Hot water was added into it to prepare its paste. It was assured that no lumps were produced. Binder was muggy, it did strengthen the samples. The volume of binder varies in different types of combinations depending that a homogeneous mixture is formed. Newspaper (NP) paste was taken and placed into brick mould box, levelled and pressed to make it compact. Afterwards, the mould box was emptied in an outdoor dry place under natural conditions. Three NP bricks were made in this way while three cardboard (CB) bricks were also prepared with the same procedure. The rice husk (RH) sample was taken and thoroughly mixed with binder until the paste was obtained. This paste was placed in brick mould box and was levelled. The same procedure was adopted and repeated for the combination of SDNP, SDCB, RHCD, RHNP, RHCB, CDNP, CDCB, and NPCB. *Table 2* shows the sample combinations in different treatments. The ratios of wastes were based on the volume. The same procedure was repeated for various ratios like 1:9, 7:3, 3:2, and 1:4 respectively. Three bricks of each combination were made. A metallic template was used for the formation of bricks. The volume of each prepared brick was 1823 cm^3 with dimensions of 22.86 cm length, 11.43 cm width and 6.98 cm height. The shape of prepared bricks is shown in *Figure 1b, c* and *d*.

Cost-effectiveness measurements of fire bricks

Market prices of the organic wastes (newspaper, sawdust, rice husk, cardboard and cow dung) were collected and then were compared with the market price of hard wood as control. Cost-effectiveness of fire bricks made of organic waste was calculated. Prices were calculated in US dollars. The price for waste material includes labor charges to prepare fuel bricks (2 persons/day and charges are 3.48 USD/day/person). The prices for dry waste are as newspaper 2.61 USD/40 kg, cow dung 0.35 USD/40 kg, cardboard 3.10 USD/40 kg, sawdust 1.66 USD/40 kg, rice husk 2.78 USD/40 kg.

Analysis of fire bricks for gaseous emissions

Combustion analysis was carried out to measure gas emissions from fire bricks. The system contained weight meter, a stove with ceramic lining and chimney. The Testo 350 flue gas analyzer connected to the chimney was used to measure the emissions, flue gas temperature, net efficiency, gross efficiency, and burning period. In present research, the fuel bricks were burned in the stove of ceramic lining. The emissions of burning of fuel bricks were observed in mg/Nm^3 and then compared with National Environmental Quality Standards (NEQS) for stack emissions. NEQS values for CO, Oxides of Nitrogen and Oxides of Sulphure are 800 mg/Nm^3, 600 mg/Nm^3 and 1700 mg/Nm^3, respectively. However, NEQS for smoke number is 2 on Ringleman Scale (GOP, 2016).

Statistical analysis

MS-Excel (2010) was used for statistical analysis. Descriptive statistics such as percentage, average, were applied to process results of density, porosity, gas emissions and cost-effectiveness of fire bricks. ANOVA was performed for different parameters at significance level of 1%. Degree of freedom (df), sum of squares (SS) and mean sum of squares (MSS) were calculated for different source of variation (SoV).

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Table 2. *Effect of organic waste types, their combinations and soaking time on the quality of fire bricks*

Combinations	Treatments		Abbreviations	Soaking time (hours)	Quality
Individual Material	T1	Newspaper	NP	48	Good
	T2	Cardboard	CB	48	Good
	T3	Sawdust	SD	00	Average
	T4	Rice husk	RH	00	Average
	T5	Cow dung	CD	00	Good
Combinations (1:1)	T6	Sawdust and rice husk	SDRH	00	Average
	T7	Sawdust and cow dung	SDCD	00	Good
	T8	Sawdust and newspaper	SDNP	24	Good
	T9	Sawdust and cardboard	SDCB	24	Good
	T10	Rice husk and cow dung	RHCD	00	Good
	T11	Rice husk and newspaper	RHNP	24	Good
	T12	Rice husk and cardboard	RHCB	24	Good
	T13	Cow dung and newspaper	CDNP	24	Good
	T14	Cow dung and cardboard	CDCB	24	Good
	T15	Newspaper and cardboard	NPCB	24	Good
Combinations (1:9)	T16	Sawdust and cow dung	SDCD	00	Good
	T17	Sawdust and newspaper	SDNP	48	Good
	T18	Sawdust and cardboard	SDCB	48	Good
	T19	Rice husk and cow dung	RHCD	00	Good
	T20	Rice husk and newspaper	RHNP	48	Good
	T21	Rice husk and cardboard	RHCB	48	Good
	T22	Cow dung and newspaper	CDNP	48	Good
	T23	Cow dung and cardboard	CDCB	48	Good
Combinations (1:4)	T24	Sawdust and cow dung	SDCD	00	Good
	T25	Sawdust and newspaper	SDNP	48	Good
	T26	Sawdust and cardboard	SDCB	48	Good
	T27	Rice husk and cow dung	RHCD	00	Good
	T28	Rice husk and newspaper	RHNP	48	Good
	T29	Rice husk and cardboard	RHCD	48	Good
	T30	Cow dung and newspaper	CDNP	48	Good
	T31	Cow dung and cardboard	CDCB	48	Good
Combinations (2:3)	T32	Rice husk and newspaper	RHNP	72	Best
	T33	Rice husk and cardboard	RHCB	72	Best
	T34	Sawdust and cardboard	SDCB	72	Best
	T35	Cow dung and cardboard	CDCB	72	Best
	T36	Cow dung and newspaper	CDNP	72	Best
	T37	Sawdust and cow dung	SDCD	72	Best
	T38	Sawdust and newspaper	SDNP	72	Good
	T39	Rice husk and cow dung	RHCD	72	Good
Combinations (3:7)	T40	Sawdust and cow dung	SDCD	00	Good
	T41	Sawdust and newspaper	SDNP	48	Good
	T42	Sawdust and cardboard	SDCB	48	Good
	T43	Rice husk and cow dung	RHCD	00	Good
	T44	Rice husk and cardboard	RHCB	48	Good
	T45	Cow dung and newspaper	CDNP	48	Good
	T46	Cow dung and cardboard	CDCB	48	Good
	T47	Rice husk and newspaper	RHNP	48	Good

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Results and discussion

Fire bricks of individual materials were found to be difficult to form besides their quality remained average. However, when all treatments were used in combinations, it not only improved their quality but also made their formation easy. Bricks of individual materials: newspaper (T1), cardboard (T2) and cow dung (T5) were made. Out of these treatments, bricks with T1 and T2 were of good quality, while T5 fire bricks were of best quality. It was difficult to make fire bricks from treatments T3 (sawdust) and T4 (rice husk) as individual materials due to their physical properties as both were in powder form. Even the quality of fire bricks prepared from sawdust and rice husk in the presence of binder was also very poor. Sawdust and rice husk can be used along with other waste materials in order to make fire bricks. Apparently, the surface of fire bricks was smooth and homogeneous (*Fig. 1*).

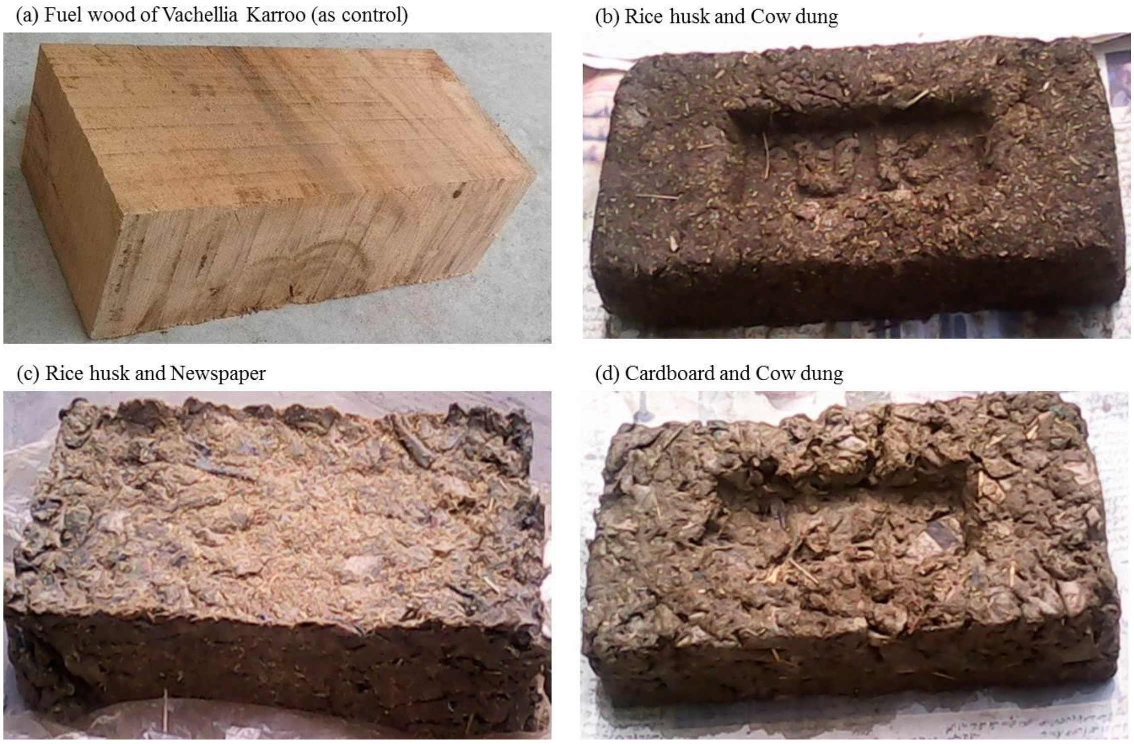

Figure 1. *Fuel wood (as control) and samples of prepared bricks from organic wastes*

It was found that fresh cow dung did not require soaking. Sawdust and rice husk were not soaked because they were used with paste of others. Only newspapers and cardboard were soaked. Various soaking periods were used to create homogeneous moisture in the mixture of materials for formation of bricks. Quality of six combinations (T32-T37) with a soaking period of 72 h was found to be the best, which was further studied (details in *Table 2*).

Amount of carbon monoxide and carbon dioxide in fire brick exhaust

Figure 2 shows the amount of oxygen, CO and CO_2 in the exhaust of fire bricks. Amount of CO varied significantly with treatments from T32 to T37. The lowest CO was observed in T32 (RHNP) and the highest CO emissions were found in T35

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(CDCB). Emission of CO was within NEQS in case of T32 (RHNP), T33 (RHCB) and T37 (SDCB). Carbon monoxide (CO) emissions were the lowest as 265 mg/Nm^3 in T32 and the highest as 3002 mg/Nm^3 in T35 (*Fig. 2a*) however, NEQS for CO is 800 mg/Nm^3. All values were lower than the ones of NEQS (GOP, 2016) while only T37 (SDCD) emission value meets the ones of NEQS because of their composition. Ishrat and Lakshami (2014) conducted a study at Hyderabad, India to make fuel briquettes from mixture of paper, rice husk and sawdust and emission analysis of combustion of these briquettes was 84.77 mg/Nm^3 CO that is much lower than that of this study.

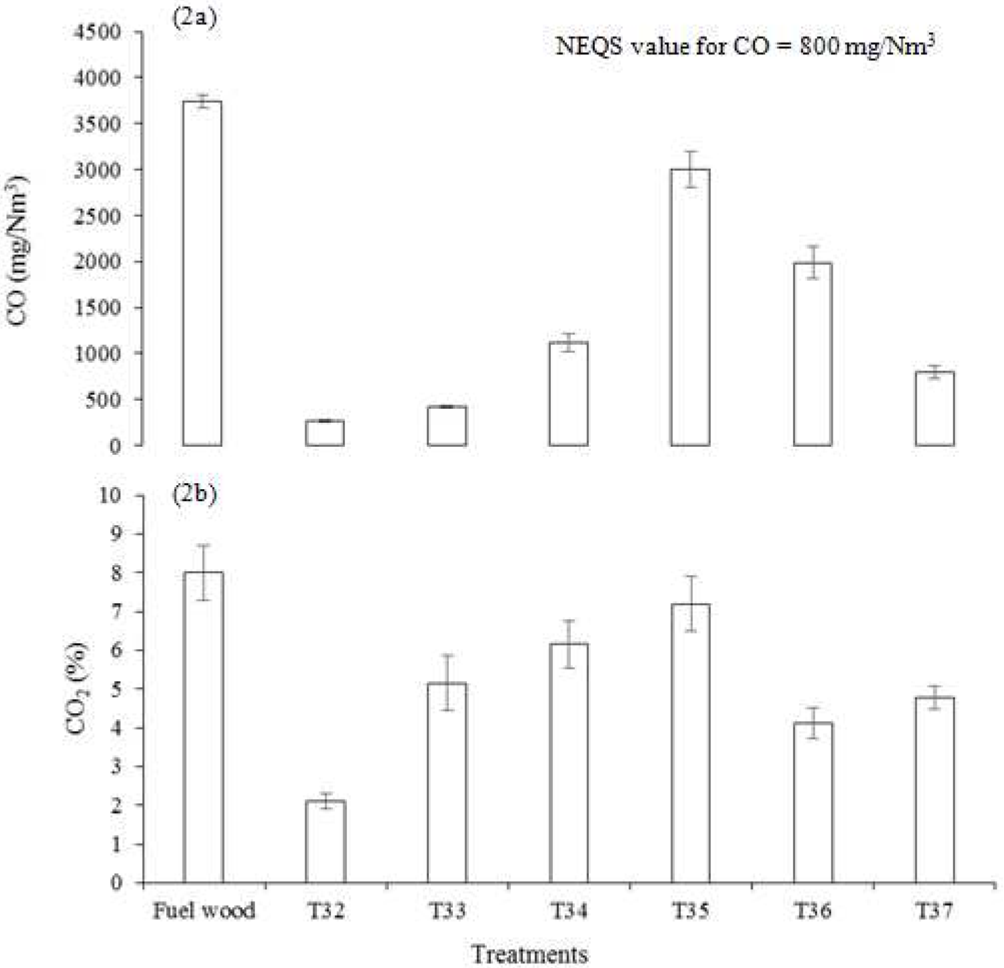

Figure 2. *Amount of CO and CO$_2$ in the fire brick exhaust*

Figure 2b indicates the results of CO_2 emission from fire bricks. It was observed that the highest carbon dioxide emission was seen in T35 (CDCB) and the lowest in T32 (RHNP). Carbon dioxide (CO_2) emission was the lowest 2.1% in T32 and the highest 7.2% in T35. The lower CO_2 emission was due to the combination of biomass NPRH in T32. According to Maia et al. (2014), when biomass fuel bricks were analyzed for emissions of CO_2 the resultant value was 18.56%, which is much higher than that of the present study. In another study, carbon dioxide values were found as 50% for wood chips and paper, and sawdust and paper, and 30% for sugarcane and paper. Evans, S (2014) reported 10 times lower carbon emissions from burning organic residues like

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branches, twigs and sawdust than in case of fuel wood coals. However, Fernandes et al. (2013) revealed that the biomass briquettes showed a greater release of CO_2 with a peak of 0.48%. Lower oxygen concentration caused higher CO concentration during combustion.

Amount of NO and NO_x in fire brick exhaust

It was observed that NO emission from fire bricks slightly varied from one another. T32 (RHNP) and T35 (CBCD) showed almost similar and the highest emission of NO than others, while T33 (RHCB) showed the lowest emission of NO (*Fig. 3a*). The minimum observed nitric oxide (NO) emission value was 106.7 mg/Nm^3 in T33 and the maximum was 324 mg/Nm^3 in T35. The relevant literature shows 2.45 mg/Nm^3 when biomass fuel bricks were analyzed for NO that is much lower than that of the present study. High nitrogen contents, resulting in an elevated emission of dust and NOx, are still a matter of investigation on the use of herbaceous biomass at small-scale household appliances (Energy Commission of Ghana, 2008).

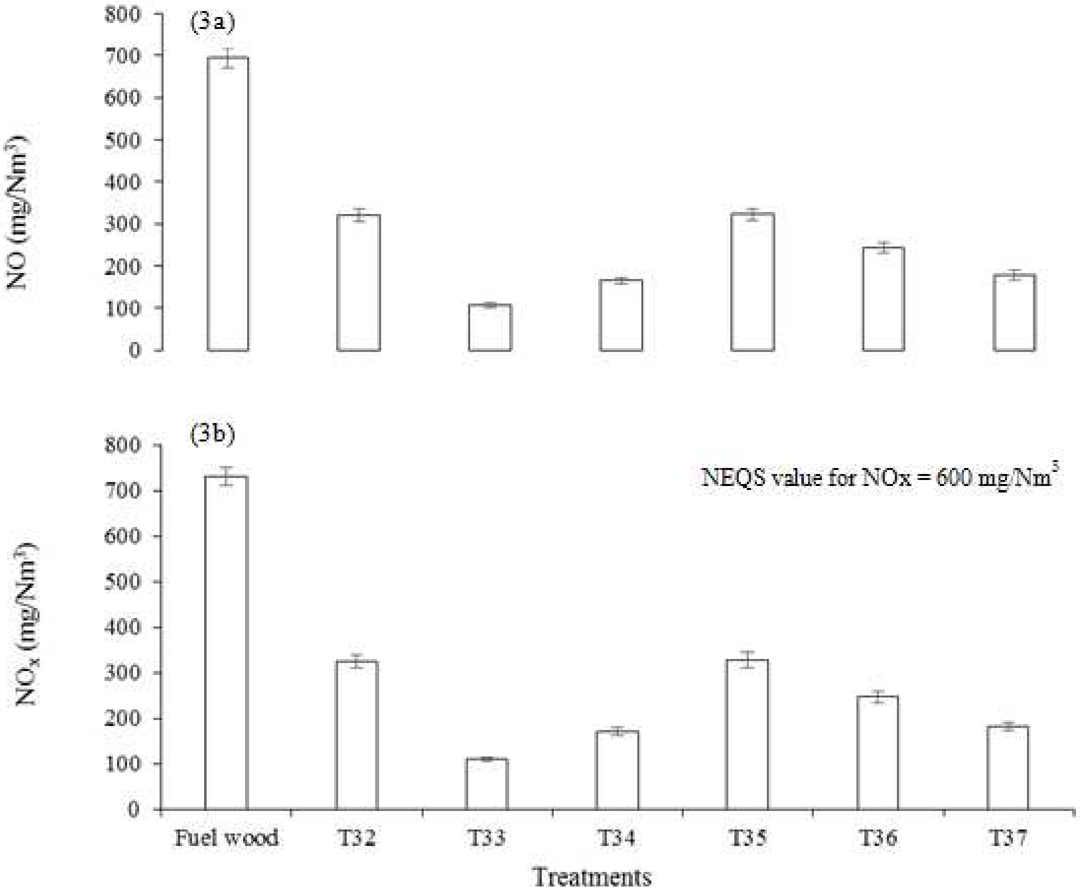

Figure 3. *Amount of NO and NO_x in fire brick exhaust*

Figure 3b shows NOx emissions from fire bricks. It was observed that NOx emissions varied significantly from T32 to T37. The highest NOx emissions (327 mg/Nm^3) were observed in T32 (RHNP) and T35 (CDCB), while T33 (RHCB)

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(3b)

showed the lowest (109.3 mg/Nm^3) NOx emissions. It is stated that all of the NOx emissions during burning the bricks were below those of the NEQS.

Amount of SO_2 in fire brick exhaust

Figure 4 indicates the SO_2 released from burning the fire bricks. It was observed that T35 (CDCB) liberated the highest emissions of SO_2. The lowest emission was observed in T32 (RHNP).

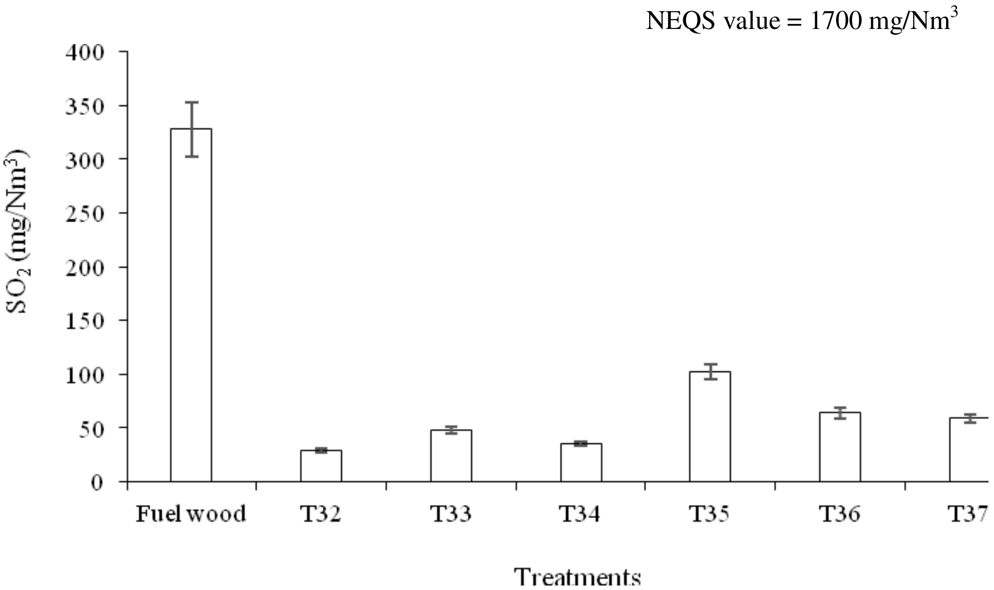

Figure 4. *Amount of SO_2 in fire brick exhaust*

The minimum sulphur dioxide (SO_2) emissions – 29 mg/Nm^3 - were also observed in T32 and the maximum -102.6 mg/Nm^3 - in T35 (*Fig. 4*). Even the maximum observed value was lower than that of the NEQS which is 1700 mg/Nm^3. The reason behind this is the composition of fire bricks. When biomass of fire bricks was analyzed to determine their SO_2 emissions, they were 167.7 mg/Nm^3 (Enweremadu, et al., 2004) which were greater than those of the present study. Less concentration of nitrogen and sulphur was found in fire bricks smoke, which means less polluted atmosphere. This shows that if fire bricks will be used for heat, they will not harm the environment (Enweremadu, et al., 2004). The oxides of nitrogen and sulphur produce nitric and sulphuric acid which cause acid rain (Chaney, 2010).

Combustion of wood and coal as fuel causes air pollution, which in turn damages the health of human beings. One cubic meter of fuel wood release 61-73 kg of CO_2. Longer exposures to the gases such as carbon monoxide (CO), sulphur oxides (SOx) and nitrogen oxide (NOx) cause harm to human health (Raymer, 2006).

Table 3 shows results of the statistical analysis (ANOVA) for different gases in exhaust from burning of different types of fuel bricks. It is clear that there is significant difference between gaseous emissions from burning of fuel bricks. Emission of gases (CO, CO_2, NO, NOx and SO_2) has been significantly reduced for fuel bricks as compared to that of commonly used fuel wood.

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Table 3. *Statistical analysis (ANOVA) for different gases in exhaust*

Parameter	SOV	df	SS	MSS	$F_{Cal.}$	$F_{Tab.}$
CO	Treatments	6	30272032.34	5045338.72	101.83*	4.46
	Error	14	693667.79	49547.70	--	--
	Total	20	30965700.13	--	--	--
CO_2	Treatments	6	78.29	13.05	12.27*	4.46
	Error	14	14.89	1.06	--	--
	Total	20	93.19	--	--	--
NO	Treatments	6	692193.87	115365.64	319.35*	4.46
	Error	14	5057.53	361.25	--	--
	Total	20	697251.40	--	--	--
NOx	Treatments	6	782367.46	130394.58	988.39*	4.46
	Error	14	1846.97	131.93	--	--
	Total	20	784214.43	--	--	--
SO_2	Treatments	6	203766.11	33961.02	592.95*	4.46
	Error	14	801.85	57.27	--	--
	Total	20	204567.96	--	--	--

* indicates significant difference between treatment

Net efficiency, flue gas temperature and burning time for fire bricks

Figure 5a indicates that the net efficiency of fire bricks slightly varied from T32 to T37. It was observed that the highest net efficiency was found in T37 (SDCD), and the lowest net efficiency was observed with treatment T36 (CDNP). The net efficiency which is the efficiency of solid burned particles of fire bricks was minimum (39.7%) with treatment T36 and maximum 58.9% with T37. In Latvia, a study was conducted to make fire briquettes from woody and non-woody herbaceous resources like grain husk. According to combustion analysis of those fire briquettes, the net energy production was 15.6 to 17.7MJ/Kg.

Flue gas temperature was measured to determine the efficiency of fire bricks. *Figure 5b* shows flue gas temperature while burning the fire bricks. It was observed that flue gas temperature was the highest in case of T35 (CDCB). In case of T36 (CDNP) and T37 (SDCD) same flue gas temperature was found, and it was the lowest for T32 (RHNP). Minimum temperature was 230 °C in T32 and maximum was 450.2 °C. The combustion analysis of flue bricks gave flue gas temperature variation from 331 to 474 °C.

Fernandes et al. (2013) found that carbon, hydrogen and oxygen are the major constituents of biomass fuels. Carbon and hydrogen are oxidized to make CO_2 and H_2O which are responsible for high calorific value of the bricks. According to Ringleman scale, the smoke number indicates the apparent density of smoke, the greater the smoke number the greater the amount of particulate matter in the bricks. Minimum smoke number was 1.4 with treatment T33 (RHCB) and maximum 5.3 with T35 (CDCB) as shown in *Figure 5b*. While the NEQS for smoke number is 2 on the Ringleman scale. Maia et al. (2014) produced banana leaves fuel briquettes and found that in combustion tests, heavy smoke production was attributed to the fact that oxygen was supplied in

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uncontrolled way. Therefore, it is important to provide sufficient oxygen to convert CO to CO_2 and minimize the smoke.

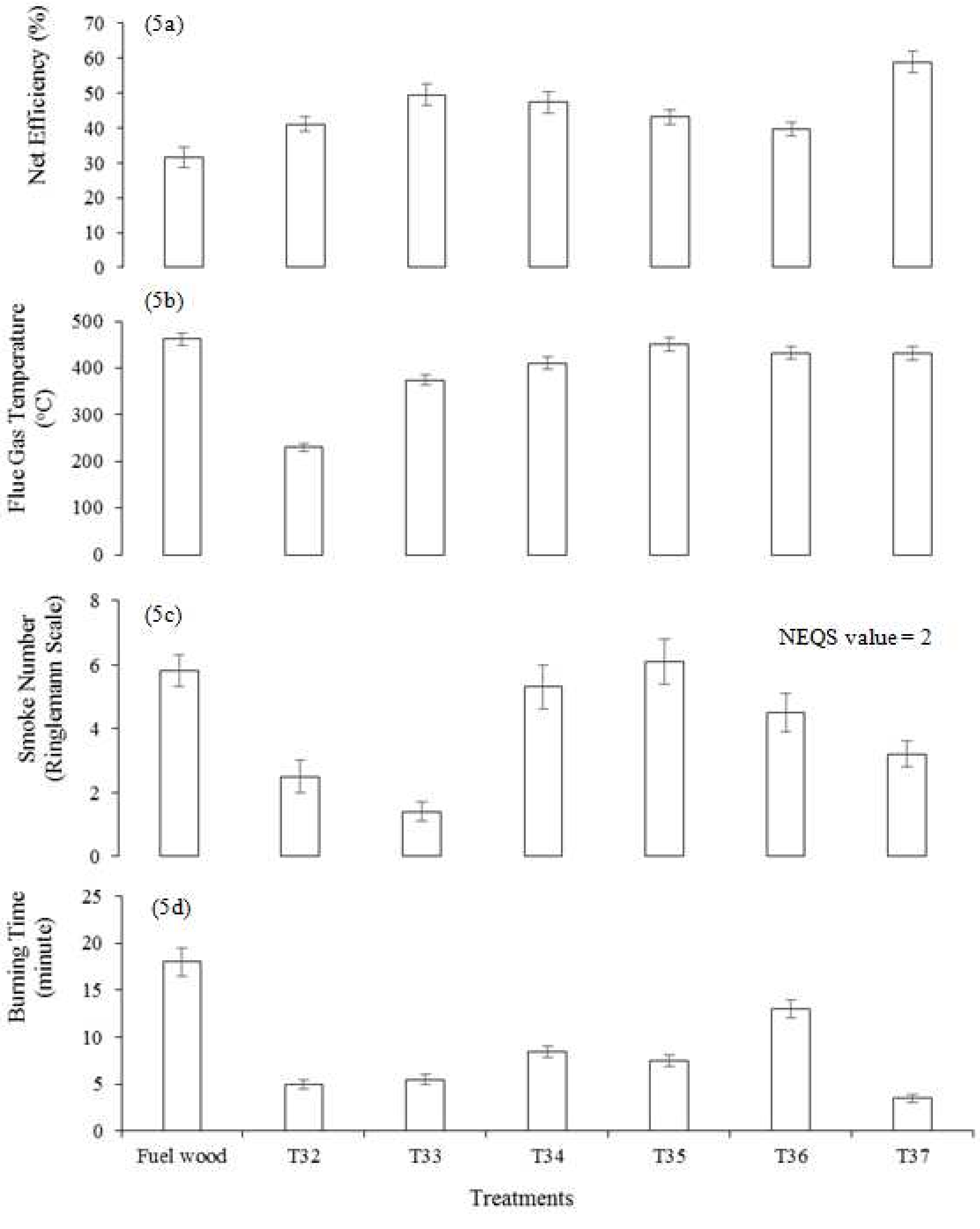

Figure 5. *Net efficiency, flue gas temperature and burning time for fire bricks*

Figure 5d shows burning time or duration of burning of fire bricks. It was observed that burning period was the highest for T36 (CDNP) and the lowest in case of T37 (SDCD). Burning period was observed as the lowest in T37 (4 min) and the highest was 13 min with treatment T36.

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Table 4 shows results of statistical analysis of variance (ANOVA) for net efficiency, flue gas temperature and burning time of bricks. It is clear that there is significant difference between treatments for net efficiency, smoke number and burning time. However, there is non-significant difference between treatments in case of flue gas temperature. It shows that there is no effect on flue gas temperature for different types of fuel bricks.

Table 4. *Statistical analysis (ANOVA) for net efficiency, flue gas temperature and burning period of bricks*

Parameter	SOV	df	SS	MSS	$F_{Cal.}$	$F_{Tab.}$
Net efficiency	Treatments	6	1325.53	220.92	19.53*	4.46
	Error	14	158.34	11.31	--	--
	Total	20	1483.87	--	--	--
Flue gas temperature	Treatments	6	133200.59	22200.10	3.05^{ns}	4.46
	Error	14	102041.30	7288.66	--	--
	Total	20	235241.89	--	--	--
Smoke number	Treatments	6	57.40	9.57	58.40*	4.46
	Error	14	2.29	0.16	--	--
	Total	20	59.69	--	--	--
Burning time period	Treatments	6	454.00	75.67	56.75*	4.46
	Error	14	18.67	1.33	--	--
	Total	20	472.67	--	--	--

* indicates significant difference between treatment; ns indicates non-significant difference between treatment

Cost-effectiveness of fire bricks

Cost-effectiveness of the fire bricks manufactured from various organic waste was calculated based on local market prices. The price of fuel bricks prepared from various combinations of these materials is also less than that of the control (fuel wood). The number above bars indicates (%) the fuel bricks prepared from the waste material are less expensive than the control (fuel wood) used as fuel (*Fig. 6*).

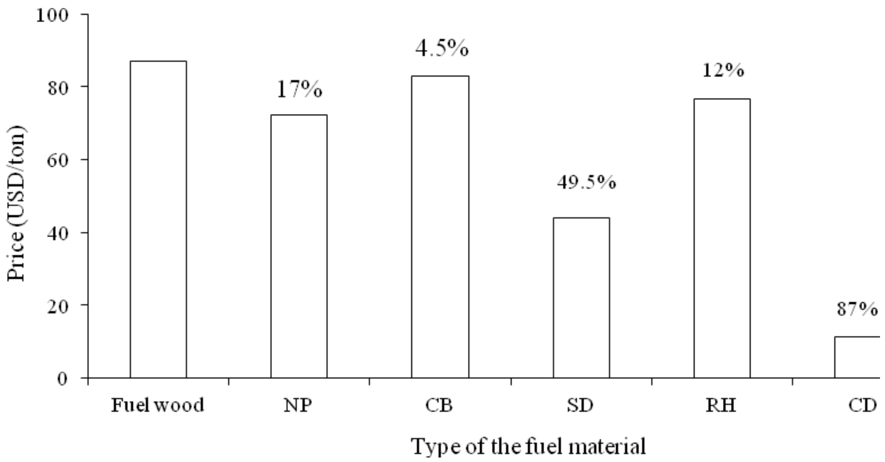

Figure 6. *Cost-effectiveness of using organic waste for manufacturing fire bricks*

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It was assessed that cow dung is 87% cheaper than the fuel wood followed by sawdust that is 49.5% less expensive than the fuel wood.

Conclusion

High quality and durable fire bricks can be made while using a combination of RHNP, RHCB, SDCB, CDCB, CDNP, SDCD in a ratio of 2:3 by volume. Moreover, the fire bricks produced from different organic wastes meet the recommended fuel characteristics as there was no significant difference in the flue gas temperature. The net burning efficiency range of fire bricks ranges from 39.7 to 58.9%, significantly higher than that of the fuel wood. Contrary to fuel wood, fire brick emissions like CO, CO_2, SO_2, NOx are within the permissible limits of NEQS. It is observed that fire bricks are eco-friendly and the use of these bricks will minimize the harmful effects of traditional fuel wood burning which would further minimize pressure on fuel budget of rural households. Fire bricks have the market potential due to their easy way of making, cost effectiveness and environment friendly nature. Similarly, this method recycles the waste into eco-friendly fuel bricks that are more efficient and reduce the burden on wood for energy. These bricks have high durability and can be transported anywhere without any damage to them.

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SOYBEAN (*GLYCINE MAX* L.) GERMPLASM SCREENING AND GEOGRAPHICAL DETERMINATION BASED ON TARGETED ISOFLAVONE METABOLOMICS

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Abstract. In this study, the combination of high-performance liquid chromatography (HPLC) based isoflavone profiling with multivariate analysis was used to screen specific soybean germplasms from Southwestern China, and simultaneously to investigate the influence of genotype, the geographical environment and the germplasm community size on isoflavone biosynthesis. A total of 144 soybean germplasms were evaluated for 12 isoflavones, which varied markedly due to genotypes and locations. Partial least squares analysis (PLS) results exhibited little variability among the 144 samples of four different locations. Five samples were separated from other genotypes based on the first PLS principal component that had high isoflavones. Daidzin, genistin, malonyldaidzin, malonylgenistin and acetylgenistin were the discriminating metabolites. Later the bidirectional orthogonal projection to latent structures-discriminant analysis (O2PLS-DA) was applied for geographical determination of five specific germplasms samples. Geographical environment influenced mainly acetylgenistin, acetyldaidzin, glycitein and malonylglycitin. Our results suggest that genetic factors play a vital role in the isoflavones biosynthesis of a major community, whereas the environmental factors can effectively regulate the isoflavones accumulation especially in the evaluation of small groups of soybean germplasms. These results demonstrate the use of the HPLC based metabolite profiling combined with multivariate analysis (PLS and O2PLS-DA) as a tool for the determination of specific germplasm, biomarker metabolite and geographical origin.
Keywords: *community size, environment, latitude, multivariate analysis, metabolite*

Introduction

Soybean (*Glycine max* L.) is a highly consumable legume crop on the basis of its nutritional value in the world. Soybean is regarded as a functional food with a variety of beneficial composition, especially in China. Soybeans contain several phytochemicals such as isoflavones, tocopherols, saponins and functional protein that promote and maintain human health (Sugano, 2005). Among all, isoflavones are presented in large amount and have gained more attention globally. Soybean isoflavones are an important secondary plant metabolite, which have variety of physiological activities. Having similar structure of endo-estrogen, isoflavones can exhibit weak estrogen-like activities so as to regulate the hormonal level (Wu et al., 2009). It is reported that isoflavones can reduce the occurrence of breast and prostate cancer, risk of cardiovascular diseases and relieve the osteoporosis and menopausal symptoms (Clubbs and Bomser, 2007; Howes et al.,

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2006; Wu et al., 2009). The major isoflavones can be divided into four groups: malonyl glycosides (malonyldaidzin, malonylgenistin, and malonylglycitin), aglycones (daidzein, genistein, and glycitein), acetyl glycosides (acetyldaidzin, acetylgenistin, and acetylglycitin), and glycosides (daidzin, genistin, and glycitin) (Kim et al., 2014). Each isoflavone has different physiological activities. For example, isoflavone aglycones have stronger biological activities and are absorbed faster, and in greater amounts by the body than corresponding glucosides (Izumi et al., 2000; Setchell et al., 2001). Moreover, daidzein and genistein exhibit high antioxidant activities (Devi et al., 2009; Heim et al., 2002), while genistein has high estrogenic potential, and daidzein has lower estrogenic effects (Kuiper et al., 1998).

Due to benefits described above, much effort has been spent to improve the nutritional quality of soybean by transgenic engineering approaches in recent years. For example, soybean genotype has been engineered to contain lower or higher levels of isoflavones (Lee et al., 2008; Paucar et al., 2010). However, phytochemicals are affected by genotype, weather and geographical sowing location, and they can be varied among soybean germplasms and within germplasm when planted in various geographical regions (Eldridge and Kwolek, 1983). Wang and Murphy observed that isoflavone contents varied across the year, and across growing locations within the same year for single soybean germplasms (Wang and Murphy, 1994). Similar studies confirm the results of previous research by studying multi cultivars over multi years and environment (Hoeck et al., 2000; Lee et al., 2003). However, the effects of germplasm community size on isoflavone biosynthesis are still need to be investigated.

China is regarded as the place of origin of soybean with the history of more than 5,000 years. China has built the largest repository where until 2000, 25,144 soybean germplasms have been collected. Southwestern China was identified as core origin, and most germplasms were provided by Yunnan, Sichuan (including Chongqing) and Guizhou provinces (Xinan et al., 1998). It shows the importance of soybean growth in this region, which provides abundant basic material for the selection and breeding of high quality soybean germplasms.

Although the soybean has been a major crop in Southwest of China, but few studies screened specific soybean germplasm, and investigated the correlation of isoflavone with genotype and geographical environment by metabolomics method previously. Therefore, it was significant to evaluate soybean germplasm from South-western China by using metabolomics method.

In the new era of metabolomics, metabolite profiling combined with chemometrics has become a useful tool for determining phenotypic variations, assessing food quality and identifying metabolic networks in biological systems that can lead to direct breeding strategies (Kim et al., 2014; Park et al., 2013). Thus, in the present study, the combination of HPLC based metabolic profiling with multivariate analysis (PLS and O2PLS-DA) was used to screen the specific isoflavone germplasms, and to investigate the influence of genotype, geographical environment and germplasm community size on isoflavone biosynthesis.

Materials and methods

Plant materials and chemicals

In this study, 144 soybean germplasms were collected from Southwest China in 2014. These germplasms include 102 from Sichuan province, 25 from Chongqing, 15

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from Guizhou province and 2 from Yunnan province (*Table S1* in the *Appendix*). Geographical locations and detailed information of these soybean germplasms are shown in *Figure 1* and *Table S1,* respectively. HPLC grade acetonitrile was provided by Thermo Fisher scientific inc. (NYSE: TMO). Six of the isoflavone standards (daidzein, gentstein, glycitein, dadzin, genistin and glyctin) were purchased from Weikeqi Biological Technology Co., Ltd. (China). Six external isoflavone standards (malonyldaidzin, malonylgenistin, malonylglycitin, acetyldaidzin, acetylgenistin and acetylglycitin) were purchased from Wako Pure Chemical Industries, Ltd. Japan.

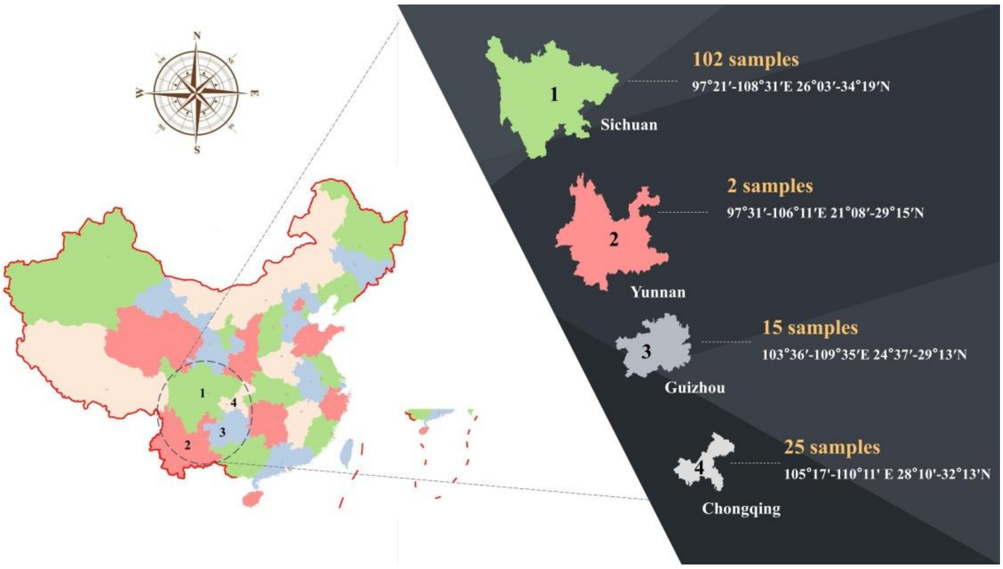

Figure 1. *Geographical locations of soybean germplasms from Southwest China*

Sample preparation

Soybean isoflavones were extracted using a protocol adapted from Liu et al. (2016) with small modifications. All soybeans samples were ground with cyclone miller (Cyclotec 1093 Foss, Denmark), screened through a mesh (size 250 µm) and heated at 40 °C for 24 h. Three extraction replications from soybeans were prepared for each sample. Approximately 200 mg of soya flour was dissolved into 5 mL of a pre chilled MeOH/H_2O (80/20 v: v). The mixture was extracted with ultrasonic for 3 h at 40 °C, and then centrifuged at 11,000 g for 10 min. About 1.5 mL supernatant was filtered through a 0.22 µm organic membrane filter before injecting in a sample bottle. The samples were stored at -20 °C before subjected to HPLC analysis.

Quantification

Agilent 1260 series high performance liquid chromatography (HPLC) system equipped with a mass spectrometric detector (Agilent Quadrupole LC/MS 6120) was used to identified and quantified twelve isoflavones. Three time quantitative HPLC analysis was performed on YMC-Pack ODS-AQ column (4.6 i.d. × 250 mm, RP-18, 5 µm). Previously described chromatographic conditions were used with small modifications (Liu et al., 2016). Linear gradient mobile phase was used, which includes 100% acetonitrile (solution A) and 0.1% acetic acid aqueous (solution B). The linear gradients were as follows: 0 min 15% A, 0~30 min 20% A， 30~60 min 40% A， 60~70

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min 40% A. The flow rate, column temperature and injection volume were 0.8 ml/min, 30 °C and 5 μL, respectively. The UV detector was set to 260 nm. Selected ion monitoring (SIM) mode was used in the mass spectral acquisition with scan range 100−700 amu. The following conditions were used for mass spectra: desolvation pressure, 35 psig; desolvation temperature, 350 °C; electrospray ionization (ESI), positive ion mode and capillary voltage, 3.8 kV. Nitrogen was used in the ion source and as the collision gas, and the desolvation gas flow rate was 10 L min^{-1}.

Each of the twelve isoflavones was identified by comparing the sample retention times with those of the isoflavone standards. However, the isoflavone concentrations were calculated in absolute term via linear regression.

Statistical analysis

Metabolomics analysis combined with chemometrics was used to investigate the quantification data acquired from HPLC. Partial least squares analysis (PLS) was performed to screen the specific soybean germplasms, and to evaluate the among-groups and within-groups variabilities of multivariate data. Score plot and loading plot were provided by the SIMCA-P 13.0 software (Umetrics, MKS Instruments Inc., Umea, Sweden), the former visualized the variation of all samples by observing the clustering or scattering of the point in score plot and the latter explained which variables contribute most to this difference. Specific soybean germplasms were subjected to O2PLS-DA to evaluate the relationships in terms of similarity or difference among them and predict the geographical origin. Furthermore, score-loading correspondence identified the biomarker metabolites which distinguished the samples from different regions.

Results and discussion

Variation of isoflavone in soybean germplasms

Twelve isoflavones including malonylglycosides (malonyldaidzin, malonylgenistin, and malonylglycitin), aglycones (daidzein, genistein, and glycitein), acetyl glycosides (acetyldaidzin, acetylgenistin, and acetylglycitin), and glycosides (daidzin, genistin, and glycitin) were detected in the 144 soybean germplasms (*Fig. 2*), and then analyzed by SPSS 20.0 to obtain the mean value ± standard error. The total isoflavone levels identified in 144 soybean germplasms are shown in *Table S2* (see *Appendix*). The total isoflavone contents varied significantly among all germplasms due to different genotypes and locations. As shown in *Table S2,* the total isoflavone contents of soybean germplasms varied from 0.612 $mg.g^{-1}$ to 7.635 $mg.g^{-1}$. Among the identified isoflavones, glycosides and malonylglycosides derivatives were observed predominant.

However, in Northern China the total isoflavone contents in cultivated and wild soybean germplasms ranged from 1.462-6.115 $mg.g^{-1}$ and 3.896-7.440 $mg.g^{-1}$, respectively (Cui et al., 2013). The reason for that could be the environmental conditions of Southwest China (Sichuan, Chongqing, Guizhou and Yunnan) are relatively cool and wet compared to Northern China. Therefore, high isoflavone accumulation was shown in this study, because earlier studies in warm and dry environments have shown low isoflavone accumulation (Lozovaya et al., 2005). A similar study has confirmed the change in soy isoflavone content with subtle changes in environmental factors (Caldwell et al., 2005).

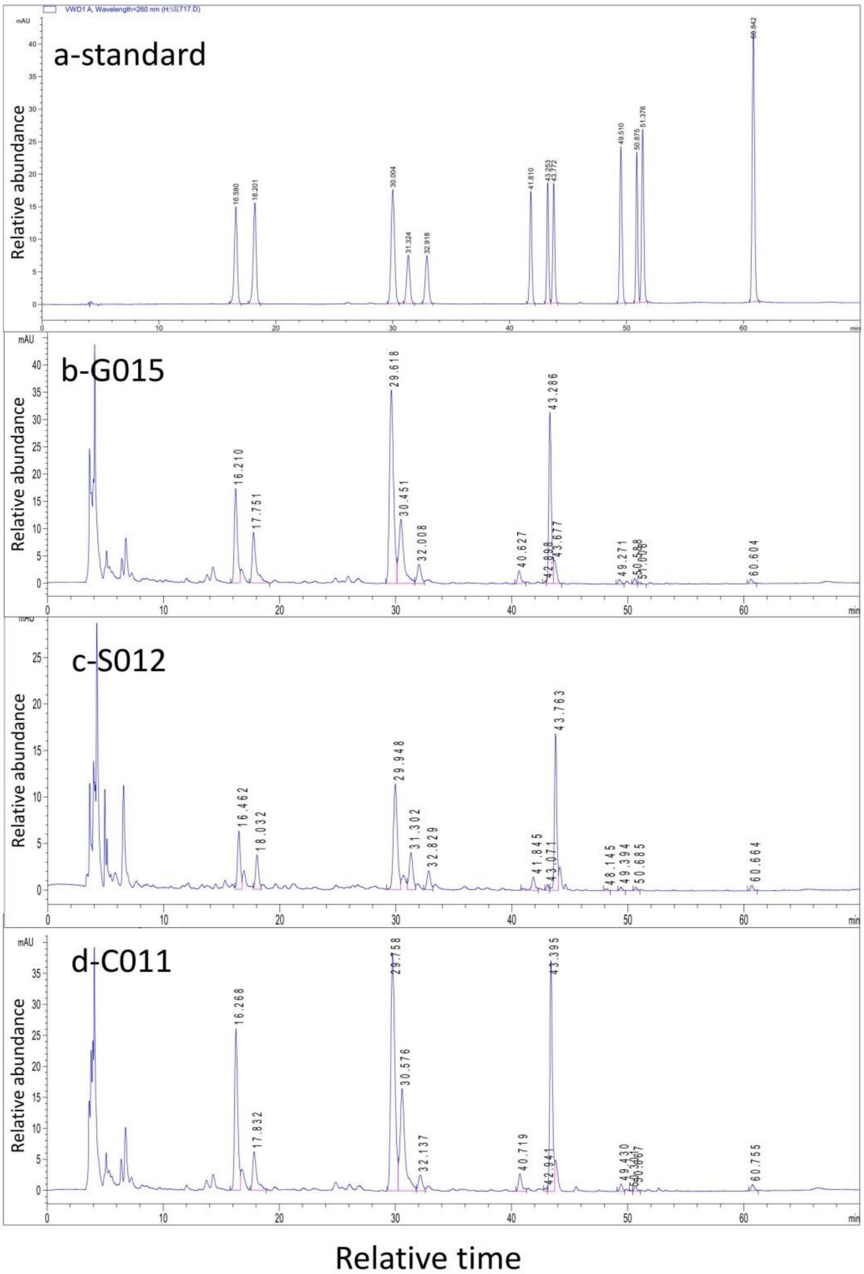

Figure 2. *Total ion chromatograms (TICs) of HPLC analysis of soy isoflavone standards and soybean samples. a, standard chromatogram; b, soybean germplasm "G015" (high MIS, high MIP); c, soybean germplasm "S012" (high MIS, low MIP); d, soybean germplasm "C011" (low MIS, low MIP); Peak assignment: peak 1-daidzin (DG); 2-glycitin (GLG); 3-genistin (GEG); 4-malonyldaidzin (MD); 5-malonylglycitin (MGL); 6-acetyldaidzin (AD); 7-acetylglycitin (AGL); 8-malonylgenistin (MG); 9-daidzein (DE); 10-acetylgeni.stin (AG); 11-glycitein (GLE); 12-genistein (GE); X axis names is Relative time; Y axis names is Relative abundance*

Particularly in recent years, more researches about genotype × environment interaction, especially about the secondary metabolism of plants to investigate the correlation with soybean isoflavones of different varieties and regions. Ge et al. (2011) measured the

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isoflavone of 100 Chinese soybean varieties of different origin, and indicated that the soybean grown in southern China had higher isoflavone content than in northern China. Similar study by Sun et al. (2004) measured the isoflavone content of 249 Chinese soybean varieties, and indicated the great variation among varieties and locations. Moreover, the total isoflavones of wild soybean were noted higher than cultivated soybean (Chune et al., 2010). Undoubtedly, genotype × environment interaction decides the most of the plant secondary metabolism, such as soy isoflavone biosynthesis.

In addition, comparative analysis of total isoflavone contents in soybean genotypes from four different locations showed higher isoflavone contents in samples grown in Chongqing (*Table S2*). This is because, Chongqing is located at a high longitude area, has a subtropical humid climate that promotes isoflavones accumulation in soybean seed. This is consistent with previous study that high longitudes are favourable for isoflavone biosynthesis (Wu et al., 2017).

Screening of germplasm by PLS-DA

HPLC-based isoflavone profiling was conducted to assess the variations in isoflavone contents of various soybean germplasms. Data regarding the 12 isoflavones identified by HPLC analysis were subjected to PLS-DA to identify the differences among soybean germplasms and four different regions of origin (*Fig. 3*).

The PLS-DA score plot showed the profile differences of samples, which are different in genotypes and source of regions (*Fig. 3A*). The corresponding loadings scatter plots of the isoflavone profiling is presented in *Figure 3B*. PLS-DA score plot demonstrated a little variability among samples of four regions of origin. However, five samples (C002, G010, G011, G013 and S075) clearly stood out from other genotypes in PLS component 1 (*Fig. 3A*). Previous studies have indicated the effects of genetic variation on isoflavone biosynthesis (Eldridge and Kwolek, 1983; Wang and Murphy, 1994). In our study, this could confirm that genotype plays an important role in the synthesis of isoflavones. However, PLS could not distinguish the influence of geographical environment.

The corresponding loading plot is used to identify those compounds that can exhibit maximum variability within a population (Kim et al., 2013). The five specific isoflavone germplasms were screened-out from all samples, resulted from their higher scores in component 1 of the PLS-DA. Daidzin, genistin, malonyldaidzin, malonylgenistin and acetylgenistin led to major contribution in component 1, for which the eigenvectors were 0.40, 0.42, 0.39, 0.40 and 0.36, respectively. The loading plot showed that isoflavone contents were higher in these five samples than others. Moreover, daidzin, genistin, malonyldaidzin, malonylgenistin and acetylgenistin were the discriminating metabolites which distinguished the specific isoflavone germplasms from the others. The isoflavone contents were controlled by genotype which contributed to the variation of specific germplasms in component 1 of the PLS-DA. The result is similar to Kim et al. (2014), however more studies confirmed the influence of the both the genotype and the environment on the isoflavones synthesis (Lee et al., 2003; Murphy et al., 2009). Several studies have shown the individual and total isoflavone contents of genotypes at different locations. Hoeck et al. (2000) found the significant differences among locations in one or more years for total and individual isoflavone contents. However, the consistency of the ranking among genotypes for the contents of individual and total isoflavones seems to depend on the magnitude of the differences in their inherent genetic potential for the traits (Zhang et al., 2014; Carter et al., 2018).

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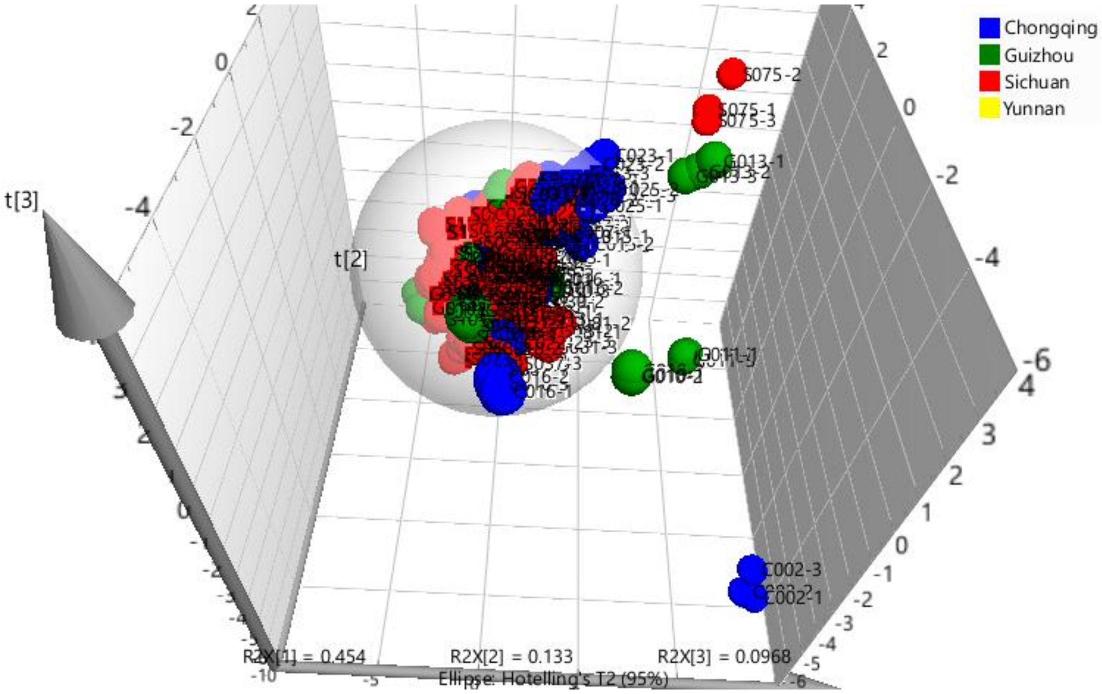

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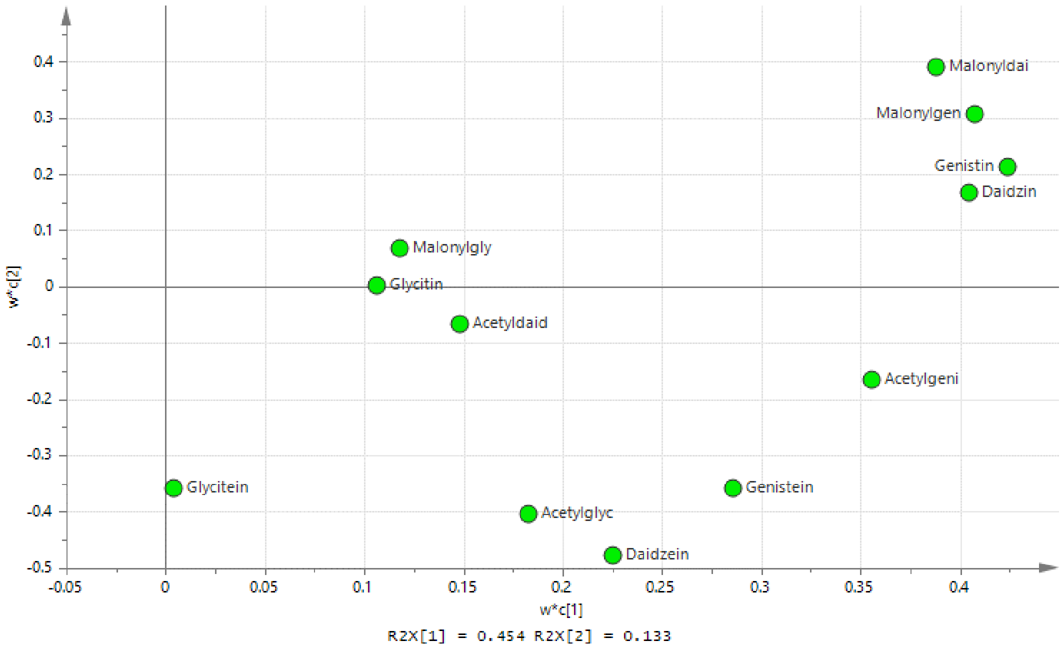

B

Figure 3. *PLS-DA score plots (A) and corresponding loadings scatter plots (B) of 144 geographical different soybean seeds*

To further elaborate the differences among 12 isoflavones in different samples, a heat map was created (*Fig. 4*). Different colors indicate the relative concentrations of corresponding metabolites in each sample. Red color indicates higher isoflavone level. The results clearly show that the content of daidzin, genistin, malonyldaidzin, malonylgenistin and acetylgenistin were higher in five specific germplasms (C002,

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G010, G011, G013 and S075) than others. These five specific germplasms had large amount of total isoflavone contents. Moreover, it also shows the daidzin, genistin, malonyldaidzin, malonylgenistin and acetylgenistin as discriminating metabolites which distinguished the specific isoflavone samples from the others.

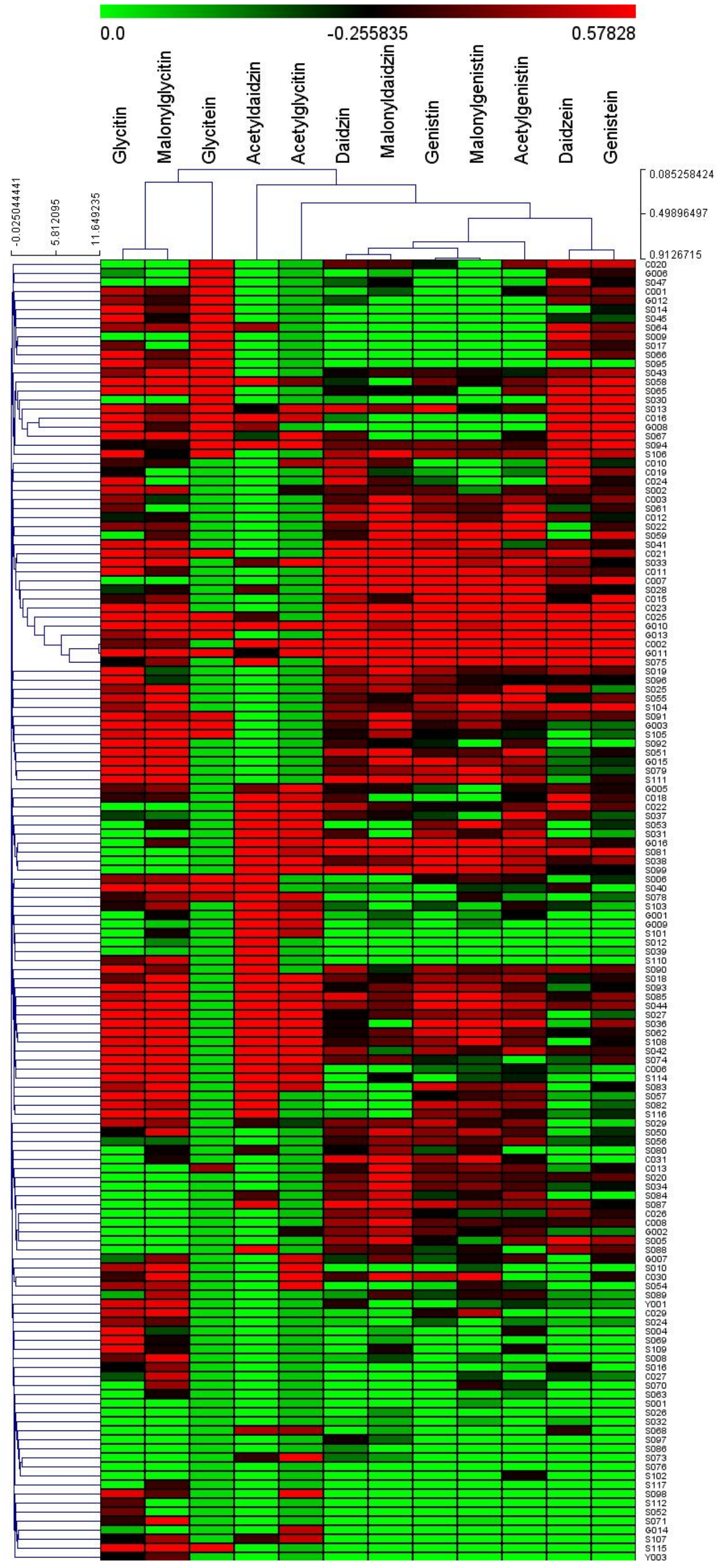

Figure 4. *Heat map and cluster analysis of 12 isoflavones in 144 soybean genotypes*

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Geographical determination

O2PLS-DA is a good multivariate projection method, which separates the structured noise in bidirectional way X and Y. O2PLS divides systematic variation into two parts (X and Y). One is regarded as the predictive part which is related to both X and Y (covarying), whereas the other is regarded as parallel part that is not related to orthogonal. As a result, O2PLS improves the interpretation of the predicted model and simplify it as well. It has been reported that O2PLS performed very well for geographical determination of different samples (Consonni et al., 2010). We therefore applied O2PLS-DA for geographical determination of five specific germplasms (*Fig. 5*).

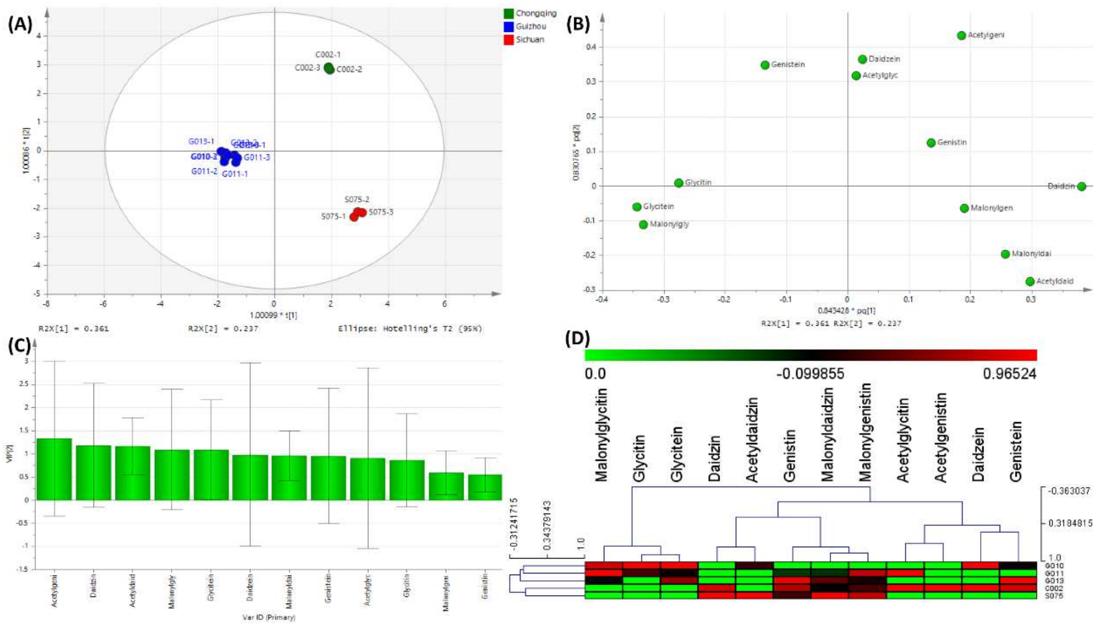

Figure 5. *O2PLS-DA score plots (A) and corresponding loadings scatter plots (B), important features identification (C), and heat map of cluster analysis (D) of 5 geographical different soybean seeds from Chongqing, Guizhou and Sichuan*

These five specific germplasm samples came from clustering and scattering in score scatter plot (*Fig. 5*). The parameters of cross-validation plot, i.e., R2(X) = 0.995, R2(Y) = 0.995, and Q2 = 0.989, indicated a valid model. All samples which had different geographical origins showed a clear separation into three groups: Chongqing, Guizhou and Sichuan (*Fig. 5A*). The different geographical environment led to the sample variations. The score scatter plot indicated that the samples grown in Guizhou were clearly separated from others. This variation was mainly attributed to glycitin, glycitein, and malonylglycitin, of which the corresponding loading scatter plot was negative and contributed mostly to the first O2PLS principal component (*Fig. 5B*). Combining with the VIP value (*Fig. 5C*), glycitein and malonylglycitin with VIP more than 1.0 were determined as biomarker metabolites for soybean seed from Guizhou. In addition, samples from Chongqing (component 2 dimension) and Sichuan (component 2 dimension) were distinguished based on the second O2PLS principal component. The corresponding loading scatter plot was positive for daidzein, acetylglycitin and

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acetylgenistin which contributed most to second O2PLS principal component. As a result, samples from Chongqing were separated from others. Combining the VIP values with the loading plot, the acetylgenistin with VIP more than 1.0 was determined as a biomarker metabolite to distinguish samples of Chongqing from others. However, as the loading plots showed that the acetyldaidzin had a negative loading value and contributed most to second O2PLS principal component. In addition, the VIP value of acetyldaidzin was more than 1.0. This confirmed that acetyldaidzin was a biomarker metabolite that distinguished samples of Sichuan from others.

The results suggest that the content of glycitein and malonylglycitin were higher in samples from Guizhou while the content of acetylgenistin and acetyldaidzin were higher in samples from Chongqing and Sichuan, respectively. Moreover, the comparative analysis of total isoflavone contents in five samples from different locations showed higher isoflavone contents in samples grown in Sichuan province followed by Chongqing (*Table S3* in the *Appendix*). This could be due to the high latitude of both Sichuan and Chongqing areas with favorable environment to promote soybean isoflavone accumulation (*Fig. 1*). It is consistent with earlier study that the geographical latitude of the genotype location plays an important role in separating soybean genotypes with different chemical profiles (Wu et al., 2017). A similar study was conducted by Zhang et al. (2007) in Northern China also confirmed the positive correlation between latitude and soybean isoflavone.

In addition, specific isoflavones were screened from all samples because their genotypes expressed higher isoflavone contents than the others. Therefore, the difference in regions led to the difference of isoflavone contents, which distinguished the five specific samples. It confirmed that geographical environment also influence the isoflavones synthesis besides genotypes. However, the geographical environment could distinguish small community size of specific samples.

A heat map was created to further explain the geographical determination of soybean genotypes based on the targeted isoflavone metabolomics, and to interpret the influence of geographical environment on isoflavone contents (*Fig. 5D*). Samples from different regions clustered different groups in the heat map. However, they were not completely divided into three groups on the base of regions. Samples variability could be distinguished by difference of 12 isoflavones presented in the heat map. As results show, the content of glycitein and malonylglycitin were higher in samples from Guizhou while the content of acetylgenistin and acetyldaidzin were found higher in samples from Chongqing and Sichuan, respectively. The results are the same as the conclusion drawn from O2PLS-DA. It confirmed that glycitein, malonylglycitin, acetylgenistin and acetyldaidzin were biomarker metabolites that could interpret the influence of geographical environment to the isoflavone contents.

Conclusion

In conclusion, the synthesis of isoflavone was affected by both the genotype and the geographical environment. When the community size of germplasm samples was large, the isoflavone contents were mainly subjected to the effect of genotypes which distinguished samples from the others. The genotype mainly influenced the content of daidzin, genistin, malonyldaidzin, malonylgenistin and acetylgenistin. These metabolites differentiated the specific isoflavone germplasms from others. However, the geographical environment also played an important role in distinguishing the small groups of soybean germplasms. Different geographical environments had different influence on the synthesis of isoflavones. The geographical environment affected the acetylgenistin, acetyldaidzin, glycitein and malonylglycitin, which were considered as biomarker metabolites. In addition, these distinguished specific samples from three different regions could be used for future breeding programs.

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APPENDIX

Table S1. A. *Detailed information of soybean germplasms. Schedule soybean germplasm resources details -1*

Code	Name	Origin	Code	Name	Origin	Code	Name	Origin
*G001	E119	Liu Hongchang collected from Guizhou	*S013	E237-5	Jiuzhaigou County, Sichuan Province	*S041	GYQC	Qingchuan County, Sichuan Province
*G002	E193	Dafang County, Guizhou Province	*S014	E238	Jiuzhaigou County, Sichuan Province	*S042	E233	Wangcang County, Sichuan Province
*G003	E194	Dafang County, Guizhou Province	*S016	E247	Wenchuan County, Sichuan Province	*S043	E316	Leshan City, Sichuan Province
*G005	E157	Daozhen County, Guizhou Province	*S017	E246-1	Wenchuan County, Sichuan Province	*S044	E133	Danling County, Sichuan Province
*G006	E159	Daozhen County, Guizhou Province	*S018	E248	Wenchuan County, Sichuan Province	*S045	E129	Leshan City, Sichuan Province
*G007	E160	Daozhen County, Guizhou Province	*S019	E327	Bazhong City, Sichuan Province	*S047	E294	Yanyuan County, Sichuan Province
*G008	E41	Kaige County, Guizhou Province	*S020	E331	Dujiangyan County, Sichuan Province	*S050	A12-3	Luzhou City, Sichuan Province
G009	14033	Guizhou Oil Research Institute	*S022	E5	Chengdu city, Sichuan Province	*S051	LLZ	Luzhou City, Sichuan Province
*G010	E276	Qinglong County, Guizhou Province	*S024	E278-2	Xinjin county, Sichuan Province	*S052	14030	Pengshan County, Sichuan Province
*G011	E180	Songtao County, Guizhou Province	*S025	E244-2	Xuanhan County, Sichuan Province	*S053	E333	Renshou County, Sichuan Province
*G012	E171	Zunyi City, Guizhou Province	*S026	E183	Zhongjiang County, Sichuan Province	*S054	E334	Renshou County, Sichuan Province
*G013	E252	Zunyi City, Guizhou Province	*S027	E184	Zhongjiang County, Sichuan Province	*S055	E188	Renshou County, Sichuan Province
*G014	E25	Zunyi City, Guizhou Province	*S028	E337	Zhongjiang County, Sichuan Province	*S056	E189	Renshou County, Sichuan Province
*G015	E172	Zunyi City, Guizhou Province	*S029	E338	Zhongjiang County, Sichuan Province	*S057	E347	An County, Sichuan Province
*G016	E173	Nanbai County, Guizhou Province	*S030	E339	Zhongjiang County, Sichuan Province	*S058	A17	Jiangyou County, Sichuan Province
S001	14063	Sichuan Agricultural University	*S031	A13	Zhongjiang County, Sichuan Province	*S059	E199-2	Pingwu County, Sichuan Province
S002	E197	Sichuan Agricultural University	*S032	14067	Deyang City, Sichuan Province	*S061	E205-2	Pingwu County, Sichuan Province
S004	14031	Sichuan Agricultural University	*S033	A45	Guang'an City, Sichuan Province	*S062	A36	Langzhong County, Sichuan Province
S005	E210	Sichuan Agricultural University	*S034	E178	Linshui County, Sichuan Province	*S063	14008	Langzhong County, Sichuan Province
S006	E209	Sichuan Agricultural University	*S036	A40	Yuechi County, Sichuan Province	S064	E318	Nanchong Institute of Agricultural Sciences
S008	14072	Sichuan Agricultural University	*S037	E288-2	Guangyuan City, Sichuan Province	S065	E319	Nanchong Institute of Agricultural Sciences
S009	E282	Sichuan Agricultural University	*S038	E317	Changxi County, Sichuan Province	S066	E320	Nanchong Institute of Agricultural Sciences
S010	14045	Sichuan Agricultural University	*S039	E312	Qingchuan County, Sichuan Province	S067	E324	Nanchong Institute of Agricultural Sciences
*S012	E340	Sichuan Province	*S040	E313	Qingchuan County, Sichuan Province	S068	14038	Nanchong Institute of Agricultural Sciences

Note: The code with * are landraces, and the code without * are cultivars

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Table S1. B. *Detailed information of soybean germplasms. Schedule soybean germplasm resources details -2*

Code	Name	Origin	Code	Name	Origin	Code	Name	Origin
S069	14050	Nanchong Institute of Agricultural Sciences	*S095	14091	Yibin City, Sichuan Province	*C002	E346	Chongqing
S070	14047	Nanchong Institute of Agricultural Sciences	*S096	E207-1	Yibin City, Sichuan Province	*C003	E242	Chongqing
S071	14060	Nanchong Institute of Agricultural Sciences	*S097	E207-2	Yibin City, Sichuan Province	*C006	14069	Dazu County, Chongqing
S073	14024	Nanchong Institute of Agricultural Sciences	*S098	14029	Anyue County, Sichuan Province	*C007	E175-1	Dianjiang County, Chongqing
S074	C103	Nanchong Institute of Agricultural Sciences	*S099	A18	Lezhi County, Sichuan Province	*C008	E175-2	Dianjiang County, Chongqing
S075	E352-3	Yingshan County, Sichuan Province	S101	14019	Zigong Institute of Agricultural Sciences	*C010	E176	Dianjiang County, Chongqing
*S076	14090	Neijiang City, Sichuan Province	S102	14023	Zigong Institute of Agricultural Sciences	*C011	E176-1	Dianjiang County, Chongqing
*S078	14020	Zizhong County, Sichuan Province	S103	E203	Zigong Institute of Agricultural Sciences	*C012	E176-3	Dianjiang County, Chongqing
*S079	YCQ-1	Weiyuan County, Sichuan Province	S104	E204	Zigong Institute of Agricultural Sciences	*C013	E176-4	Dianjiang County, Chongqing
*S080	YCQ-2	Weiyuan County, Sichuan Province	S105	14048	Zigong Institute of Agricultural Sciences	*C015	E66	Dianjiang County, Chongqing
*S081	E345	Shuangliu County, Sichuan Province	S106	E204-1	Zigong Institute of Agricultural Sciences	*C016	E241	Jiangjin District, Chongqing City
*S082	E281	Suining City, Sichuan Province	S107	14042	Zigong Institute of Agricultural Sciences	*C018	E145	Qujiang District, Chongqing City
*S083	E281-1	Suining City, Sichuan Province	S108	14052	Zigong Institute of Agricultural Sciences	*C019	14001	Qujiang District, Chongqing City
*S084	A25-1	Suining City, Sichuan Province	S109	14014	Zigong Institute of Agricultural Sciences	*C020	E148	Qujiang District, Chongqing City
*S085	A25-2	Suining City, Sichuan Province	S110	14056	Zigong Institute of Agricultural Sciences	*C021	E150	Qujiang District, Chongqing City
*S086	14037	Shehong County, Sichuan Province	S111	14028	Zigong Institute of Agricultural Sciences	*C022	E144	Qujiang District, Chongqing City
*S087	E326	Ya'an City, Sichuan Province	S112	14058	Zigong Institute of Agricultural Sciences	*C023	E164	Qujiang District, Chongqing City
*S088	E343	Ya'an City, Sichuan Province	S114	14071	Zigong Institute of Agricultural Sciences	*C024	E149	Qujiang District, Chongqing City
*S089	E285	Ya'an City, Sichuan Province	S115	14049	Zigong Institute of Agricultural Sciences	*C025	E151	Qujiang District, Chongqing City
*S090	E161	Ya'an City, Sichuan Province	S116	14068	Zigong Institute of Agricultural Sciences	*C026	E243	Rongchang County, Chongqing City
*S091	E162	Ya'an City, Sichuan Province	S117	14070	Zigong Institute of Agricultural Sciences	*C027	14025	Tongnan County, Chongqing City
*S092	E163	Ya'an City, Sichuan Province	*Y001	14161	Yunnan Province	*C029	14035	Tongnan County, Chongqing City
*S093	E165	Ya'an City, Sichuan Province	*Y003	14003	Chuxiong City, Yunnan Province	*C030	Y-1	Yunyang County, Chongqing City
*S094	E212	Ya'an City, Sichuan Province	*C001	E274	Chongqing	*C031	Y-2	Yunyang County, Chongqing City

Note: The code with * are landraces, and the code without * are cultivars

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Table S2. *The total isoflavone levels identified in 144 soybean germplasms*

Name	DG	GLG	GEG	MD	MGL	AD	AGL	MG	DE	AG	GLE	GE.	Total
	Daidzin	Glycitin	Genistin	Malonyldaidzin	Malonylglycitin	Acetyldaidzin	Acetylglycitin	Malonylgenistin	Daidzein	Acetylgenistin	Glycitein	Genistein	
C001	0.330±0.017	0.184±0.004	0.423±0.005	0.286±0.005	0.114±0.002	0.000±0.000	0.000±0.000	0.248±0.005	0.029±0.000	0.011±0.000	0.043±0.003	0.026±0.000	1.695±0,004
C002	1.823±0.059	0.165±0.011	2.253±0.091	0.939±0.035	0.116±0.003	0.044±0.001	0.158±0.015	1.288±0.062	0.119±0.001	0.064±0.001	0.000±0.000	0.077±0.002	7.044±0.144
C003	0.467±0.010	0.174±0.004	0.767±0.016	0.491±0.006	0.097±0.001	0.000±0.000	0.000±0.000	0.439±0.005	0.025±0.000	0.014±0.000	0.000±0.000	0.026±0.000	2.500±0.038
C006	0.287±0.008	0.252±0.006	0.527±0.013	0.191±0.003	0.169±0.003	0.046±0.003	0.020±0.001	0.323±0.008	0.020±0.000	0.010±0.001	0.000±0.000	0.019±0.000	1.864±0.042
C007	0.932±0.036	0.051±0.003	1.255±0.031	0.562±0.030	0.036±0.001	0.000±0.000	0.000±0.000	0.751±0.035	0.035±0.002	0.016±0.001	0.000±0.000	0.035±0.002	3.674±0.134
C008	0.544±0.015	0.037±0.001	0.684±0.020	0.529±0.013	0.000±0.000	0.000±0.000	0.000±0.000	0.407±0.012	0.026±0.001	0.011±0.001	0.000±0.000	0.024±0.000	2.262±0.062
C010	0.436±0.015	0.101±0.006	0.683±0.023	0.506±0.028	0.072±0.007	0.000±0.000	0.000±0.000	0.450±0.019	0.019±0.000	0.013±0.000	0.019±0.010	0.022±0.000	2.320±0.095
C011	0.587±0.006	0.157±0.002	0.477±0.004	0.362±0.022	0.107±0.005	0.000±0.000	0.017±0.000	0.246±0.004	0.049±0.002	0.009±0.001	0.000±0.000	0.021±0.000	2.032±0.037
C012	0.729±0.021	0.217±0.009	1.036±0.023	0.708±0.021	0.110±0.006	0.000±0.000	0.000±0.000	0.569±0.019	0.030±0.000	0.014±0.000	0.000±0.000	0.024±0.000	3.437±0.099
C013	0.604±0.002	0.141±0.001	0.822±0.005	0.540±0.007	0.103±0.001	0.000±0.000	0.000±0.000	0.417±0.002	0.029±0.002	0.016±0.000	0.000±0.000	0.021±0.000	2.693±0.012
C015	0.559±0.001	0.154±0.002	1.803±0.030	0.334±0.001	0.119±0.006	0.000±0.000	0.000±0.000	1.145±0.009	0.023±0.001	0.027±0.000	0.000±0.000	0.041±0.001	4.206±0.031
C016	0.357±0.002	0.214±0.002	0.482±0.004	0.188±0.002	0.123±0.002	0.040±0.001	0.019±0.000	0.242±0.003	0.093±0.002	0.008±0.000	0.157±0.004	0.051±0.001	1.975±0.015
C018	0.461±0.001	0.153±0.001	0.469±0.007	0.255±0.007	0.109±0.006	0.039±0.001	0.021±0.001	0.249±0.006	0.051±0.005	0.010±0.000	0.000±0.000	0.023±0.000	1.841±0.022
C019	0.592±0.030	0.146±0.020	0.504±0.006	0.291±0.040	0.058±0.029	0.000±0.000	0.000±0.000	0.26055±0.000	0.041±0.001	0.010±0.002	0.000±0.000	0.026±0.000	1.928±0.019
C020	0.488±0.022	0.126±0.005	0.566±0.017	0.353±0.012	0.070±0.003	0.000±0.000	0.000±0.000	0.244±0.005	0.037±0.001	0.013±0.000	0.035±0.001	0.029±0.000	1.959±0.065
C021	0.862±0.024	0.196±0.006	0.986±0.020	0.692±0.016	0.127±0.003	0.000±0.000	0.000±0.000	0.496±0.007	0.049±0.001	0.014±0.000	0.040±0.001	0.028±0.000	3.489±0.074
C022	0.572±0.024	0.116±0.003	0.589±0.023	0.348±0.014	0.079±0.002	0.054±0.004	0.023±0.001	0.355±0.015	0.047±0.002	0.013±0.001	0.000±0.000	0.024±0.001	2.219±0.089
C023	1.121±0.034	0.241±0.009	1.253±0.041	0.723±0.025	0.176±0.008	0.000±0.000	0.000±0.000	0.832±0.032	0.054±0.002	0.016±0.001	0.000±0.000	0.030±0.001	4.445±0.152
C024	0.611±0.010	0.193±0.006	0.532±0.005	0.354±0.005	0.080±0.003	0.000±0.000	0.000±0.000	0.259±0.002	0.037±0.000	0.008±0.000	0.000±0.000	0.022±0.000	2.096±0.029
C025	1.15±0.049	0.294±0.011	1.441±0.056	0.509±0.002	0.151±0.001	0.020±0.001	0.000±0.000	0.683±0.009	0.060±0.002	0.020±0.000	0.050±0.002	0.038±0.000	4.421±0.112
C026	0.514±0.003	0.059±0.002	0.577±0.001	0.420±0.003	0.000±0.000	0.000±0.000	0.000±0.000	0.322±0.005	0.032±0.001	0.010±0.000	0.000±0.000	0.023±0.000	1.956±0.007
C027	0.199±0.012	0.138±0.005	0.373±0.017	0.219±0.012	0.129±0.013	0.000±0.000	0.000±0.000	0.328±0.009	0.022±0.001	0.006±0.000	0.000±0.000	0.020±0.001	1.434±0.065
C029	0.332±0.036	0.186±0.006	0.610±0.044	0.256±0.016	0.134±0.006	0.000±0.000	0.000±0.000	0.490±0.051	0.010±0.005	0.009±0.003	0.000±0.000	0.018±0.000	2.047±0.021
C030	0.460±0.009	0.155±0.019	0.808±0.021	0.483±0.042	0.147±0.012	0.000±0.000	0.082±0.002	0.855±0.016	0.018±0.001	0.008±0.003	0.000±0.000	0.022±0.000	3.038±0.114
C031	0.612±0.022	0.098±0.003	0.776±0.024	0.557±0.017	0.103±0.002	0.000±0.000	0.000±0.000	0.716±0.024	0.018±0.000	0.011±0.000	0.000±0.000	0.018±0.000	2.910±0.015

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	Daidzin	Glycitin	Genistin	Malonyldaidzin	Malonylglycitin	Acetyldaidzin	Acetylglycitin	Malonylgenistin	Daidzein	Acetylgenistin	Glycitein	Genistein	
G001	0.339±0.037	0.126±0.004	0.479±0.007	0.285±0.022	0.101±0.002	0.040±0.000	0.022±0.000	0.304±0.003	0.016±0.000	0.010±0.000	0.000±0.000	0.018±0.000	1.740±0.053
G002	0.515±0.028	0.042±0.001	0.694±0.039	0.428±0.024	0.000±0.000	0.000±0.000	0.000±0.000	0.351±0.017	0.020±0.001	0.012±0.001	0.000±0.000	0.020±0.001	2.088±0.107
G003	0.455±0.006	0.219±0.003	0.623±0.008	0.464±0.008	0.149±0.006	0.000±0.000	0.000±0.000	0.473±0.007	0.020±0.000	0.011±0.000	0.038±0.001	0.020±0.000	2.472±0.028
G005	0.418±0.004	0.162±0.002	0.540±0.003	0.326±0.002	0.119±0.002	0.024±0.007	0.024±0.001	0.273±0.010	0.029±0.001	0.011±0.000	0.000±0.000	0.022±0.000	1.949±0.008
G006	0.280±0.013	0.133±0.005	0.414±0.014	0.265±0.010	0.080±0.004	0.000±0.000	0.000±0.000	0.223±0.008	0.026±0.001	0.008±0.000	0.030±0.002	0.023±0.000	1.482±0.056
G007	0.379±0.011	0.136±0.003	0.538±0.013	0.377±0.011	0.120±0.003	0.000±0.000	0.021±0.000	0.360±0.008	0.012±0.000	0.012±0.000	0.000±0.000	0.022±0.000	1.976±0.038
G008	0.273±0.002	0.227±0.003	0.421±0.002	0.128±0.002	0.108±0.001	0.027±0.001	0.000±0.000	0.195±0.005	0.068±0.003	0.008±0.000	0.141±0.005	0.044±0.000	1.641±0.003
G009	0.310±0.002	0.113±0.001	0.412±0.000	0.271±0.003	0.090±0.002	0.044±0.000	0.020±0.000	0.309±0.003	0.012±0.000	0.008±0.000	0.000±0.000	0.016±0.000	1.604±0.008
G010	1.163±0.008	0.282±0.004	1.316±0.013	0.419±0.015	0.150±0.004	0.073±0.001	0.025±0.000	0.509±0.009	0.111±0.000	0.021±0.000	0.088±0.002	0.060±0.000	4.216±0.009
G011	0.925±0.085	0.147±0.012	1.422±0.128	0.676±0.059	0.118±0.009	0.136±0.013	0.031±0.002	1.032±0.096	0.029±0.002	0.016±0.001	0.000±0.000	0.034±0.001	4.566±0.407
G012	0.371±0.006	0.175±0.002	0.437±0.002	0.232±0.022	0.106±0.002	0.000±0.000	0.000±0.000	0.190±0.008	0.031±0.001	0.009±0.000	0.044±0.002	0.024±0.000	1.620±0.032
G013	0.875±0.017	0.175±0.003	2.353±0.047	1.092±0.024	0.133±0.001	0.000±0.000	0.000±0.000	1.210±0.027	0.058±0.001	0.026±0.001	0.055±0.006	0.084±0.001	6.062±0.127
G014	0.081±0.001	0.131±0.003	0.142±0.004	0.073±0.003	0.083±0.001	0.000±0.000	0.018±0.001	0.062±0.002	0.005±0.005	0.002±0.000	0.000±0.000	0.015±0.000	0.612±0.018
G015	0.467±0.018	0.303±0.010	0.924±0.040	0.510±0.019	0.148±0.003	0.000±0.000	0.000±0.000	0.472±0.017	0.020±0.000	0.014±0.001	0.000±0.000	0.021±0.000	2.880±0.106
G016	0.822±0.005	0.122±0.001	0.987±0.008	0.538±0.009	0.111±0.002	0.097±0.001	0.021±0.000	0.625±0.010	0.031±0.001	0.018±0.001	0.000±0.000	0.022±0.000	3.395±0.013
S001	0.181±0.004	0.095±0.004	0.302±0.004	0.231±0.003	0.087±0.003	0.000±0.000	0.000±0.000	0.306±0.006	0.012±0.001	0.009±0.000	0.000±0.000	0.016±0.001	1.239±0.013
S002	0.479±0.020	0.188±0.003	0.682±0.005	0.357±0.016	0.128±0.006	0.000±0.000	0.000±0.000	0.307±0.003	0.029±0.000	0.012±0.001	0.000±0.000	0.023±0.001	2.218±0.012
S004	0.323±0.001	0.206±0.002	0.472±0.002	0.267±0.001	0.096±0.000	0.000±0.000	0.000±0.000	0.246±0.011	0.018±0.000	0.011±0.000	0.000±0.000	0.016±0.000	1.656±0.013
S005	0.546±0.006	0.057±0.000	0.588±0.004	0.460±0.008	0.000±0.000	0.000±0.000	0.000±0.000	0.305±0.004	0.049±0.002	0.013±0.000	0.000±0.000	0.027±0.001	2.045±0.022
S006	0.331±0.007	0.174±0.006	0.611±0.012	0.227±0.002	0.123±0.003	0.061±0.002	0.000±0.000	0.408±0.003	0.018±0.000	0.011±0.000	0.039±0.001	0.021±0.000	2.023±0.032
S008	0.340±0.005	0.165±0.002	0.472±0.008	0.290±0.002	0.143±0.004	0.000±0.000	0.000±0.000	0.312±0.003	0.013±0.000	0.009±0.001	0.000±0.000	0.018±0.000	1.761±0.025
S009	0.299±0.009	0.100±0.002	0.289±0.005	0.132±0.003	0.060±0.001	0.000±0.000	0.000±0.000	0.118±0.001	0.047±0.001	0.006±0.000	0.053±0.003	0.026±0.000	1.131±0.018
S010	0.283±0.004	0.180±0.002	0.450±0.006	0.211±0.004	0.137±0.002	0.000±0.000	0.029±0.002	0.3234±0.007	0.015±0.001	0.008±0.000	0.000±0.000	0.018±0.000	1.656±0.025
S012	0.150±0.001	0.113±0.002	0.343±0.002	0.132±0.004	0.093±0.009	0.037±0.002	0.000±0.000	0.244±0.007	0.013±0.000	0.007±0.000	0.000±0.000	0.018±0.000	1.150±0.024
S013	0.610±0.013	0.204±0.006	0.917±0.020	0.398±0.037	0.116±0.002	0.013±0.007	0.021±0.001	0.343±0.008	0.061±0.002	0.012±0.000	0.048±0.001	0.058±0.001	2.801±0.036
S014	0.255±0.005	0.229±0.001	0.432±0.008	0.110±0.001	0.114±0.001	0.000±0.000	0.000±0.000	0.182±0.002	0.017±0.000	0.009±0.000	0.047±0.001	0.021±0.000	1.418±0.017

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	Daidzin	Glycitin	Genistin	Malonyldaidzin	Malonylglycitin	Acetyldaidzin	Acetylglycitin	Malonylgenistin	Daidzein	Acetylgenistin	Glycitein	Genistein	
S016	0.287±0.020	0.144±0.001	0.404±0.003	0.182±0.002	0.120±0.004	0.000±0.000	0.000±0.000	0.268±0.002	0.023±0.001	0.009±0.000	0.000±0.000	0.018±0.000	1.455±0.018
S017	0.226±0.007	0.166±0.005	0.240±0.006	0.123±0.002	0.073±0.002	0.000±0.000	0.000±0.000	0.099±0.002	0.030±0.001	0.006±0.000	0.062±0.005	0.023±0.000	1.047±0.028
S018	0.492±0.012	0.165±0.003	0.761±0.016	0.316±0.004	0.147±0.003	0.063±0.002	0.025±0.001	0.453±0.007	0.022±0.000	0.014±0.000	0.000±0.000	0.022±0.000	2.479±0.046
S019	0.521±0.013	0.213±0.004	0.778±0.017	0.469±0.007	0.095±0.001	0.000±0.000	0.000±0.000	0.401±0.005	0.033±0.000	0.012±0.001	0.000±0.000	0.025±0.000	2.548±0.043
S020	0.495±0.021	0.087±0.003	0.726±0.016	0.474±0.010	0.055±0.001	0.000±0.000	0.000±0.000	0.416±0.008	0.026±0.001	0.012±0.000	0.000±0.000	0.024±0.000	2.317±0.037
S022	0.474±0.009	0.172±0.003	1.019±0.016	0.492±0.030	0.117±0.003	0.000±0.000	0.000±0.000	0.539±0.010	0.018±0.000	0.016±0.000	0.000±0.000	0.023±0.000	2.870±0.068
S024	0.336±0.007	0.175±0.003	0.534±0.008	0.177±0.002	0.113±0.003	0.000±0.000	0.000±0.000	0.273±0.003	0.019±0.000	0.010±0.000	0.000±0.000	0.019±0.000	1.656±0.019
S025	0.517±0.015	0.177±0.005	0.689±0.017	0.428±0.009	0.141±0.003	0.000±0.000	0.000±0.000	0.387±0.008	0.033±0.002	0.018±0.000	0.000±0.000	0.020±0.001	2.411±0.057
S026	0.274±0.004	0.101±0.002	0.414±0.004	0.273±0.004	0.065±0.002	0.000±0.000	0.000±0.000	0.234±0.002	0.017±0.000	0.007±0.000	0.000±0.000	0.017±0.000	1.403±0.011
S027	0.402±0.035	0.225±0.005	0.740±0.027	0.358±0.031	0.186±0.003	0.061±0.003	0.024±0.001	0.476±0.026	0.015±0.000	0.013±0.000	0.000±0.000	0.020±0.000	2.520±0.055
S028	0.957±0.004	0.140±0.002	1.305±0.017	0.846±0.004	0.104±0.002	0.022±0.003	0.000±0.000	0.629±0.012	0.025±0.000	0.021±0.001	0.000±0.000	0.021±0.000	4.069±0.031
S029	0.509±0.034	0.184±0.002	0.823±0.016	0.390±0.009	0.120±0.002	0.018±0.000	0.000±0.000	0.379±0.003	0.014±0.000	0.013±0.001	0.000±0.000	0.019±0.000	2.471±0.044
S030	0.344±0.012	0.120±0.005	0.398±0.007	0.218±0.006	0.053±0.002	0.000±0.000	0.000±0.000	0.176±0.005	0.059±0.002	0.006±0.001	0.043±0.003	0.051±0.001	1.469±0.044
S031	0.464±0.011	0.081±0.001	0.790±0.019	0.248±0.004	0.064±0.001	0.057±0.001	0.021±0.000	0.384±0.008	0.017±0.003	0.015±0.001	0.000±0.000	0.019±0.000	2.160±0.044
S032	0.334±0.015	0.065±0.002	0.380±0.018	0.267±0.009	0.058±0.001	0.000±0.000	0.000±0.000	0.300±0.008	0.019±0.001	0.009±0.000	0.000±0.000	0.017±0.000	1.449±0.053
S033	1.015±0.019	0.230±0.007	0.966±0.015	0.703±0.009	0.163±0.004	0.023±0.000	0.024±0.001	0.490±0.008	0.032±0.001	0.017±0.001	0.000±0.000	0.021±0.000	3.684±0.064
S034	0.520±0.004	0.068±0.001	0.699±0.004	0.559±0.004	0.055±0.001	0.000±0.000	0.000±0.000	0.449±0.001	0.021±0.000	0.012±0.000	0.000±0.000	0.021±0.000	2.404±0.009
S036	0.412±0.002	0.220±0.004	1.099±0.004	0.236±0.003	0.165±0.002	0.073±0.001	0.024±0.001	0.555±0.008	0.019±0.001	0.018±0.00	0.000±0.000	0.027±0.000	2.848±0.011
S037	0.463±0.026	0.139±0.002	0.549±0.022	0.320±0.032	0.094±0.010	0.040±0.005	0.021±0.001	0.289±0.009	0.028±0.003	0.017±0.005	0.000±0.000	0.020±0.000	1.980±0.093
S038	0.473±0.007	0.057±0.001	1.029±0.018	0.389±0.038	0.045±0.001	0.071±0.002	0.023±0.001	0.603±0.044	0.026±0.000	0.014±0.000	0.000±0.000	0.026±0.000	2.759±0.039
S039	0.220±0.006	0.128±0.002	0.388±0.010	0.154±0.004	0.090±0.001	0.040±0.002	0.000±0.000	0.235±0.007	0.018±0.000	0.009±0.001	0.000±0.000	0.019±0.000	1.300±0.031
S040	0.350±0.005	0.212±0.002	0.475±0.007	0.269±0.003	0.164±0.001	0.050±0.001	0.000±0.000	0.332±0.005	0.026±0.001	0.010±0.000	0.042±0.001	0.018±0.000	1.946±0.024
S041	0.724±0.007	0.190±0.004	0.817±0.011	0.525±0.018	0.144±0.007	0.000±0.000	0.000±0.000	0.606±0.012	0.029±0.001	0.010±0.000	0.000±0.000	0.022±0.000	3.069±0.038
S042	0.541±0.007	0.257±0.004	0.782±0.015	0.280±0.007	0.158±0.003	0.054±0.000	0.022±0.000	0.385±0.004	0.026±0.001	0.015±0.000	0.000±0.000	0.023±0.000	2.543±0.033
S043	0.407±0.020	0.172±0.006	0.650±0.032	0.345±0.016	0.143±0.008	0.000±0.000	0.000±0.000	0.366±0.017	0.031±0.001	0.010±0.000	0.038±0.002	0.028±0.001	2.190±0.102
S044	0.536±0.008	0.205±0.009	0.882±0.015	0.374±0.017	0.158±0.001	0.068±0.000	0.024±0.000	0.608±0.004	0.030±0.000	0.015±0.000	0.000±0.000	0.026±0.000	2.927±0.027

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S045	0.303±0.002	0.224±0.003	0.317±0.007	0.197±0.002	0.101±0.002	0.000±0.000	0.000±0.000	0.157±0.001	0.022±0.000	0.008±0.000	0.041±0.000	0.021±0.000	1.391±0.007
S047	0.367±0.023	0.118±0.006	0.421±0.023	0.307±0.014	0.054±0.001	0.000±0.000	0.000±0.000	0.252±0.012	0.038±0.001	0.009±0.001	0.040±0.001	0.022±0.000	1.628±0.083
S050	0.487±0.005	0.145±0.004	0.740±0.019	0.454±0.043	0.132±0.005	0.000±0.000	0.000±0.000	0.503±0.011	0.018±0.000	0.012±0.000	0.000±0.000	0.021±0.000	2.512±0.034
S051	0.590±0.013	0.240±0.005	0.647±0.010	0.473±0.005	0.174±0.001	0.000±0.000	0.000±0.000	0.414±0.007	0.020±0.001	0.024±0.001	0.000±0.000	0.022±0.000	2.604±0.040
S052	0.133±0.016	0.166±0.006	0.283±0.007	0.162±0.004	0.084±0.002	0.000±0.000	0.000±0.000	0.187±0.007	0.009±0.000	0.007±0.000	0.000±0.000	0.015±0.000	1.047±0.015
S053	0.289±0.012	0.126±0.006	0.724±0.024	0.238±0.010	0.105±0.000	0.069±0.002	0.023±0.004	0.549±0.020	0.015±0.001	0.013±0.000	0.000±0.000	0.021±0.000	2.172±0.078
S054	0.197±0.004	0.177±0.007	0.489±0.010	0.161±0.005	0.126±0.002	0.000±0.000	0.047±0.001	0.384±0.007	0.013±0.002	0.010±0.001	0.000±0.000	0.017±0.000	1.620±0.026
S055	0.479±0.009	0.187±0.002	0.819±0.014	0.320±0.009	0.144±0.005	0.000±0.000	0.000±0.000	0.568±0.013	0.022±0.000	0.016±0.000	0.000±0.000	0.025±0.000	2.581±0.044
S056	0.444±0.007	0.136±0.003	0.741±0.015	0.400±0.004	0.094±0.001	0.000±0.000	0.000±0.000	0.405±0.006	0.021±0.000	0.014±0.000	0.000±0.000	0.021±0.000	2.275±0.030
S057	0.335±0.007	0.217±0.004	0.576±0.011	0.250±0.002	0.162±0.001	0.055±0.002	0.000±0.000	0.390±0.003	0.019±0.000	0.012±0.000	0.000±0.000	0.019±0.000	2.035±0.024
S058	0.382±0.006	0.260±0.005	0.719±0.008	0.193±0.003	0.143±0.001	0.054±0.001	0.013±0.007	0.357±0.005	0.035±0.000	0.013±0.000	0.078±0.005	0.031±0.000	2.278±0.017
S059	0.442±0.003	0.128±0.001	1.192±0.017	0.525±0.003	0.111±0.005	0.000±0.000	0.000±0.000	0.605±0.006	0.015±0.000	0.018±0.000	0.000±0.000	0.029±0.002	3.065±0.021
S061	0.556±0.011	0.166±0.004	0.718±0.014	0.510±0.010	0.088±0.001	0.000±0.000	0.000±0.000	0.406±0.014	0.021±0.000	0.015±0.000	0.000±0.000	0.023±0.000	2.504±0.041
S062	0.415±0.009	0.197±0.005	0.853±0.012	0.369±0.004	0.166±0.004	0.083±0.001	0.031±0.000	0.660±0.002	0.023±0.000	0.012±0.000	0.000±0.000	0.022±0.000	2.833±0.028
S063	0.284±0.010	0.110±0.003	0.396±0.010	0.205±0.006	0.102±0.003	0.000±0.000	0.000±0.000	0.288±0.010	0.015±0.000	0.010±0.000	0.000±0.000	0.016±0.000	1.426±0.040
S064	0.244±0.006	0.177±0.004	0.366±0.008	0.139±0.004	0.124±0.003	0.029±0.001	0.000±0.000	0.164±0.002	0.037±0.000	0.007±0.000	0.058±0.000	0.025±0.000	1.371±0.020
S065	0.409±0.014	0.222±0.013	0.596±0.014	0.308±0.002	0.147±0.007	0.000±0.000	0.000±0.000	0.262±0.005	0.051±0.001	0.013±0.001	0.058±0.002	0.038±0.002	2.104±0.051
S066	0.313±0.018	0.200±0.011	0.382±0.021	0.145±0.015	0.114±0.008	0.000±0.000	0.000±0.000	0.167±0.007	0.040±0.002	0.009±0.000	0.058±0.002	0.025±0.000	1.451±0.081
S067	0.479±0.006	0.213±0.002	0.482±0.003	0.241±0.002	0.138±0.001	0.011±0.000	0.040±0.000	0.262±0.002	0.070±0.001	0.011±0.000	0.080±0.002	0.033±0.000	2.06±0.015
S068	0.294±0.002	0.050±0.001	0.280±0.002	0.268±0.001	0.038±0.001	0.030±0.000	0.016±0.000	0.215±0.001	0.025±0.007	0.008±0.002	0.000±0.000	0.015±0.000	1.239±0.006
S069	0.319±0.010	0.199±0.005	0.441±0.012	0.268±0.007	0.102±0.002	0.000±0.000	0.000±0.000	0.244±0.005	0.011±0.000	0.009±0.000	0.000±0.000	0.017±0.000	1.610±0.041
S070	0.187±0.008	0.120±0.007	0.367±0.011	0.200±0.005	0.122±0.003	0.000±0.000	0.000±0.000	0.376±0.010	0.014±0.000	0.010±0.000	0.000±0.000	0.018±0.000	1.415±0.034
S071	0.136±0.012	0.153±0.005	0.264±0.016	0.169±0.010	0.141±0.009	0.000±0.000	0.000±0.000	0.290±0.017	0.009±0.005	0.007±0.000	0.000±0.000	0.015±0.000	1.185±0.073
S073	0.363±0.032	0.000±0.000	0.278±0.007	0.271±0.007	0.000±0.000	0.018±0.000	0.051±0.001	0.251±0.007	0.018±0.000	0.007±0.000	0.000±0.000	0.016±0.000	1.272±0.045
S074	0.485±0.034	0.254±0.008	0.560±0.018	0.376±0.023	0.173±0.006	0.043±0.001	0.026±0.001	0.326±0.020	0.021±0.001	0.008±0.002	0.000±0.000	0.024±0.000	2.295±0.088
S075	2.008±0.073	0.144±0.002	2.05±0.057	1.502±0.058	0.119±0.003	0.185±0.009	0.000±0.000	1.513±0.047	0.050±0.000	0.024±0.001	0.000±0.000	0.036±0.001	7.635±0.224

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Name	DG	GLG	GEG	MD	MGL	AD	AGL	MG	DE	AG	GLE	GE.	Total
	Daidzin	Glycitin	Genistin	Malonyldaidzin	Malonylglycitin	Acetyldaidzin	Acetylglycitin	Malonylgenistin	Daidzein	Acetylgenistin	Glycitein	Genistein	
S076	0.279±0.016	0.033±0.001	0.232±0.010	0.160±0.007	0.000±0.000	0.000±0.000	0.000±0.000	0.124±0.004	0.010±0.000	0.005±0.000	0.000±0.000	0.014±0.000	0.857±0.037
S078	0.204±0.005	0.154±0.004	0.471±0.011	0.190±0.006	0.122±0.004	0.048±0.001	0.020±0.000	0.374±0.010	0.016±0.000	0.009±0.000	0.023±0.012	0.020±0.000	1.653±0.051
S079	0.483±0.006	0.263±0.013	0.818±0.012	0.403±0.005	0.201±0.005	0.000±0.000	0.000±0.000	0.787±0.011	0.021±0.001	0.013±0.000	0.000±0.000	0.020±0.000	3.009±0.046
S080	0.400±0.014	0.093±0.004	0.539±0.019	0.404±0.016	0.101±0.002	0.019±0.000	0.000±0.000	0.376±0.016	0.013±0.001	0.008±0.000	0.000±0.000	0.017±0.000	1.970±0.071
S081	0.754±0.023	0.115±0.002	0.970±0.021	0.477±0.022	0.077±0.002	0.083±0.002	0.019±0.003	0.582±0.017	0.056±0.001	0.014±0.000	0.000±0.000	0.048±0.001	3.195±0.084
S082	0.312±0.009	0.191±0.005	0.830±0.019	0.193±0.004	0.127±0.003	0.070±0.003	0.000±0.000	0.492±0.011	0.015±0.000	0.013±0.001	0.000±0.000	0.020±0.000	2.263±0.054
S083	0.316±0.004	0.180±0.004	0.849±0.004	0.181±0.003	0.148±0.003	0.058±0.001	0.020±0.002	0.444±0.017	0.014±0.001	0.014±0.000	0.000±0.000	0.021±0.000	2.245±0.009
S084	0.489±0.006	0.036±0.000	0.551±0.008	0.448±0.005	0.061±0.030	0.020±0.000	0.000±0.000	0.368±0.007	0.015±0.000	0.014±0.000	0.000±0.000	0.016±0.000	2.018±0.007
S085	0.610±0.027	0.184±0.008	0.974±0.037	0.378±0.019	0.147±0.004	0.078±0.004	0.020±0.001	0.579±0.030	0.024±0.000	0.014±0.000	0.000±0.000	0.026±0.000	3.033±0.129
S086	0.356±0.007	0.012±0.002	0.348±0.000	0.140±0.066	0.065±0.000	0.000±0.000	0.000±0.000	0.256±0.002	0.017±0.001	0.007±0.000	0.000±0.000	0.017±0.000	1.217±0.059
S087	0.638±0.0008	0.047±0.001	0.826±0.016	0.533±0.003	0.052±0.001	0.036±0.015	0.000±0.000	0.456±0.025	0.033±0.001	0.013±0.000	0.000±0.000	0.021±0.000	2.655±0.018
S088	0.480±0.002	0.039±0.001	0.543±0.001	0.350±0.000	0.027±0.000	0.056±0.001	0.000±0.000	0.369±0.002	0.033±0.001	0.008±0.000	0.000±0.000	0.024±0.000	1.930±0.002
S089	0.351±0.013	0.132±0.004	0.545±0.020	0.354±0.022	0.126±0.002	0.000±0.000	0.000±0.000	0.377±0.007	0.020±0.000	0.012±0.000	0.000±0.000	0.019±0.000	1.934±0.059
S090	0.566±0.006	0.201±0.004	0.778±0.012	0.295±0.003	0.118±0.002	0.060±0.004	0.000±0.000	0.411±0.005	0.032±0.001	0.013±0.001	0.000±0.000	0.025±0.000	2.499±0.021
S091	0.529±0.015	0.198±0.014	0.721±0.022	0.547±0.016	0.126±0.007	0.000±0.000	0.000±0.000	0.471±0.017	0.031±0.000	0.012±0.000	0.026±0.013	0.024±0.000	2.684±0.092
S092	0.450±0.035	0.286±0.001	0.560±0.010	0.304±0.008	0.168±0.003	0.000±0.000	0.000±0.000	0.241±0.005	0.013±0.000	0.012±0.001	0.000±0.000	0.016±0.000	2.051±0.059
S093	0.408±0.005	0.221±0.003	0.819±0.004	0.378±0.040	0.167±0.001	0.059±0.002	0.024±0.001	0.487±0.026	0.020±0.000	0.012±0.000	0.000±0.000	0.021±0.000	2.616±0.030
S094	0.491±0.008	0.146±0.003	0.727±0.013	0.367±0.049	0.104±0.001	0.053±0.001	0.023±0.000	0.425±0.019	0.036±0.000	0.012±0.000	0.056±0.000	0.031±0.000	2.470±0.045
S095	0.271±0.006	0.215±0.004	0.403±0.006	0.255±0.003	0.119±0.001	0.000±0.000	0.000±0.000	0.266±0.002	0.009±0.005	0.008±0.000	0.032±0.001	0.018±0.000	1.596±0.020
S096	0.565±0.006	0.200±0.004	0.717±0.008	0.433±0.007	0.097±0.002	0.000±0.000	0.000±0.000	0.367±0.024	0.023±0.000	0.010±0.000	0.000±0.000	0.021±0.000	2.434±0.051
S097	0.412±0.027	0.051±0.001	0.400±0.009	0.282±0.003	0.041±0.001	0.000±0.000	0.000±0.000	0.193±0.008	0.015±0.000	0.008±0.000	0.000±0.000	0.016±0.000	1.419±0.015
S098	0.148±0.005	0.190±0.006	0.369±0.005	0.192±0.007	0.113±0.003	0.000±0.000	0.023±0.003	0.195±0.008	0.015±0.000	0.007±0.001	0.000±0.000	0.016±0.000	1.268±0.026
S099	0.658±0.006	0.036±0.000	0.890±0.008	0.584±0.013	0.054±0.001	0.052±0.002	0.024±0.001	0.524±0.013	0.023±0.000	0.014±0.000	0.000±0.000	0.021±0.000	2.879±0.020
S101	0.276±0.003	0.118±0.002	0.379±0.002	0.215±0.005	0.102±0.008	0.039±0.002	0.018±0.001	0.255±0.004	0.010±0.005	0.008±0.000	0.000±0.000	0.016±0.000	1.437±0.023
S102	0.228±0.018	0.033±0.001	0.213±0.014	0.190±0.010	0.039±0.001	0.000±0.000	0.000±0.000	0.125±0.007	0.009±0.004	0.011±0.000	0.000±0.000	0.014±0.000	0.862±0.046
S103	0.365±0.007	0.151±0.000	0.544±0.010	0.207±0.003	0.123±0.003	0.047±0.001	0.017±0.001	0.292±0.006	0.022±0.000	0.011±0.000	0.000±0.000	0.019±0.000	1.797±0.024

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	Daidzin	Glycitin	Genistin	Malonyldaidzin	Malonylglycitin	Acetyldaidzin	Acetylglycitin	Malonylgenistin	Daidzein	Acetylgenistin	Glycitein	Genistein	
S104	0.552±0.009	0.217±0.003	0.794±0.018	0.508±0.008	0.100±0.002	0.000±0.000	0.000±0.000	0.447±0.010	0.052±0.000	0.014±0.000	0.045±0.001	0.028±0.000	2.757±0.048
S105	0.420±0.024	0.189±0.002	0.576±0.006	0.421±0.006	0.159±0.008	0.000±0.000	0.000±0.000	0.368±0.005	0.017±0.000	0.010±0.000	0.035±0.001	0.020±0.000	2.215±0.040
S106	0.512±0.014	0.175±0.005	0.758±0.026	0.353±0.003	0.136±0.007	0.000±0.000	0.000±0.000	0.542±0.008	0.043±0.001	0.015±0.000	0.000±0.000	0.031±0.000	2.564±0.045
S107	0.092±0.002	0.144±0.003	0.182±0.001	0.096±0.002	0.123±0.004	0.019±0.000	0.019±0.001	0.122±0.002	0.013±0.000	0.006±0.000	0.000±0.000	0.014±0.000	0.830±0.011
S108	0.437±0.020	0.192±0.008	0.759±0.035	0.364±0.011	0.190±0.005	0.080±0.002	0.022±0.002	0.606±0.018	0.018±0.000	0.013±0.000	0.000±0.000	0.022±0.000	2.703±0.090
S109	0.312±0.004	0.207±0.003	0.404±0.007	0.321±0.010	0.103±0.004	0.000±0.000	0.000±0.000	0.250±0.008	0.013±0.000	0.011±0.000	0.000±0.000	0.017±0.000	1.638±0.034
S110	0.194±0.013	0.163±0.020	0.373±0.037	0.144±0.007	0.130±0.016	0.038±0.001	0.000±0.000	0.236±0.023	0.012±0.001	0.007±0.000	0.000±0.000	0.016±0.000	1.313±0.046
S111	0.667±0.010	0.216±0.011	0.837±0.005	0.487±0.009	0.166±0.005	0.000±0.000	0.000±0.000	0.724±0.011	0.019±0.000	0.015±0.001	0.000±0.000	0.023±0.000	3.154±0.034
S112	0.253±0.004	0.161±0.004	0.305±0.005	0.244±0.003	0.086±0.001	0.000±0.000	0.000±0.000	0.199±0.003	0.013±0.000	0.007±0.000	0.000±0.000	0.014±0.000	1.283±0.017
S114	0.314±0.005	0.213±0.004	0.392±0.004	0.308±0.020	0.210±0.016	0.044±0.003	0.023±0.001	0.326±0.004	0.018±0.000	0.010±0.001	0.000±0.000	0.016±0.000	1.875±0.021
S115	0.144±0.002	0.220±0.005	0.220±0.000	0.131±0,005	0.174±0.007	0.000±0.000	0.000±0.000	0.143±0.002	0.000±0.000	0.006±0.001	0.037±0.002	0.015±0.000	1.090±0.009
S116	0.342±0.013	0.209±0.005	0.733±0.023	0.214±0.007	0.144±0.008	0.059±0.004	0.000±0.000	0.441±0.017	0.020±0.001	0.011±0.001	0.000±0.000	0.021±0.000	2.194±0.073
S117	0.212±0.018	0.110±0.003	0.301±0.011	0.216±0.011	0.108±0.006	0.000±0.000	0.000±0.000	0.259±0.010	0.016±0.001	0.007±0.000	0.000±0.000	0.016±0.000	1.245±0.054
Y001	0.430±0.028	0.189±0.003	0.478±0.003	0.245±0.004	0.133±0.002	0.000±0.000	0.000±0.000	0.315±0.004	0.020±0.000	0.010±0.000	0.000±0.000	0.019±0.000	1.839±0.035
Y003	0.322±0.006	0.144±0.002	0.338±0.004	0.237±0.004	0.113±0.002	0.000±0.000	0.000±0.000	0.210±0.000	0.018±0.000	0.007±0.000	0.000±0.000	0.018±0.000	1.407±0.014

***Table S3.** The total isoflavone contents of five specific germplasm samples from different locations. Chongqing (C002), Sichuan (S075) and Guizhou (G010, G011 and G013)*

Name	Daidzin	Glycitin	Genistin	Malonyldaidzin	Malonylglycitin	Acetyldaidzin	Acetylglycitin	Malonylgenistin	Daidzein	Acetylgenistin	Glycitein	Genistein	Total
C002	1.823±0.059	0.165±0.011	2.253±0.091	0.939±0.035	0.116±0.003	0.044±0.001	0.158±0.015	1.288±0.062	0.119±0.001	0.064±0.001	0.000±0.000	0.077±0.002	7.044±0.144
G010	1.163±0.008	0.282±0.004	1.316±0.013	0.419±0.015	0.150±0.004	0.073±0.001	0.025±0.000	0.509±0.009	0.111±0.000	0.021±0.000	0.088±0.002	0.060±0.000	4.216±0.009
G011	0.925±0.085	0.147±0.012	1.422±0.128	0.676±0.059	0.118±0.009	0.136±0.013	0.031±0.002	1.032±0.096	0.029±0.002	0.016±0.001	0.000±0.000	0.034±0.001	4.566±0.407
G013	0.875±0.017	0.175±0.003	2.353±0.047	1.092±0.024	0.133±0.001	0.000±0.000	0.000±0.000	1.210±0.027	0.058±0.001	0.026±0.001	0.055±0.006	0.084±0.001	6.062±0.127
S075	2.008±0.073	0.144±0.002	2.05±0.057	1.502±0.058	0.119±0.003	0.185±0.009	0.000±0.000	1.513±0.047	0.050±0.000	0.024±0.001	0.000±0.000	0.036±0.001	7.635±0.224

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SYNTHESIS OF CHEMICALLY MODIFIED CARBON EMBEDDED SILICA AND ZEOLITE FROM RICE HUSK TO ADSORB CRYSTAL VIOLET DYE FROM AQUEOUS SOLUTION

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Abstract. Toxic dyes contaminated aqueous solutions discharged from different industries should be treated before releasing to the environment. In the present study adsorbents manufactured from an easily available waste rice husk (RH) has been used to treat dyes contaminated water. Hereby, two adsorbents, carbon embedded silica (CES) and rice husk ash (RHA) mediated zeolite (Z-RHA) have been synthesized from rice husk by chemical modification, and a comparative study has been performed for decontaminating crystal violet (CV) dye laden aqueous solution. Characterization of the manufactured RHA, CES and Z-RHA has been done by using several techniques like Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), Scanning electron microscope (SEM), Energy dispersive X-ray (EDX) and Brunauer-Emmett-Teller (BET). The specific surface area for RHA, CES and Z-RHA was 28 m^2/g, 110 m^2/g and 122 m^2/g respectively. Additionally, batch experiments have been performed to evaluate the effect of different parameters like initial concentrations of the adsorbate, initial pH, adsorbent dosages and contact time. The adsorption capacity of the CES and Z-RHA is 18.75 mg/g and 19.28 mg/g respectively, which are satisfactorily higher than the adsorption capacity of their parent material RHA (8.3 mg/g). In the optimization study, the maximum adsorption has been found at the initial concentration of 100 mg/l of the dye, solution pH 8, adsorbent dosages 0.6 mg/50 ml and contact time 10 min. This study stated that the chemically modified adsorbents manufactured from RHA can be an easy solution to remove CV dye from wastewater.
Keywords: *toxic dye removal, polluted water, carbon embedded silica, modified rice husk, zeolite*

Introduction

Nowadays, synthetic dyes are used in different industries like the garment dye industries, textile, plastic, leather, paper, food, mineral processing and cosmetic industries (Dalaran et al., 2011; Kumari et al., 2017). There are more than 10 thousand dyes that are currently used in different industries and the annual production of the dyes all over the world is around 280 thousand tons, which is a huge amount (Mass and Chaudhari, 2005). Alarming amount of dyes are being released into the environment from these industries each year. A small amount of dyes can cause serious pollution of water due to their harmful characteristics such as high visibility, low biodegradability and toxicity (Arenas et al., 2017; O'Neill et al., 1999). Dye-contaminated water can be carcinogenic as well as mutagenic due to their obstruction of light penetration into water which inhibits the photosynthesis activity of aquatic biota (Vakili et al., 2014).

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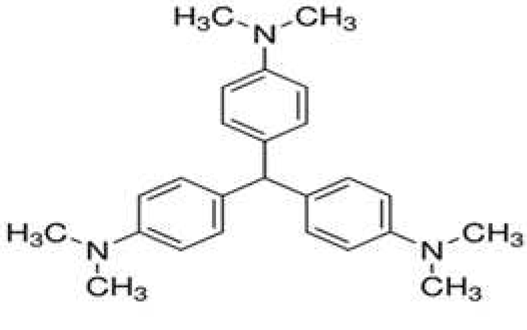
Consequently those dyes can have harmful effects on human beings through aquatic animals and alimentary chain (Secula et al., 2011). Removal of dyes is a great concern not only to protect the environment but also to make it safe for the human health. Among different types of toxic dyes crystal violet (CV) represents one of the mostly used cationic dyes in different industries. The characteristics of the CV dyes are shown in *Table 1*. Cationic dyes are colored organic base salts and they have no water solubilizing agent. Due to these characteristics the cationic dyes are more toxic than the anionic dyes (Loqman et al., 2017; Hao et al., 2000). CV is also known as hexamethyl pararosaniline chloride. There are a lot of established techniques for removing toxic dyes from wastewater approved by different studies such as electrocoagulation, electrochemical oxidation and advanced oxidation process (Arenas et al., 2017). Moreover, the physical treatment like adsorption is very much useful technique to remove toxic compounds from wastewater, if the adsorbents are readily available and cost effective (Banerjee et al., 2017; Lakshmi et al., 2009). There are lots of studies on the activated carbon from different available cheap materials like coconut husk, coconut shell, bamboo ash etc. to reduce toxicity from water or wastewater (Arenas et al., 2017; Yadav et al., 2015; Zhang et al., 2011; Yadanaparthi et al., 2009; Xu et al., 2009). In this study we are using the rice husk ash (RHA) to make zeolite (Z-RHA) and carbon embedded silica (CES) to remove the toxic dye CV from the wastewater. Rice husk (RH) is an easily available material with no price. The amount of the RH is assumed to be approximately twenty percent of the gross weight of grain produced (Arenas et al., 2017). In the disposal point of view, the end material of paddy is very much harmful for environment. RHA has higher efficiency to remove either biodegradable or non-biodegradable toxic pollutants from wastewater due to its highly porous surface and large surface area (Naiya et al., 2009). On the other hand, RHA mainly contained SiO_2 which is very much useful to remove different kinds of heavy metal and dyes after certain modification of them (Lakshmi et al., 2009; Totlani et al., 2012). From the literature review we found that the adsorbents manufactured with modification of rice husk are still not applied on the toxic dye CV. Adsorption study of manufactured RHA mediated Z-RHA and CES on CV dye has been done in the present study. CES is a material made from RHA, with embedding the silica contained by RHA with unburnt carbon. Z-RHA is another manufactured material composed of silica, aluminium and oxygen present in RHA. Finally the adsorption performance of CES and Z-RHA to CV dye removal from aqueous solution has been investigated in this study.

***Table 1.** Properties of CV dye*

Name of the dye	Molecular Structure	Formula	Molecular weight (g mol^{-1})	Ion	Maximum wavelength (nm)
Crystal Violet		$C_{25}H_{30}ClN_3$	407,98	Cationic	590

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Materials and methods

Collection of rice husk and preparation of RHA

Rice husk (RH) sample has been collected from the local market of the Qingdao city in Shandong province of China without any cost. Collected RH sample has been sorted to separate from foreign materials like dust, broken parts of rice shoots and roots, stones etc. followed by sieving and washing under running tap water. Finally, after washing with distilled water the RH sample has been dried in room temperature to remove all the extra moisture present on the surface of the husk. In order to remove the metal impurities the sample has been treated with hydrochloric acid. Then the treated sample has been stirred with sodium hydroxide to remove the excess acid. Concurrently, the treated RH has been thoroughly washed again by distilled water to remove both the acid and the alkali from the surface (Arenas et al., 2017). After drying the rice husk, ash has been produced by pyrolyzing the RH at the temperature from 500 to 700 °C for 5 h in a muffle furnace to remove excess organic contents. Consequently, the RHA has been stored in sterile zip-lock bag for further use. Previously, it has been reported that after acid treatment, calcination and pyrolisis of RH, major portion of final material has been represented by silica (Banerjee et al., 2017).

Preparation of Z-RHA

Zeolite RHA has been prepared from the sodium silicate (Na_2SiO_3) obtained by RHA. Firstly, seed and feedstock gel have been prepared by mixing RHA with sodium alluminate ($NaAlO_2$). A homogeneous seed gel has been prepared by mixing Na_2SiO_3 presented in rice husk, with $NaAlO_2$ in distilled water under continuous agitation (Banerjee et al., 2017). Then the prepared gel has been left at ambient temperature up to 24 h for aging. Consequently, homogeneous gel of Al_2O_3:$10SiO_2$ has been formed as seed gel. As well as, feedstock gel has been prepared by following same procedure without any aging. Then the feedstock gel and seed gel have been mixed and stirred vigorously to make a homogeneous mixture and again kept in room temperature for 24 h for aging. Additionally, the aged solution has been crystallized at 100 °C for another 24 h in room temperature. Finally the prepared sample has been washed, dried and stored for further analysis (Goyal et al., 2005).

Preparation of CES

CES has been prepared from the sodium silicate obtained by RHA, after digestion in the solution of NaOH. RHA has been digested with NaOH solution for 1 h at 80 °C temperature to form Na_2SiO_3. Then the alkaline solution has been acidified with phosphoric acid (H_3PO_4) to neutralize the pH and silicic acid has been formed (Totlani et al., 2012). Silicic acid is a chemical compound of silicon, hydrogen and oxygen, also it is the hydrate of SiO_2. Due to heating they lose water to precipitate silica gel as an active form of SiO_2. The partially unburnt carbon present in the RHA has been made a duel substrate like CES including mesoporous silica and partially activated carbon (Totlani et al., 2012). Finally, the samples have been kept at 100 °C for 24 h and preserved for further analysis. The detailed procedure has been reported previously by Shelke et al. (2010).

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Characterization of adsorbents

The physico-chemical characteristics of prepared RHA, CES and Z-RHA have been investigated. Gravimetric methods have been used for the proximate analysis. The surface area and the pore size of prepared RHA, CES and Z-RHA have been measured by Brunauer–Emmett–Teller (BET) surface area analyzer. The functional groups present in the adsorbents have been determined by using Fourier transform infrared (FTIR) spectroscopy. To know the structure and orientation of the prepared adsorbents the x-ray diffraction (XRD) analysis has been conducted. The features and the elements present in the prepared adsorbents have been studied by scanning electron microscope (SEM) images and energy dispersive analysis of X-rays (EDX).

Batch kinetic and adsorption studies

In the adsorption study of CV dye, the 1000 mg/l dye solution has been prepared by dissolving 1 gm chemical dye in 1000 ml distilled water. Then the stock solution has been used to make different concentrations (50 mg/l, 100 mg/l, 150 mg/l, 200 mg/l) of dye for further study. 0.5 g of adsorbent (RHA, CES, Z-RHA) has been added into conical flask with different concentrations of dye solutions (Kumari et al., 2017). Then the flasks have been shaken in a rotary shaker at 30 °C temperature and 150 rpm, and the concentrations have been observed after each 10 min. The pH of the samples has been adjusted from 2-10 by adding either 0.1 M HCl or 0.1 M NaOH solutions. The supernatant liquid has been collected carefully from the flasks by using pipette and the concentrations have been measured by comparing the absorbance from spectrophotometer (Setthaya et al., 2017). The percentages of dye removal, and adsorption capacity at equilibrium phase q_e (mg/g) have been calculated as *Equation 1* (Langmuir, 1981):

$$\%\,Dye\,Removal = \frac{\mathrm{Co} - \mathrm{Ce}}{Co} \times 100 \qquad \text{(Eq.1)}$$

where, C_0 denotes initial concentrations and Ce denotes equilibrium concentrations of CV dye (mg/l). Distilled water has been used to blank before each set of the experiments as control. The adsorption capacity of the unit mass of the adsorbents at equilibrium q_e (mg/g) has been calculated by *Equation 2* (Langmuir, 1981):

$$qe = \frac{(\mathrm{Co} - \mathrm{Ce})\ \mathrm{V}}{W} \qquad \text{(Eq.2)}$$

where, V is the volume of the dye solution (ml) and W is the weight of the adsorbent (g) added to the volume V.

Each of the experiment has been done three times for the exact and authenticated value of the measured parameters for kinetic and adsorption studies. For the dosage optimization also three replications per each treatment have been used and the average value of data has been plotted.

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Results and discussion

Characterization of synthesized adsorbents

FTIR analysis

The major peaks in the FTIR spectra (*Fig. 1*) for RHA at 3475 cm^{-1}, 2370 cm^{-1}, 1735 cm^{-1}, 1143 cm^{-1}, 810 cm^{-1} and 468 cm^{-1} are representing the presence of the following functional groups Si-OH, O=C=O, C=O, Si-O-Si, C-H and C-Cl in the sample (Kumari et al., 2017). On the other hand the FTIR report for CES sample shows the peaks at 3463 cm^{-1}, 2881 cm^{-1}, 2374 cm^{-1} representing Si-OH, C-H, O=C=O, have broaden. More peaks at 1604 cm^{-1}, 1564 cm^{-1}, 1392 cm^{-1}, 1118 cm^{-1}, 615 cm^{-1} represent C=C, N-O, C-H, C-O, C-Cl respectively (Uddin et al., 2009; Chowdhury et al., 2011; Kumari et al., 2017). Whereas the spectra of Z-RHA sample (*Fig. 1*) show the major peaks at 3363 cm^{-1}, 2374 cm^{-1}, 2137 cm^{-1}, 1666 cm^{-1}, 1371 cm^{-1}, 887 cm^{-1} represent Si-OH, O=C=O, N=N=N, C=O, N-O, C-Cl functional groups respectively (Setthaya et al., 2017). The presence of the polar group on the surface of CES and Z-RHA likely gives the cation exchange capacity for both of them.

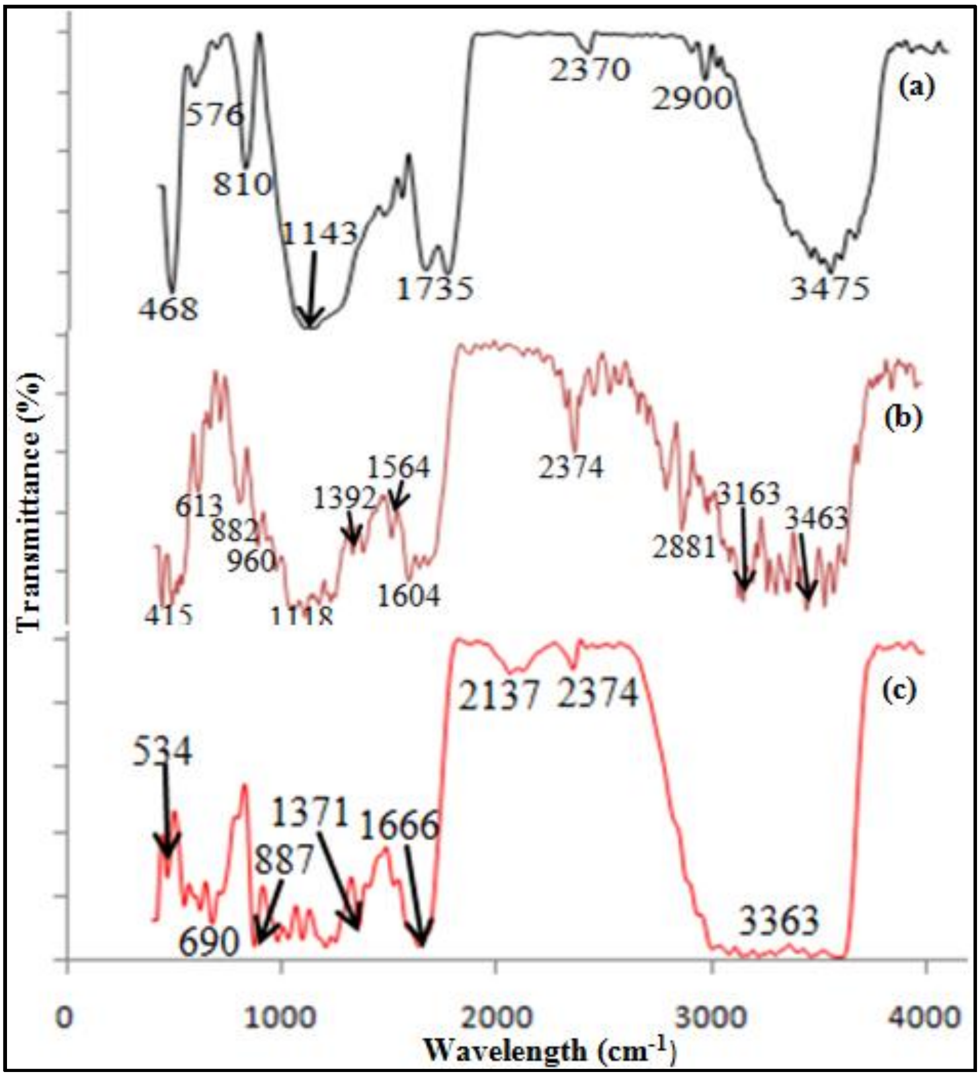

***Figure 1.** FTIR spectra of (a) RHA (b) CES and (c) Z-RHA*

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XRD analysis

The XRD pattern of the RHA, CES and Z-RHA are shown in *Figure 2a, b* and *c* respectively. Both *Figure 2a* and *b* show intense peaks at 23 A° which are indicating the presence of carbon in the samples. As well as *Figure 2b* shows the intense peaks at 33 A° due to the presence of the SiO_2 in CES sample (Kumari et al., 2017). Consequently, XRD pattern for Z-RHA sample shows peaks at 25 A°, 32 A°, and 35 A° (*Fig. 2c*) indicating the presence of AlO_2 and Zeolite ($Na_2Al_2Si_3.3O_{10}.6(H_2O)_7$) (Setthaya et al., 2017; Geetha et al., 2015).

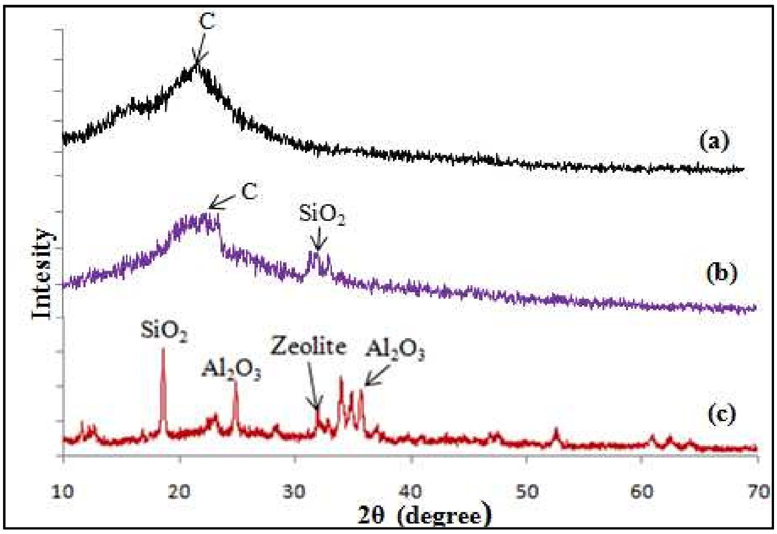

Figure 2. *XRD pattern of (a) RHA (b) CES and (c) Z-RHA*

SEM and EDX

Figure 3a, b and *c* are showing the SEM images of RHA, CES and Z-RHA respectively. Surface structure of the CES and Z-RHA are shown to be more porous than that of the RHA, which is evidence for more adsorption of dyes by CES and Z-RHA compared to their parent material, RHA (Setthaya et al., 2017; Geetha et al., 2015). From the Brunauer–Emmett–Teller (BET) analysis it is also found that the surface area of the RHA, CES and Z-RHA is 28 m^2/g, 110 m^2/g and 122 m^2/g respectively. In EDX analysis of the CES (*Fig. 3e*) sample shows presence of more amount of silica than the RHA (*Fig. 3d*). As well as Z-RHA sample (*Fig. 3f*) shows the presence of Al which is evidence for the presence of zeolite (Banerjee et al., 2017; Loqman et al., 2017; Lee et al., 2017).

Effect of the initial concentrations

In this study it has been found that the concentration of the pollutants plays important role in the adsorption of dyes by synthesized adsorbent. Hence, the amount of removal percentages and adsorption capacity of the materials has been tested with different initial concentrations of dyes. The removal percentages of dye by manufactured adsorbents for different concentrations have been calculated by *Equation 1* and plotted in *Figure 4*. Steep increase has been found, maximum removal percentages for CES (90.69%) and Z-RHA (96.45%) have been noted at the initial concentrations of 100

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mg/l. Whereas, the maximum removal percentages for RHA (53.66 %) have been achieved comparatively gradually and found at the initial concentrations of 150 mg/l. Adsorption capacity (Qe) has been measured by *Equation 2* and plotted with the initial concentrations of dyes (*Fig. 5*). Hereby, it shows that the adsorption capacity of the adsorbent depends on the initial concentrations of the adsorbate. The initial concentrations of the adsorbate act as a driving force to be adhered to an adsorbent (Menya et al., 2018; Lee et al., 2017; Li et al., 2014). Due to increasing the amount of the adsorbate concentrations the removal percentages as well as the adsorption capacity of the adsorbent materials increased due to the existing driving force (Lee et al., 2017; Li et al., 2014).

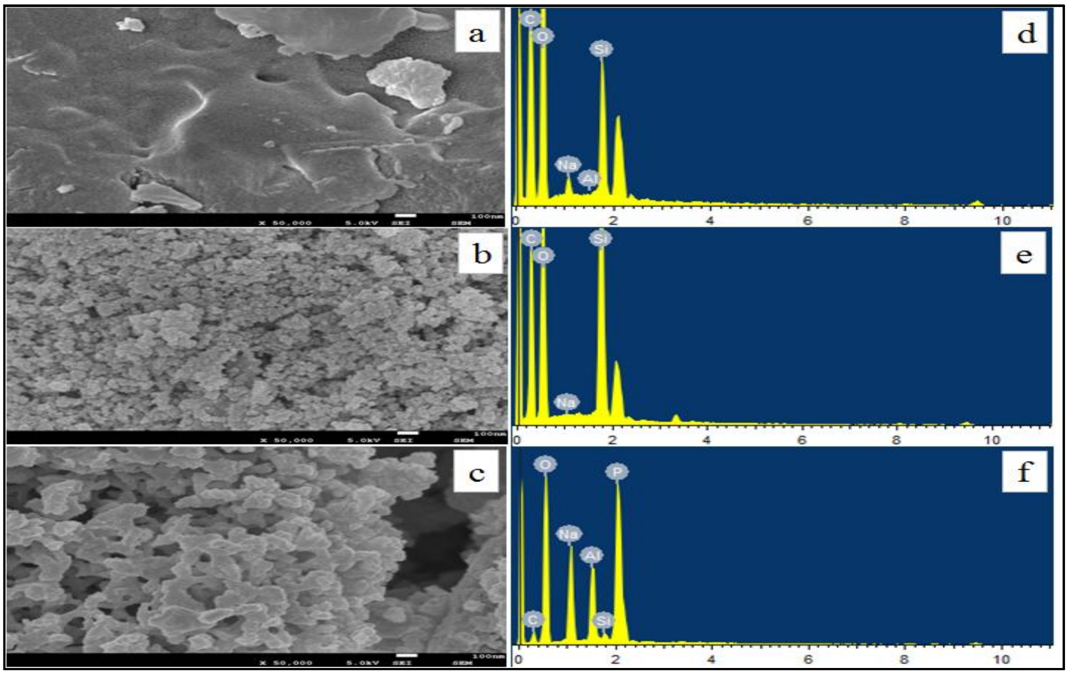

Figure 3. *SEM images of (a) RHA (b) CES and (c) Z-RHA and EDX spectra of (d) RHA (e) CES and (f) Z-RHA*

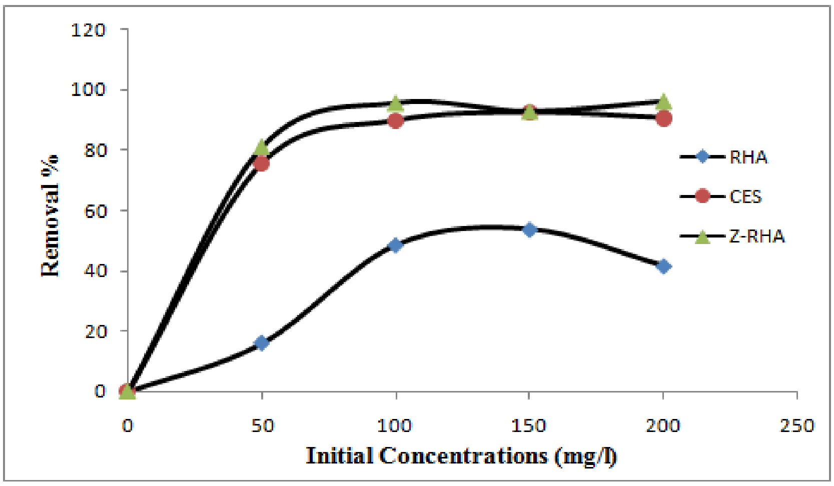

Figure 4. *Effect of the initial concentrations on dyes removal*

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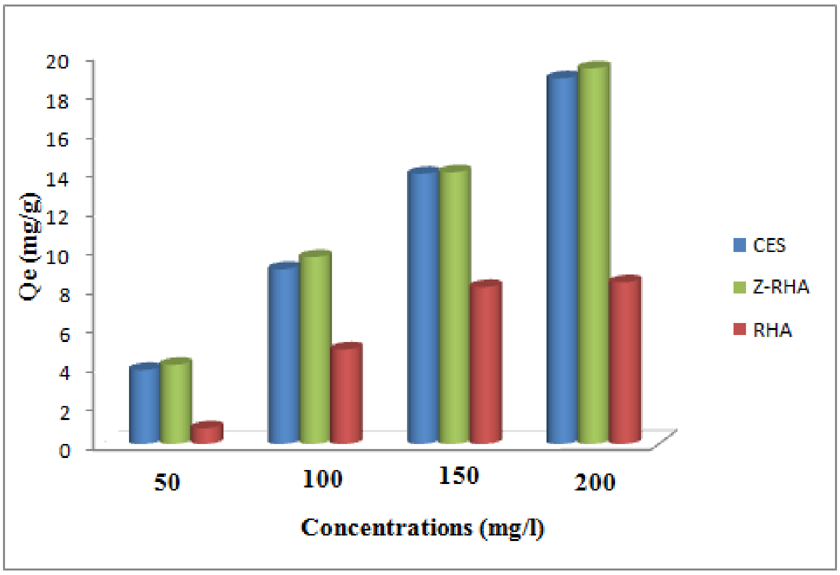

Figure 5. *Adsorption capacities of the adsorbents in different concentrations of dyes*

The maximum adsorbent capacity for RHA, CES and Z-RHA is 8.3 mg/g, 18.78 mg/g and 19.28 mg/g respectively which values are significantly more reasonable compared to those of other investigations (*Table 2*). Different studies have investigated the adsorption capacity of RHA and chemical modified RHA on different heavy metals. Adsorption of CES and Z-RHA on dye like CV is a noble approach which has been investigated in this study. However the adsorption capacity of the manufactured adsorbents is very much reasonable in comparison with other published study.

Table 2. *Comparative adsorption capacity of different relative adsorbents*

Type of adsorbent	Adsorbate	Adsorption capacity (mg/g)	References
Natural modernite (zeolite)	As	17.33	Chutia et al., 2009
Bulgarian natural zeolite	Cu	6.74	Panayatova et al., 2001
Zeolite	Fe	3.93	Ostroski et al., 2009
Rice husk derived zeolite (Z-RHA)	CV dye	19.28	Present study
Mesoporous RHA	Cd	11.7	Srivastava et al., 2006
Rice husk	Hg	6.7	Khalid et al., 1999
Rice husk ash	Pb	12.3	Doner and Akman, 2003
RHA	CV dye	8.3	Present study
Carbon embedded silica from RHA (CES)	CV dye	18.78	Present study

Effect of pH

The pH of the adsorbate solution is considered a very important parameter in adsorption studies due to two reasons, namely that hydrogen ions have strong competing ability as well as they can ionize the adsorbent surfaces (Rattanapan et al., 2017; Totlani et al., 2012). The change in solution pH may cause to dissociate the functional groups present on the surface of the adsorbent which ultimately affects the

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adsorption processes of the adsorbent. The effect of the pH on dye adsorption using manufactured materials has been studied by varying pH 2 to 10. The data on the removal percentages of dyes with different pH of the solutions plotted in *Figure 6*. It can be concluded that the removal percentages of dyes for CES and Z-RHA show higher removal percentages from the starting up to pH 8, whereas the removal percentage for RHA sample was less at lower pH. These phenomena might happen due to the high surface area of CES and Z-RHA samples rather than the RHA, which may nullify acidic effects during adsorption (Rattanapan et al., 2017; Mor et al., 2016; Totlani et al., 2012).

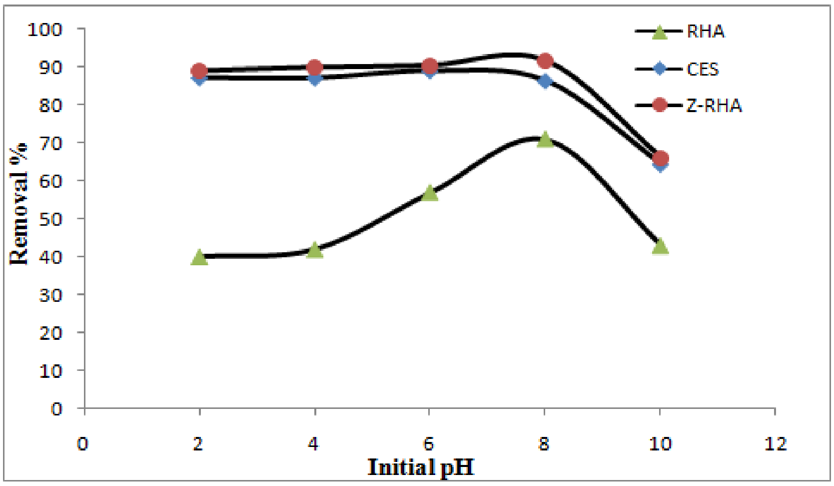

Figure 6. *Effect of the initial pH on dyes' removal*

Effect of dosages

The effect of the different dosages on CV dyes' removal has been studied and plotted in *Figure 8* between removal percentage against the adsorbent dosage 0.2-1.2 mg/50 ml. Usually, the adsorption or removal percentages increase with increasing dosages of adsorbents due to large surface area and consequently, more availability of the vacant sites for adsorption (Rattanapan et al., 2017; Totlani et al., 2012). From *Figure 7,* it is shown that the maximum removal percentages for all materials RHA (62.74%), CES (92.24%) and Z-RHA (94.61%) are at the dosages 0.6 mg/50 ml. After that the removal percentages did not increase with the increase of dosages. This might have happened due to the saturation of the adsorbent in the pollutant solutions (Sawasdee et al., 2017; Kumari et al., 2017; Sharma et al., 2010). Finally it can be concluded from *Figure 8* that the Z-RHA shows more removal efficiency than the other two due to its large surface area.

Effect of contact time

The effect of the contact time on CV dye adsorption has been plotted in *Figure 8*. From *Figure 8* it has been revealed that the adsorption capacity for all of three manufactured adsorbents increases steeply within short time (10 min), and then there is no noticeable increase with time. In this time the adsorption capacities of RHA, CES and Z-RHA are 8.3 mg/g, 18.75 mg/g and 19.28 mg/g respectively. This result can be explained as, at the initial stages of the adsorption the large number of the vacant places

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are available on the surface of the adsorbents (Yadav et al., 2015; Totlani et al., 2012; Xu et al., 2009). With the increase of the time a repulsive force also starts between the molecules on the adsorbent surface and the bulk phase which is the reason to stop the further adsorption with more lapse time (Singh et al., 2015; Saravanane et al., 2002). The experiment has been carried up to 70 min but no further changes in adsorption have been noticed.

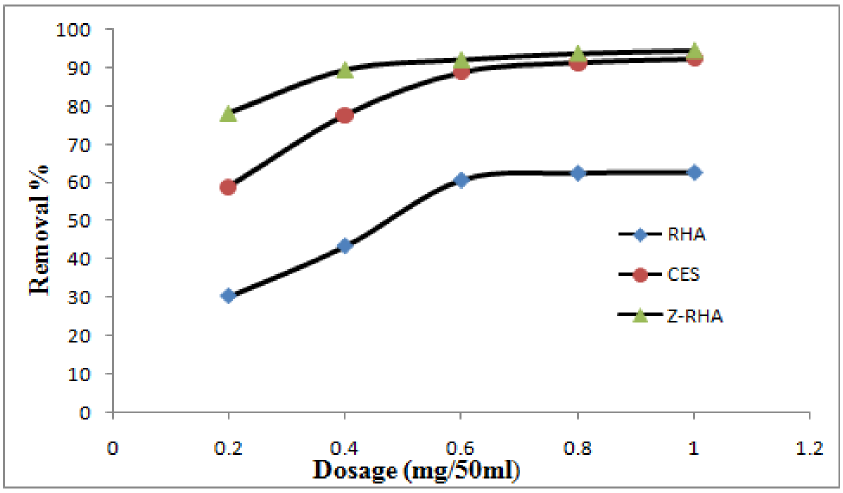

Figure 7. *Removal percentages of dyes with different dosages of RHA, CES and Z-RHA*

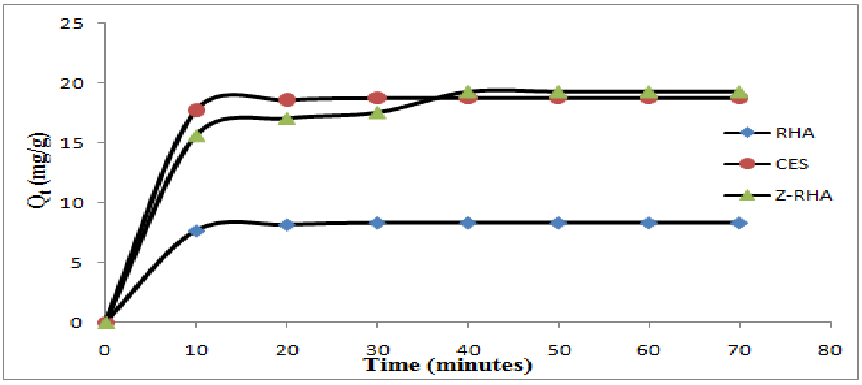

Figure 8. *Effect of the contact time on adsorption capacity of the adsorbents*

Conclusion

Rice husk is a common waste which is locally available in large quantity with no price. Chemically modified rice husk ash is basically used by many researchers over the world to decontaminate different kinds of the heavy metals but still there has been a lack of research on removal of textile dyes from aqueous solutions. In this study CES and Z-RHA have been synthesized from RHA after chemical modification and applied to remove CV dyes from aqueous solution. The percentages of removal of CV dyes have been found for RHA, CES and Z-RHA are 53.6%, 90.69% and 96.45% respectively. The optimization of adsorption has been studied and maximum adsorption of the adsorbents are found at initial concentration of adsorbate 100 mg/l, solution pH 8, adsorbent dosages 0.6 mg/50 ml and contact time 10 min. FTIR and XRD analyses

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indicate the presence of silica and aluminum in the CES and Z-RHA which are evident to synthesis of carbon embedded silica and zeolite. From the SEM images it has been also found that the surface of the CES and Z-RHA samples are more rough than that of their parent materials, which evidently adsorb more pollutants than RHA. Thus, from the result of this study it is clear that the chemically modified rice husk can be an alternate solution to treat textile wastewater. Due to the easy availability of the rice husk it has the opportunity to be more popular adsorbent in water treatment and it can help managing the huge amount of rice husk produced as agro waste over the world. Based on this study it can be recommended for future research to examine if the RHA can be further used to remove both anionic and cationic dyes with more chemical modification. Chemically modified RHA adsorbents can possibly be applied in large scale for industrial wastewater treatment which can be a tonic of research for the future researchers.

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CHANGES OF PETROLEUM HYDROCARBON IN JIAOZHOU BAY 1984-1988

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Abstract. Petroleum hydrocarbon (PHC) pollution in marine bays has been one of the critical environmental issues in many countries and regions. Jiaozhou Bay is a semi-closed bay in the fast-growing region in eastern China and has been contaminated by a variety of pollutants after the 1980s. Using investigation data in this bay during 1981-1988, this paper analyzed the temporal, spatial and seasonal changes of PHC. Results showed that PHC contents in surface waters during 1984-1988 were 0.01-0.16 mgL^{-1}, 0.025-0.124 mgL^{-1}, 0.005-0.122 mgL^{-1}, 0.014-0.091 mgL^{-1} and 0.005-0.178 mgL^{-1}, respectively. The pollution level of PHC during 1984-1988 was moderate and was changing with seasons due to the variations of source input. Low values of PHC contents during 1984-1988 were all closed to 0.005 mgL^{-1}, and this value could be considered as the 'background' value of PHC. High values of PHC contents in spring during this period tended to be stable, yet in summer and autumn during this period tended to be increasing. Stream discharge was the major source of PHC, and marine traffic and marine itself were also responsible. The source strengths were still slight/moderate in according to Chinese Sea Water Quality Standard (GB 3097-1997). Stream discharge was one of the major sources of PHC in every year, and the source strengths were increasing along with time. PHC in this bay was mainly input from rainfall runoff. Marine traffic had been one of the important sources since the 1980s, indicated that the oil leaking from marine traffic should be paid attention to. Marine current was also one of the important sources, and the source strengths could be as high as 0.122 mgL^{-1}. Rainfall runoff was the major force of various pollutants to the marine bay, the source input of PHC was also in order of summer > spring > autumn > winter. This was the major reason to explain the seasonal variations of PHC contents in Jiaozhou Bay. The background value of PHC in the marine bay was 0.005 mgL^{-1}, and the increase of PHC contents in the ocean could be calculated as 0.122-0.005 = 0.117 mgL^{-1}. This was the results of the storage of PHC in the ocean. The control and management of anthropogenic source input of PHC in the marine bay were necessary. The outcome of this paper is to identify the major sources, to define the annual change trend and the spatial-seasonal variations, to assess the storage of PHC in the marine bay, and to provide a basis for environmental management decision-making.
Keywords: *spatial, seasonal, annual, pollution, source*

Introduction

Along with the rapid development of industry, agriculture and traffic, a great deal of waste gas, water and residue are generated and discharged to air, soil and water environment (Yang et al., 2002, 2013a). The problem of marine pollution is tending to be more and more serious since the ocean is the "sink" of pollutants (Yang and Miao, 2010; Yang and Gao, 2010). PHC is widely used in industry, agriculture and traffic (Yang et al., 2014). By means of stream discharge, atmospheric deposition and oil spilling, many marine bays have been polluted by PHC and have caused serious environmental problems (Yang et al., 2014, 2015a). Identifying the major anthropogenic sources, the annual change trend and the spatial-seasonal variations is essential to marine environmental protection.

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Jiaozhou Bay is a semi-closed bay located in the south of Shandong Peninsula in eastern China and is surrounded by cities of Jiaozhou, Jiaonan and Qingdao in the north, west and east, respectively. This bay has been contaminated after the 1980s, and previous studies showed that this bay has been polluted by various pollutants including Pb (Yang et al., 2008a, 2011a), Hg (Yang et al., 2008b, 2013b), HCH (Yang et al., 2011b, 2015b, 2015c), Cu (Yang et al., 2015d, 2015e). Using investigation data of the bay during 1981-1988, this paper analyzed the temporal, spatial and seasonal changes of PHC. The aim of this paper is to provide a basis for environmental management decision-making.

Materials and methods

Study area

Jiaozhou Bay (120°04′-120°23′ E, 35°55′-36°18′ N) is in the south of Shandong Province, eastern China (*Fig. 1*). It is a semi-closed bay with the total area, average water depth and bay mouth width of 446 km^2, 7 m and 3 km, respectively. There are more than ten inflow rivers such as Haibo River, Licun River, Dagu River, and Loushan River etc., most of which have seasonal features (Yang et al., 2005, 2004).

Data collection

Data on PHC contents in Jiaozhou Bay were provided by North China Sea Environmental Monitoring Center. The investigation was conducted in July, August and October 1984 (*Fig. 1*), April, July and October 1985 (*Fig. 1*), April, July and October 1986 (*Fig. 1*), May, July and November 1987 (*Fig. 1*), and April, July and October 1988 (*Fig. 2*). Surface water samples were collected and measured by the National Specification for Marine Monitoring (China’s State Oceanic Administration, 1991).

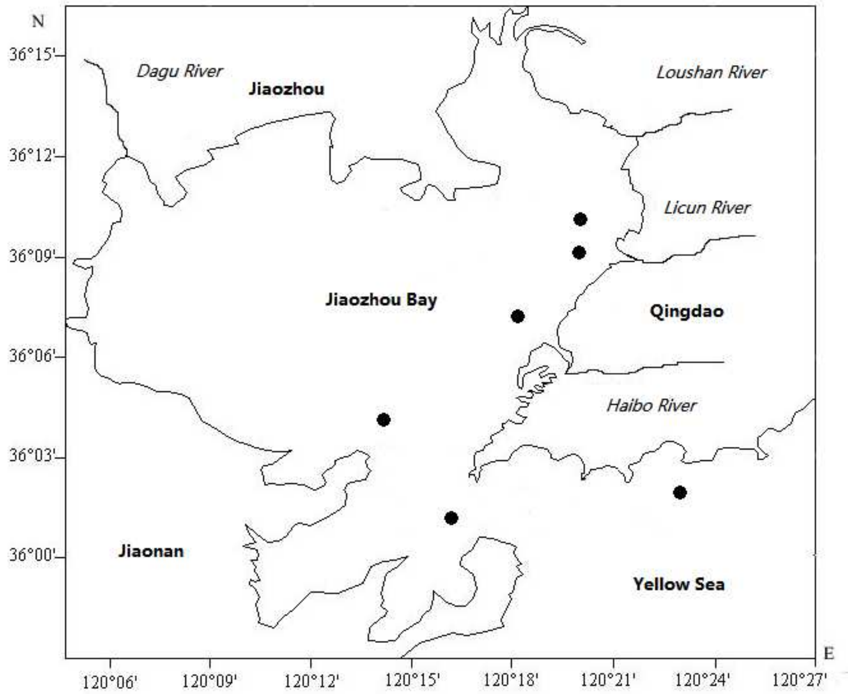

Figure 1. *Geographic location of Jiaozhou Bay and monitoring sites in 1984-1987*

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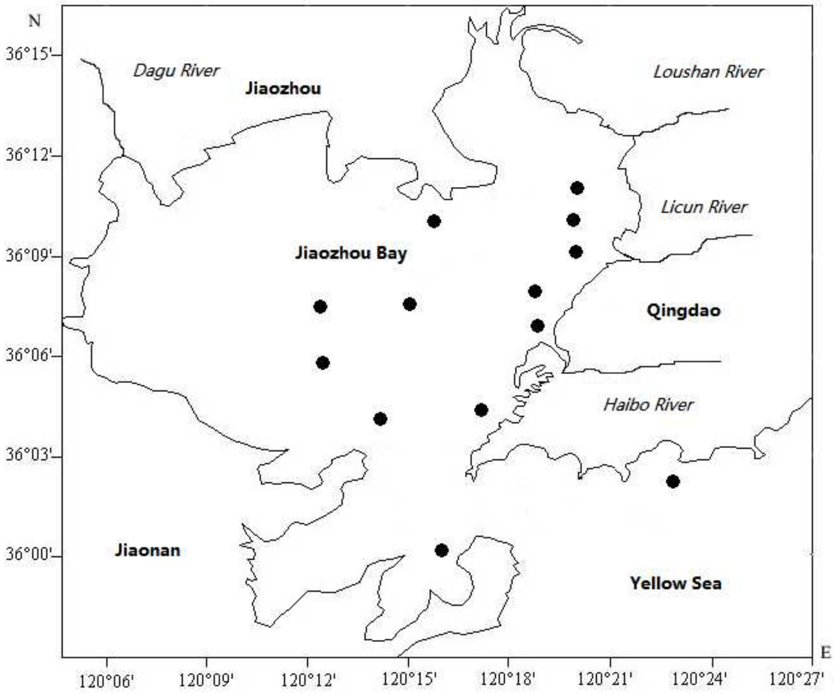

Figure 2. *Geographic location of Jiaozhou Bay and monitoring sites in 1984-1987*

Results and discussion

Annual changes of PHC

PHC contents in surface waters during 1984-1988 were 0.01-0.16 mgL^{-1}, 0.025-0.124 mgL^{-1}, 0.005-0.122 mgL^{-1}, 0.014-0.091 mgL^{-1} and 0.005-0.178 mgL^{-1}, respectively. In the guidelines for PHC in Chinese Sea Water Quality Standard (GB 3097-1997), there are 4 classes of water quality: Class I (and II), III and IV (*Table 1*). PHC contents in surface waters in July, August and October 1984 were 0.050-0.060 mgL^{-1}, 0.090-0.160 mgL^{-1} and 0.010-0.050 mgL^{-1}, and were Class I (and II), Class III and Class I (and II), respectively. PHC contents in surface waters in April, July and October 1985 were 0.025-0.064 mgL^{-1}, 0.059-0.124 mgL^{-1} and 0.010-0.121 mgL^{-1}, belonged to Class I (and II), Class III and Class I (and II), respectively. PHC contents in surface waters in April, July and October 1986 were 0.005-0.066 mgL^{-1}, 0.022-0.122 mgL^{-1} and 0.005-0.017 mgL^{-1}, belonged to Class I to III, Class I to III and Class I (and II), respectively. PHC contents in surface waters in May, July and November 1987 were 0.014-0.060mgL^{-1}, 0.016-0.066 mgL^{-1} and 0.030-0.091 mgL^{-1}, all ranging from Class I to III. PHC contents in surface waters in April, July and October 1988 were 0.014-0.064 mgL^{-1}, 0.005-0.178 mgL^{-1} and 0.022-0.169 mgL^{-1}, all ranging from Class I to III. In general, the pollution level of PHC during 1984-1988 was moderate (*Table 2*) and was changing with seasons due to the seasonal variations of source input.

Table 1. *Guidelines for PHC in Chinese Sea Water Quality Standard (GB 3097-1997)*

Class	I (and II)	III	IV
Guideline	0.05	0.30	0.50

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Table 2. *Water quality of PHC in different seasons in Jiaozhou Bay 1984-1988*

Year	Spring	Summer	Autumn
1984	-	I, II, III	I, II, III
1985	I, II, III	I, II, III	I, II, III
1986	I, II, III	I, II, III	I, II
1987	I, II, III	I, II, III	I, II, III
1988	I, II, III	I, II, III	I, II, III

Temporal changes of PHC

In the area of study, April, May and June belong to spring, July, August and September belong to summer, and October, November and December belong to autumn. During 1984-1988, PHC contents in spring, summer and autumn were 0.005-0.066 mgL^{-1}, 0.005-0.178 mgL^{-1} and 0.005-0.169 mgL^{-1}, respectively. According to the high values, PHC contents in different seasons were in order of summer > autumn > spring. The high values of PHC contents in different seasons during 1984-1988 were showed in *Figure 3*. It could be seen that high values of PHC contents in spring during this period tended to be stable, yet in summer and autumn during this period tendes to be increasing. In general, high values of PHC contents during 1984-1988 tended to be increasing. The seasonal changes of PHC contents indicated that the source inputs of PHC in this bay were relative high in summer and autumn yet was relatively low in spring. The reason was that summer and autumn were the wet seasons and a lot of PHC was discharged to Jiaozhou Bay via rainfall-runoff. Meanwhile, the atmospheric deposition of PHC to Jiaozhou Bay was increasing in the wet seasons. In according to the temporal changes of PHC contents in different seasons, it could be found that the pollution levels of PHC tended to be more and more serious along with time. It should be noticed that the low values of PHC contents during 1984-1988 were all closed to 0.005 mgL^{-1}, and this value could be considered as the ‘background’ value of PHC in Jiaozhou Bay. PHC contents in surface waters were directly impacted by source inputs and these trends indicated that the anthropogenic input of PHC was increasing along with the rapid development of industry around this bay, and the source control of PHC was essential to improve the water quality.

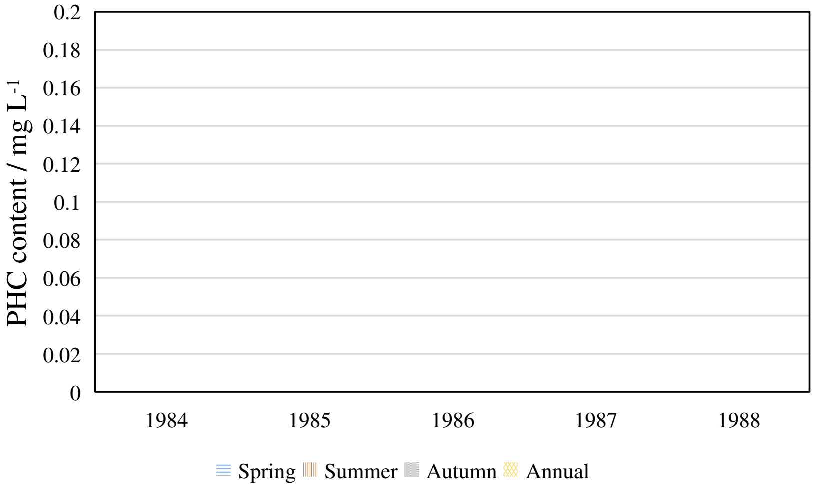

Figure 3. *High values of PHC contents in different seasons in Jiaozhou Bay 1984-1988*

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Horizontal distributions of PHC

The contents of PHC in surface waters are mainly impacted by the source input, and the high-value regions are the key evidence to identify the sources. In July, August and October 1984, the high-value regions of PHC contents were in the estuary of the Haibo River (0.060 mgL^{-1}), the estuary of the Loushan River (0.160 mgL^{-1}) and the estuary of the Loushan River (0.050 mgL^{-1}), respectively. In April, July and October 1985, the high-value regions of PHC contents were in the estuary of the Licun River (0.064 mgL^{-1}), the estuary of the Haibo River (0.124 mgL^{-1}) and the estuary of the Licun River (0.121 mgL^{-1}), respectively. In April, July and October 1986, the high-value regions of PHC contents were in the estuary of the Loushan River (0.066 mgL^{-1}), open waters (0.122 mgL^{-1}) and the estuary of the Licun River (0.017 mgL^{-1}), respectively. In May, July and November 1987, the high-value regions of PHC contents were in the southwest coast (0.060 mgL^{-1}), the estuary of the Loushan River (0.066 mgL^{-1}) and the southwest coast (0.091 mgL^{-1}), respectively. In April, July and October 1988, the high-value regions of PHC contents were in the estuary of the Licun River (0.064 mgL^{-1}), the estuary of the Haibo River (0.178 mgL^{-1}) and the estuary of the Haibo River (0.169 mgL^{-1}), respectively.

Sources of PHC

In general, the horizontal distributions of PHC in surface waters are mainly impacted by the source input, and PHC contents are always decreasing along the way from the high-value regions towards areas far away. In July 1984, PHC contents were decreasing from the estuary of Haibo River in the northeast of the bay to the south of the bay (*Fig. 4*), indicating that stream discharge was one of the major sources. In April 1985, PHC contents were decreasing from the estuary of Licun River in the northeast of the bay to the south of the bay (*Fig. 5*), indicating that stream discharge was one of the major sources.

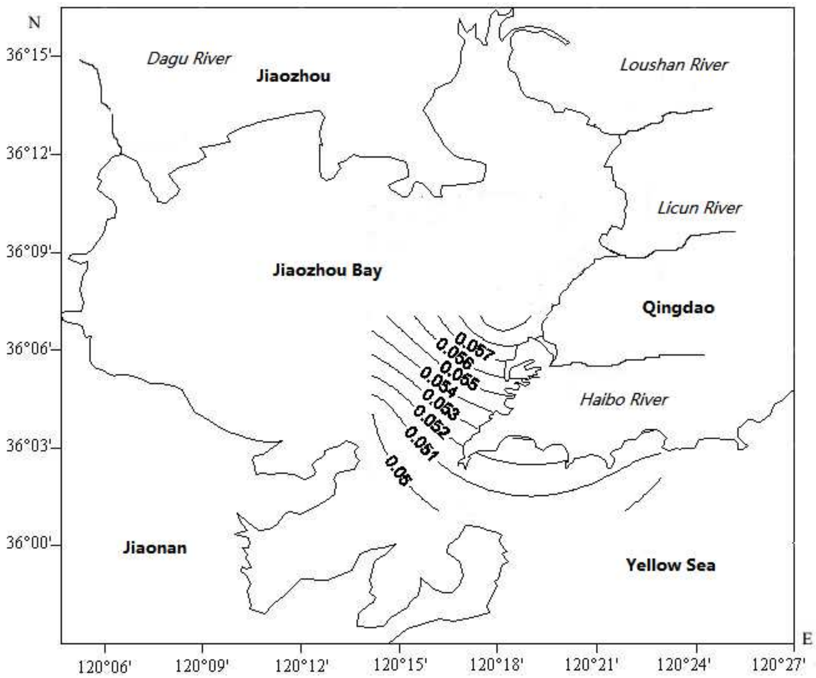

Figure 4. *Horizontal distribution of PHC in surface waters in Jiaozhou Bay in July 1984/mgL^{-1}*

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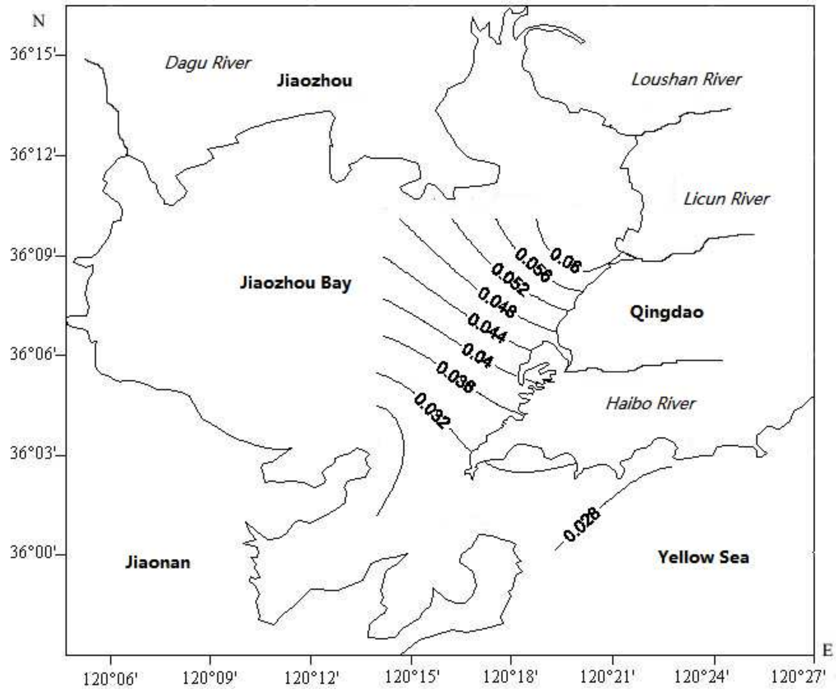

Figure 5. *Horizontal distribution of PHC in surface waters in Jiaozhou Bay in April 1985/mgL*$^{-1}$

In July 1986, PHC contents were decreasing from the open waters to the inside of the bay (*Fig. 6*), indicating that marine current was one of the major sources. In November 1987, PHC contents were decreasing from the southwest coast where there was an important harbour to the northeast of the bay (*Fig. 7*), indicating that marine traffic was one of the major sources.

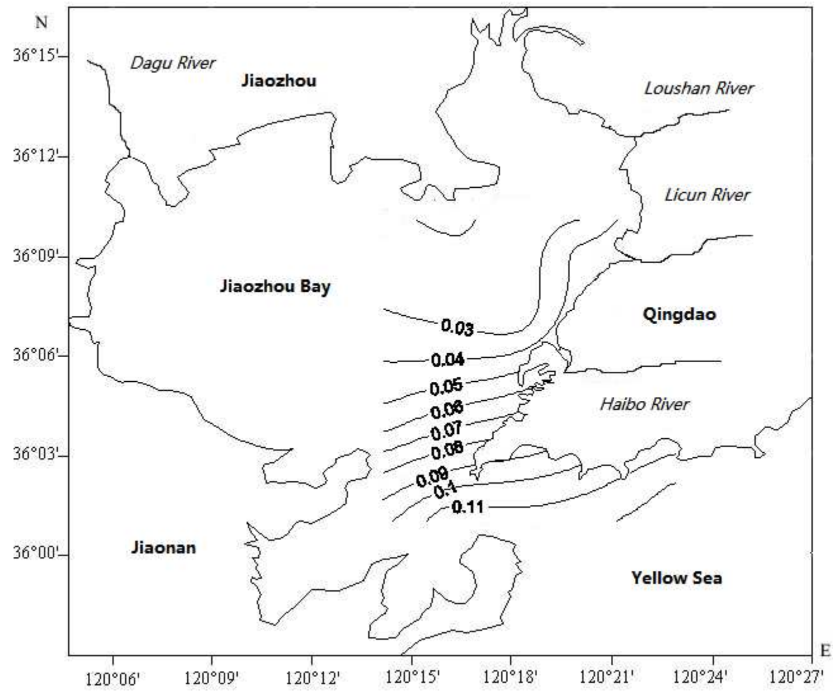

Figure 6. *Horizontal distribution of PHC in surface waters in Jiaozhou Bay in July 1986/mgL*$^{-1}$

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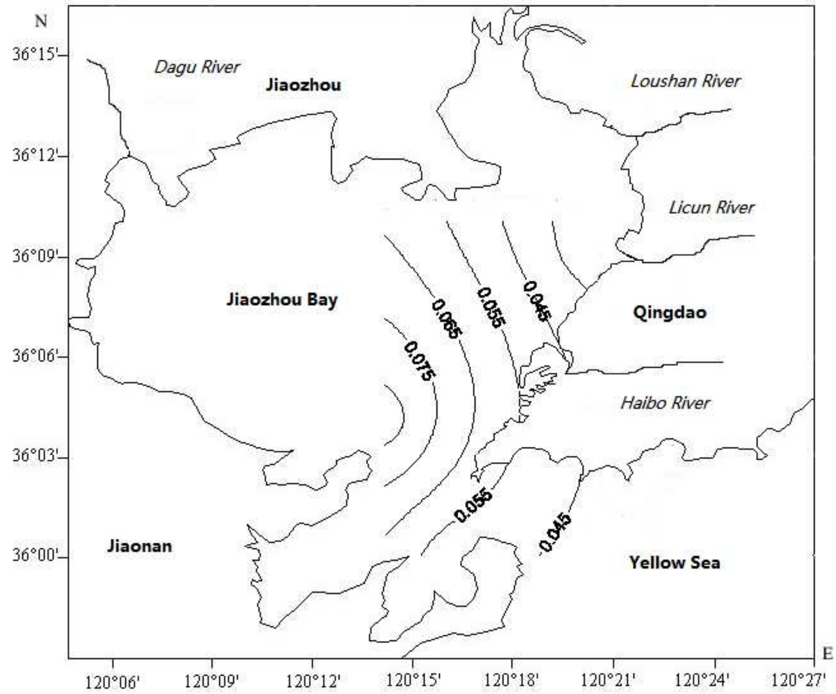

Figure 7. *Horizontal distribution of PHC in surface waters in Jiaozhou Bay in November 1987/mg L^{-1}*

In July 1988, PHC contents were decreasing from the estuary of Haibo River to the center of the bay (*Fig. 8*), indicating that stream discharge was one of the major sources. In general, stream discharge was the major source of PHC, and marine traffic and the sea itself were also responsible (*Table 3*).

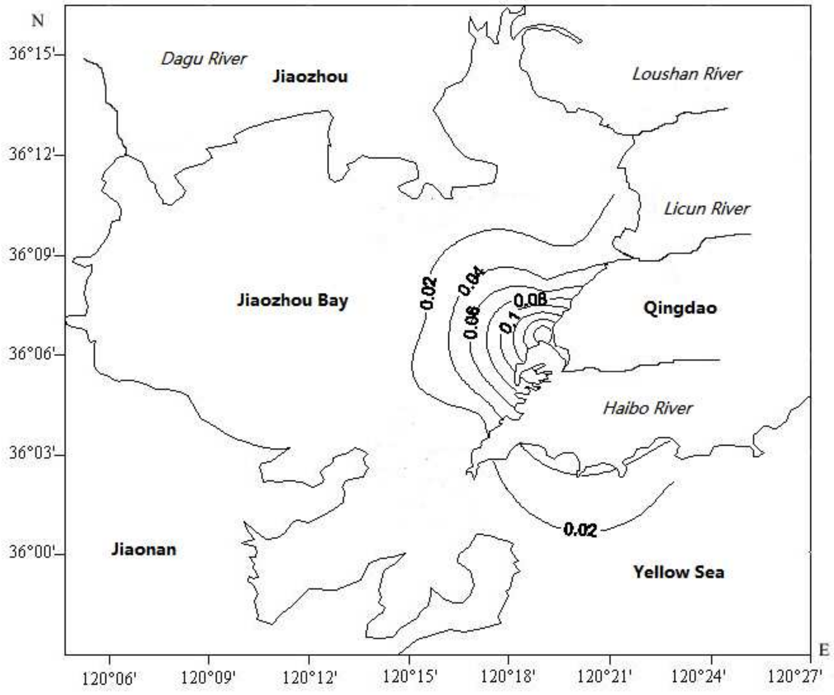

Figure 8. *Horizontal distribution of PHC in surface waters in Jiaozhou Bay in July 1988/mg L^{-1}*

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Table 3. *Sources and source strengths of PHC in Jiaozhou Bay 1984-1988*

Year	Month	Source	Source strength
	July	Haibo River	0.060
1984	August	Loushan River	0.160
	October	Loushan River	0.050
	April	Licun River	0.064
1985	July	Haibo River	0.124
	October	Licun River	0.121
	April	Loushan River	0.066
1986	July	Marine current	0.122
	October	Licun River	0.017
	May	Marine traffic	0.060
1987	July	Loushan River	0.066
	November	Marine traffic	0.091
	April	Licun River	0.064
1988	July	Haibo River	0.178
	October	Haibo River	0.169

Changes of PHC's sources

In 1984, the major source of PHC in Jiaozhou Bay was stream discharge, whose source strength was 0.05-0.16 mgL^{-1}. In 1985, the major source of PHC in Jiaozhou Bay was stream discharge, whose source strength was 0.064-0.124 mgL^{-1}. In 1986, the major sources of PHC in Jiaozhou Bay were stream discharge and marine current, whose source strength was 0.017-0.066 mgL^{-1} and 0.122 mgL^{-1}, respectively. In 1987, the major sources of PHC in Jiaozhou Bay were stream discharge and marine traffic, whose source strength was 0.066 mg L^{-1} and 0.060-0.091 mg-L^{-1}, respectively. In 1988, the major source of PHC in Jiaozhou Bay was stream discharge, whose source strength was 0.064-0.178 mgL^{-1}. In general, there were three sources of PHC, i.e., stream discharge, marine current and marine, and the source strength was still slight/moderate in according to Chinese Sea Water Quality Standard (GB 3097-1997) (*Table 4*). For annual changes, stream discharge was one of the major sources of PHC in every year, and the source strengths were increasing along with time. Hence, it could be concluded that PHC in this bay was mainly input from rainfall runoff. However, marine traffic had been one of the important sources since the 1980s, indicating that oil leaking from marine traffic should be paid attention to. Furthermore, the marine current was also one of the important sources, and the source strengths could be as high as 0.122 mg L^{-1} during the 1980s. This indicated the ocean had been strongly impacted by anthropogenic source input of PHC. Once the ocean was polluted, the remediation of water quality would be a task and long-term work. As a whole, source control should be promoted.

Table 4. *Pollution levels of PHC's sources in Jiaozhou Bay 1984-1988*

Source	Stream discharge			Marine current	Marine traffic
	Loushan River	Licun River	Haibo River		
Source strength	0.050-0.160	0.017-0.121	0.060-0.178	0.122	0.060-0.091
Pollution level	Slight	Slight	Moderate	Moderate	Slight

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During the period of study, the seasonal variation of precipitation was very significant (*Fig. 9*). The maximum and minimum amount of precipitation occurred in summer and winter, respectively. For monthly variations, the amount of precipitation was the lowest in in January (11.8 mm), and was increasing to a relatively high value in April (33.4 mm), and was reaching the maximum amount in August (150.3 mm), and then was decreasing to a relatively low in November (23.4), and finally was reaching the minimum in January in January again. In general, the periods of precipitation were in order of summer > spring > autumn > winter, resulting in a river discharge which was also in order of summer > spring > autumn > winter. Since rainfall runoff was the major force of various pollutants to the marine bay, the source input of PHC was also in order of summer > spring > autumn > winter. This was the major reason for the seasonal variations of PHC contents in Jiaozhou Bay.

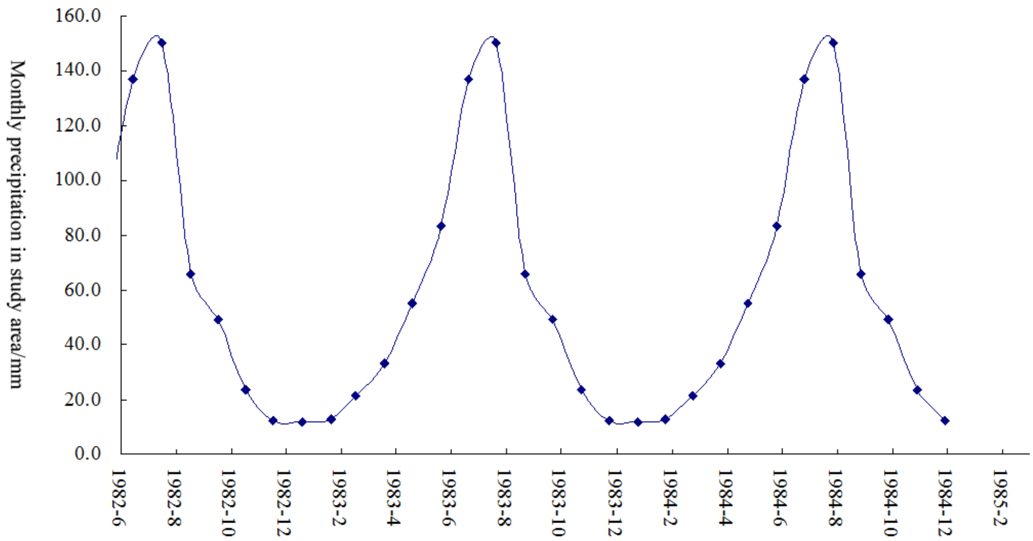

Figure 9. *Monthly precipitation in study area/mm*

Storage of PHC

In general, precipitation determined the river discharge, river discharge determined the source input of PHC, resulting in the seasonal variations of PHC in Jiaozhou Bay waters. By means of the continuous source input of PHC from river discharged, and the continuous accumulation of PHC in waters, a great deal of PHC was stored in Jiaozhou Bay, resulting in the increasing trend of PHC contents in waters. However, in 1987, PHC contents in waters were mainly impacted bay source input of marine traffic, resulting in different seasonal distribution patterns. In case of little source input, the background value of PHC in this bay was 0.005 mgL^{-1}, while in case of source input from marine current, the high-value was 0.122 mgL^{-1}. In consideration that the background value of PHC was 0.005 mgL^{-1} and the background value of PHC in marine was mgL^{-1}, the increase of PHC contents in the ocean could be calculated as 0.122-0.005 = 0.117 mgL^{-1}. This was the results of the storage of PHC in the ocean. Furthermore, a block diagram model was provided to demonstrate that PHC contents in marine waters were increasing continuously by means of continuous source input (*Fig. 10*). Hence, the control and management of anthropogenic source input of PHC in the marine bay were necessary.

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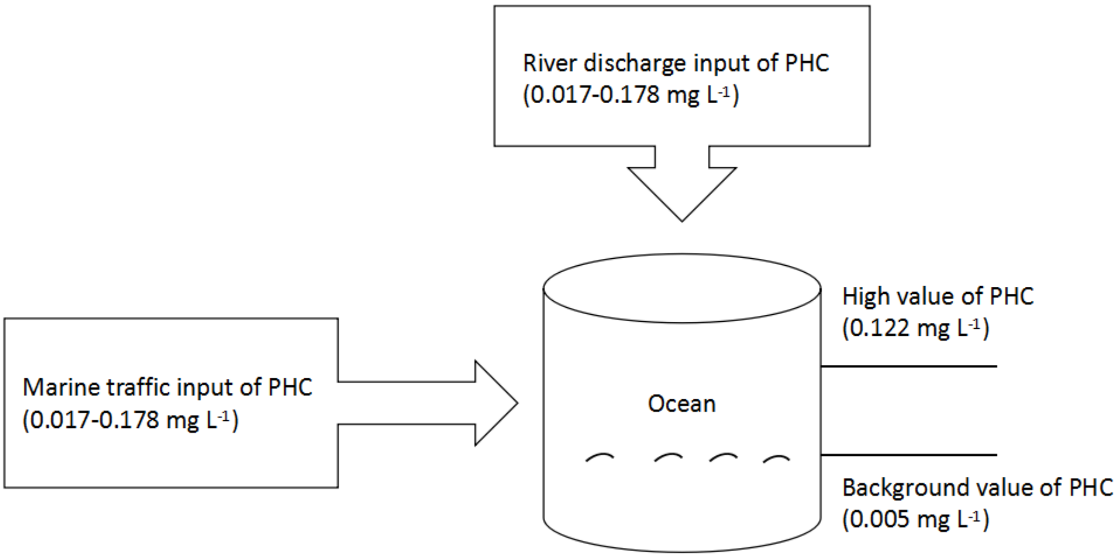

Figure 10. *Block diagram model for the influence of PHC input on marine PHC content*

Conclusion

PHC during 1984-1988 was 0.01-0.16 mgL^{-1}, 0.025-0.124 mgL^{-1}, 0.005-0.122 mgL^{-1}, 0.014-0.091 mgL^{-1} and 0.005-0.178 mgL^{-1}, respectively. The pollution level of PHC during 1984-1988 was moderate and was changing with seasons due to the seasonal variations of source input. Low values of PHC contents during 1984-1988 were all closed to 0.005 mgL^{-1}, and these values could be considered as the 'background' values of PHC in Jiaozhou Bay. High values of PHC contents in spring during this period tends to be stable, yet in summer and autumn during this period were tending to be increasing. The pollution levels of PHC tends to be more and more serious along with time, and the source control of PHC is essential to improve the water quality.

The sources of PHC in Jiaozhou Bay during 1984-1988 were identified. Stream discharge was the major source of PHC, and marine traffic and marine were also responsible. The source strengths were still slight/moderate in according to Chinese Sea Water Quality Standard (GB 3097-1997). Stream discharge was one of the major sources of PHC in every year, and the source strengths were increasing along with time. PHC in this bay was mainly input from rainfall runoff. Marine traffic had been one of the important sources since the 1980s, indicated that oil leaking from marine traffic should be paid attention to. Marine current was also one of the important sources, and the source strengths could be as high as 0.122 mgL^{-1} during the 1980s. Source control should be promoted.

Rainfall runoff was the major force of various pollutants to the marine bay, the source input of PHC was also in order of summer > spring > autumn > winter. This was the major reason for the seasonal variations of PHC contents in Jiaozhou Bay. The background value of PHC was 0.005 mgL^{-1} and that in marine was mgL^{-1}, the increase of PHC contents in the ocean could be calculated as 0.122-0.005 = 0.117 mgL^{-1}. This was the results of the storage of PHC in the ocean. Furthermore, a block diagram model was provided to demonstrate that PHC contents in marine waters were increasing continuously by means of continuous source input. Hence, the control and management of anthropogenic source input of PHC in the marine were necessary.

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THE EFFECTS OF SOIL CONDITIONERS ON GRASS COLOUR THROUGHOUT THE GROWING SEASON

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Abstract. The aim of this study is to assess soil conditioners' effects on the colour intensity of lawn grass. The field experiment was conducted in the experimental facility between 2013 and 2015 in Poland. The following grass species were used in the experiment (factor B): *Lolium perenne*, *Festuca rubra*, and *Poa pratensis*. Another experimental factor tested in the research was soil conditioners (factor A) as Substral, Humus Active Papka, Eko-Użyźniacz, UGmax. At the end of each growing season between 2013 and 2015 the colour of grasses was assessed in accordance with the methodology using a 9-point rating scale. This assessment was conducted in three seasons: spring, summer, and autumn. In all seasons of the year smooth-stalked meadow-grass had the most favourable colour when treated with Substral in spring and summer seasons, and with Eko-Użyźniacz in autumn. Comparing all soil conditioners, grass treated with UGmax had the best colour. From a practical point of view, of all grass species tested in the study, perennial ryegrass had the smallest, most favourable, colour variation during the whole period.
Keywords: *leaf-blade greenness, lawns, soil conditioners, turf grasses, growing season*

Introduction

Green spaces positively affect human well-being and mental state not only in dwelling areas but also in workplaces. In addition, to some degree they satisfy inhabitants' aesthetic needs. Regardless of the purpose of a green area, grass is the basic plant to cover the ground in a lawn (Grabowski et al., 2003a; Prończuk and Żurek, 2008; Pooya et al., 2013; Knot et al., 2017). With its richness of species and forms, grass has an amazing ability to adapt to changing weather conditions and different ways of its use (Wolski, 2003; Knot et al., 2017). A lawn should be composed of several species of grass, complementing each other in terms of their properties. Selection of those species should depend on habitat conditions and on the lawn's user capacity to provide means to meet maintenance requirements (Salehi and Khosh-Khui, 2004; Czeluściński et al., 2017). Each species of grass has a unique role in the environment, as well as in human life and economic activity (Pooya et al., 2013; Knot et al., 2017). In Poland there are some 160 species of grass, but out of this considerable number only 16 of them are suitable to plant lawns (Grabowski et al., 2003a; Prończuk and Żurek, 2008). The most common grass species used for extensive lawns include: red fescue (*Festuca rubra*), smooth-stalked meadow-grass (*Poa pratensis*), and perennial ryegrass (*Lolium perenne*). These species have a varied degree of leaf-blade greenness. In general, red fescue, as opposed to the other two

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species, is a dark green plant. Naturally, an important element in determining the appearance of a lawn is its colour (Jankowski et al., 2012a, b; Braun et al., 2016), which can change under the influence of the weather, habitat, and other conditions. Difficulty in maintaining a proper grass colour under adverse moisture conditions or with insufficient nutrients in the soil can cause a loss of a lawn's natural greenness and, consequently, its attractiveness can be significantly reduced. Green is a calming and beneficial colour for the human eye, positively affecting mental health. In degraded areas there is a much higher rate of people suffering because of the direct action of pollutants, but there is also a significant increase in mental health problems resulting from stress caused by a lack of contact with nature (Stępczak, 1997; Salehi et al., 2004; Knot et al., 2017).

As a result of the presence of green spaces, negative effects of long lasting droughts can be reduced. However, longer periods with little rainfall lead to a change of lawn colour from green to yellow, with some grass leaves turning discoloured (Prończuk and Żurek, 2008; Braun et al., 2016). When adequate care and conditions cannot be provided, an application of soil conditioners before setting up a lawn can be a good solution. The quality of the turf with its colour depends to a large extent on a frequent application of plant nutrients which can stimulate quality and colour of the grass (Jankowski et al., 2010, 2011a, b, c). Additionally, leaf colour, apart from leaf fineness, is an indicator most often assessed in lawns. Grass with narrow dark green leaves looks the best (Jankowski et al., 2012a), and this is the colour most desired by lawn users. Plant physiological processes can be modified by mineral compounds stimulating plant growth. Soil conditioners are based on natural substances such as plant or humus extracts and phytohormones (Hamza and Suggars, 2001). Those bio fertilizers demonstrate a positive impact on grass metabolism, stimulate life processes, and reduce the effects of adverse environmental conditions (drought, salinity, temperature fluctuations) and pathogens (Chen et al., 2004; Gąbka and Wolski, 2008). Soil conditioner application contributes to the decomposition of organic matter. This is a very important effect due to the release of mineral forms, which are an important source of nutrients for arable crops (Hamza and Suggars, 2001; Chen et al., 2004; Calvo et al., 2014). Soil conditioners used instead of mineral fertilizers can play an essential role in grass ecological maintenance, and the aim of this study is to assess their effects on the colour intensity of lawn grass.

Materials and methods

Experimental design

Set up in 2012 the field experiment was conducted in Poland 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 $g \cdot m^2$. 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*.

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Table 1. *Composition of soil conditioners applied in the experiment*

Soil conditioner	Macronutrients (g·kg^{-1})						Micronutrients (mg·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	-
g·m^{-2}	4.4	0.44	1.66									
Humus Active Papka (HAP)	0.2	1.3	4.6	3.0	0.5	-	15	500	3	1	-	Active humus with useful microorganisms
g·m^{-2}	0.05	0.33	1.15									
Eko-Użyźniacz (EU)	0.6	0.3	0.7	-	-	-	-	-	-	-	-	Endo micorhiza, fungi, bacteria, enzymes of earthworms
g·m^{-2}	0.06	0.03	0.07									
Ugmax (UG)	1.2	0.2	2.9	-	0.1	0.2	0.3	-	-	-	-	Lactic acid bacteria, photosynthetic bacteria, *Azotobacter*, *Pseudomonas,* yeast, *Actinomycetes*
g·m^{-2}	0.03	0.01	0.07									

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.

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 m^{-2} (0.6 l in 250 l of water), Eko-Użyźniacz - 100 ml m^{-2} (10 l in 100 l of water), and Humus Active Papka - 250 ml 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 m^{-2}. The tested fertilizers were used in doses recommended by the manufacturer.

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

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things its colour was assessed in accordance with the methodology using a 9-point rating scale (*Table 2*). This assessment was conducted in three seasons: spring, summer, and autumn. In each year of the research spring assessment was made around 20 May, summer assessment around 20 August, and autumn assessment around 10 October.

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, 2011). Chemical analysis showed that the soil was of neutral pH (pH = 6.8), with assimilable phosphorus in a form of $H_2 PO_4$ - standing at 170 mg·kg^{-1}, magnesium Mg^{2+} at 84 mg·kg^{-1}, potassium K^+ at 114 mg·kg^{-1}, total nitrogen N at 1.3 g·kg^{-1}, and organic carbon at 13.5 g·kg^{-1} DM.

Table 2. *Lawn grass colour rating with a scale of 1 to 9 according to Domański (1998)*

Rating	Grass colour
1	Bare ground with no grass
2	Dry grass
3	Yellowish to brownish green
4	Yellowish green with discolouration
5	Greyish green
6	Bluish green
7	Light green
8	Lush green
9	Dark green

Statistical analysis

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 interaction. Based on lawn colour 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 (about 4 km from experimental station). In order to determine the temporal variation of meteorological elements and their effects on vegetation, Sielianinov's hydrothermal coefficient 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 (ed);

$0.4 < K \leq 0.7$ very dry (vd);

$0.7 < K \leq 1.0$ dry (d);

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1.0 < K ≤ 1.3 quite dry (qd);
1.3 < K ≤ 1.6 optimal (o);
1.6 < K ≤ 2.0 quite wet (qw);
2.0 < K ≤ 2.5 wet (w);
2.5 < K ≤ 3.0 very wet (vw);
K > 3.0 extremely wet (ew).

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).

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.

Table 3. *Sielianinov's hydrothermal coefficient (K) during the growing season*

Years	Month						
	Apr.	**May**	**June**	**July**	**Aug.**	**Sept.**	**Oct.**
2013	2.56 (vw)	3.07 (ew)	2.11 (w)	0.84 (d)	0.78 (d)	2.53 (vw)	0.60 (vd)
2014	1.36 (o)	1.87 (qw)	1.64 (qw)	0.59 (vd)	1.92 (qw)	0.64 (vd)	0.12 (ed)
2015	1.22 (qd)	2.63 (vw)	0.87 (d)	1.08 (qd)	0.18 (ed)	1.46 (o)	1.94 (qw)

Results

One of the most important characteristics rated in lawn quality assessment is its colour. In the present study, assessment done in spring seasons (*Table 4*) proved that grass colour was dependent on the species, the type of soil conditioner, and meteorological conditions.

Comparing soil conditioner effects it was observed that in spring grass was the most intensely green (7.86^o) on plots where Substral was applied, being the least intensely green as a result of Eko-Użyźniacz (6.05^o) or Humus Active Papka (6.08^o) application. Despite that, statistical analysis showed that differences between colour ratings of lawns treated with different kinds of soil conditioner were not significant.

Similarly, the differences between the colour ratings of grass species were not statistically significant either, although smooth-stalked meadow-grass and red fescue had slightly more intense colour than perennial ryegrass, with their ratings of 6.97^o and 6.94^o, which is bright green.

The forms of soil conditioners affected the colour of different grass species to a different extent. It was observed that Substral application resulted in leaf blades with the darkest shade of green for all grass species, but smooth-stalk meadow-grass was of the most intense colour (8.13^o). In spring seasons, out of all conditioners UGmax application resulted in the most favourable colour of leaf blades in all species, with the average rating of above 7, indicating light green colour. Throughout all growing seasons it turned out that in the spring, grass was the greenest in 2015, with the rating of 7.45^o, as the average score of all three types of soil conditioners.

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Table 4. *Assessment of grass colour (a 9-point scale) between 2013 and 2015 in the spring*

Year (C)	Species (B)	Soil conditioner (A)				$\bar{x}$
		(S)	(EU)	(HAP)	(UG)	
2013	Smooth-stalked meadow-grass	6.6	5.9	3.8	4.2	5.13
	Perennial ryegrass	7.2	4.9	7.1	5.3	6.13
	Red fescue	7.8	8.1	8.2	5.9	7.5
2014	Smooth-stalked meadow-grass	8.8	6.1	5.9	8.9	7.43
	Perennial ryegrass	8.8	3.9	3.7	8.9	6.33
	Red fescue	8.7	3.8	4.1	9	6.4
2015	Smooth-stalked meadow-grass	9	8	7.8	8.7	8.38
	Perennial ryegrass	7.2	6.8	7.2	7	7.05
	Red fescue	6.7	7	7	7	6.93
		Species effect-mean values				
Smooth-stalked meadow-grass		8.13	6.66	5.83	7.26	6.97
Perennial ryegrass		7.73	5.2	6.0	7.07	6.5
Red fescue		7.73	6.3	6.43	7.3	6.94
		Fertilizer effect-mean values				
		7.86	6.05	6.08	7.21	6.8
		Growing season effect-mean values				
2013		7.2	6.3	6.36	5.13	6.25
2014		8.76	4.6	4.56	8.93	6.71
2015		7.63	7.26	7.33	7.56	7.45

$LSD_{0,05}$
A = ns B = ns C = ns
A/B = ns B/A = ns A/C = 1.96
C/A = 1.76 B/C = 1.53 C/B = 1.53

ns – not significant

Grass colour considerably improved in summer seasons compared with spring periods (*Table 5*). The results indicated that in summer seasons grass treated with Substral (8.95°) and UGmax (8.84°) had dark green colour, with lawns treated with those two soil conditioners having the most favourable appearance. Grass colour in plots with Eko-Użyźniacz and Humus Active Papka was rated considerably lower, with a score statistically different from the ratings of the other two soil conditioners.

Comparing the colours of different grass species, it was observed that in summer seasons there was no statistically significant difference between them, with the average rating of above 8 for all species, although red fescue had the best colour (8.23°). Grass strongly responded with increased greenness of leaf blades to the application of Substral and UGmax, which was proved by comparing average colour ratings of the three grass species treated with those two conditioners. Those ratings were over 8.8° for both soil conditioners, indicating dark green colour.

Additionally, the weather conditions in summer seasons affected colour intensity of the grass tested in the study. In the 2014 and 2015 growing seasons grass had the most intense dark green colour (above 8.8°), while in 2013 it was much lighter, with the rating of 6.62°, indicating bluish green. The difference in grass colour between all the above growing seasons was statistically significant.

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Table 5. *Assessment of grass colour (a 9-point scale) between 2013 and 2015 in the summer*

Year (C)	Species (B)	Soil conditioner (A)				$\bar{x}$
		(S)	(EU)	(HAP)	(UG)	
2013	Smooth-stalked meadow-grass	9	4.2	3.9	8.7	6.45
	Perennial ryegrass	8.9	3.9	4.3	9	6.53
	Red fescue	9	5	4.9	8.7	6.9
2014	Smooth-stalked meadow-grass	9	8.8	9	8.9	8.93
	Perennial ryegrass	8.9	9	8.9	9	8.95
	Red fescue	8.9	9	9	8.8	8.93
2015	Smooth-stalked meadow-grass	9	8.8	8.9	8.9	8.9
	Perennial ryegrass	8.9	8.9	8.6	8.7	8.78
	Red fescue	9	8.9	8.7	8.9	8.88
		Species effect-mean values				
Smooth-stalked meadow-grass		9	7.26	7.26	8.83	8.09
Perennial ryegrass		8.9	7.26	7.26	8.9	8.08
Red fescue		8.96	7.63	7.53	8.8	8.23
		Fertilizer effect-mean values				
		8.95	7.38	7.35	8.84	8.13
		Growing season effect-mean values				
2013		8.96	4.36	4.36	8.8	6.62
2014		8.93	8.93	8.96	8.9	8.93
2015		8.96	8.86	8.73	8.83	8.85

$LSD_{0,05}$		
A = 1.56	B = ns	C = 1.23
A/B = ns	B/A = ns	A/C = 0.55
C/A = 0.49	B/C = ns	C/B = ns

ns – not significant

During autumn seasons quite variable grass colours were observed (*Table 6*). Comparing the types of soil conditioners, Eko-Użyźniacz application resulted in the best colour of grass (6.51°). Grass fertilised with Substral had the least favourable colour during in the autumn (5.87°). However, the differences between the effects of different conditioners were not statistically significant. Contrary to that, there were significant differences between grass species in their shades of green in autumn seasons. Perennial ryegrass had the best colour, with the rating of 7.07°, which was significantly better than for other species of grass.

The grass had the best dark-green colour in 2013 with the rating of above 8.7 as the average of the effects of all conditioners. High rainfall in September 2013 had a huge impact on grass colour, but in the autumn of 2014 and 2015 grass had significantly lower colour rating. The difference in colour ratings between the grass in autumn of 2013 and of the other two years was statistically significant. Comparing the average colour ratings of grass species in all growing seasons (*Table 7*) it was found that perennial ryegrass had the most advantageous colour (7.22°). Taking into account the type of applied soil conditioner, perennial ryegrass treated with Substral (7.89°) and smooth-stalked meadow-grass treated with Humus Active Papka had the highest colour rating of 6.07°.

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Table 6. *Assessment of grass colour (a 9-point scale) between 2013 and 2015 in the autumn*

Year (C)	**Species (B)**	**Soil conditioner (A)**				$\bar{x}$
		(S)	**(EU)**	**(HAP)**	**(UG)**	
2013	Smooth-stalked meadow-grass	9	8.9	8.8	8.6	8.83
	Perennial ryegrass	8.9	8.8	8.5	9	8.8
	Red fescue	9	8.9	8.8	8.6	8.83
2014	Smooth-stalked meadow-grass	3	3	2.8	4.1	3.23
	Perennial ryegrass	4.9	6.2	6	5.1	5.55
	Red fescue	5	6	5.9	4.7	5.4
2015	Smooth-stalked meadow-grass	4	9	3.8	4.2	5.25
	Perennial ryegrass	7.2	6	6.9	7.4	6.88
	Red fescue	1.9	1.8	2	2.3	2
		Species effect-mean values				
Smooth-stalked meadow-grass		5.33	6.96	5.13	5.63	5.76
Perennial ryegrass		7	7	7.13	7.16	7.07
Red fescue		5.3	5.56	5.56	5.2	5.41
		Fertilizer effect-mean values				
		5.87	6.51	5.94	6	6.08
		Growing season effect-mean values				
2013		8.96	8.86	8.7	8.73	8.81
2014		4.3	5.07	4.9	4.63	4.72
2015		4.36	5.6	4.23	4.63	4.71

$LSD_{0,05}$
A = ns B = 1.55 C = 1.55
A/B = ns B/A = ns A/C = ns
C/A = ns B/C = 2.12 C/B = 2.12

ns – not significant

Table 7. *Effect of soil conditioners on grass colour (a 9-point scale) between 2013 and 2015 mean values*

Species (B)	**Soil conditioner (A)**				$\bar{x}$
	(S)	**(EU)**	**(HAP)**	**(UG)**	
Smooth-stalked meadow-grass	7.49	6.96	6.07	7.24	6.94
Perennial ryegrass	7.89	6.49	6.8	7.71	7.22
Red fescue	7.33	6.5	6.5	7.1	6.86
	Fertilizer effect-mean values				
	7.56	6.65	6.46	7.35	7.01
$LSD_{0,05}$ A = 0.81 B = ns					
	Growing season effect-mean values				
2013	8.37	6.51	6.47	7.55	7.23
2014	7.33	6.2	6.14	7.49	6.79
2015	6.98	7.24	6.76	7.01	7.0
$LSD_{0,05}$ A=ns B = ns					

ns – not significant

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Grass treated with Substral had the most favourable lush green colour, with the rating of 7.56°, as the average for all the species, but when it was treated with Humus Active Papka the rating was the lowest, with blue shade of green (6.46°).

Comparing the three years of research, without a division into seasons of the year, it was observed that the grass had the highest colour rating in 2013 (7.23°) and the lowest in 2014 (6.79°). The coefficient of variation of grass colours calculated during the research (*Table 8*) indicated that during the whole research period perennial ryegrass had the smallest variation of the intensity of greenness, and, at the same time, it was characterised by the highest average colour rating of 7.22°, which is light green colour.

Table 8. *Standard deviation and coefficient of variation of grass colour*

Species	Min. value of colour rating	Max. value of colour rating	Mean values of colour rating	Standard deviation	Coefficient of variation
Smooth-stalked meadow-grass	2.8	9	6.94	2.35	33.86
Perennial ryegrass	3.7	9	7.22	1.77	24.52
Red fescue	1.8	9	6.86	2.38	34.69

Coefficient of variation: 0–20% small variation, 20–40% moderate variation, 40–60% large variation, >60% very large variation

Discussion

In this study the differences between the colour ratings of grass species in spring were not significant, although smooth-stalked meadow-grass and red fescue had slightly more intense colour than perennial ryegrass. But in the studies, of Starczewski and Affek-Starczewska (2011) the cultivars of perennial ryegrass were characterized by the most intensive green at the beginning of the growing season (vivid green in May), and the least favorable in the summer month (grey, dirty green) than other species.

In summer seasons the best green colour had red fescue, but not significant to the other species. In this study in autumn seasons, there were significant differences between grass species in their shades of green but perennial ryegrass had the best colour. Also in the studies by Grabowski et al. (1999) and Jankowski et al. (1999) cultivars of perennial ryegrass achieved the highest marks of colour assessment in autumn periods.

Comparing the average colour ratings of grass species in all growing seasons, it was found that perennial ryegrass had the most advantageous colour. In regard to soil conditioner effects it was observed that in spring and in summer grass was the most intensely green on plots where Substral was applied, being the least intensely green as a result of Eko-Użyźniacz or Humus Active Papka application. According to Jankowski et al. (2012a) the colour of a lawn is an unstable characteristic, depending, among others, on habitat conditions, the content of macro- and micronutrients in the soil, and on their availability for plants. Soil conditioners make absorption of nutrients easier, which affects metabolism of plants (Calvo et al., 2014). Studies conducted by Talar-Krasa and Świerszcz (2015) confirm stimulating properties of humic acids present in soil conditioners on plant growth and development as well as grass colour. In the case of the colour of the species studied in the present experiment, application of soil conditioners resulted in a significant increase in leaf blade greenness. In the spring and in summer the best green colour was after Substral application.

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Comparing the types of soil conditioners in autumn, Eko-Użyźniacz application resulted in the best colour of grass. Also in the study of Wiśniewska-Kadżajan (2013) the intensity of the green colour of turf lawns has improved with the increase of the applied dose of mushrooms substrate. According to Jankowski et al. (2012b) the type and amount of fertilizers applied had a big impact on the intensity of the colour. In the other study of Jankowski et al. (2011d) a significantly better effect of fertilizer Sierrablen on the colour of monoculture turfs was observed as compared with Trawovit Komplet.

As the average for all the species, grass treated with Substral had the most favourable lush green colour, but when it was treated with Humus Active Papka the rating was the lowest, with blue shade of green. Braun et al. (2016) studied the stability of dark-green colour of grass leaves over the entire period of vegetation together with greenness durability into late autumn, and observed that it was also dependent on the application of proper fertilizers.

The weather conditions in spring, summer and autumn seasons of each experimental year affected colour intensity of the grass tested in the study. Also the results of Wiśniewska-Kadżajan (2013) clearly indicate that the colour of turf lawns strongly affected by weather conditions.

Throughout all growing seasons it turned out that in the spring, grass was the greenest in 2015, in summer in 2014 and 2015 but in autumn in 2013. In the studies by other authors (Jankowski et al., 1999; Grabowski et al., 1999, 2003b) a similar tendency was noticeable at the assessment of lawn colour.

For example, in autumn period high rainfall in September 2013 had a huge impact on grass colour. In the other study of Jankowski et al. (2011d), the lawn colour was also largely dependent on the meteorological conditions. The results obtained indicate that more favorable weather conditions in 2003 had an effect on more intensive and more desirable colour of lawns. The intensity of green colour of leaf blades of lawn turfs in 2003 was to a large extend affected by the most even distribution of precipitation during the growing period.

In the literature concerning lawn turfs there are few studies on the assessment of their colouring. Consequently, it is difficult to relate the results of the present study to other authors.

Conclusion

In the study grass colour varied over the growing seasons in relation to the grass species (smooth-stalked meadow-grass, perennial ryegrass, red fescue) and the soil conditioner applied. Of all the bio products, Substral application resulted in the most favourable grass colour when used in spring and summer, and Eko-Użyźniacz when applied in autumn. This means that differences in the effects of soil conditioners in different seasons under different weather conditions were difficult to evaluate. However, those results also indicate an interaction between the types of soil conditioner and the meteorological conditions in their effect on grass colour. In all seasons of the year smooth-stalked meadow-grass had the most favourable colour when treated with Substral in spring and summer seasons, and with Eko-Użyźniacz in autumn. Comparing all soil conditioners, grass treated with UGmax had the best colour. This conditioner is composed not only of minerals, but also contains such micro-organisms as lactic acid bacteria, photosynthetic bacteria, *Azotobacter*, *Pseudomonas*, yeast, and *Actinomycetes.*

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From a practical point of view, of all grass species tested in the study, perennial ryegrass had the smallest, most favourable, colour variation during the whole period. In regard to obtained results for future studies should be recommended UGmax for lawn fertilization as well as the other new soil conditioners.

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RANDOM AND SYSTEMATIC LAND USE/LAND COVER TRANSITIONS IN SEMI-ARID LANDSCAPES OF ETHIOPIAN CENTRAL RIFT VALLEY LAKES REGION (EAST AFRICA)

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Abstract. The analysis of land use/land cover (LULC) change has always been a topic of interest in land dynamics research. The majority of previous studies used the conventional method of "net change" analysis to show spatiotemporal LULC transitions. However, such analysis failed to indicate whether the transition is clearly systematic or due to an apparently random process. Hence, this study aimed to identify the most prominent signals of landscape transitions over the last three decades, using the landscapes of East African Rift Valley Region. We used Remote Sensing and GIS to quantify and map the changes in LULC for 1986 and 2016, and then the two maps compared to produce transition matrices. Results show that net change and swap change accounted for 43% and 57% of total change on the landscape respectively. Accordingly, 6% of scattered acacia woodland and 5% of bush land have been converted to agricultural land, whereas 7% and 3% of scattered acacia woodland have been degraded towards grazing land and bush land respectively. These changes were found to be clearly systematic and hence indicate the dominant and prominent signals of landscape transformation. Hence, future land use policies need to consider such prominent signals of LULC change in order to plan an integrated approach to safeguard the fragile ecosystems of the region, while searching for alternative livelihood options.
Keywords: *gain, loss, net change, persistence, swap change*

Introduction

Anthropogenic-induced changes in land use/land cover (LULC) have occurred in the past, is presently ongoing, and is likely to continue in the future, with its related ecological, environmental and socioeconomic impacts worldwide (MEA, 2005; FAO, 2016). Unregulated LULC change for example has brought local, regional and global impact on biotic life, soil, hydrological balance, climate and ecological processes (Lambin et al., 2003; MEA, 2005; Yan et al., 2016; Rai et al., 2017). In addition, LULC change has threatened regional food supply, livelihood systems, and global sustainability (Reid et al., 2000). Such impacts of LULC change are more serious in developing countries where economic, political and sociocultural backgrounds further aggravate the complexity of the dynamics (Kalacska et al., 2017). These entire concerns make the analysis of land dynamics always been a topic of interest in researches at various fields and levels (Manandhar et al., 2010; Li et al., 2017; Yirsaw et al., 2017),

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which have spawned a flurry of research on its extent, drivers and consequences in recent decades globally.

Based on causative factors, the transitions in LULC could be classified as ‘random’ or ‘systematic’ (Pontius et al., 2004; Briones and Sepúlveda-Varas, 2017). Random transitions are those influenced by accidental or unique processes of change (Tucker et al., 1991; Geist and Lambin, 2002; Lambin et al., 2003). These transitions are usually caused by the interaction of land use factors such as: spontaneous migration, internal conflicts, changes in microeconomic conditions or loss of ownership to land resources (Lambin et al., 2003; Braimoh and Vlek, 2005). On the other hand, systematic transitions are those which occur due to regular or common processes of change. They tend to evolve in a consistent, progressive or gradual manner driven by natural population growth, increase in commercialization, border development, lack of public awareness on the environment or changes in institutions governing access to natural resources (Lambin et al., 2003; Geist et al., 2006).

Pontius et al. (2004) and Alo and Pontius Jr (2008) pointed out that though a conventional cross-tabulation matrix is a fundamental starting point in the analysis of LULC change, several related research works failed to analyze the matrix based on its various components, and thus failed to gain as much insight as possible concerning the potential processes that determine a pattern of landscape changes. This is because the majority of earlier landscape change studies used a ‘net change’ to compare a spatiotemporal LULC change trends between two different periods. However, though a net change indicates a definite change on the landscape, the absence of net change does not necessarily indicate the absence of change on the landscape. This is ascribed to the fact that the net change fails to capture a gross gain of a land category in one location with a gross loss of the same category in other location (Manandhar et al., 2010). This type of simultaneous loss and gain for a land category in spatial allocation is termed as the ‘swapping’ component of a change (Pontius et al., 2004; Braimoh, 2006; Briones and Sepúlveda-Varas, 2017). In addition, since the dominant landscape change signal is usually that of persistence, net change undermines the total change on the landscape, and hence fails to indicate the most prominent signals of LULC changes (Versace et al., 2008).

Due to such important limitations of ‘net change’, scientists recommend extending of the usual way of land transition matrices analysis beyond reporting net change, to gain an in-depth information that is important for detecting the most important signals of landscape changes (Braimoh, 2006; Manandhar et al., 2010). Pontius et al. (2004) strongly recommended detecting the most prominent signals of change, and ultimately linking pattern to process when analyzing LULC changes. Similarly, Geist et al. (2006) pointed the importance of considering two fundamental steps in any study of land dynamics. These are detecting changes in the landscape first, and then ascribing that change to some set of casual factors. The importance of addressing systematic and random LULC transitions has been further evidenced by recent studies (Burmeister and Schanze, 2016; Zewdie and Csaplovics, 2016; Briones and Sepúlveda-Varas, 2017). Linking the studies of changes in landscape pattern to the processes underlying helps to better understand the mechanisms of change; generate predictions about future rates of change, identify potential vulnerable places to change, and to design appropriate policy responses (Lambin, 1997; Nagendra et al., 2004; Alo and Pontius Jr, 2008).

Ethiopia is one of the typical countries in the sub-Saharan Africa confronted with the multidirectional impacts of extensive and rapid LULC dynamics since the beginning of the twentieth century (Kindu et al., 2013; Lemenih and Kassa, 2014). For example,

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empirical studies shown that deforestation in Ethiopian highlands has changed the landscape from 40% of forest cover in 1900 to 3.2% cover in 1980 (Dessie and Kleman, 2007; Eshetu, 2014), though some unpublished sources reported an increase in forest coverage of the country since 2000. On the other hand, significant increase in agricultural lands in expense of losses in forestlands, woodlands and grasslands has been reported in arid and semi-arid areas of the country including the Central Rift Valley (CRV) Lakes Region (Muzein, 2006; Garedew et al., 2009; Temesgen et al., 2013; Meshesha et al., 2014; Ariti et al., 2015; Zewdie and Csaplovics, 2016). In addition, in recent years, expansion in built-up areas has also been accelerated in Ethiopia since the government change and subsequent land use policy reforms in 1991 (Meire et al., 2013). Nonetheless, among such previous land dynamics studies in different parts of the country, only study by Zewdie and Csaplovics (2016) in north western Ethiopia, addressed random and systematic transition analysis. In addition, these studies did not emphasize the degree of landscape categories persistence relative to gross losses and gains. Hence, this study is aimed at: 1) detecting and mapping spatiotemporal LULC changes for the years 1986 and 2016; 2) analyzing LULC change matrices according to their various components (net change, persistence, gross gain/loss and swap); and 3) identifying systematic and random transitions, and the most prominent signals of LULC changes, and relating this to some possible causative factors, taking the CRV lakes region of Ethiopia as ideal case study site. This way of analysis helps in linking patterns to process and in designing appropriate policy interventions aimed at reducing the adverse effects of spectacular LULC changes in developing countries like East Africa.

Materials and methods

The study area

The study site, also known as Ziway-Shalla basin, is one of the four sub basins under the Rift Valley Lakes Basin, located about 170 km south of the capital city, Addis Ababa, Ethiopia. It is geographically found in the limits of 7°20'–8°00'N latitude and 38°20'– 38°50'E longitude (*Fig. 1*). The study area belongs to the main African Rift system (Benvenuti et al., 2002). The African Rift originates from Aden Junction (Arabian Plate) in the Middle East and continues in the direction of south-west, traversing longitudinally eastern African countries such as Djibouti, Eritrea, Ethiopia, Kenya, Uganda, and Tanzania (JICA, 2012). The CRV is part of the Main Ethiopian Rift system (Molin and Corti, 2015) which is topographically characterized by a depression zone with steep marginal faults along its eastern and western edges (JICA, 2012). Altitude ranges from 1554 to 2069 m a.s.l. and bounded by north-western and south-eastern highlands (Hengsdijk and Jansen, 2006). The total area of the basin is about 13,401 km^2 (JICA, 2012). It is situated in the current regional administrative boundaries of Southern Nations, Nationalities and Peoples (SNNP) and Oromia (*Fig. 1*).

The climate in CRV lakes region varies markedly with altitude and season (Jansen et al., 2007). It is characterized by warm and wet summer (June to September) and dry, cold and windy winter (October to May). The climate of the main valley in the central portion is dominantly semi-arid, whereas that of highland portion is sub-humid (Fritzsche et al., 2007). Based on the data obtained from National Meteorological Agency (NMA), Ziway Station, mean annual rainfall is about 739 mm, while mean monthly minimum and maximum temperatures are 14 and 27 °C respectively (*Fig. 2*),

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and this figure significantly varies throughout the basin depending on elevation. In general, the region is highly vulnerable to climate change as it encompasses a dryland zone which has been repeatedly hit by drought (Jansen et al., 2007; Biazin and Sterk, 2013).

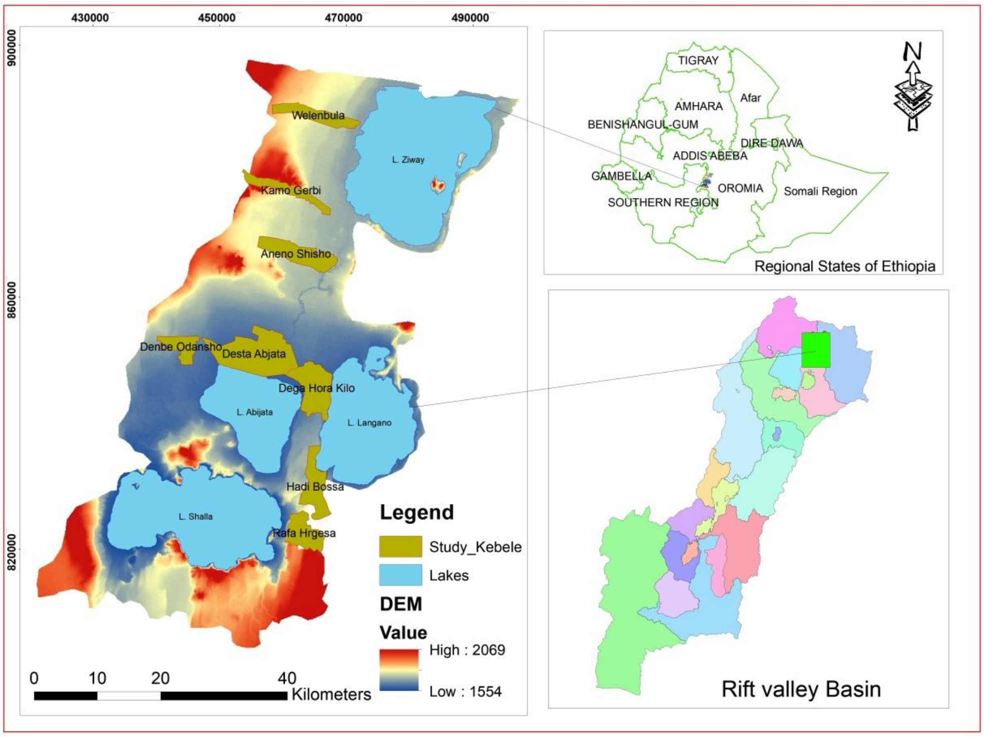

Figure 1. *Location map of the study area: The map also indicates agro ecological areas and Kebeles (Villages), the lowest administrative units in Ethiopia, selected for households' socioeconomic survey*

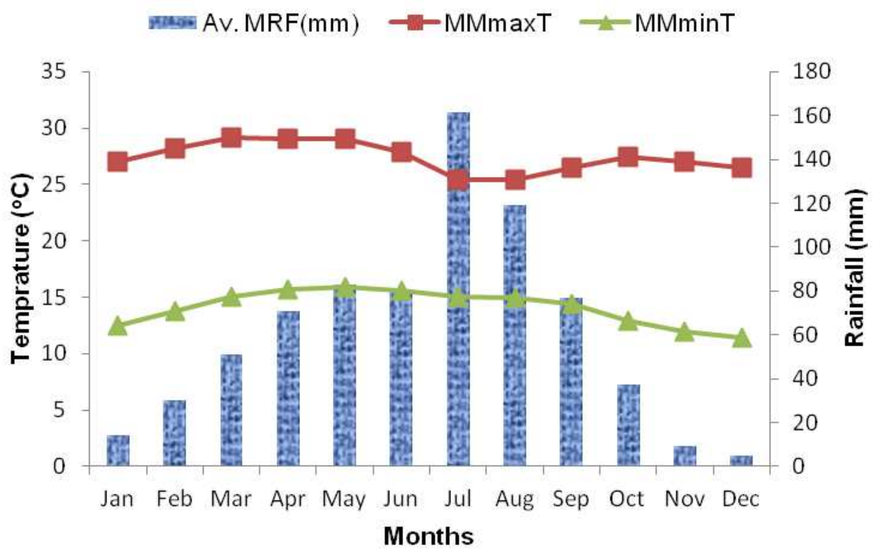

Figure 2. *Mean monthly maximum ($MM_{max}T$) and minimum ($MM_{min}T$) temperatures (°C), and average monthly rainfall (MRF) distribution (mm) (1982-2017) at Ziway Meteorological Station. (Source: NMA)*

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Soil in CRV lakes region is largely derived from recent volcanic rocks (Itanna, 2005). It is generally shallow in depth, and texture ranges between loamy sand and sandy loam that readily compacts and is susceptible to crusting, and also sensitive to drought (Biazin et al., 2011). The soil is weak in its physical structure (Abdelkadir and Yimer, 2011; Temesgen et al., 2013). Above all, the area is known for its highly fragile ecosystems subjected to high natural and anthropogenic-induced pressures. One of such human-induced pressures on environment among others in the region is the rapid changes in LULC.

Based on interpolation of 1994 and 2007 national census (CSA, 1994 and 2007), the total population for two Districts in the study area, namely Adami Tulu-Jido Kombolcha and Arsi Negele, is 155 382 in 1986, 319 484 in 2000 and 521 124 in 2016, which indicates a sharp increase in population growth in the region. According to Melka et al. (2015), more than 85% of rural farmers in the study area mainly practice mixed farming, predominantly rain-fed, while the rest practice agro-pastoral livelihood system. The major field crops are maize, wheat, ‘teff’ (*Eragrostis tef*), barley, lentil, horse bean, chickpea and field pea (Hengsdijk and Jansen, 2006), whereas acacia woodland dominates the vegetation type of the region. Due to erratic nature of rainfall and poor farming system, agricultural productivity is still low and subsistent in the region (Garedew et al., 2009). With such highly variable climate and soil moisture stress, feeding the ever-growing population becomes a major challenge to government rural poverty reduction program in the region.

Data sources

Satellite imageries and field survey are the two main data sources used for this study (*Table 1*). For the purpose of temporal LULC change detection of 1986 and 2016, cloud-free Landsat5 Thematic Mapper (TM) and Landsat8 Operational Land Imager (OLI) imageries were respectively used after downloading from online United States Geological Survey (USGS) data base archive (http://glovis.usgs.gov). Satellite imagery from the same season of the year (January to February) was used to minimize discrepancies in reflectance caused by seasonal vegetation fluxes and sun angle differences. In addition, Google earth service, topographic map and administrative map of the study area were used for boundary delineation, navigation purpose, support in ground truthing and training site establishment.

Table 1. *Data used and sources*

Data source	Path/Row	Resolution	Analysis period and date of acquisition
Landsat5 Thematic Mapper (TM)	168/055	30 m	1986 (21/01/1986)
Landsat8 Operational Land Imager (OLI)	168/055	30 m	2016(04/02/2016)
Aster Global Digital Elevation Model (ASTGM)			2016
Topographic map/sheet (EMA) Scale:1: 250000			1986
Google Earth/varying resolution			2017 (1986-2016)
Sketch map/GCPs/Ground observation/Key informants			Field work in 2017

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Field survey was carried out from January to May 2017 to collect information about households' socioeconomic background, historical LULC change, driving forces, impacts and adaptation strategies of farmers who were knowledgeable about LULC dynamics during the respective study period (1986-2016). Focus group discussions with five to ten participants per group were carried out in a total of eight Kebeles (Villages) randomly selected from two adjacent study Woredas (Districts) (five at Adami Tulu-Jido Kombolcha District and three at Arsi Negele District) (*Fig. 1*). Additional land use information also gathered from at least one elder key informant (age at least 60) from each village. Ground control points (GCP) were collected through interpretation of topographic map, Google earth, focus group discussion/key informants' interview, and visual observation carried out during field verification.

Image processing and LULC classification

Landsat images were pre-processed for geometric and atmospheric corrections using 30 m by 30 m Aster Global Digital Elevation Model (ASTGM) of the study area, and the commonly used dark subtraction technique respectively (Jensen, 1996). Each image was georectified to Universal Transverse Mercator (UTM) WGS 1984 Zone 37 North coordinates using ground control points collected during field work (Hall et al., 1991; Wijedasa et al., 2012). A first-order affine transformation and nearest-neighbour resampling method was applied for LULC classification (Jensen, 1996), resulting in a root mean square error (RMSE) below 15 m for all Landsat images. The procedure involved radiometric rectification of the 1986 image to the 2016 image, followed by a tasseled cap orthogonal transformation of the original six bands in each image in to three new dimensional spaces, corresponding to soil brightness, green vegetation and moisture indices (Hall et al., 1991). Apart from yielding relevant training data for LULC classification, the transformation also improved visual discrimination of LULC types. Ground-truth GPS points recorded in the field were used for training of the 2016 imagery, to determine the LULC classes during the image classification process, and to assess the accuracy of the classification.

LULC classification was carried out using the supervised maximum likelihood algorithm approach which is generally recognized as the popular classifier technique (Booth and Oldfield, 1989; Liu and Zhou, 2004; Verburg et al., 2004; Lillesand et al., 2014). It requires prior knowledge of the area to set training sites and use of spectral information contained in individual pixels to generate LULC classes. Accordingly, we classified the LULC categories of the study area into: dense and scattered acacia woodlands, grass/grazing land, agricultural land, shrub/bush land, water body, marsh/swampy area and bare land. The operational definition of each LULC category is given in *Table 2*.

Accuracy assessment was done using a separate set of points randomly generated using a stratified random sampling approach to determine the precision of the classified image (Jensen, 1996) which is important for post-classification change detection analysis (Liu and Zhou, 2004). The reference points were transferred to a GIS software program, in which they were overlaid with the classified images. A field check was made to test the accuracy of the reference points. The accuracy of a classification was assessed by comparing the classification with some reference data that was believed to reflect accurately the true LULC classes. The overall accuracy was measured by counting the number of pixels classified consistently in the satellite image and on the ground and dividing this by the total number of sample pixels in each class. The post-

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classification comparison (PCC) change detection technique was used for change detection analysis (Jensen, 1996). This was done by comparison of independently produced classified images, by properly coding the classification results of 1986 and 2016, from which a change map that indicates a complete matrix of change was produced (Singh, 1989). Actual change was obtained by a direct comparison between classified image from 1986 with that obtained for 2016 and results described by LULC change in percentage.

Table 2. *Operational definitions of different LULC types of CRV lakes region of Ethiopia*

LULC type	General description
Dense acacia woodland	Acacia dominated woodlands where the trees cover approximately more than 40% of the ground surface. It also includes other dense broad-leaved tress like riverine forests and plantations
Scattered acacia woodland	Acacia dominated woodlands where the trees cover approximately between 10-40% of the ground surface and the open areas under the tree canopy covered with grasses and herbs
Agricultural land	Areas used for rain-fed and irrigated cultivation (small or large scale, seasonal or perennial), including fallow plots and a complex unit, i.e. cultivated land mixed with bushes, trees and rural homesteads
Grass/grazing land	Grass being the dominant plants with the canopy of scattered acacia trees approximately covering less than 10%
Water body	Lakes, reservoirs/ponds, rivers and streams
Swampy/marshy land	Permanently waterlogged areas covered by long grasses, other aquatic plants, and mainly found near lakes and river banks
Bush/shrub land	Land covered by scattered small trees, bushes and shrubs (less than 5 m in height) and occasionally found mixed with grass
Bare land	Surface not covered by any type of vegetation, mainly including: sands, rock outcrops, cattle tracks, or exposed soils not used by any of the above LULC types

The LULC transition matrix

For the simplicity of explanation of the analysis methods used for LULC transition in this study, the general cross-tabulation matrix for comparison of two maps from two different points in time (1986 and 2016) was adopted from the initial work of Pontius et al. (2004). In a cross-tabulation matrix, the rows display the classes of 1986 and the columns display the classes of 2016. Entries on the diagonal indicate ‘persistence’, proportion of the landscape that did not undergo change during 1986-2016, and it usually dominates most landscape changes (Pontius et al., 2004). Persistence helps to compute two types of changes: gross gains and gross losses (Pontius et al., 2004). The gains are the differences between the column totals and persistence, whereas losses are the differences between row totals and persistence.

LULC persistence, net change and swap

Persistence is simply the proportion of the landscape that did not undergo change during the respective study period, represented by entries on the diagonal of a cross-tabulation matrix. Swap, as elaborated by Pontius et al. (2004), is a change attributed to location which implies simultaneous gain and loss of a land class on the landscape. Thus, the computation of swap requires the pairing of each pixel that loses with a pixel

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those gains. The amount of swap (S_j) of land class j is defined as two times the minimum of the gain and the loss (*Eq. 1*) as in Pontius et al. (2004).

For each class j in cross-tabulation matrix, the absolute value of net change during 1986-2016 is simply calculated as $|C_{+j} - C_{j+}|$. *Equation 2* defines the absolute value of the net change, denoted N_j, for class j as the maximum of the gain and loss minus the minimum of the gain and loss (Braimoh, 2006). This net change is the remaining unpaired gain or loss after all gains and losses have been paired to compute the amount of swap. *Equation 3* shows that one can express total change (T_j) for each category as either the sum of the net change and swap or the sum of the gains and losses as in Pontius et al. (2004) and Braimoh (2006).

$$S_j = 2\min\left(C_{j+} - C_{jj}, C_{+j} - C_{jj}\right) \quad \text{(Eq.1)}$$

$$N_j = \mathrm{Max}\left(C_{j+} - C_{jj}, C_{+j} - C_{jj}\right) - \mathrm{Min}\left(C_{j+} - C_{jj}, C_{+j} - C_{jj}\right) = \left|C_{+j} - C_{j+}\right| \quad \text{(Eq.2)}$$

$$T_j = D_j + S_j = \mathrm{Max}\left(C_{j+} - C_{jj}, C_{+j} - C_{jj}\right) + \mathrm{Min}\left(C_{j+} - C_{jj}, C_{+j} - C_{jj}\right) \quad \text{(Eq.3)}$$

In addition to these indices of land change, other important indicators of LULC vulnerability to transition (Ouedraogo et al., 2011; Zewdie and Csaplovics, 2016) like: gross loss to persistence ratio (l/p) which assesses the exposure of a land cover for a change, gross gain to persistence ratio (g/p) which evaluates the gain of land cover in comparison to its 1986 size, net change to persistence ratio (n/p), the tendency of land classes to undergo change, and gain to loss ratio (g/l) were analyzed based on baseline data in in cross-tabulation matrix.

Identification of dominant signals of LULC changes

Even though the analysis of persistence, swap, gross gain and gross loss is very important, still it fails to inform whether systematic transitions exist or not among the land classes, as this general analysis fails to examine the dynamics among the off-diagonal entries of cross-tabulation matrix. In other words, it fails to identify the dominant signals of land change. To relate the concept of ‘random’ and ‘systematic’ LULC transitions to land change transition matrix, it is important to define the terms in terms of statistical concept of Chi-square test as explained by Pontius et al. (2004). Statistically, a landscape transition is said to be ‘random’ if a LULC class gains from other categories in proportion to the availability of those other losing classes, or if a class loses to other class in proportion to the size of those other gaining classes (Pontius et al., 2004; Braimoh, 2006; Versace et al., 2008; Ouedraogo et al., 2011), and any large deviation from these proportions is referred to as ‘systematic’ transitions.

The identification of systematic inter-category transitions generally consists of four steps. The *first step* computes the expected gain, g_{ij} for each class under a random process of gain using the formula given in *Equation 4* (Pontius et al., 2004). *Equation 4* assumes that the gain of each class and the proportion of each class in 2016 are given a priori. The gain is then distributed in each column among the off-diagonal entries in the column according to their relative proportions in 1986. This makes the gain fairly random.

$$g_{ij} = \left(C_{+j} - C_{jj}\right)\left(\frac{C_{i+}}{100 - C_{j+}}\right), \forall i \neq j \quad \text{(Eq.4)}$$

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The *second step* computes the differences between the observed proportions and the expected proportions under a random process of gain (Pontius et al., 2004). Large positive or negative deviations from zero indicate systematic inter-category transitions rather than random transitions occurring between two LULC classes. The higher a positive difference between the observed proportion and the expected proportion under a random process of gain for the transition between class 1 and class 2, the larger the area affected by systematic gain of 2 from 1. The higher a negative difference between the observed proportion and the expected proportion under a random process of gain for the transition between class 1 and 2, the higher the tendency of class 2 to avoid gaining systematically from class 1.

The *third step* computes the expected loss, l_{ij} under a random process of loss using the formula in *Equation 5* (Pontius et al., 2004). *Equation 5* assumes the loss of each class is fixed, and distributes the loss in each row across the other classes relative to their proportions in 2016. The *fourth* and the *last step* compute the differences between the observed and the expected proportions under a random process of loss (Pontius et al., 2004). Similarly, large positive and negative deviations from zero indicate systematic inter-category transitions rather than random transitions occurring between two LULC types. In general, according to Alo and Pontius Jr (2008), to arrive at a conclusive evidence of a dominant signal of landscape transformation, class 1 must systematically gain from class 2, and class 2 must systematically lose to class 1 simultaneously.

$$l_{ij} = (C_{i+} - C_{ii})\left(\frac{C_{+j}}{100 - C_{+i}}\right), \forall i \neq j \quad \text{(Eq.5)}$$

Results

Accuracy assessment

Results of the overall classification accuracy, producer accuracy, user accuracy and kappa statistics for each LULC class, derived from the error matrix, were used to determine the degree of accuracy of the LULC classification (*Table 3*). The lowest producer accuracy of 61% and 60% were obtained for bare land and swampy/marsh land classes for 1986 and 2016 respectively. On the other hand, an overall accuracy of greater than 90% was obtained for both study periods which could be acceptable for proceeding to further data analysis in this study. The lowest producer accuracy assessment value obtained for bare land in 1986 could be due to classification error with grazing land, as both could share similar spectral signature especially in arid landscapes (Zhang et al., 2015). Similarly, it is difficult to differentiate between swampy and water body, and scattered and dense acacia woodlands in some circumstances due to absence of clear cut boundaries between these classes which could also be possible source of classification error.

Major LULC transitions

From the analysis of results, we observed an increase in human pressure and a decrease in natural and semi-natural landscapes in CRV lakes region of Ethiopia. Spatiotemporal pattern of LULC change in the study area for 1986 and 2016 is shown in *Figure 3,* while *Table 4* summarizes the 1986 and 2016 proportion, gain, loss, swap, net

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change and total change of each LULC class. The largest portion of the landscape has been covered by water body in both 1986 (31%) and 2016 (29%), whereas dense acacia woodland and swampy/marshy land covered the least percentage (<3%) during both periods.

Table 3. *Accuracy assessment results of LULC classification*

LULC type	1986		2016	
	Producer accuracy	**User accuracy**	**Producer accuracy**	**User accuracy**
Agricultural land	88.24	85.71	90.20	93.88
Scattered acacia woodland	100.00	96.36	95.65	91.67
Bare land	61.11	84.62	76.92	83.33
Bush/Shrub land	92.59	80.65	94.12	80.00
Dense acacia woodland	75.00	90.00	66.67	66.67
Grass/Grazing land	75.86	75.86	96.77	92.31
Swampy/Marshy land	90.91	90.91	60.00	85.71
Water body	100.00	100.00	97.40	98.68
Overall accuracy	90.63		92.58	
Kappa Statistics	0.89		0.91	

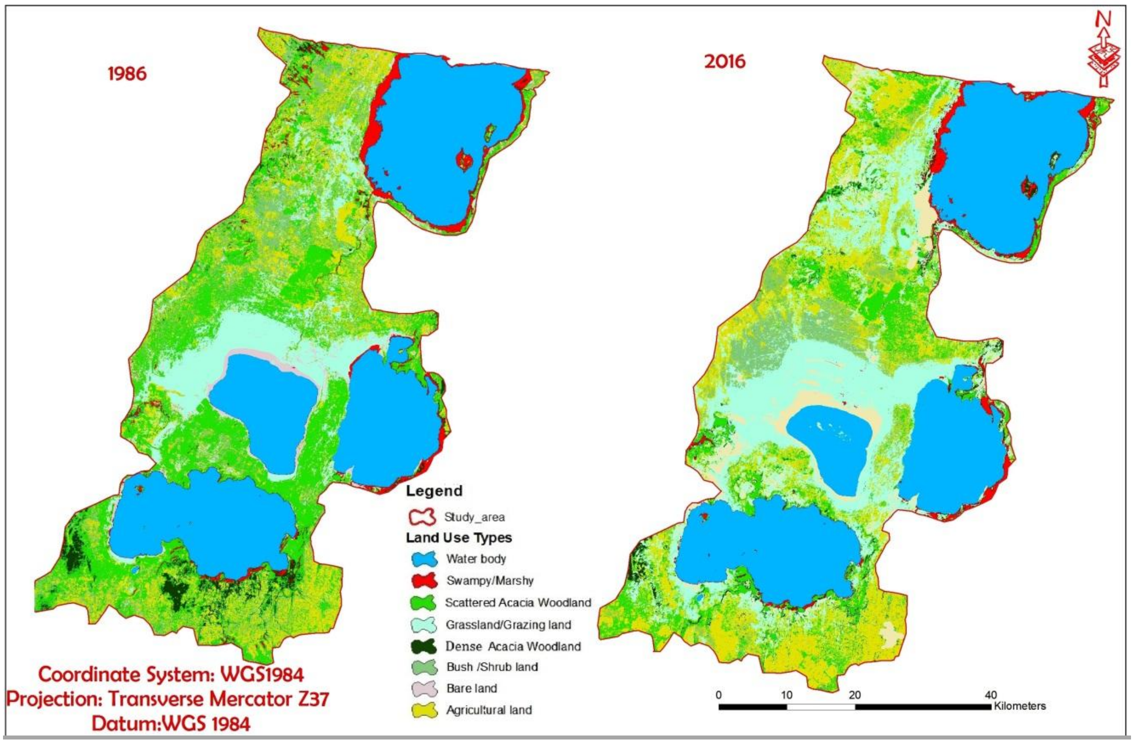

Figure 3. *LULC maps for the years 1986 and 2016*

Over the last three decades, grass/grazing land, agricultural land and bare land have increased by 124%, 42% and 34% respectively, whereas scattered acacia woodland, bush/shrub land and swampy/marshy land have declined by 52%, 50% and 31% respectively (*Table 4*). Scattered acacia woodland, which was the second in terms of

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proportion in 1986 (23%), declined by about half percentage in 2016 (11%). Due to this, scattered acacia woodland experienced the highest area loss (18%) followed by bush/shrub land (11%) during the same period. On the other hand, grazing land, which was only 11% in 1986, has shown an increment and took the second position in terms of area percentage proportion in 2016 (25%). Hence, grazing land experienced the highest gain in over 17% of the landscape followed by agricultural land (14%) during the study period. The gain-to-loss ratio was also highest (5.2) for grazing land, indicating that grazing land experienced five times more gain than loss. In general, total change occurred in about 48% of the landscape during the study period while the rest, 52%, persisted (*Table 4*).

Table 4. *Landscape changes (%)*

LULC types	Total 1986	Total 2016	Persistence	Gain	Loss	Total change	Swap	Absolute value of net change
Agricultural land	13.94	19.85	6.15	13.70	7.79	21.50	15.59	5.91
Scattered acacia woodland	22.87	11.08	5.15	5.93	17.72	23.65	11.86	11.79
Bare land	3.79	5.08	0.59	4.50	3.21	7.71	6.41	1.29
Bush/Shrub land	11.90	5.92	1.20	4.72	10.70	15.42	9.43	5.98
Dense acacia woodland	2.48	2.23	0.50	1.73	1.97	3.70	3.45	0.25
Grass /Grazing land	11.06	24.81	7.76	17.05	3.30	20.35	6.60	13.76
Swampy/Marshy land	2.99	2.07	1.44	0.62	1.54	2.17	1.24	0.92
Water body	30.97	28.96	28.80	0.16	2.17	2.34	0.32	2.01
Total	100.00	100.00	51.59	48.41	48.41	48.41	27.45	20.96

Net change and swap changes

Results showed both swap changes and net changes occurred for most of LULC categories in the study area. The summary for changes attributed to net change and swap is given in *Table 4*. Changes in all land categories except for water body consists both swap and net changes, whereas change in dense acacia woodland is nearly a pure swap type. This is to mean that for dense acacia woodland the net change is zero, and hence each cell gained can be paired with each cell lost. The change attributed to quantitative net change is highest for grazing land (about 68% of total change for grazing land); followed by scattered acacia woodland (about 50% of its total change). Net change overall accounted for 43% of total change on the landscape. On the other hand, the change attributed to location (swap) is highest for agricultural land (72% of total change for its whole area), followed by scattered acacia woodland (50% of its total change). Swap of land change dynamics overall accounted for 57% of total landscape change in the study area.

Persistence of LULC classes

We observed all LULC categories showed relative persistence during the study period which is in consistent with the usual trend in most LULC change studies. About

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52% of landscape remained unchanged during the study period (*Fig. 4* and *Table 4*). The proportion of different LULC classes that were unchanged between 1986 and 2016 are also shown in the diagonal cells of *Table 5*. Water body experienced the highest resistance to change during the study period. About 93% of landscape occupied by water body in 1986 remained unchanged in 2016.

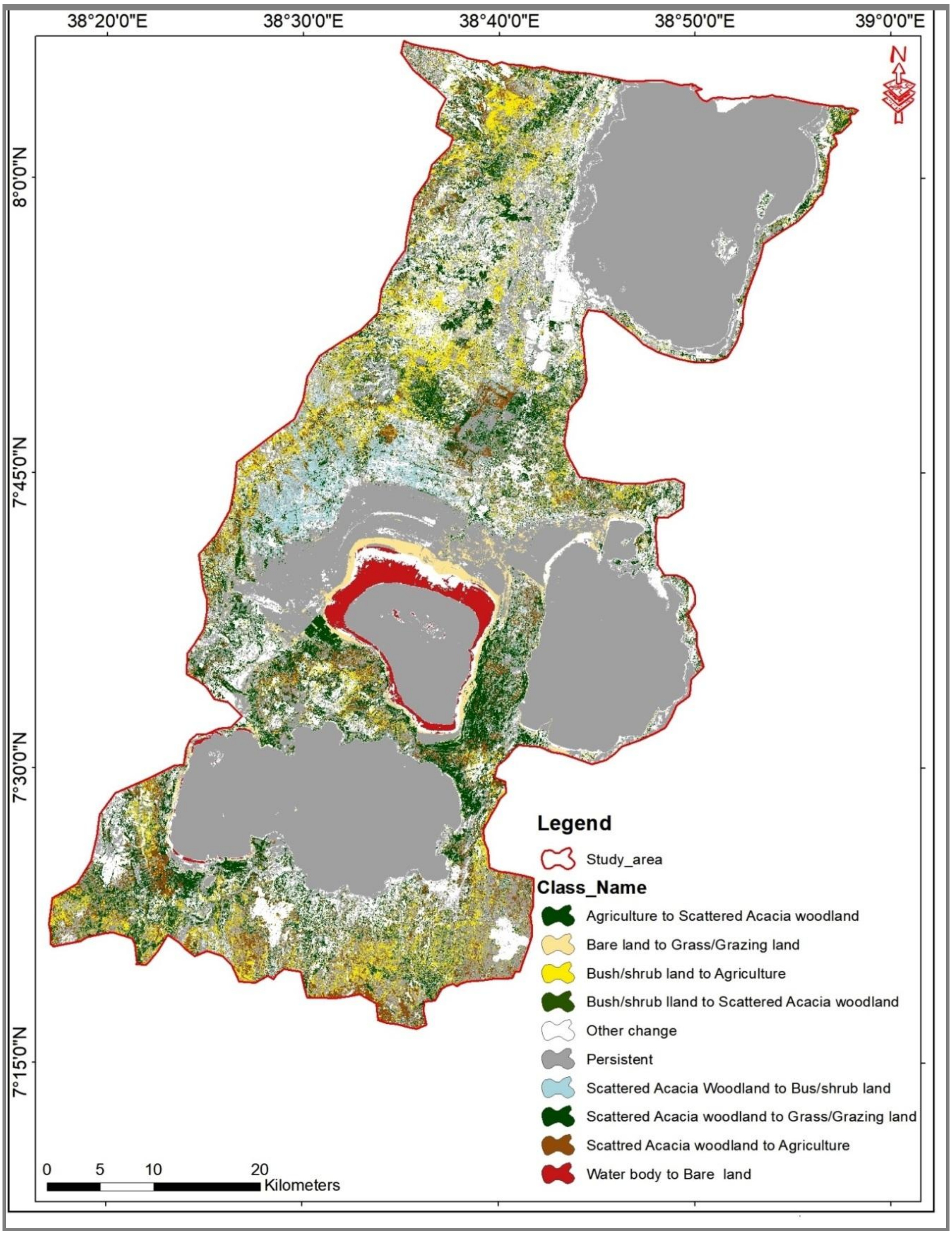

Figure 4. *Map for major LULC transitions and persistence (1986-2016)*

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Table 5. *LULC transition matrix for 1986-2016 (%)*

1986	2016									
	AL	SA	BL	BS	DA	GL	SM	WB	Total 1986	Loss
Agricultural land (AL)	6.15	2.42	0.62	1.20	0.17	3.35	0.03	0.00	13.94	7.79
Scattered acacia woodland (SA)	6.26	5.15	0.97	2.55	1.06	6.69	0.20	0.00	22.87	17.72
Bare land (BL)	0.56	0.28	0.59	0.08	0.04	2.21	0.04	0.00	3.79	3.21
Bush/Shrub land (BS)	5.05	1.97	0.58	1.20	0.13	2.94	0.03	0.00	11.90	10.70
Dense acacia woodland (DA)	0.63	0.55	0.06	0.05	0.50	0.56	0.12	0.00	2.48	1.97
Grass/Grazing land (GL)	1.03	0.43	0.96	0.82	0.04	7.76	0.02	0.00	11.06	3.30
Swampy/Marshy land (SM)	0.17	0.26	0.09	0.01	0.28	0.57	1.44	0.16	2.99	1.54
Water body (WB)	0.00	0.01	1.22	0.00	0.02	0.74	0.18	28.80	30.97	2.17
Total 2016	19.85	11.08	5.08	5.92	2.23	24.81	2.07	28.96	100.00	48.41
Gain	13.70	5.93	4.50	4.72	1.73	17.05	0.62	0.16	48.41	

Bush/shrub land on the other hand showed the lowest persistence in 1.20% of the landscape. As shown in *Table 6,* the loss-to-persistence ratio (l/p) for all classes is greater than 1 except for grazing land and water body. This indicates all land classes except grazing land and water body showed a higher tendency to lose than to persist. The highest (8.88) and lowest (0.08) l/p ratio was obtained for bush/shrub land and water body classes respectively. On the other hand, the gain-to-persistence ratio (g/p) is greater than 1 for all classes except aquatic habitats (*Table 6*), indicating that these land classes experienced more gain than persistence. Bare land experienced the highest g/p ratio (7.68), whereas water body and swampy/marshy lands experienced the least values.

Table 6. *Gain to persistence (g/p), loss to persistence (l/p), net change to persistence (n/p) and gain to loss (g/l) ratios of LULC classes*

LUC class	Gain to persistence (g/p)	Loss to persistence (l/p)	Net change to persistence (n/p)	Gain to loss (g/l)
Agricultural land	2.23	1.27	0.96	1.76
Scattered acacia woodland	1.15	3.44	-2.29	0.33
Bare land	7.68	5.47	2.20	1.40
Bush/Shrub land	3.92	8.88	-4.97	0.44
Dense acacia woodland	3.43	3.92	-0.49	0.87
Grass /Grazing land	2.20	0.43	1.77	5.17
Swampy/Marshy land	0.43	1.07	-0.64	0.40
Water body	0.01	0.08	-0.07	0.07

Systematic and random LULC transitions

Results have clearly showed that systematic as well as random LULC transitions occurred in the study area during 1986 to 2016. In *Table 7a,* the off-diagonal numbers

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represent expected values of the gain in LULC categories at the given persistence under a random process of change. The difference between the observed proportions (*Table 5*) and the expected proportions (*Table 7a*) is given in *Table 7b*. The observed value for gain is greater than the corresponding expected value for all land classes except for swampy and water body (*Fig. 5*). Relatively large positive values in *Table 7b* indicates systematic gain transitions. Hence, agricultural land systematically gained from both bush land (3.16%) and scattered acacia wood land (2.62%) transitions. Similarly scattered acacia woodland gained systematically from both agricultural land (1.35%) and bush land (1.05%) transitions. In addition, grazing land systematically gained from both scattered acacia woodland (2.30%) and bare land (1.48%). Bush land also systematically gained from scattered acacia wood land (1.33%). On the other hand, grazing land, agricultural land, scattered acacia woodland and bush land, all systematically avoided gaining from water bodies (indicated by large negative values in *Table 7b*), which also mean that water body avoided systematically losing to these LULC categories.

Table 7. *LULC transition matrix for inter-class gains*

1986	2016									
	AL	**SA**	**BL**	**BS**	**DA**	**GL**	**SM**	**WB**	**Total 1986**	**Loss**
(a) Expected gains under a random process of gain (%)										
Agricultural land (AL)	6.15	1.07	0.65	0.75	0.25	2.67	0.09	0.03	11.66	5.51
Scattered acacia woodland (SA)	3.64	5.15	1.07	1.22	0.40	4.39	0.15	0.05	16.07	10.93
Bare land (BL)	0.60	0.29	0.59	0.20	0.07	0.73	0.02	0.01	2.51	1.93
Bush/Shrub land (BS)	1.90	0.91	0.56	1.20	0.21	2.28	0.08	0.03	7.17	5.96
Dense acacia woodland (DA)	0.39	0.19	0.12	0.13	0.50	0.47	0.02	0.01	1.83	1.33
Grass/Grazing land (GL)	1.76	0.85	0.52	0.59	0.20	7.76	0.07	0.03	11.77	4.01
Swampy/Marshy land (SM)	0.48	0.23	0.14	0.16	0.05	0.57	1.44	0.01	3.08	1.64
Water body (WB)	4.93	2.38	1.45	1.66	0.55	5.94	0.20	28.80	45.90	17.10
Total 2016	19.85	11.08	5.08	5.92	2.23	24.81	2.07	28.96	100.00	48.41
Gain	13.70	5.93	4.50	4.72	1.73	17.05	0.62	0.16	48.41	
(b) Differences between observed landscape transitions and the expected gains (%)										
Agricultural land (AL)	0.00	1.35	-0.03	0.45	-0.08	0.67	-0.06	-0.03	2.28	2.28
Scattered acacia woodland (SA)	2.62	0.00	-0.10	1.33	0.65	2.30	0.05	-0.05	6.80	6.80
Bare land (BL)	-0.05	-0.01	0.00	-0.12	-0.03	1.48	0.01	-0.01	1.28	1.28
Bush/Shrub land (BS)	3.16	1.05	0.02	0.00	-0.08	0.66	-0.04	-0.03	4.73	4.73
Dense acacia woodland (DA)	0.23	0.36	-0.06	-0.08	0.00	0.08	0.11	-0.01	0.64	0.64
Grass/Grazing land (GL)	-0.73	-0.42	0.44	0.23	-0.16	0.00	-0.05	-0.02	-0.71	-0.71
Swampy/Marshy land (SM)	-0.30	0.03	-0.05	-0.15	0.23	0.00	0.00	0.15	-0.09	-0.09
Water body (WB)	-4.93	-2.37	-0.23	-1.66	-0.53	-5.19	-0.02	0.00	-14.93	-14.93
Total 2016	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gain	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	

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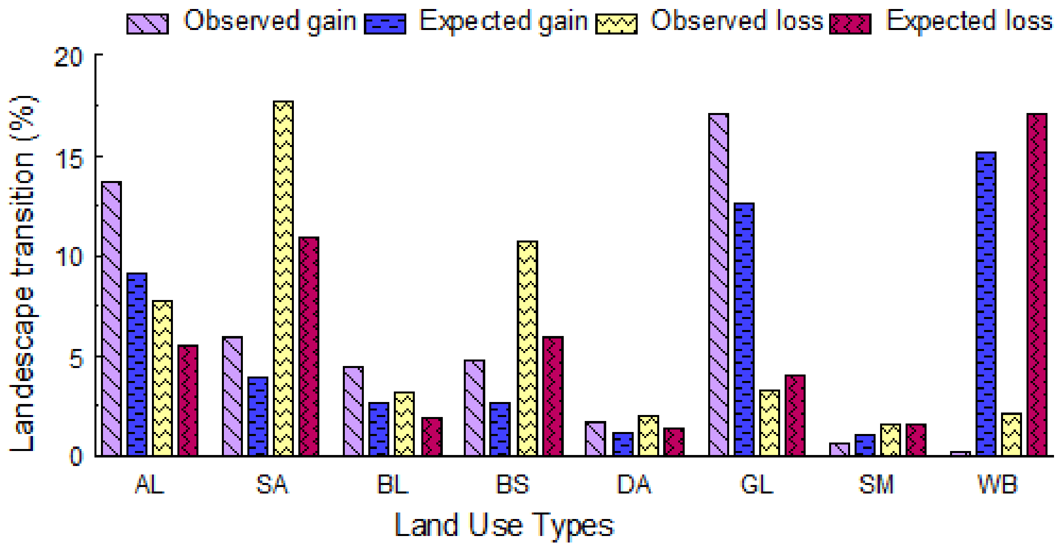

Figure 5. *Observed and expected LULC gross gains and losses for 1986-2016 (%): AL = agricultural land, SA = scattered acacia woodland, BL = bare land, BS = bush/shrub land, DA = dense acacia woodland, GL = grass/grazing land, SM = swampy/marshy land, and WB = water body*

The values for expected losses under a random process of loss are given in *Table 8a,* whereas the differences between the observed and expected losses are given in *Table 8b*. Relatively large positive values (>0) in *Table 8b* indicate systematic loss transitions. Accordingly, scattered acacia woodland systematically loses to agricultural land (**2.31**%), grazing land (**1.74**%) and bush land classes (**1.37**%). Similarly bush land systematically loses to agricultural land (**2.80**%), agricultural land systematically loses to scattered acacia woodland (**1.35**%), bare land systematically loses to grazing land (**1.37**%) and water body systematically loses to bare land (**1.07**%). On the other hand, scattered acacia woodland, bush land, agricultural land and grazing land systematically avoided losing to water bodies which is indicated by relatively large and negative values in *Table 8b*.

In general, major interclass systematic gains and losses observed for this study can be summarized as follows (*Fig. 4* and *Table 5*). About 6% of scattered acacia woodland and 5% of bush land has been converted to agricultural land, whereas about 7% and 3% of scattered acacia woodland has been respectively degraded and shifted to grazing land and bush/shrub lands during the study period. On the other hand, there has been an increase in vegetation biomass from agricultural land and bush land to scattered acacia woodland in over 2% of the landscape from each class. Similarly, there has been a vegetation restoration from bare land to grass land via ecological succession on lake retreat areas in over 2% of the landscape during the study period.

Discussions

Our result indicated that there has been an increased pressure from anthropogenic-induced LULC dynamics in the study region during the last three decades. This is evidenced by substantial gains/losses observed for major LULC categories during the

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study period (*Table 4*). For instance, expansion in agricultural land, grazing land and bare land by 124%, 42% and 34% respectively are among the changes attributed to gain, whereas decline in acacia woodlands and bush/shrub lands by 52% and 50% respectively are the major changes attributed to loss. Gain in grazing land is mainly due to land transition from lake retreat bare lands, particularly of Lake Abijata, that gradually shifts to grass/grazing land through ecological succession (Biazin and Sterk, 2013; Temesgen et al., 2013). With current rate of retreat, studies predicted that Lake Abijata will totally dry within coming 50 years (Temesgen et al., 2013). The other probable source for gain in grazing land is that in most cases acacia woodlands have been subjected to free access of firewood extraction and charcoal making by the community in rural Ethiopia, which gradually degrade the trees and shifts the land to either bush/shrub lands or open grazing areas. Coupled with natural pressures (frequent draught and fragile ecosystems), overgrazing is one basic proximate forcing factor behind high land degradation (critical water and wind erosion) observed during field work in CRV lakes region.

Table 8. *LULC transition matrix for inter-class losses*

1986	2016									
	AL	**SA**	**BL**	**BS**	**DA**	**GL**	**SM**	**WB**	**Total 1986**	**Loss**
(a) Expected losses under a random process of loss (%)										
Agricultural land (AL)	6.15	1.08	0.49	0.58	0.22	2.41	0.20	2.82	13.94	7.79
Scattered acacia woodland (SA)	3.96	5.15	1.01	1.18	0.44	4.95	0.41	5.77	22.87	17.72
Bare land (BL)	0.67	0.37	0.59	0.20	0.08	0.84	0.07	0.98	3.79	3.21
Bush/Shrub land (BS)	2.26	1.26	0.58	1.20	0.25	2.82	0.23	3.29	11.90	10.70
Dense acacia woodland (DA)	0.40	0.22	0.10	0.12	0.50	0.50	0.04	0.58	2.48	1.97
Grass /Grazing land (GL)	0.87	0.49	0.22	0.26	0.10	7.76	0.09	1.27	11.06	3.30
Swampy/Marshy land (SM)	0.31	0.17	0.08	0.09	0.04	0.39	1.44	0.46	2.99	1.54
Water body (WB)	0.61	0.34	0.16	0.18	0.07	0.76	0.06	28.80	30.97	2.17
Total 2016	15.22	9.08	3.23	3.81	1.69	20.43	2.56	43.97	100.00	48.41
Gain	9.08	3.93	2.65	2.61	1.19	12.67	1.11	15.17	48.41	
(b) Differences between observed landscape transitions and the expected losses (%)										
Agricultural land (AL)	0.00	1.35	0.13	0.62	-0.05	0.93	-0.17	-2.82	0.00	0.00
Scattered acacia woodland (SA)	2.31	0.00	-0.05	1.37	0.61	1.74	-0.21	-5.77	0.00	0.00
Bare land (BL)	-0.11	-0.09	0.00	-0.12	-0.03	1.37	-0.03	-0.98	0.00	0.00
Bush/Shrub land (BS)	2.80	0.71	0.00	0.00	-0.13	0.12	-0.20	-3.29	0.00	0.00
Dense acacia woodland (DA)	0.23	0.33	-0.04	-0.07	0.00	0.06	0.08	-0.58	0.00	0.00
Grass land/Grazing land (GL)	0.16	-0.05	0.74	0.56	-0.06	0.00	-0.07	-1.27	0.00	0.00
Swampy/Marshy land (SM)	-0.14	0.08	0.01	-0.09	0.25	0.18	0.00	-0.30	0.00	0.00
Water body (WB)	-0.60	-0.33	1.07	-0.18	-0.05	-0.01	0.12	0.00	0.00	0.00
Total 2016	4.63	1.99	1.85	2.11	0.54	4.39	-0.49	-15.01	0.00	0.00
Gain	4.63	1.99	1.85	2.11	0.54	4.39	-0.49	-15.01	0.00	

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Agricultural land expansion in the region on the other hand is mainly in expense of losses in acacia woodlands and bush/shrub lands. Gain in agricultural land is associated with continual expansion of agricultural frontier as population increase (Garedew et al., 2009; Ariti et al., 2015), as population growth is the main underlying driver behind LULC change in developing countries like sub-Saharan Africa (Braimoh, 2006; Ouedraogo et al., 2011). This indicates agricultural land, bush/shrub land as well as grazing land targeted mainly the peculiar acacia woodland vegetation of the region for their gains. The result is in consistent with the results of previous LULC change studies in the region (Biazin and Sterk, 2013; Temesgen et al., 2013; Ariti et al., 2015) who reported agricultural land expansion as root cause for woodland vegetation decline in the region. Study by Zewdie and Csaplovics (2016) in dry lands of north western Ethiopia, an area with comparable agro ecological condition, also reported similar results. However, study by Braimoh (2006) and Ouedraogo et al. (2011) in western Sub-Saharan Africa reported decline in closed woodland mainly due to logging activities than due to crop land expansion. These studies also reported the systematic conversion of grazing land to crop land which is different from the case of our study. The loss in agricultural land observed for this study might be due to fallowing and abandonment of highly degraded farm lands as confirmed during field verification.

The other principal LULC change observed for this study is the substantial loss in acacia woodlands and bush/shrub land of the region (*Table 4*). Proximate causes for loss in scattered acacia woodland and bush/shrub land is most likely deforestation for firewood, charcoal making, and cultivation land expansion, owing to population pressure, and partly due to recent agricultural investment expansion in the region (Biazin and Sterk, 2013; Temesgen et al., 2013). The government of Ethiopia targeted the CRV lakes region as one potential agricultural investment zone due to its accessible lake water for irrigation and the proximity to Addis Ababa for ease of agricultural products export. Overgrazing pressure from pastoralist herds seasonally coming from neighbouring Districts in search of pasture grass is also another main factor for degradation of both woodlands and bush/shrub lands. Slight gain observed in scattered acacia woodland on the other hand could be associated with either degradation of dense acacia woodlands or government rehabilitation initiatives of degraded lands from either abandon agricultural lands or bush/shrub lands through area closure in recent years. This indicates part of the degraded and abandoned agricultural lands could shift to scattered acacia woodlands, following regeneration of remnant acacia trees after rehabilitation treatment like area closure. Currently the government has given strong attention to implement degraded land rehabilitation program, mainly through social mobilization, throughout the country though such practices are criticized for effectiveness and sustainability in most cases.

Analysis for changes attributed to persistence, net change and swap was another objective for this study. Results indicated that changes at landscape level are more attributed to swap (57% of total change) than to net change (43% of total change). However, the majority of LULC classes showed the tendency to persist (52%) than to change during the same period (*Table 4* and *Fig. 4*). Persistence is the resistance of the landscape to resist change and it usually dominates most landscape changes (Pontius et al., 2004). In line to this fact, our result also showed that the majority of the landscape area persisted than to change. It is normal to see more persistence than change in LULC change analysis (Pontius et al., 2004). Our result also showed that the overall percent

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change attributed to location (swap) is greater than the change attributed to net change at landscape level. This indicates the importance of analyzing swap beyond net change analysis in order to see the changes attributed to location which otherwise overlooked by 'net change' analysis alone (Pontius et al., 2004; Braimoh, 2006).

Finally, our result also identified systematic and random LULC changes occurred during the study period by comparing the observed change to the expected change arising from chance for any given degree of persistence (*Table 7b* and *8b*). Accordingly, the gain in agricultural land from scattered acacia wood land and bush/shrub land is systematic. This implies when agricultural land gains, new agricultural land tends to gain systematically from these classes (Braimoh, 2006). Similarly, scattered acacia woodland systematically gained from agricultural lands and bush/shrub lands; and both bush/shrub land and grazing lands systematically gained from scattered acacia woodland. This implies there is a systematic exchange of land portion among some LULC classes. On the other hand, the scattered acacia woodland systematically losing to agricultural land, bush/shrub land and grazing lands, whereas by reverse, agricultural land systematically losing to scattered acacia woodlands. In addition, bush/shrub lands systematically losing to agricultural land, and water body systematically losing to bare land. All the rest gain/loss observed in this study is depicted as random changes. However, it should be noted that a change is random does not necessarily downplay its worth as factors for random change could also lead to systematic change and vice versa (Braimoh, 2006). *Figure 5* shows that the observed value for gain/loss is greater than the corresponding expected value for all land classes, except for swampy/marshy and water body. According to Braimoh (2006), the expected gain or loss could be different from the observed gain or loss due to various reasons: like changes in spatial determinants of land use distribution, such as population growth, soil suitability and the opening up of new roads, or it could be due to competition between LULC types.

This study might have limitations, for instance by using Landsat data, changes that are below pixel size might have been missed. Hence, in order to generate more information from the method, first, it is important to use algorithms that reduce map errors (Verburg et al., 2004; Alba, 2014; Agarwal et al., 2002) as accuracy assessment alone may not warrant for absence of map errors. For example, it is recommended to use other modeling algorithms like agent based modeling and spatial land change models for better results. Second, it might be important performing the method at multiple map resolutions in order to measure the sensitivity of the results to changes in map scale (Pontius et al., 2004). In addition, the similarity in spectral signature of some LULC classes could be another source of classification error in this study.

Conclusions

This study followed an approach that moves from broad to more detailed level of analysis and tried to highlight the importance of an in depth analysis of systematic and random LULC transition beyond the conventional net change analysis. It also indicated the importance of focusing on most prevalent systematic process of land change. Hence, future studies in the fields of geography and landscape ecology need to incorporate this method since existing popular methods fail to segregate LULC change according to its different components and thus fail to gain maximum insight in to the processes driving these changes.

Our result clearly shown that, there have been active LULC dynamics in CRV lakes region of Ethiopia for the period of 1986 to 2016. From the concrete data of systematic gains and losses, we conclude that there have been most dominant and prominent signals of change in terms of conversion, degradation and restoration of LULC in CRV lakes region of Ethiopia. The majority of land conversion as well as degradation signals targeted the acacia woodlands of the region. To arrive at a conclusive evidence of a dominant signal of landscape transformation, class 1 must systematically gain from class 2, and class 2 must systematically lose to class 1 simultaneously. Accordingly, the conversion of both scattered acacia woodlands and bush/shrub lands to agricultural land; the degradation of scattered acacia woodlands towards bush/shrub lands and grass/grazing lands; and the restoration in vegetation biomass from agricultural land and bare land towards scattered acacia woodland and grazing land respectively are found to be clearly systematic, and hence indicates the dominant signals of landscape transformation in this study. All the rest transitions are found to be either random or not fully systematic.

The result of this study implicates the continued land degradation in developing countries, particularly East Africa, where the livelihood of the majority of population is directly dependent on natural resources. The loss in acacia woodlands and bush/shrub lands either by conversion or through degradation, calls for means to reduce such adverse pressures. The decline in water bodies and shift to bare land, particularly of Lake Abijata, requires urgent watershed management measures on its catchment area. Increased land use intensity due to increased population pressure calls for agricultural intensification related policies to prevent crop land expansion on fragile lands. The pressure on woodlands from firewood extraction and charcoal making requires availing affordable alternative energy source technologies to poor households. The restoration in vegetation biomass requires further study to know where and how it occurs, how long it takes and factors underlying it. Future studies also need to investigate the local and national level drivers behind; and the impacts imposed by such continued LULC changes to rural livelihood, natural ecosystem and biodiversity in the region. In general, future land use policies need to consider such prominent signals of land change in order to plan an integrated approach to safeguard the fragile acacia woodland ecosystems while searching for alternative livelihood options to feed the ever growing population in the region.

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HABITAT PREFERENCES OF *OXYCOCCUS PALUSTRIS* PERS. ON PEATLANDS IN EAST POLAND IN THE PERSPECTIVE OF SHAPING THE CONDITIONS OF ECOLOGICAL CULTIVATION OF THE SPECIES

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Abstract. The objective of the paper was the determination of values of selected habitat conditions optimal for *Oxycoccus palustris Pers.* on peatlands in the central part of East Poland in the aspect of provision of proper conditions in ecological cultivations of the plant. The obtained results showed a broad range of ecological tolerance towards the analysed physical-chemical properties of the habitat, however not the exceeding values typical of the occupied habitat, as evidenced by the relatively common occurrence of the species. Statistical analyses of the study results showed that important factors causing variability in the abundance and therefore condition of the population of the species at particular study sites include: P_{total}, $P\text{-}PO_4$, and DOC (dissolved organic carbon). Ranges of values of the factors optimal for the functioning of the *O. palustris* population on the studied habitats were specified (P_{total} = 0.17 - 0.36; P-PO4 = 0.1 and DOC = 33.81 – 55.90 [mg dm^{-3}]).
Keywords: *small cranberry, herbs, physical-chemical properties, phytotherapy*

Introduction

The medicinal effect of active natural substances of plants on the patient's organism and the complex provision of easily available biofactors (e.g. vitamins, enzymes, or micro- and macroelements) in phytotherapy constitutes a desirable alternative for synthetic pharmacology, related to unhealthy side effects and dangerous polypharmacy (Nowiński, 1983; Brinkmann et al., 2007; Buettner et al., 2009; Drozd, 2012; Oliveira et al., 2012; Skotnicka, 2013; Senderski, 2015).

One of the most valued herbal plants obtained from nature and to a lower degree from cultivation is small cranberry (*Oxycoccus palustris* Pers.). In morphological terms, it is a shrub from the ericaceous family (*Ericaceae*) with delicate, creeping shoots reaching a height of 100 cm. It has small, leathery, evergreen leaves with elliptical shape. The flowers usually grow by 3-4 on long stalks. They are pink with petals folded downwards. The fruit is a spherical, multi-seed, juicy, red berry. It blooms in the moderate climate from June to August. The fruit matures in September, remaining on the shoot until early spring. It occurs in the countries of North and Central Europe, North Asia, and North America, and in Poland on wetlands related to ombrogenic peatlands (Jaquemart, 1997; Arnal et al., 2008; Senderski, 2015).

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In terms of life strategy, *Oxycoccus palustris* is a perennial chamephyte preferring sites on organogenic soils characteristic of raised or transitional bogs, oligotrophic, with a lower reaction and parameters of circumboreal climate, moisturised, with moderate light conditions (Jaquemart, 1997; Zarzycki and Korzeniak, 2002; Senderski, 2015).

The herbal resource is the cranberry fruit containing many bioactive substances, i.e. acids: benzoic, citric, malic, gallic, quinic, ascorbic; anthocyanins, carotenoids, flavonoids, pectins, tannins, carbohydrates, mineral substances, including iodine salts, and leaves containing glycoside arbutin (Česonienė et al., 2006; Adamczak et al., 2009; Lyutikova and Turov, 2011; Senderski, 2015). Extracts from cranberry fruit are applied as a refreshing and immunosuppressive agent, and as auxiliary medicine in urinary infections. The leaves are used as astringent and disinfectant, and to stop bleeding (Jepson and Craig, 2008; Ruzik-Kuklińska, 2010; Senderski, 2015).

Herbal plants at natural sites of occurrence, in the conditions of low human pressure, show a natural content and proportion of biologically active components translating into high quality herbal resources (Lyutikova and Turov, 2011). Medicinal products manufactured in accordance with the rules of Good Manufacturing Practice (GMP) from such plants meet the optimum assumptions of the natural herbal medicine pursuant to the guidelines of WHO (Borkowski, 1994; Drozd, 2012). Due to the above, an alternative worth considering can be ecological cultivations conducted under conditions adjusted to the natural preferences of plants.

The objective of this paper was the determination of optimal values of selected habitat conditions important for the proper functioning of *Oxycoccus palustris* in the peatlands of the central part of East Poland in the aspect of provision of proper conditions in ecological cultivations of the plant. The lack of available source texts regarding the qualitative analysis of the habitat factors of cranberry at natural sites of its occurrence is an additional argument for undertaking this research.

Material and methods

Fieldwork

The research was conducted in the area of peatlands of the central part of East Poland included in the Łęczna-Włodawa Lakeland (Polesie Podleskie). After preliminary reconnaissance, six representative sites were selected with plant species composition characteristic of the analysed region, where the presence of the population of *O. palustris* was observed within or near the Poleski National Park: lake-peatland complexes: Lake Bikcze (B), Lake Karaśne (K), Lake Długie (D), Lake Moszne (M), forest peatland Blizionki (BZ) and Dekowina (DK) – *Figure 1*.

Research areas (plots) of 100 m^2 (10 m × 10 m) were designated at study sites. Their phytocoenoses were characterised by variable contribution of *O. palustris*. In the vegetation season 2011, to confirm the correctness of the selection of study sites, the species composition of phytocoenoses of the plots was determined with consideration of percent contribution of particular species of vascular plants.

Piezometers were installed in the central part of the sites (perforated PVC pipe with a length of 1 m and diameter of 10 cm, dug into the ground) for sampling peatland water. Each year (in the years 2011-2013) in the vegetative season (from March to April), water was sampled from the piezometers seven times, and transported to the laboratory. Water reaction and electrolytic conductivity were also measured *in situ* by means of portable multiparameter meters.

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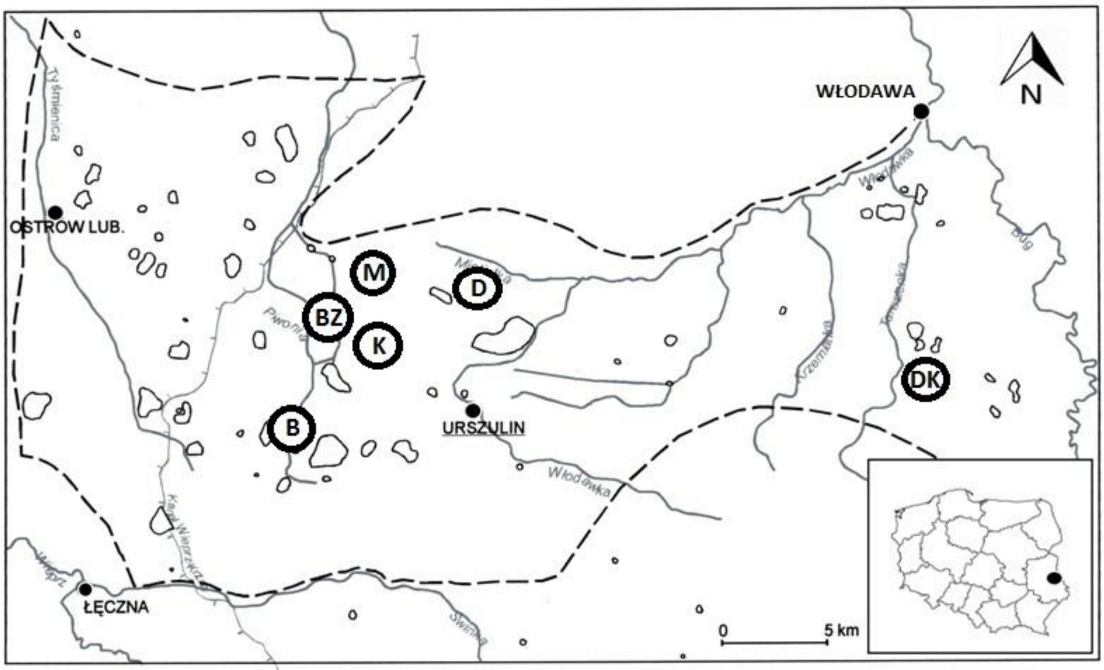

Figure 1. *Location of the study sites in the Polesie Podlaskie Region in Poland (Serafin et al., 2017). (B- Bikcze, M- Moszne, K- Karaśne, D- Długie, BZ- Blizionki, DK- Dekowina)*

Laboratory analysis

Laboratory analyses of the sampled piezometric waters were performed at the Central Agroecological Laboratory of the University of Life Sciences in Lublin (CLA) by means of certified methods. They covered 14 physical-chemical properties of peatland waters: reaction (pH), electrolytic conductivity (CON), dissolved organic carbon content (DOC), content of nitrogen fractions: total nitrogen (N_{total}), ammonia nitrogen ($N\text{-}NH_4$), nitrates ($N\text{-}NO_3$), nitrites ($N\text{-}NO_2$), and content of phosphorus fractions: total phosphorus (P_{total}), phosphates ($P\text{-}PO_4$), and concentration of sulphates ($S\text{-}SO_4$) and basic cations: potassium (K), sodium (Na), calcium (Ca), and magnesium (Mg).

Data analysis

The coefficient of species similarity of the phytocoenoses of plots was calculated by means of the Jaccard's method. It provided the basis for the classification analysis as the distance measure with the application of the classification algorithm of minimum variance (Ward clustering) for the species composition, and algorithm of complete linkage for habitats.

For the purpose of comparison of distributions of the values of the analysed properties in particular habitats, due to the lack of normality of their distribution and non-uniformity of variance within data sets, a non-parametric Kruskal-Wallis test was applied.

The ranges of values of selected environmental factors with statistical testing are presented graphically in the form of box-plots presenting particular properties of empirical distributions for two groups of study sites: B+BZ (sites with a low %

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contribution of occurrence of *O. palustris*) and M+K+D+DK (sites with a high % contribution of occurrence of *O. palustris*).

All the obtained values were analysed by means of direct ordinance methods. Based on the obtained value of gradient length (1.56), redundancy analysis (RDA) was applied. Selection of environmental variables was performed, adopting the absolute value of the correlation coefficient > 0.6 as the rejection criterion (Zuur et al., 2007).

All statistical analyses were performed using packages: vegan (version 2.4-2), nortest, MASS and ggdendro in R environmental (R Core Team, 2015; Oksanen et al., 2016).

Results and discussion

The specificity of habitat conditions of the peatlands of Polesie Podlaskie in the central part of East Poland, involving specific microclimate, rich water relations, and relatively low human pressure, determines the possibilities of occurrence of many species of environmentally valuable plants that are also useful for man, including herbs.

Oxycoccus palustris is a native species of utility peatland plants occurring quite commonly in the Łęczna-Włodawa Lakeland, as confirmed by field reconnaissance research performed in the years 2011-2013.

Other species of cranberry, i.e. *Oxycoccus microcarpus* and *O. macrocarpus*, are domesticated anthropophytes. They are slightly less common in nature, but more frequently cultivated (Stobnicka and Gniewosz, 2010). They were not observed at the selected natural study sites.

Due to its characteristic sweet-bitter taste and attractive colour, the fruit of all three species of cranberry has been used for culinary purposes for centuries (jams, confitures, juices, addition to sauces or beverages). It has also been an important component of phytotherapy, with effectiveness confirmed by traditional application and pharmacological studies (Hong and Wrolstad, 1986; Rodowski, 2001; Stobnicka and Gniewosz, 2010; Senderski, 2015).

The culinary and medicinal usefulness of cranberry is also manifested by the popularity of harvesting the herbal resource from nature, the common cultivation of popular cultivars, particularly the big fruit cranberries, e.g. Ben Lear, Bergman, Pilgrim, or Stevens (Mazur et al., 2009), as well as the popularity of sale of ready food products and herbal medicines (Stobnicka and Gniewosz, 2010).

Similarly as the content of bioactive substances and mass of the resource during vegetation in the case of herbs, the abundance of the population of many plant species can be determined by the variability of the environment. The effect of climatic (e.g. temperature, day light duration, precipitation) and soil-habitat factors, i.e. reaction, fertility, water relations, soil structure, content of organic substances, or content of different elements in the soil solution, and biocoenotic relations determine both the individual development and the abundance of individuals in the population (Stackevičiené and Labokas, 2000; Falińska, 2004; Kazimierczak et al., 2010; Lyuticova and Turov, 2011; Senderski, 2015; Usmanov et al., 2016; Serafin et al., 2017).

Observations conducted in the years 2011-2013 showed that in spite of the relatively common occurrence, the abundance of the population of small cranberry on peatlands in the central part of East Poland differs from site to site. In the case of the selected sites representative of the region, numerous populations of the species occurred in the area of

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lake-peatland complexes Karaśne, Dekowina, Moszne, and Długie (K, DK, M, and D – group I), where their contribution in phytocoenoses of the analysed areas varied from 10% (D) to 30% (K). In the remaining study areas, the contribution of *O. palustris* in phytocoenoses was considerably lower (from 2% - B to 4% - BZ – group II).

A total of 43 spermatophytes (description *Fig. 2*) from 19 botanical families were identified in the assemblages of both groups of sites, in different phytocoenotic configurations. Species such as *Carex rostrata*, *Equisetum limosum*, *Betula pubescens*, as well as *Comarum palustre* and *Menyanthes trifoliata* – herbs subject to simultaneous habitat research (Serafin et al., 2017; and unpublished data) – occurred constantly, although with different percent contributions at all of the study sites.

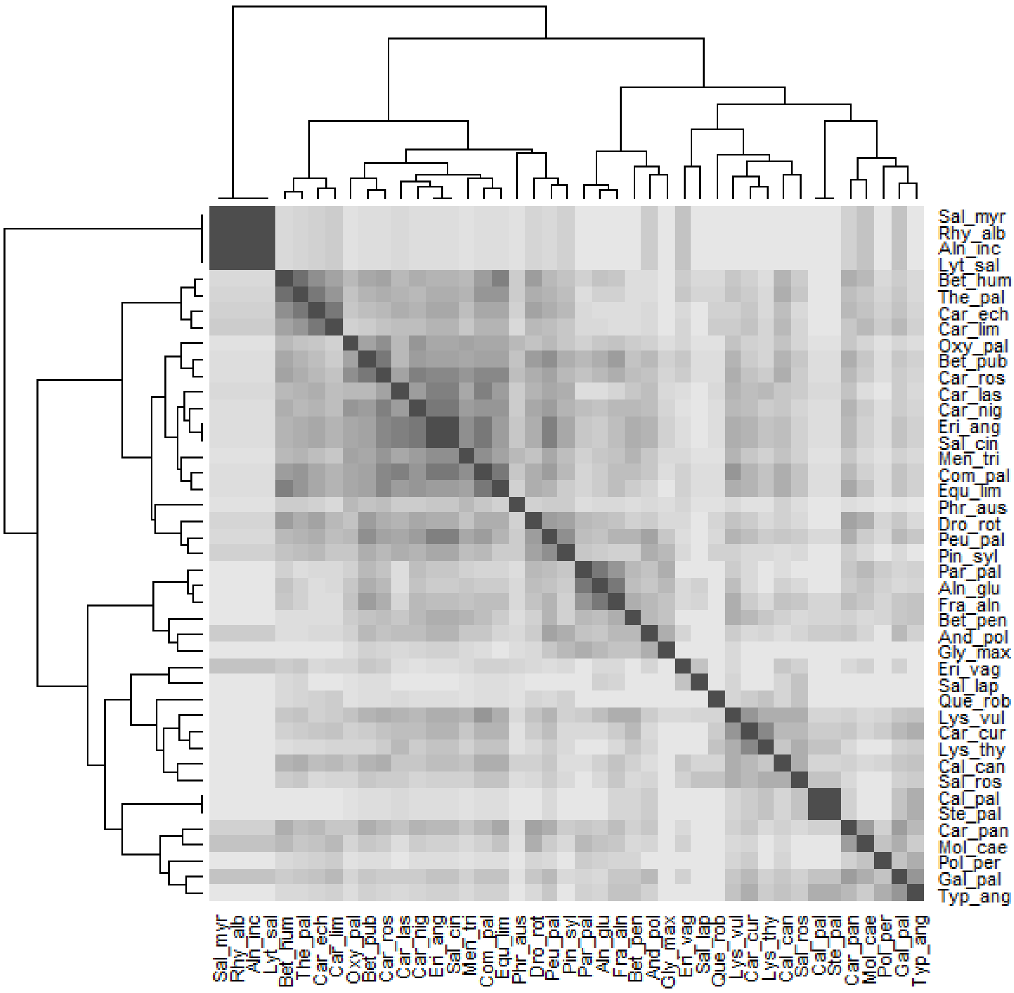

Figure 2. *Dendrogram and heatmap of hierarchical cluster analysis of 43 species based on Jaccard's coefficient and Ward method.* ***Species composition of phytozenoses****: Alnus glutinosa, Alnus incana, Andromeda polifolia, Betula humilis, Betula pendula, Betula pubescens, Calamagrostis canescens, Calla palustris, Carex curta, Carex echinata, Carex lasiocarpa, Carex limosa, Carex nigra, Carex panacea, Carex rostrata, Comarum palustre, Drosera rotundifolia, Equisetum limosum, Eriophorum angustifolium, Eriophorum vaginatum, Frangula alnus, Galium palustre, Glyceria maxima, Lysimachia thyrsiflora, Lysimachia vulgaris, Lythrum salicaria, Menyanthes trifoliata, Molinia caerulea, Oxycoccus palustris, Quercus robur, Parnassia palustris, Peucedanum palustre, Phragmites australis, Pinus sylvestris, Polygonum persicaria, Rhynchospora alba, Salix cinerea, Salix lapponum, Salix myrtilloides, Salix rosmarinifolia, Stellaria palustris, Thelypteris palustris, Typha angustifolia*

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The combination of the classic dendrogram and heatmap, for which colour intensity is directly proportionate to the degree of similarity, permitted a more thorough classification analysis of the species composition. Here, a division covering four assemblages of plant species occurs (*Fig. 2*). They were also analysed in phytosociological terms.

Assemblage I – includes species *Alnus incana, Lythrum salicaria, Rhynchospora alba*, and *Salix myrtilloides*, grouped together due to their single occurrence at individual sites (*Fig. 3*). Assemblage II – includes species showing a stronger tendency of joint occurrence at the same sites than the remaining species in other groups: *Betula humilis, Comarum palustre, Equisetum limosum, Eriophorum angustifolium, Menyanthes trifoliata, Peucedanum palustre,* (class: *Scheuchzerio-Caricetea* – fens and transitional bogs); *Betula pubescens*, *Drosera rotundifolia, Oxycoccus palustris, Pinus sylvestris* (class: *Oxycocco-Sphagnetea* – raised bogs), *Phragmites australis, Salix cinerea, Thelypteris palustris* (class: *Alnetea glutinosae* – marshy forests with black alder and thickets), and *Carex echinata, Carex lasiocarpa, Carex limosa, Carex nigra*, and *Carex rostrata* (*Fig. 3*) with variable phytosociological association. This assemblage includes the analysed species *Oxycoccus palustris.* Associations III and IV include species with variable phytosociological associations, with a weaker tendency of joint occurrence (according to Zarzycki and Korzeniak, 2002).

The Jaccard's coefficient of species similarity of the analysed phytocoenoses rarely and inconsiderably exceeded the value of 0.5, which suggests variability of the flora between both groups of sites and within each of the groups (Serafin et al., 2017).

Somewhat more complete information is provided by the classification analysis with the Jaccard's coefficient as the similarity measure, performed for sites (*Fig. 3*).

In spite of the typological similarity, characteristic of the peatlands of the central part of East Poland, in the case of the classification of sites, a division into three groups is designated: I- D, M, B – sites with high similarity of species composition; II- DK, K – sites showing mutual similarity of species composition and considerably lower towards group I; III- BZ – site with the lowest similarity of species composition towards the remaining groups (*Fig. 3*).

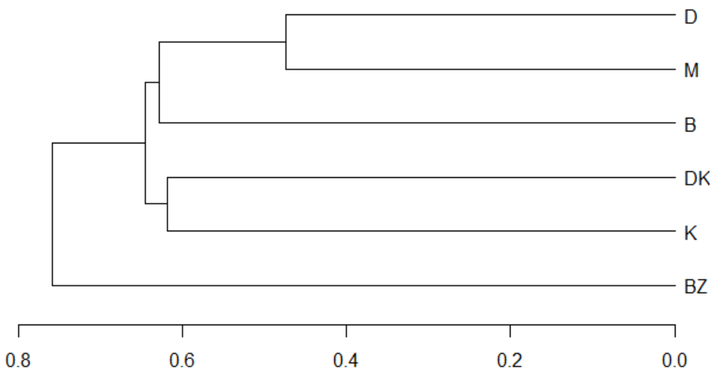

Figure 3. *Dendrogram of hierarchical cluster analysis of 6 phytocoenoses based on Jaccard's coefficient and complete linkage method*

Group 1 includes sites within active, typologically similar lake-peatland complexes (lakes: Długie (D), Moszne (M), and Bikcze - B), which determines their species similarity. Group 2 includes sites variable in terms of genesis – peatland complex not

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directly related to a water body – Dekowina (DK) and lake-peatland complex Karaśne (K), where the final stages of ecological succession occur leading to complete overgrowing of the water body – perhaps the common basis for species similarity in the group. The site in group 3, namely the Blizionki range (BZ), is a small area remaining after a shallow water body, which although typologically corresponds with preferences of the analysed species, is characterised by the lowest species similarity towards the remaining groups. In combination with the low % contribution of occurrence of *O. palustris*, this encourages searching the explanation of the fact in the abiotic properties of the habitat.

In phytosociological terms, the presented plant species compositions are dominated by species from classes: *Scheuchzerio-Caricetea, Alnetea glutinosae*, and *Oxycocco-Sphagnetea* closely associated with the ombrogenic habitats of raised and transitional bogs (Matuszkiewicz, 2001). This confirms the adequacy of selection of the study sites. Small cranberry is a species characteristic of shrub-peat assemblages from class: *Oxycocco-Sphagnetea* occurring on raised bogs, in acidic oligotrophic and dystrophic habitats fed primarily or exclusively by precipitation waters (Matuszkiewicz, 2001).

The botanical and the phytosociological analyses, however, do not constitute a significant factor affecting the greater abundance of phytocoenoses in small cranberry (classification of sites in relation to a higher % share of *O. palustris* does not correspond to the classification of maximum species similarity). Therefore, the other factors favoring a better condition of the studied species should be found.

Therefore, values of the physical-chemical properties of the habitat, analysed in groundwaters in the years 2011-2013, provide a more complete image of habitat preferences of the analysed species, additionally determining the ranges of its ecological tolerance towards the analysed physical-chemical factors based on its variable population abundance (*Table 1*).

Table 1. *Values of physical-chemical factors of piezometric waters at study sites on peatlands in the central part of East Poland (Polesie Podlaskie) with preliminary statistical analysis (Serafin et al., 2017).* N_{total}, $N\text{-}NH_4$, $N\text{-}NO_3$, $N\text{-}NO_2$, P_{total}, $P\text{-}PO_4$, $S\text{-}SO_4$, *DOC, Na, K, Ca, Mg [mg dm*$^{-3}$*], CON [*$\mu S\ cm^{-1}$*]*

FACTOR	Min	Max	Median	Mean	SD	V%
N_{Total}	0.05	182.8	7.14	22.56	35.48	157
$N\text{-}NH_4$	0.10	5.00	0.21	0.75	1.17	157
$N\text{-}NO_3$	0.05	0.12	0.05	0.05	0.01	24
$N\text{-}NO_2$	0.10	0.68	0.10	0.15	0.13	89
P_{Total}	0.04	1.78	0.34	0.42	0.40	96
$P\text{-}PO_4$	0.10	4.55	0.10	0.53	0.86	162
$S\text{-}SO_4$	0.10	40.19	0.27	2.15	6.70	311
DOC	16.90	84.10	42.54	45.15	18.46	41
CON	32.60	381.30	105.10	129.04	85.06	66
pH	4.62	6.50	5.50	5.61	0.45	8
Na	3.30	249.60	6.70	18.53	43.92	237
K	0.85	9.55	3.16	3.76	2.29	61
Ca	0.96	75.10	17.10	24.26	18.36	76
Mg	0.14	6.03	1.88	2.05	1.34	65

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Due to considerable deviations of individual results, mean values of the majority of factors (e.g. Na, Ca, DOC, CON, N_{total}, P_{total}, $S\text{-}SO_4$,) sometimes exceeded the range of typical observation distribution. The fact determined the somewhat misleading image of the intensity of a given property irrespective of the group of sites (*Table 1*), however not the exceeding values typical of the preferred habitat (following Zarzycki and Korzeniak, 2002).

The ranges of values of some of the factors (i.e. N_{total}, $P\text{-}PO_4$, DOC, and to a lower degree K and Ca) depending on the group of sites variable in terms of population abundance of the analysed species were sometimes incoherent, additionally influencing the broader amplitude of environmental valency for the small cranberry. For the group of sites with a higher percent contribution of the analysed species (M+K+D+DK), lower values in comparison to a group with its low contribution (B+BZ) were observed for P_{total}, $P\text{-}PO_4$, and DOC, and higher for Ca and Mg ions, respectively (*Fig. 4*).

Values of the remaining factors (e.g. Na, $S\text{-}SO_4$, N_{total}, CON, etc.) were characterised by similar ranges in reference to two designated groups of sites, therefore they had no limiting effect (compare *Fig. 4*).

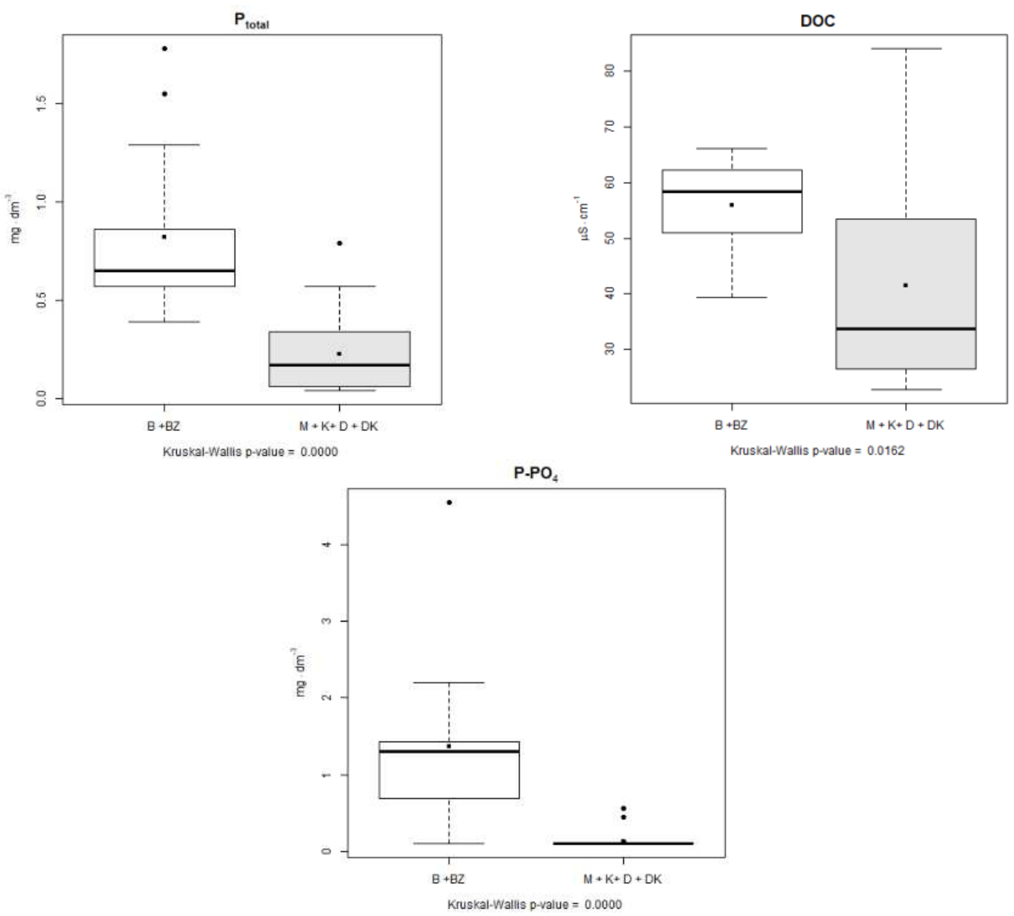

Figure 4. *Distribution of values of the investigated physical-chemical factors of piezometric groundwater which may have a limiting effect on the studied population at the study sites in the period 2011-2013. The x axis label provides the p-value derived by the Kruskal-Wallis test. The horizontal line across the central region of the box represents the median. The mean value of the data is marked by a filled square. Any observation not included between the whiskers is considered as an outlier and represented by a filled circle*

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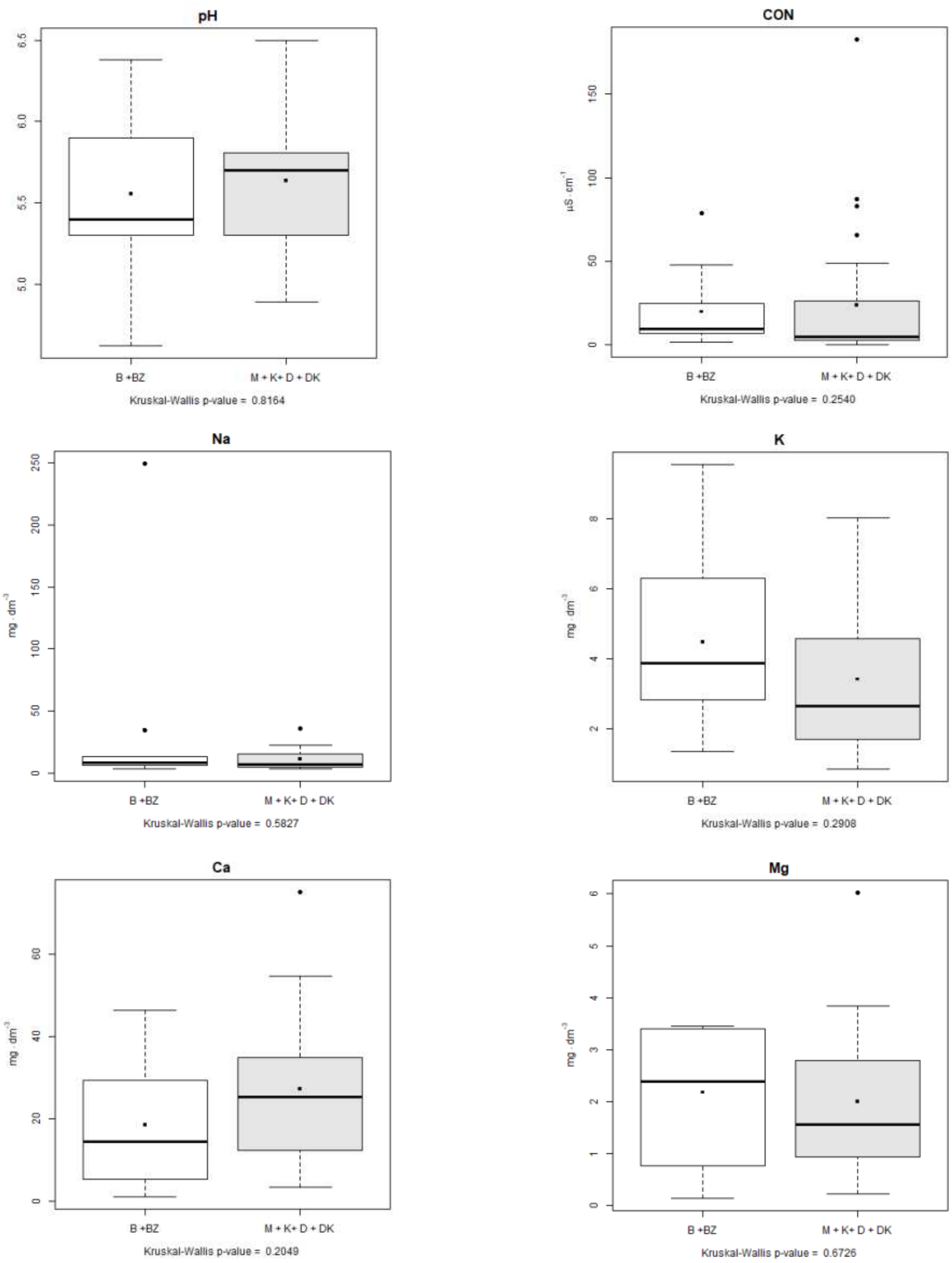
A non-parametric Kruskal-Wallis test was performed to verify the variation in the distribution of values of the investigated parameters at particular sites. Statistically significant differences were only observed in the case of distribution of values of three factors: P_{total}, P-PO_4, and DOC (*Fig. 4*), which may have a limiting effect on the population size of *O. palustris*. The reduced values of the above parameters may be particularly considered a condition favouring higher occurrence of the studied species. Statistics of distribution of values of the factors (upper and lower quartile) therefore suggest ranges optimal for the functioning of the population of the studied species: P_{total} = 0.17 - 0.36; P-PO_4 = 0.1, and DOC = 33.81 - 55.90 [mg·dm^{-3}]. In the case of factors N_{total}, N-NO_2, N-NO_3, N-NH_4, S-SO_4, pH, CON as well as Mg, Ca, Na and K, the distribution of each of the factors was found to be invariant in relation to the sites studied (*Figs. 4* and *5*).



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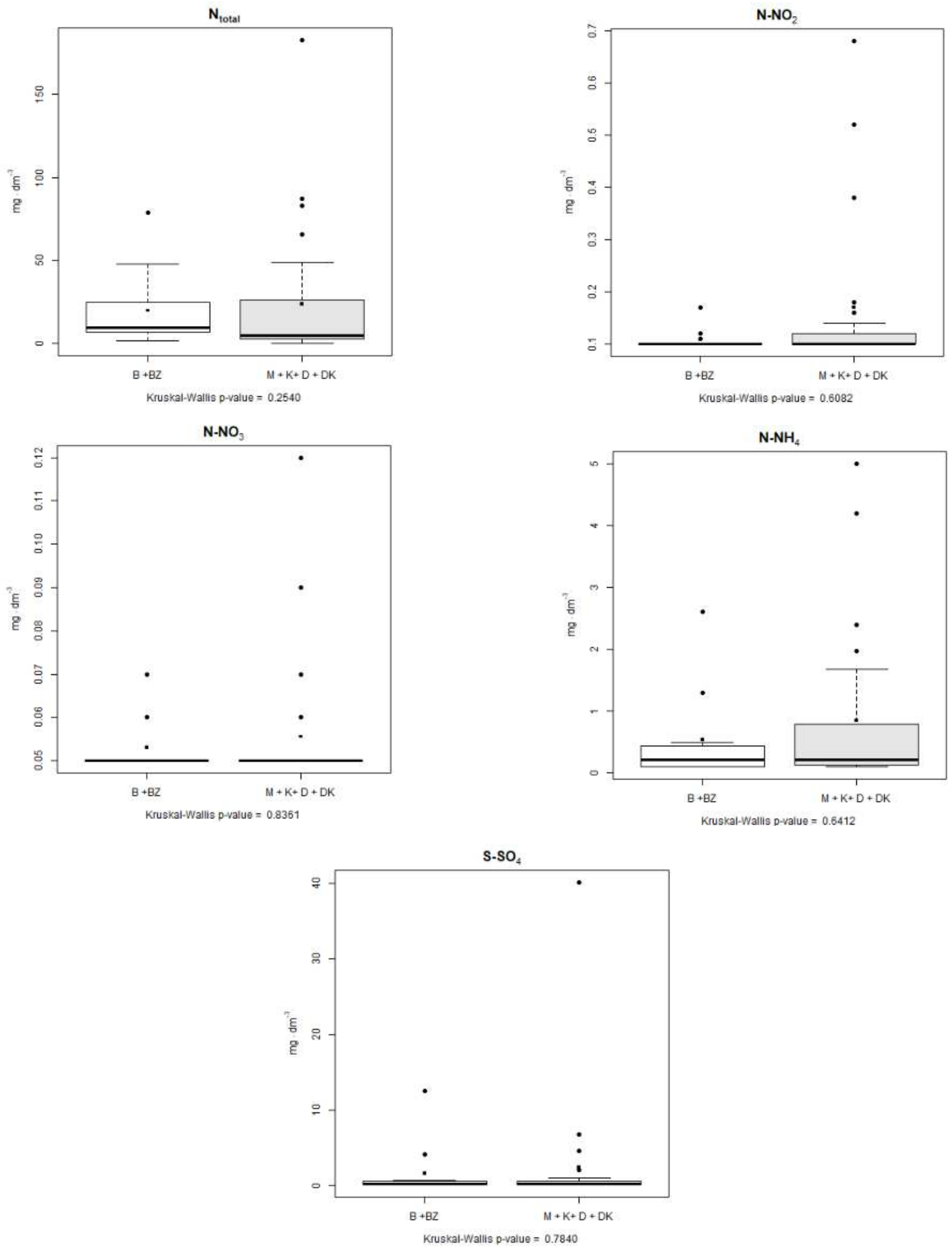

Figure 5. *Distribution of values of the other investigated physical-chemical factors of piezometric groundwater at the study sites in the period 2011-2013*

For comparison, simultaneous research conducted in the area for another species of herbal plant *Menyanthes trifoliata* showed that although both species prefer the same type of habitat, factors determining better condition of the population of the latter include: higher level of N_{total}, P_{total}, and low values of pH and CON. Values of the remaining analysed factors, invariant in relation to different sites, had no limiting effect for the occurrence of the species (Serafin et al., 2017).

The next stage of the analysis employed direct ordination methods. The ordination analysis included 34 species (species characterised by the occurrence of single

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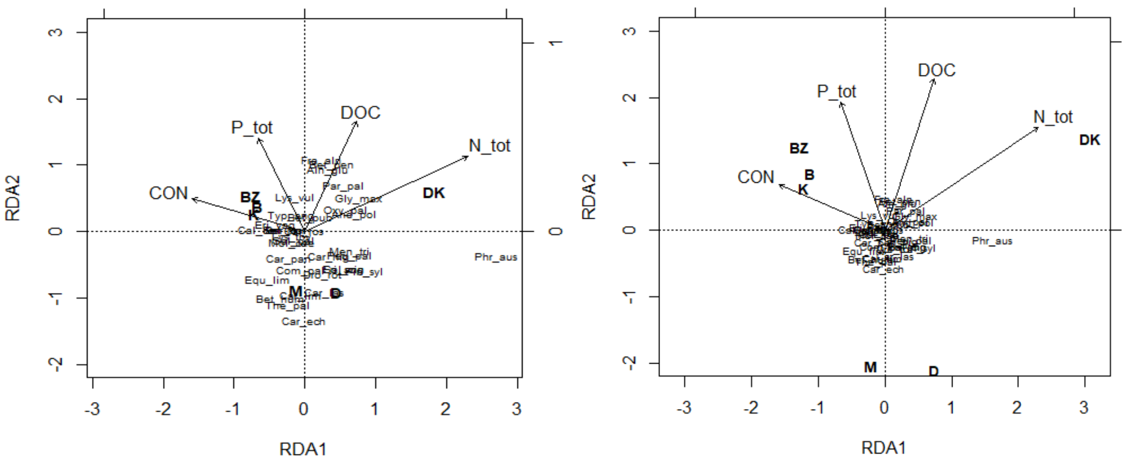
individuals at only one or two sites were excluded), 6 study sites, and 14 environmental variables.

The data set was first subject to detrended correspondence analysis (DCA) which showed a first-axis gradient length of 1.16 in standard deviation units. Therefore, we decided to apply redundancy analysis (RDA) using the rda function in the vegan package (Oksanen et al., 2016).

Prior to the analysis, redundant environmental variables (with linear correlation coefficient |r| > 0.6) were omitted to avoid collinear rite (Blanchet et al., 2008). For this reason and also taking into account the Kruskal-Wallis test results, we carried out RDA with 4 selected environmental explanatory variables: P_{total}, N_{total}, DOC and CON. Before performing further analyses, environmental data were log-transformed (using $\log(y + 1)$) and standardised to meet the assumption of normality. Furthermore, we decided to apply Hellinger transformation to the species data (Legendre and Gallagher, 2001).

RDA results were used to produce ordination diagrams with scaling = 1 and scaling = 2 (*Fig. 6a* and *b,* respectively). All environmental variables accounted for 78.58% of the total variation. The first two RDA axes explained 53.86% of the total variation in the species data (RDA1 = 35.17%, RDA2 = 18.69%). The distances between the sites were approximate Euclidean distances (*Fig. 6a*). Therefore, sites ordinated closer together (like B, K, BZ and also D, M) can be expected to have a similar species composition and similar values of environmental variables. In this context, site DK is not similar to any of the other sites. Moreover, DK is characterised by high level of N_{total} (*Fig. 6b*). Sites B, K, BZ are characterised by high CON and P_{total} levels. Variable N_{total} appeared to be associated to the positive part of axis 1. CON occupies the opposite part of the axis. The second axis is positively correlated with DOC and P_{total}.



(a) scaling = 1 (b) scaling = 2

Figure 6. *Triplot of the RDA with fitted site scores, species and environmental variables as arrows*

The triplot (*Fig. 6b*) indicates a positive correlation between N_{total} and DOC, and a similar magnitude correlation between CON and P_{total}, and between P_{total} and DOC. We found a near-zero correlation between DOC and CON, as well as between as P_{total} and

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N_{total}. In terms of species-environment associations, we observed that the studied species *Oxycoccus palustris* was strongly positively associated with N_{total}.

The habitat research and statistical analyses concerning selected physical-chemical parameters of peatland waters confirm the relatively broad spectrum of ecological tolerance for small cranberry. This is manifested by the common occurrence of the species on peatlands in East Poland. Nonetheless, the condition of its population can be determined by different habitat and biocoenotic factors determining the growth and development of the population, and therefore its abundance at different sites. More abundant occurrence of *O. palustris* in places with reduced human impact allows for the presumption that due to the evolutionally developed optimum habitat preferences, the composition and content of biologically active substances in its organs are more natural. Obtaining the herbal resource from such natural sites in accordance with the rules of Good Manufacturing Practice permits the production of natural herbal medicines pursuant to the guidelines of WHO (Borkowski, 1994; Drozd, 2012). In many cases, it is impossible to determine which biologically active compound has medicinal importance. Therefore, the properties of the herbal resource such as natural composition and the natural composition of medicinal factors, confirmed by the traditional application and effective treatment, seem to be of importance.

In the case of harvesting the herbal resource from nature, area restrictions exist, and sometimes also legal restrictions related to forms of nature protection areas. Ecological cultivations retaining optimum natural values of physical-chemical properties of habitats seem to be an excellent alternative, particularly in reference to conventional cultivations (Serafin et al., 2017) dominated by other species of cranberry more seldom occurring in nature (Stobnicka and Gniewosz, 2010).

The consequence of the habitat research will be the laboratory analysis of the content of biologically active substances obtained from the therapeutic raw material of small cranberry at all study sites for comparative purposes, and the perspective of the experiment of organic cultivation of this species in conditions optimised by these study results.

Conclusions

1. In the area of peatlands of Central-East Poland, *Oxycoccus palustris* is a species with a broad range of ecological tolerance in reference to the majority of the analysed physical-chemical habitat properties.
2. Values of the analysed habitat parameters, although variable depending on the study site, did not exceed values typical of preferences of the species, which translated into its common occurrence.
3. Lower values of concentration of important habitat parameters in ranges: $P_{total} = 0.17 - 0.36$; $P\text{-}PO_4 = 0.1$, and $DOC = 33.81 - 55.90$ [$mg \cdot dm^{-3}$] can be considered as a condition favourable for proper functioning of individuals of the analysed species.

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INVESTIGATION OF ENVIRONMENTAL DAMAGES CAUSED BY EXCAVATED MATERIALS AT FOREST ROAD CONSTRUCTION IN THE MEDITERRANEAN REGION OF TURKEY

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Abstract. Forest road constructions have been investigated in terms of damage to trees below road construction areas and in stream beds. In this study, constructing a road using a bulldozer and environmental damages caused by this technique were investigated in the forested regions of Antalya in Turkey. Decision variables were collected from 52 cross sections in a road section that was selected as a sample. In these cross sectional areas, the number of damaged and undamaged trees was determined in each cross section. Another damage type, the damage in stream beds was investigated based on deposits of excavated materials dumped during the forest road construction operation. The slope in this research area varied between 25–80%. Moreover, the maximum and the minimum length of fill areas in different cross sections was found to be approximately between 2 and 16 m. In this study, the rate of bending damage to trees below the road construction areas was 26.3%, and the rate of wounding damage was 6.1%. The number of damaged trees in various gradient was also determined in the study area. Along the chosen road section, debris were found to cover the stream bed in some areas. The wounding of tree barks was serious in this forest region although there was also significant damage caused by bark beetles.
Keywords: *road, tree damages, cross section, stream bed*

Introduction

Forest roads are necessary to provide access to forests for general management, maintenance, timber extraction, recreation (Ryan et al., 2004), regeneration, production (Demir and Hasdemir, 2005), and fire and pest control (Tehrani et al., 2015). Forest roads are associated with economic growth and national wealth. However, they have various direct and indirect effects on their adjacent environment (Tehrani et al., 2015). Construction of a forest road network is considered as the key element for a successful forest management (Krc and Begus, 2013). Planning a forest road network depends on social requirements since such roads provide access to forest villages, rural settlements and recreational areas (Acar and Eker, 2003). When forest roads are routed, construction methods and equipment selection directly affect their economical, functional and ecological efficiency (Ozturk et al., 2010). A proper design and routing of forest roads will reduce the need for major repairs and save on maintenance costs over time (Edwards, 2011).

Forest roads also have major environmental impacts. The environmental impacts of forest road constructions vary according to conditions of a terrain, that is whether it is a

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rocky, loose rock, soil, gentle or steeper terrain, and so forth. In addition, in general, environmental damage may increase depending on situations such as stands (average tree diameters, average tree lengths, stand ages, canopy closures, etc.), choice of machines, tree species, and road construction techniques. Building forest roads involves removal of vegetation and soil, thus favoring run-offs, pollution of streams, and the risk of erosion and mass movement on steeper terrains (Edwards, 2011; Hernandez-Diaz et al., 2015). Moreover, stream beds can fill up with excavated materials, which is very concerning in terms of freshwater ecology, fish health, and rerouted stream beds.

Various types of bulldozers and excavators are generally used in forest road construction operations in Turkey. Bulldozers are commonly preferred on terrains with gentle to moderate hillside slopes and on soil and loose rock grounds. However, in steep and rocky terrain conditions, the efficiency of bulldozers diminishes, and excessive environmental damages may occur because of the difficulty to keep excavated materials alongside the road (Ozturk, et al., 2009).

In this study, a forest road construction was investigated in the southern part of Turkey. The construction was carried out by using a bulldozer. Environmental damages to trees and to the stream bed caused by the forest road construction were investigated. In addition, productivity and cost of the bulldozer were evaluated, and some suggestions were offered.

Materials and methods

Study area

The study area is selected from the office zone Gursu Forest Enterprise in Kas Forest Management in Antalya (*Fig. 1*). In this enterprise, dominant commercial tree species include *Pinus brutia*, *Cedrus libani* and *Juniperus* sp. The elevation ranges from 500 m to 1200 m with ground slopes of 25% to 80%. The study area consists of Type B forest roads with a density of 16 m/ha. Total length of the sample road examined in this study was about 1575 m with an average road width of 5 m. The study area is located on the Taurus Mountainous, which is the largest and most important karst region in Turkey. Due to immediate penetration of rainfall and snowmelt into the rock crack system, surface soil formation very slowly occurs along the cracks and statification surfaces of the limestone. In this study area, the road alignment consists of soil and losses rock.

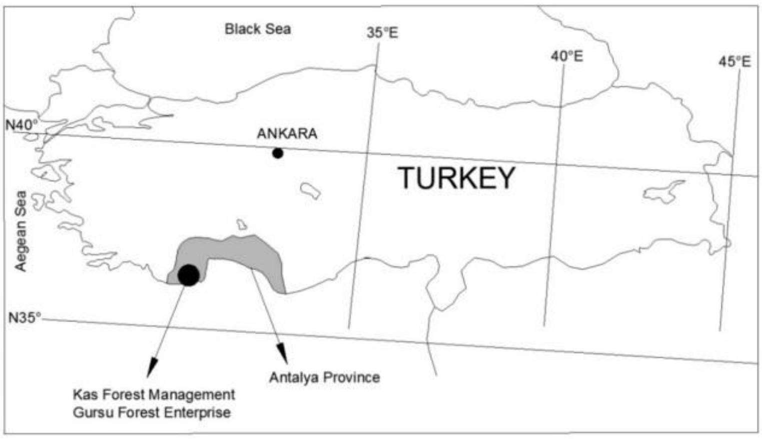

Figure 1. *The location of the study area in Antalya region*

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The equipment specifications

Komatsu D85ex type bulldozer was used in forest road construction operations. This bulldozer is a very strong machine and it is suitable for forest road operations on soil and loose rock region. The technical specifications of the bulldozer are shown in *Table 1*.

Table 1. *Technical features of the Komatsu D85ex (Anonymous, 2015)*

Specifications		Values
Bulldozer weight		21220 kg
Operating weight		28100 kg
Engine model		SAA6D125E-5
Engine type		4 cycle, water cooled, direct injection
Engine power		266 HP
Maximum travel speed	Forward	10.1 km/h
	Reverse	13.0 km/h
Blade capacity		7.0 m³
Minimum turning radius		1.99 m
Fuel tank		490 l
Bulldozer height		3330 mm
Bulldozer length		5795 mm
Bulldozer width		3635 mm
Ground pressure		73.6 kPa

Methods

The area and the trees in it during the forest road construction were measured to determine and investigate the damage caused by the excavated materials. Also, environmental damages to the stream bed caused by the excavated materials were investigated. In this study, primarily, the cross sections were determined along the forest road. Every cross section included different variables. These variables include cut-slope height (Ch), cut-slope width (Crw), road width (Road + Ditch) (Rw), fill-slope width (Frw), fill-slope length (Fl), road construction zone width (Czw), cut slope length (Cl) and ground slope (S) (*Fig. 2*).

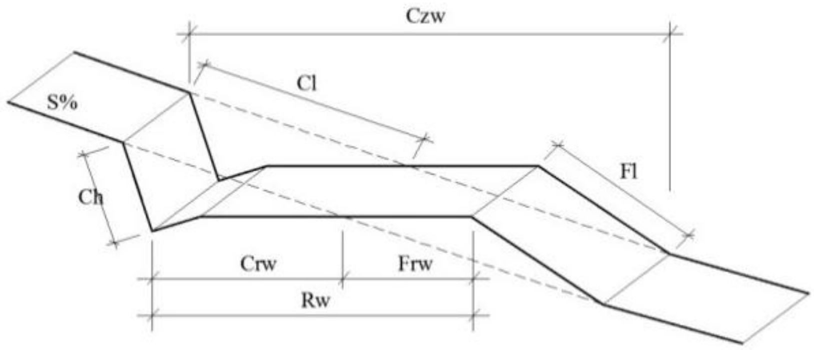

Figure 2. *The decision variables measured from each cross section along the forest road*

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In this study, surveying instruments such as a steel tape, a measuring batten, clinometers, an altimeter, and a compass were used in the research area. Along the 1,575 m of road section, decision variables were collected from 52 cross sections, which were 30 m apart from each other. The number of damaged and undamaged trees were determined in each cross section as a gradient group. The types of damage such as bending of a tree and wounding of tree bark were also observed. Using the data that were collected, distribution of damage types in relation to the construction techniques associated with the bulldozer and the effect of positional values of damaged trees were investigated. The data were entered into a spread-sheet program to compute simple statistics such as arithmetic averages and standard deviations. The average excavation speeds and productivities of the bulldozer were found throughout the forest road operation. Every cross section was shown in percent slopes, differently for cut-slopes and fill-slopes. The shapes of the cross sections were measured, and the difference between the lengths of cut-slopes and fill-slopes was shown.

Results and discussion

In the first stage of the road construction operation, the trees on the route were cut, and removed. In this route, 386 m^3 of logs were extracted from 452 fallen trees that were collected from a single cross section that was selected as a sample along the construction zone. The trees that were cut were mostly *Pinus brutia*. The results indicated that the total amount of excavated materials along the roadway was 12,195.00 m^3, The percentages of soil, loose rock and soft rock of those materials were 59%, 36% and 5%, respectively. The average operation time of the bulldozer was 8 h per day. The research was conducted in August 2015. The research area is very hot in the summer season, which affected the forest road operation.

Cross sections

In this study, the data from the cross sections were collected on the sample road. In this region, the types of materials on the ground are soil and loose rock along the forest road construction route. The excavation operation using the bulldozer was easy because the amount of rock on the ground was very small. *Table 2* lists the values of the specific variables measured from the cross sections.

Table 2. *The values of decision variables measured on the cross sections*

Variables	Symbol	Average	Standard deviation	Max. values	Min. values
Ground slope (%)	S	58.8	17.73	80	25
Cut-slope height (m)	Ch	3.51	1.43	7.8	1.7
Cut length on road width (m)	Crw	4.31	1.10	6.5	2.9
Fill length on road width (m)	Frw	2.34	1.79	8.5	0.5
Road width (m)	Rw	6.56	1.72	12.0	4.2
Cut-slope length (m)	Cl	6.55	1.85	10.8	3.9
Fill-slope length (m)	Fl	5.67	3.82	16.2	1.9
Construction zone width (m)	Czw	11.32	3.40	19.5	6.9

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The average construction zone width was 11.32 m; therefore, the sample road section affected approximately 1.78 ha of the forested area (11.32 m × 1,575 m = 17,829 m² = 1.78 ha) during the road construction activity.

In this study, the rates of cut-slopes in the forest road were mostly between 3/1 and 5/1 depending on the cut-slope heights and road widths. Excavated materials were scattered down the side of the roadway, and the fill-slope lengths varied between 1.9 and 16.2 m depending on the fill-slope gradients. The gradient of study area was found to be between 25 and 80%.

Environmental damages on trees and stream bed

Some of the cross sections did not have any damaged trees, whereas the others had damaged trees (*Fig. 3*). The damaged trees usually had bending and wounding damages (*Figs. 4* and *5*). The number and rate of the damaged trees in the study area is shown in *Table 3*.

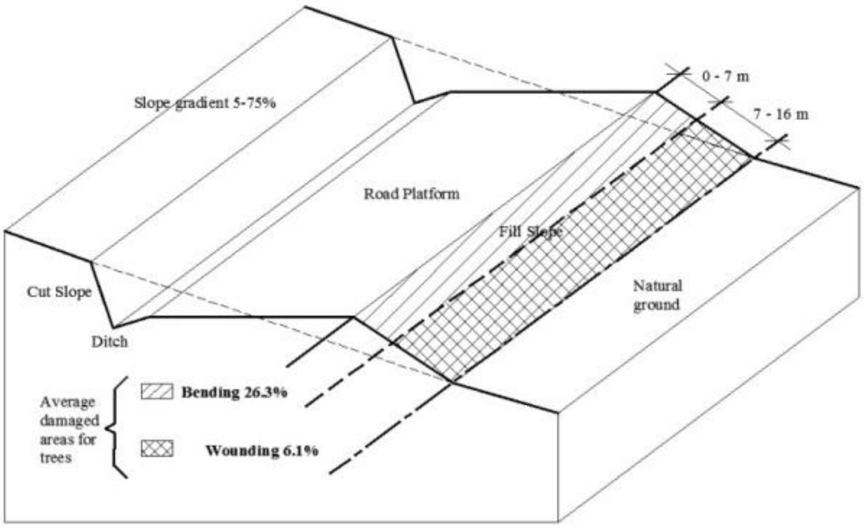

Figure 3. *Determination of cross sections along the road alignment*

Figure 4. *Bending damages of trees*

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Figure 5. *Wounding damages of trees*

The wounding of the tree barks is a very serious issue for this forest region. For many years, bark beetles have been devastating this region. The damage caused by bark beetles is denser especially on *Pinus brutia*. The environmental damage may be increased in these regions due to bark beetles. In addition, direct economic losses are rising due to the timber quality lowered by bark beetles.

Table 3. *Number and rate of damaged trees in study areas*

Cross section number	Distance (m)	Average of grade fill slope (%)	Total number of trees	Number of bending trees	Number of wounding trees	Average of distance rolling (m)	Bending percent (%)	Wounding percent (%)
1	30	65	24	6	-	9	25	0
2	30	65	29	9	1	9.5	31	3.4
3	30	75	65	20	3	22	30.8	4.6
4	30	70	22	8	2	9.5	36.4	9
5	30	45	5	-	-	5.5	0	0
6	30	72	38	14	5	18.5	36.8	13.2
7	30	63	20	4	1	7.5	20	5
8	30	55	18	8	3	6	44.4	16.6
9	30	40	13	2	-	4.5	15.4	0
10	30	58	22	5	2	8	22.7	9
Ort.	20	60.8	26	8	2	10	26.3	6.1

The number of damaged trees in the steep and gentle terrain was found to be greater. In 25–80% of the sloped areas, there were an average of 8 bent trees and 2 wounded trees. In addition, in the cross sections, the average number of undamaged trees was counted to be 26. Along the forest road, the average gradient was found to be 61%. The amount of excavated materials along the road alignment and the slope gradient are also important. The length of fill-slopes increases with increasing slope gradients, and consequently, the damage grows. When the sliding and rolling distance of excavated materials increase, the environmental damages to trees and to the river basin are increased.

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All the excavated materials were dumped down the side of the roadway by the bulldozer. As the second type of environmental damage, the stream bed was filled with excavated materials. In this case, the direction of the stream flow was diverted. This change was very serious in terms of freshwater ecology, fish health, and rerouted stream beds (*Fig. 6*).

Figure 6. *The fill with excavated material of stream bed*

Filling stream beds with excavated materials increases the risk of erosion in this area especially in the rainy season. The main reason for dumping excavated materials over the stream bed was the fact that the forest road was very close to the stream. A buffer zone should be allowed between a road and a stream so that the stream can be protected from excavated materials. According to Akgül, the width of a buffer zone should be increased depending on the slope rate. The width of a buffer zone ranges between 25 and 150 m on productive forest areas (Akgül, 2012). Stream beds are protected from flow of sediments by buffer zones, and thus, the speed of rainwater is reduced in such areas (Wenger, 1999; Wood and Armitage, 1997).

Productivity and cost of bulldozer

The production rate of the bulldozer is generally computed as the length of constructed road per hour. In this study, the average productivity of the bulldozer in soil, loose rock and soft rock were found to be 221.41, 125.00 and 86.5 m^3/h, respectively (*Table 4*).

The average slopes of soil, loose rock and soft rock in the study area were 55, 66 and 63%, respectively. The total road construction cost of the Gürsu region was 14,750.00 US$, with a unit cost of 9,370.00 US$/km. The fuel consumption of Komatsu D85ex bulldozer varied between 18–20 l/h.

In this study, the average zone width of the constructed sample road was 11.32 m. The amount of forest area impacted along the road was 1.78 ha. A study conducted in the Antalya region reported that a road construction operation in the same region resulted in a zone width of 12.18 m (Wood and Armitage, 1997). Another study conducted by Ozturk (Ozturk, et al., 2010) — in which a bulldozer was used — in the Bolu region of Turkey indicated that the average zone width of the construction was

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7.27 m, and the ground type was soil. Ozturk also reported a construction zone width of 7.47 m on loose rock in the Eskişehir region of Turkey (Ozturk, et al., 2009).

Table 4. *Productivity of bulldozer at different excavated materials*

Cross section number	Excavated materials ground type	Average slope (%)	Distance of between cross sections (m)	Productivity of bulldozer (m^3/hr)
1	Soil	55	25	221.41
2	Loose rock	66	25	125.00
3	Soft rock	63	30	86.50

There are two types of environmental damages caused by forest road constructions. These are damages to trees and damages to stream beds. The tree damages include bending and wounding of trees. The damages to stream beds include the filling of stream beds with excavated materials. In this study, in the 25–80% ground slopes, 26.3% of the trees below the forest road construction zone were bent, and 6.1% of the trees were wounded. In a similar study conducted by Tunay (Tunay and Melemez, 2004) in Antalya, 55% of trees under the forest road construction zone in a terrain with a ground slope of 51% were damaged. In a similar region, Ozturk (Ozturk, et al., 2010) reported that the types of damages that were identified on trees included bending, crushing and wounding in a karstic region. The rate of total damage including all three types was 23% in the 46–90% ground slope. Another study conducted by Caliskan in the Trabzon region of Turkey revealed that 44% of trees were damaged in soil, loose rock and rock areas of a terrain that had a ground slope of 59% (Caliskan, 2013). Damage types in those areas included bending, crushing and wounding. Although the area in this study was a sloping area, the number of trees that were damaged was less than those that were observed in the other studies. The reason might be that the types of ground were mostly soil and loose rock. The soil load usually bends trees. The rate of wounding observed on the trees was less. In this study, dumping the excavated materials on the stream bed was a more serious problem for the environment. It appears, the risk of erosion will increase in this road area in the consequent years.

In this study, the cut-slope rates ranged from 3/1 to 5/1, and the fill-slope rate was 2/3. Kramer indicated that a cut-slope rate of 3/1 is the most appropriate rate especially for steep terrains (Kramer, 2001).

Conclusion

In this study, constructing a road using a bulldozer were evaluated in soil and loose rock areas by considering economic and environmental requirements. The following are recommended:

- Bulldozers should be used in forested areas with less than 45% ground slope. Environmental damage is increased in areas where slope is higher.
- A buffer zone should be maintained between a forest road and a stream. The buffer zone protects the stream bed from excavated materials.
- Damaged trees should be removed from sensitive areas where bark beetles are abundant. Especially the wounded trees will increase the number of bark beetles. This situation is very dangerous for sensitive forest areas.

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- Cut-slope rates should be 3/1 or 5/1 on mountainous areas. Lower rates such as 1/1 or 2/1 will increase construction zone widths of forest roads.
- Bulldozer operators should be well trained to improve the efficiency of construction activities, which affect economical and environmental aspects.

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PUBLIC AWARENESS AND PERCEPTIONS OF CLIMATE CHANGE: DIFFERENCES IN CONCERN ABOUT CLIMATE CHANGE IN THE WEST MEDITERRANEAN REGION OF TURKEY

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Abstract. This study was aimed at determining perception and awareness levels about climate change in three city centers located in the West Mediterranean Region of Turkey and identified differences in public perceptions and knowledge about regional climate change. The study utilized a questionnaire method to obtain data. The results found that approximately half of the participants had inadequate climate change knowledge. In particular, knowledge levels regarding climate change adaptation were comparatively low. Women were more concerned about climate changed compared to men. Additionally, primary and high school graduates and married individuals were also more concerned. Age and income were not determining variables. There were no significant differences in terms of gender, age, income and educational status apart from marital status as to whether or not adaptation to climate change will be achieved. There are varying levels of awareness among the public regarding climate change, and thus it is especially important to organize outreach programs supported by media to increase climate change adaptation knowledge levels. Institutions need to improve their ability to promote public awareness and knowledge about climate change in order to reach a larger proportion of the public.
Keywords: *global warming, public opinion, adaptation, Turkey*

Introduction

Climate change is a key agenda item for a majority of countries due to the increased visibility of general ecological problems, developments in science and technology, and related studies by ecological economists since the 1970s. It is forecasted that important consequences, such as melting of icebergs, rising sea levels, and severe weather events may directly or indirectly impact human life and health, socioeconomic sectors and ecological systems (IPCC, 2013). It is now generally and widely agreed that global warming is accelerating in present-day conditions due to human-induced effects and that climate change is reaching a level that affects the future of society (Cook, 2013; MacCracken, 2001; IPCC, 2001). The fifth assessment report of the *Intergovernmental Panel on Climate Change* confirms changes in the frequency and intensity of some extreme events such as weather and ocean temperatures, precipitation changes, rising sea levels and drought, floods and storms (IPCC, 2014).

Policymakers face the challenge of combating climate change and determining strategies for optimal adaptation and rational policies. Strategies that focus on maintaining the healthy and effective functioning of climate-change-resistant ecosystems and managing and conserving water, land and biological resources are fundamentally important to coping with the impacts of climate change. While efforts to hinder and prevent this process continue at an international level, work aimed at implementing adaptation in societies around the world are also underway (IPCC, 1990,

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1995, 2007; WB, 2008; EC, 2009). National climate legislation and strategies have been formed in most United Nations member countries (Dubash et al., 2013).

In terms of general public awareness about climate change, visible changes such as an increase in temperatures and a decrease in precipitation are effective in impacting public perceptions (Rankoana, 2016). The most striking aspect of climate change perception is personal experience with negative natural phenomena, which is a strong determinant of individual risk perception (Frondel et al., 2017). According to many academic researchers, climate change policies have been insufficient to sensitize people in society to the meaning and importance of the problem and in particular for mobilizing people to take action (Westerhoff and Robinson, 2013: Randall, 2009; Lejano et al., 2013). The risk perception of the public regarding the possible consequences of climate change is of great importance. Apart from shaping climate policies, risk perception also plays a central role in adaptation and reduction initiatives (Lujala et al., 2015). Therefore, there is a need for more data determinants that influence people's perceptions of climate change.

Turkey is one of the countries that is most affected by climate change due to global warming. The complex climate structure in Turkey, which is surrounded by the sea on three sides, a fragmented topography, and orographic features means that different areas in Turkey will be affected to varying degrees (Ozturk, 2002). The *Turkey Climate Change Adaptation Strategy and Course of Action* policy aims to reduce the impact of climate change and adapt to global warming. Importantly, public awareness levels and institutional capacity must be increased in Turkey (MEU, 2010). Very few empirical studies have considered how the public in Turkey interprets and respond to climate change. This study investigates perceptions, concerns, and adaptation to climate change by residents of three city centers in the West Mediterranean Region of Turkey.

Materials and methods

Study area

Many of the problems caused by climate change have a tremendous effect on cities. The economic and social aspects of these problems must be tackled alongside the environmental implications (Albayrak and Atasayan, 2017). National strategies must be reorganized to a local level to improve the adaptation ability of cities against increasing risks (Warmsler et al., 2013). Over the last thirty years, the western and southern regions of Turkey have suffered from droughts due to Mediterranean precipitation patterns (Turkes, 2012). As such, this study focused on city centers (Antalya, Isparta, and Burdur) of the Western Mediterranean Region of Turkey (*Fig. 1*). The comparison of precipitation and average temperature of the study area between 1970 and 2017 was shown in *Figure 2*.

Data collection and analysis

Data were collected using a questionnaire approach. The multiple-choice questions in the questionnaire used a Likert scale. The questionnaire consists of two parts. The first part gathers profile information regarding the participants and the second part consists of questions designed to determine their level of perception and awareness about climate change. The questionnaire was conducted in face to face in the aforementioned three city centers. In order to determine the views of a group of participants with a 5%

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sampling error, the sample size was calculated by the formula (*Eq. 1*) below (Bas, 2010):

$$n = \frac{t^2 Npq}{(N-1)d^2 + t^2 pq} \qquad \text{(Eq.1)}$$

where:

n sampling size,
t t value (1.96 for 95% confidence level),
N population size,
p,q probability of availability of the mass to be measured in the main mass,
D sampling error accepted (5%).

Figure 1. *Study area*

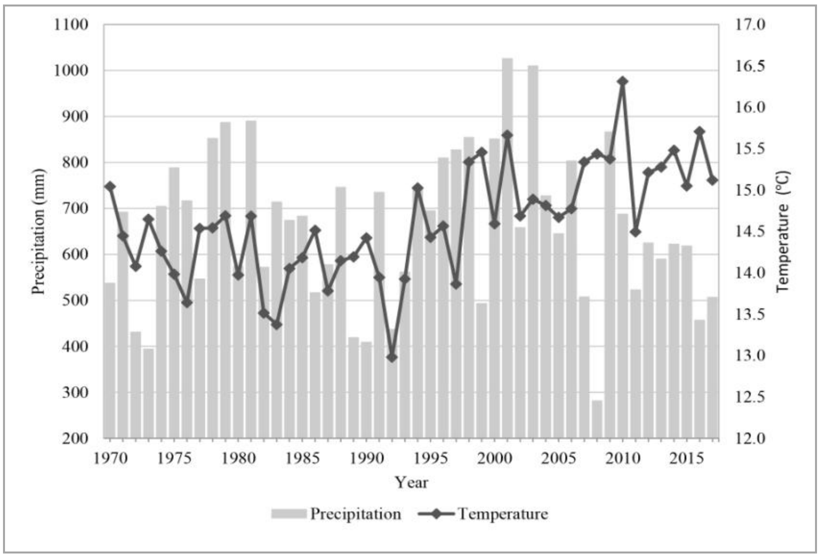

Figure 2. *Temperature and precipitation change between years 1970 and 2017 in study area*

Based on the above formula, the sample size needs to include 383 questionnaires. This study obtained 406 questionnaire forms in three cities (175 in Antalya, 118 in Isparta and 113 in Burdur) that were filled out and analyzed to increase the reliability of

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the study. In the questionnaire applications, a systematic random sampling procedure was used. Quotas were set on age, gender, education and city population. In this context, participants were selected to represent the whole population in the study area. The internal consistency (Cronbach's alpha) coefficient of the scaled statements was determined to be 0.910. Since the internal consistency coefficient was greater than 0.8, the scale was highly reliable as a statistic.

The data obtained from the questionnaires were evaluated using statistical methods. The answers given in the first stage were analyzed using the SPSS package program to convert to percentage ratios. The Shapiro-Wilk test, the most effective method of testing the hypothesis for normality, was used to determine whether or not the questionnaire data was parametric. It was determined that 95% of the data did not have normal distribution ($p < 0.05$), meaning that it is not parametric. As such, non-parametric tests were used in the data analysis. In order to check for discrepancies among socio-demographic features (age, gender, education, etc.) while determining the views of individuals participating in the questionnaires on climate change concerns and their thoughts on climate change adaptation, several statistical methods were employed, including: for bivariate features the Mann-Whitney U test (Macfarland and Yates, 2016) and for more than two variables the Kruskal-Wallis H test (Corder and Foreman, 2014).

Results and discussion

Participant profile characteristics

Participant profile characteristics are provided in *Table 1*. A majority of the participants were young and middle-aged. Approximately one third were women. The majority were university graduates. About 60% of participants were single. The proportion with moderate and low incomes was 93.6%.

Table 1. *Profile characteristic of participants*

Characteristics	Number	%
Age		
18-25	158	38.9
26-45	142	35.0
46-65	98	24.1
>65	8	2.0
Gender		
Female	149	36.7
Male	257	63.3
Education		
Primary, secondary or high school	161	39.7
Graduate	245	60.3
Marital status		
Married	150	36.9
Single	256	63.1
Monthly income		
≤ 1400[*] TRY[**]	115	28.3
1401-5000 TRY	265	65.3
> 5000 TRY	26	6.4

[*]Minimum wage, [**]Approx. 1 TRY = 0.28 US$

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Opinions regarding climate impacts and perceptions about climate change

Since climate change is an important threat, it is helpful to determine the level of knowledge and awareness in the community, in particular regarding causes, effects, how to tackle the problem, and adaptation. *Figure 3* shows that while all participants have some knowledge about climate change, about roughly half do not have enough information. The levels of knowledge in the areas of climate change and adaptation are relatively low. Ochieng and Koske (2013), Semenza (2008), Michail et al. (2007) and Papadimitriou (2004) concluded that knowledge on climate change is inadequate, which is a finding similar to our study.

Figure 3 shows that all of the people living in the cities inside the research area are aware of climate change. Although some are at low knowledge levels, almost everybody has some information on causes, impacts, how to tackle climate change, and how to adapt to it. The most important reason for this is that climate change is largely associated with changes in the air. When asked, “What is the first concept you think of when you hear the words climate change?” the most popular answers were shifting seasons (41.1%) and global warming (20.4%). Natural disasters (17.5%) and weather deterioration (7.6%) follow closely (*Fig. 4*). In studies aimed at determining perception about global warming conducted by Steentjes et al. (2017) in France, Germany, Norway and England, global warming and seasonal change were also the first concepts to come to mind. In addition, emissions, environmental pollution, and air pollution, which are the driving forces of climate change, lag behind. Such awareness supports the idea that emotional and empirical learning is more effective than intellectual-based learning to change attitudes towards climate change issues (Lujala et al., 2015).

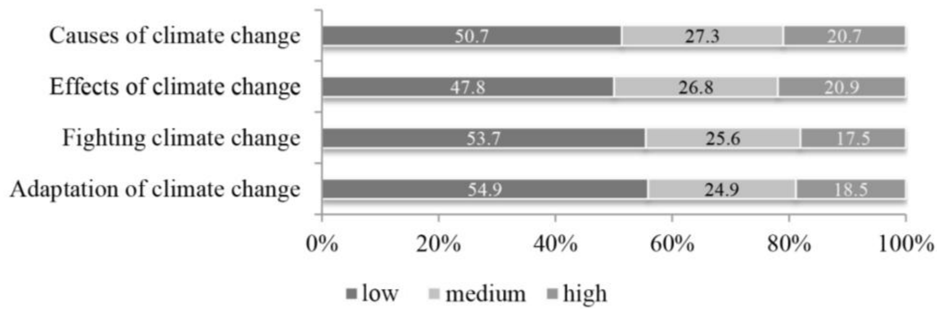

Figure 3. *Knowledge level on climate change (n = 406, only values > 5%)*

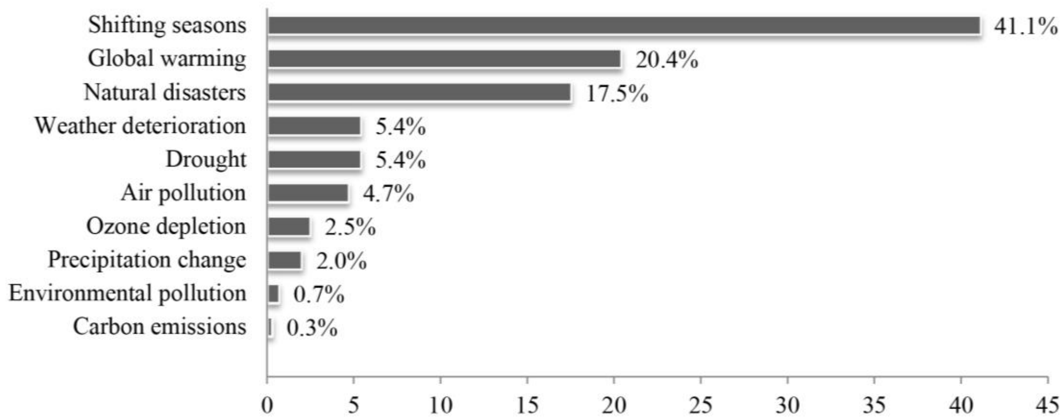

Figure 4. *What first comes to mind when you hear the phrase “climate change”?*

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Findings on the results of climate change can be found in *Figure 5*. The vast majority of respondents indicate that climate change will cause many negative consequences, ranging from melting of glaciers to high temperatures. The level of awareness for melting of glaciers was higher (40.4%) than other impacts. Sea level rise was considered relatively important at a lower level. Unlike other concepts, about 6% of participants indicated that this is not important. The most important effect of climate change shown in *Figure 5* is the risk of extreme temperatures and drought. In our study, 75.8% of respondents in Turkey believed that heat and drought in the Mediterranean basin have increased and believe that this will continue in the future.

Schmidt et al. (2013) did a comparative analysis of 27 different countries and determined that climate change is covered in the media of countries at varying levels and that the attention of the media to carbon-dependent countries, which are committed under the Kyoto Protocol, is particularly high. The findings of this research also support this. In our study, 72% of the respondents said that they were provided information and awareness about climate change through the media. The distribution of effective media tools is internet (29%), TV (27%), and newspapers and magazines (16%). Since climate change is a global phenomenon, such platforms can affect societies around the world. Importantly, the prominence of the media is clearly evident.

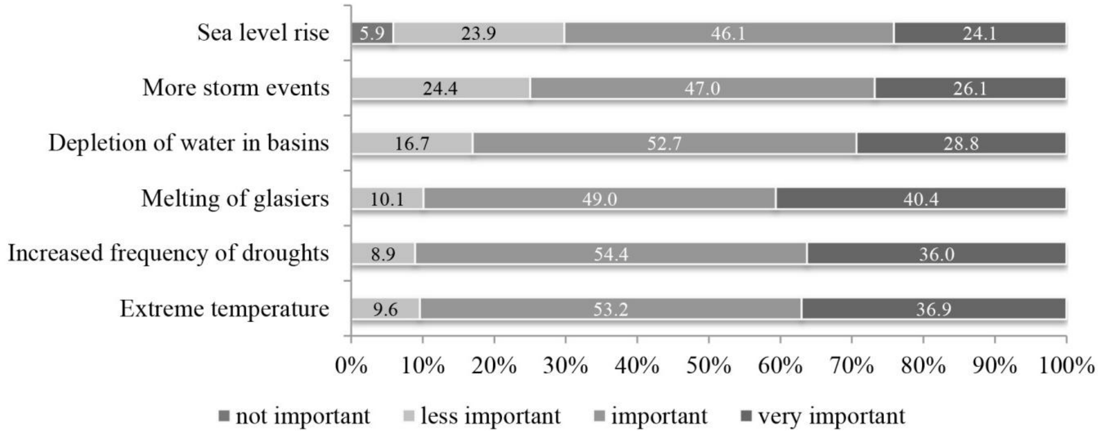

Figure 5. *Climate change impacts (n = 406, only values > 5%)*

Opinions regarding concerns about climate change

Public perceptions of climate change vary internationally. For example, people in France are more worried than those in Germany and Norway, while people in the United Kingdom are less concerned (Steentjes et al., 2017). Public perceptions also fluctuate over time. Social research shows that in the U.S., concern about climate change has fallen considerably since 2008 (Scruggs and Benegal, 2012). However, in many parts of the world in general, concerns over climate change have increased in recent years (Capstick et al., 2015).

Among the people participating in our study, women are generally more worried than men (*Table 2*). Similarly, Shi et al. (2016) also found that women in Germany and the United Kingdom were more worried than men. Recent research suggests that knowledge of climate change has limited impact on shaping climate change concerns (Malka et al., 2009; Kahan et al., 2012; Kellstedt et al., 2008). However, if measured in an area-specific and multidimensional manner, we see that knowledge is indeed an

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important driving force for climate change concerns, even when we control for human values. Likewise, different information dimensions play different roles in shaping the concerns of climate change. In some studies, women were found to have more knowledge than men (McCright, 2010). For this reason, the increased levels of anxiety in women compared to men can be explained by the varying levels of knowledge.

Worldwide, educational attainment is the most powerful determinant of climate change awareness. While understanding the anthropogenic cause of climate change is the strongest determinant of climate change risk perceptions, especially in Latin America and Europe, the perception of local temperature change is the strongest determinant in many African and Asian countries. However, public awareness and other important factors related to risk perceptions underscore the need to develop specific climate communication strategies for each country (Lee et al., 2015).

Ultimately, basic education is vital for public climate literacy, local dimensions of climate change, public participation, and support for climate action. In this study, statistically significant differences were determined between university graduates and others by educational level (*Table 2*). This difference can be explained by the high level of awareness among university students. Similarly, Yayar et al. (2014) found that level of education has a positive effect on awareness. Additionally, married people are more worried than single people (*Table 2*). The main reason for this is the concern that the children of married participants will continue to live in this world. Shi et al. (2016) found that elderly adults in the UK are less concerned about climate change compared to young people. On the contrary, no difference was found according to age groups in our study ($p > 0.05$). Similarly, income was not a variable with significant differences (*Table 2*).

Table 2. *Statistical relations on concern about climate change according to socio-economic characteristics*

Characteristic		n	Test results		
Gender	Female Male	149 257	MW-U* = 17837.0	Z = -1.969	**p = 0.049***
Age	18-25 26-45 46-65 > 65	158 142 98 8	X^{2**} = 2.777	df = 3	p = 0.427
Marital status	Married Single	150 256	MW-U = 17864.0	Z = -2.006	**p = 0.045***
Monthly Income	≤ 1400 TRY 1401-5000 TRY > 5000 TRY	115 265 26	X^2 = 0.835	df = 2	p = 0.659
Education	Graduate Primary, secondary or high school	161 245	MW-U = 18102.0	Z = -2.401	**p = 0.016***

*Mann Whitney U test, **Kruskal Wallis H test, ***$p < .05$

Perceptions of adaptation to climate change

The *Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment Report* (IPCC, 2007) indicated that harmonization studies are of great importance in managing climate change impacts and are the only means of counteracting climate change.

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Importantly, this is the sole reason precautions were taken for climate change adaptation in the past. This emphasizes the importance of adopting a strategic approach at regional and national levels as well as at the global scale. It is vital to ensure harmony between the various sectors and levels of management to ensure that measures are taken in a timely and more effective manner to address climate change adaptation.

In recent years, creating awareness in the public about climate change in Turkey has been about lowering emission levels for compliance with the effects of climate change while adaptation to the results of climate change has been very low. The situation is similar in NGOs, which are mainly active in the management of ecosystem services (MEO, 2010).

In this study, overall, only a few demographic variables were significantly related to adaptation to climate change. Accordingly, married and unmarried people have different opinions about adaptation to climate change ($p < 0.05$). Married participants stated that more adaptation can be achieved. This can be explained by the positive perceptions that married people have developed for their concern about climate change. There were no statistically significant differences ($p > 0.05$) in terms of gender, age, income and educational status (*Table 3*). Although nearly half of the participants have low knowledge levels, there are positive opinions that climate change can be harmonized to a large extent. In other words, in this study there is wide participation (57.4%) in the idea that climate change is an important subject of adaptation. Meanwhile, however, the subject is characterized by different perceptions, interpretations, and evaluations contradicting each other in many respects. For individuals with high climate change awareness, adaptation capacity was also higher (Marshall et al., 2013).

Table 3. *Statistical relations of ideas on adaptation to climate change according to socio-economic characteristics*

Characteristic		n	Test results		
Gender	Female Male	149 257	MW-U* = 18434.0	Z = -0.730	p = 0.465
Age	18-25 26-45 46-65 > 65	158 142 98 8	X^{2**} = 3.254	df = 3	p = 0.354
Marital status	Married Single	150 256	MW-U = 16707.0	Z = -2.271	**p = 0.023*****
Monthly Income	≤ 1400 TRY 1401-5000 TRY > 5000 TRY	115 265 26	X^2 = 1.831	df = 2	p = 0.400
Education	Graduate Primary, secondary or high school	161 245	MW-U = 19236.0	Z = -0.491	p = 0.623

*Mann Whitney U test, **Kruskal Wallis H test, ***p < .05

Such an understanding about the different levels of awareness, knowledge and support among various demographics is important in terms of supporting policies and strategies for harmonization. Those who indicate that they can be adapted have the perception that *"we will have to change our lifestyle"*, *"new technologies will be produced"* and *"we will learn to live with a warmer climate"*.

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One of the most important ways to adapt to the effects of climate change is to protect natural ecosystems. One of the most vulnerable sources of climate change is forest ecosystems (Detten and Faber, 2013). Withana et al. (2007) found that there is a perception that forest fires resulting from climate change will increase. In our work, there was a high perception, with 85% holding the view that forest fires will increase in the Mediterranean basin. According to public perceptions, the most important causes of climate change are air pollution (26%), deforestation (23%) and ozone depletion (15%). In this context, forests are considered as one of the most important approaches to adaptation, as forests are important resources for climate change. The survey indicated that 91.4% of respondents underline that more afforestation is needed. However, 92.6% of the respondents indicated that they would invest in alternative energy sources (wind, solar, geothermal etc.).

Conclusions

In this study, the most important determinants of concern for climate change were gender, educational status, and marital status. Age and income levels were not determinants of concern. These results are a resource for developing climate change policies and actions to increase perception and awareness regarding climate change. Governments should promote changes in perception by increasing the level of consciousness and awareness of nature, in particular about the risks of climate change and how to better manage risk factors. The greater the awareness in the community regarding the effects of climate change, the easier it will be to eliminate risks and improve adaptation.

We found that about half of the participants have little knowledge about climate change. In particular, the levels of knowledge on adaptation are relatively lower. The success of policies and actions by governments and environmental agencies to mitigate and adapt to climate change depends on support by the public. This support is closely related to public perception and awareness. In Turkey, although climate change awareness raising activities from civil society organizations have increased, the effectiveness of the NGOs working in this area is inadequate. A significant number of NGOs have focused on mitigation activities and active management of ecosystem services, but they are few in number. Importantly, many NGOs need to be aware of climate change mitigation as “mitigation” and that awareness raising is needed regarding “adaptation”. While there is considerable public awareness about climate change, it is especially important to promote programs supported by media to raise the level of knowledge regarding climate change adaptation. Increasing the capacity of institutions to address this important issue is keys. In summary, it is necessary to increase public awareness in Turkey regarding climate change.

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EFFECT OF DIFFERENT FERTILIZER TREATMENTS ON BOTANICAL COMPOSITION, HERBAGE YIELD AND HERBAGE QUALITY IN THE EASTERN ANATOLIA REGION PASTURE OF TURKEY

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Abstract. This study was carried out to reveal the effect of different doses of nitrogen, phosphorus and combinations of these doses on the botanical composition, herbage yield and herbage quality in the Eastern Anatolia Region pasture of Turkey. In the study 0, 5, 10, 15 kg da^{-1} nitrogen, 0, 4, 8, 12 kg da^{-1} phosphorus doses and different combinations of these fertilizers (5-4, 5-8, 5-12, 10-4, 10-8, 10-12, 15-4, 15-8, 15-12 kg da^{-1}) were used and the study was conducted over a natural pasture section of Research and Application Center of Bingol University. Experiments were conducted between the years 2014-2017 for 4 years in randomized blocks factorial experimental design with 3 replications and 2 factors. Weight-based botanical composition, green herbage yield, dry herbage yield, crude protein yield, acid detergent fiber (ADF), neutral detergent fiber (NDF) and relative feed values (RFV) were investigated. Present findings revealed that fertilizer treatments (nitrogen and phosphorus doses and combinations) increased legume ratio in botanical composition, green herbage yields, dry herbage yields, crude protein yields and relative feed values and reduced ADF and NDF ratios systematically during the first three years of the experiments. The greatest green herbage yield (758.9 kg da^{-1}), dry herbage yield (458.6 kg da^{-1}) and crude protein yield (63.7 kg da^{-1}) were obtained from 10 kg da^{-1} nitrogen and 8 kg da^{-1} phosphorus treatments. As compared to the control plot, the plot with 10-8 kg da^{-1} N-P treatment had about 115.7% greater green herbage yield and 154.4% greater dry herbage yield. Therefore, 10 kg da^{-1} nitrogen and 8 kg da^{-1} phosphorus treatments could be recommended for fertilization programs to be applied in pastures of Bingol province and the Eastern Anatolia pastures with the similar ecological conditions.
Keywords: *pasture, nitrogen, phosphorus, crude protein yield, relative feed value*

Introduction

Meadow and pastures are commonly used in animal feeding in Turkey. Misuse of pastures for years, excessive and uncontrolled grazing practices have made soil surfaces barren, resulted in serious soil erosion over these surfaces and ultimately resulted in significant yield and quality losses. Then pastures were not able to meet the feed needs of the present animals. Therefore, pastures should urgently be reclaimed to make them sufficient again to meet forage needs of livestock. Fertilization is among the significant methods used for pasture reclamation.

Fertilizers are the primary inputs used in modern agriculture to improve yield levels (Ergene, 1997). They can alone provide about 40% yield increase in agricultural production activities and thus may provide significant contributions to food safety (Karaman, 2012). With the efficient use of fertilizers, about 50-70% yield increase can be achieved for nitrogen and 10-15% for phosphorus (IFA, 1999). Fertilization has versatile impacts on meadow and pastures. Fertilizers can improve herbage yield and quality, provide regular distribution of herbage throughout the grazing periods, ease the establishment of seed-propagated seedlings and enrich the taste of herbage (Altin, 1992; Heady and Child, 1994).

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However, economic analyses should be performed to assess the economy of fertilization practices (level of yield increase achieved with the fertilizer quantity applied per unit area). For this purpose, the economic limit of the fertilizer to be applied should be well identified. The price of the product obtained with additional fertilizer should be greater or at least equal to the price of utilized fertilizer plus the price of other practices performed during fertilization. Otherwise, fertilizer use will not be economic (Sezen, 1995).

In previous studies carried out in Turkey and the other countries, it was stated that pasture vegetation could benefit from precipitations more efficiently, vegetation herbage quantity and quality could be improved with fertilization practices. With reclamation practices, pastures regain their previous statuses and may provide sufficient and quality herbage. To have expected benefits from the fertilizers, economic analyses should be performed, proper fertilizer types and doses should be well identified.

Previous studies also revealed that pasture class was improved from poor or risky to healthy with fertilization practices (Dasci et al., 2009), pasture herbage quality and quantities were improved with fertilization practices (Celik et al., 2001; Hatipoglu et al., 2001; Hatipoglu et al., 2005; Gur, 2007; Balabanli et al., 2010; Andic et al., 2001; Budakli and Carpici, 2011), crude protein of pasture hay was improved (Andic et al., 2001; Hatipoglu et al., 2005; Balabanli et al., 2010; Budakli and Carpici, 2011), ADF (Budakli and Carpici, 2011) and NDF ratios (Balabanlı et al., 2010) decreased and relative feed values increased with fertilization practices.

This study was conducted in Bingol province of Turkey to investigate the effects of different nitrogen doses combined with different phosphorus doses on botanical composition, herbage yield and quality of pastures and to determine proper fertilizer doses for Eastern Anatolia pastures and the other pastures with similar ecological conditions.

Materials and methods

Location, soil and climate

The studies were carried out in the province of Bingol. Bingol province is located in the Eastern Anatolia Region of Turkey. Experiments were set up in April of 2014 over the Research and Application Center of Bingol University about 15 km from Bingol province with an altitude of 1088 m (38.81256°N - 40.53551°E). The map of Turkey and a few photographs of the study area are given in *Figure 1*. Experiments were conducted between the years 2014-2017 for 4 years.

Meteorological data of the research site were received from Bingol Provincial Directorate of Meteorology. As it can be seen from *Table 1,* annual variations in temperature and relative humidity values were similar to each other, but precipitations of the years 2014 and 2017 were lower than the other years.

Soil samples were taken from 0-30 cm soil profile of different sections of the research site. Soil analyses were performed at laboratories of Soil Science and Plant Nutrition Department of Bingol University Agricultural Faculty. Soil analyses revealed that experimental soils were clay-loam in texture. Soils were slightly acidic (pH = 6.266), unsaline (0.014%), poor in organic matter (1.09%) and lime (0.41%) and sufficient in potassium (20.27 kg da^{-1}) and phosphorus (7.60 kg da^{-1}).

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Figure 1. *The map of Turkey and photographs of study area*

Table 1. *Monthly average climate data of Bingol for 2014-2017 years. (Source: General Directorate of Meteorology, Bingol)*

Months	**Average temperature (°C)**				**Total precipitation (mm)**				**Relative humidity (%)**			
	2014	**2015**	**2016**	**2017**	**2014**	**2015**	**2016**	**2017**	**2014**	**2015**	**2016**	**2017**
January	-0.4	-1.8	-2.8	-3.7	143.1	148.2	235.1	63.9	71.3	74.7	75.3	71.1
February	2.0	1.9	2.4	-2.3	82.3	115.8	86.3	32.9	57.7	73.8	73.7	61.6
March	8.6	5.4	7.0	5.9	83.5	154.4	125.5	114.5	62.9	65.9	60.4	64.7
April	13.2	10.9	13.9	10.8	41.6	66.7	45.5	166.4	53.3	58.7	48.4	58.8
May	17.2	16.6	16.3	16.4	63.2	21.2	62.2	92.4	52.1	52.0	57.4	56.2
June	22.3	22.9	22.2	22.6	25.9	8.1	34.6	9.6	36.9	37.0	43.6	39.0
July	27.8	27.9	26.9	28.0	4.0	0.0	3.5	0.0	26.3	26.8	33.4	28.1
August	28.0	27.5	28.0	27.6	0.9	0.6	0.0	2.5	24.0	29.7	28.0	26.0
September	21.3	23.4	19.9	23.5	63.7	0.8	29.1	0.0	36.2	30.2	40.3	26.4
October	13.7	14.3	15.2	13.4	87.3	220.9	4.4	52.8	62.3	68.3	43.0	48.6
November	6.3	14.4	6.4	7.3	99.0	18.9	53.7	99.5	64.3	56.4	47.9	68.5
December	4.6	1.3	-2.2	3.7	63.2	46.2	152.6	74.6	75.7	58.6	73.4	69.8
Total/Ave.	**13.7**	**13.7**	**12.8**	**12.8**	**757.7**	**801.8**	**832.5**	**709.1**	**51.9**	**52.7**	**52.1**	**51.6**

Trial design and treatments

Effects of four different nitrogen doses (0, 5, 10, 15 kg da^{-1}), four different phosphorus doses (0, 4, 8, 12 kg da^{-1}) and combination of these doses (5-4, 5-8, 5-12, 10-4, 10-8, 10-12, 15-4, 15-8, 15-12 kg da^{-1}) on herbage yield and quality of pasture

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vegetation were investigated in this study. Urea (46% N) as nitrogen source and triple super phosphate (43-44% P_2O_5) as phosphorus source were used. The chemical composition of urea is H_2N-CO-NH_2 and chemical composition of the triple super phosphate is $Ca(H_2PO_4)_2.H_2O$. For each treatment and combination, plot size was arranged as 2 x 6 = 12 m^2. Experiments were conducted in randomized blocks with three replications and factorial experimental design with 2 factors. Phosphorus treatments were applied at the beginning of October in which effective precipitations started and nitrogen treatments were applied in April of each year. In the same way nitrogen application for combinations was carried out in April and phosphorus application in October.

Measurements

Vegetation was harvested from randomly placed 33 × 33 cm frame sections (3 frames for each plot) of each plot at earing stage of the dominant vegetation plants at the last week of May. Harvested herbage was weighted to get green herbage weight. Then, samples were dried at 78 °C for 24 h and weighted to get dry herbage weights. Dry weights were used to get dry herbage yields. The dry herbage obtained from the frames was divided into three groups, namely legumes, grasses and other family plants. The rates of botanical composition according to weight were obtained by dividing the dry herbage yield values of plant groups determined in each frame into the total dry herbage yield determined in said frames.

Dried samples were ground and subjected to crude protein, ADF (acid detergent fiber), NDF (neutral detergent fiber) and related quality analyses with the aid of NIRS (Near Infrared Spectroscopy - Foss Model 6500) device at Science and Technology Implementation and Research Center of Dicle University (Basaran et al., 2011; Mut et al., 2010; Cinar and Hatipoglu, 2015). Crude protein ratios were used to get crude protein yield per decare. ADF and NDF values were used to get relative feed value (RFV = (88.9 - (0.779 × %ADF)) × (120 / %NDF) / 1.29 (Van Dyke and Anderson, 2000; Morrison, 2003).

Statistics

Experimental data were subjected to variance analysis with JMP statistical software in accordance with randomized blocks factorial experimental design with three replications and two factors. Means were separated with Tukey's HSD (honest significant difference) test (Steel and Torrie, 1980; Kalayci, 2005).

Results

Botanical composition (%)

Annual botanical composition of pastures with different nitrogen and phosphorus doses are presented in *Figure 2*. As it can be seen from *Figure 2,* fertilizer doses applied to pastures increased the ratio of legumes in botanical composition in the second and third year, but legume ratio decreased again in the fourth year. Phosphorus fertilizer was the primary reason for the increasing legume ratio of the pasture in 2015 and 2016. Since lower precipitation levels were received in 2014 and 2017, legume ratio of those years were lower than the other years. The pasture was dominantly a graminae pasture with about 93.50% graminae species.

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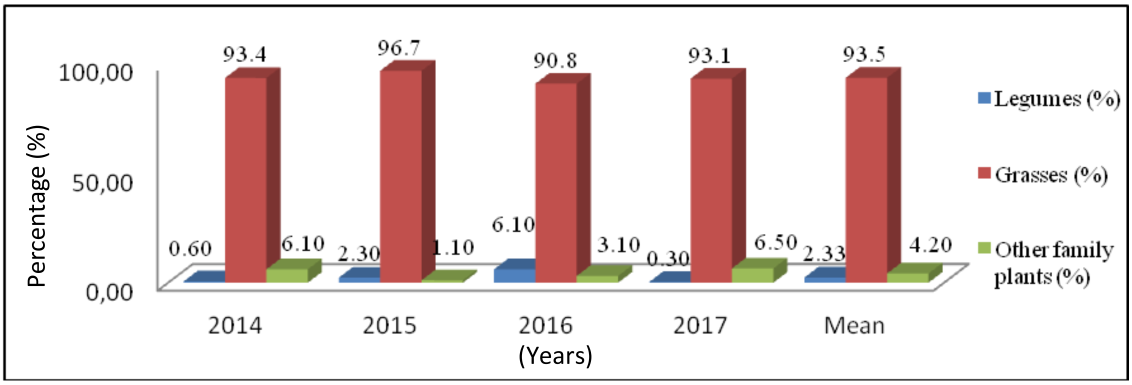

Figure 2. *The botanical composition of the pasture area (%)*

Green and dry herbage yields (kg da^{-1})

Green herbage yields and averages from the pastures treated with nitrogen and phosphorus doses for 4 years are provided in *Table 2* and dry herbage yields and averages are provided in *Table 3*. The effects of nitrogen and phosphorus treatments on green and dry herbage yields were found to be significant and the differences in green and dry herbage yields of the years were also significant (1%). As the average of 4 years, the greatest green herbage yield (758.9 kg da^{-1}) and the greatest dry herbage yield (458.6 kg da^{-1}) were obtained from the plots with 10 kg da^{-1} N and 8 kg da^{-1} P treatments; the lowest green herbage yield (351.8 kg da^{-1}) and the lowest dry herbage yield (180.3 kg da^{-1}) were obtained from the control treatment (0-0 kg da^{-1}).

Table 2. *Green herbage yields and averages of the pasture parcels (kg da^{-1})*

N-P	2014	2015	2016	2017	Mean
0-0	216.3	443.5	478.8	268.7	351.8 e**
0-4	212.5	518.7	634.6	372.7	434.6 de
0-8	359.7	573.0	739.8	528.7	550.3 bcd
0-12	255.7	957.3	936.2	559.0	677.1 abc
5-0	303.7	538.3	876.9	423.0	535.5 b-e
5-4	250.3	638.3	891.6	271.0	512.8 cde
5-8	281.5	636.3	722.9	525.7	541.6 b-e
5-12	419.8	553.3	776.6	793.3	635.8 abc
10-0	309.2	930.3	806.3	449.3	623.8 a-d
10-4	355.3	759.7	949.7	781.3	711.5 ab
10-8	348.5	549.0	1242.3	895.7	758.9 a
10-12	286.2	600.0	920.3	876.0	670.6 abc
15-0	194.8	874.3	687.1	431.3	546.9 bcd
15-4	280.7	739.0	1052.6	640.7	678.3 abc
15-8	329.4	665.7	1090.9	538.3	656.1 abc
15-12	269.5	769.8	1123.2	706.3	717.2 ab
Mean	292.1 D**	671.7 B	870.6 A	566.3 C	600.2

**P < 0.01, CV(%): 22.5

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With fertilization treatments for 4 years, average green herbage yield increased from 351.8 kg da^{-1} to 600.2 kg da^{-1} and the average dry herbage yield increased from 180.3 kg da^{-1} to 321.3 kg da^{-1}. Such values revealed that applied fertilizer doses resulted in 70.6% increase in green herbage yields and 78.2% increase in dry herbage yields. However, considering the plot with the greatest yields (10 kg da^{-1} N and 8 kg da^{-1} P), such an increase as compared to control (0-0 kg da^{-1}) was 115.7% in green herbage yield and 154.4% in dry herbage yield.

Table 3. *Dry herbage yields and averages of the pasture parcels (kg da^{-1})*

N-P	2014	2015	2016	2017	Mean
0-0	130.6	192.0	278.0	120.7	180.3 f**
0-4	147.5	230.3	416.3	166.3	240.1 ef
0-8	177.6	252.3	456.7	226.0	278.2 cde
0-12	140.1	325.3	587.0	230.7	320.8 b-e
5-0	194.0	317.3	393.3	152.7	264.3 def
5-4	157.8	359.0	520.7	129.0	291.6 b-e
5-8	169.0	341.7	523.0	233.3	316.8 b-e
5-12	221.6	430.8	431.0	326.3	352.4 bcd
10-0	154.7	331.3	686.7	152.0	331.2 b-e
10-4	212.6	423.2	561.7	331.7	382.3 ab
10-8	214.3	515.0	622.3	482.7	458.6 a
10-12	170.2	388.0	446.4	383.3	347.0 bcd
15-0	124.0	288.7	702.0	161.0	318.9 b-e
15-4	170.0	471.0	593.0	251.0	371.3 abc
15-8	198.2	498.9	516.3	245.3	364.7 abc
15-12	150.5	407.7	412.0	316.3	321.6 b-e
Mean	170.8 D**	360.8 B	509.1 A	244.3 C	321.3

**$P < 0.01$, CV(%): 21.3

With regard to yields of the years, the greatest green herbage and dry herbage yields were obtained from 2015 and 2016 and the lowest green herbage and dry herbage yields were obtained from 2014 and 2017. Lower precipitation levels of the years 2014 and 2017 were the primary reason of such lower yields in those years. Since 2014 was the first year of the experiments and thus the impacts of fertilizers did not showed up and the precipitations in March and April were lower than the other years, the lowest yields were observed in 2014.

As compared to control treatments (0-0 kg da^{-1}), all nitrogen and phosphorus treatments had greater yields (*Fig. 3*). However, only 10-0 kg da^{-1}, 5-12 kg da^{-1}, 10-4 kg da^{-1}, 10-8 kg da^{-1}, 10-12 kg da^{-1}, 15-4 kg da^{-1}, 15-8 kg da^{-1} and 15-12 kg da^{-1} nitrogen and phosphorus treatments had yield values greater than general average (321.3 kg da^{-1}). Therefore while preparing fertilization plans, cost-benefit analysis should be performed and one of those treatments should be selected.

Crude protein yield (kg da^{-1})

Crude protein ratios and averages of pasture plots treated with different nitrogen and phosphorus doses are provided in *Table 4*. The effects of nitrogen and phosphorus treatments on crude protein yields were found to be significant and the differences in crude protein yields of the years were also significant (1%). The greatest crude protein yield (63.7 kg da^{-1}) was obtained from 10 kg N and 8 kg P treatments and the lowest crude protein yield (23.8 kg da^{-1}) was obtained from the control plot (0-0 kg da^{-1}).

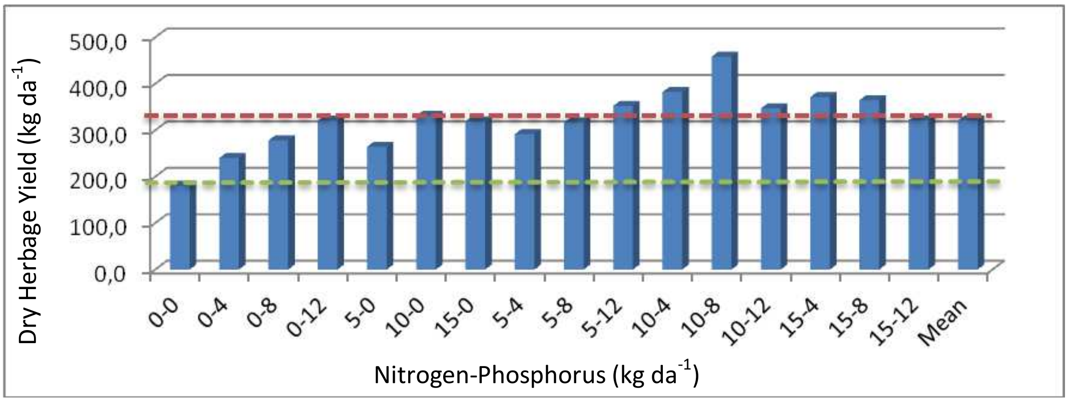

Figure 3. *The dry herbage yield of the pasture parcels (kg da^{-1})*

Table 4. *Crude protein yields and averages of the pasture parcels (kg da^{-1})*

N-P	2014	2015	2016	2017	Mean
0-0	19.4	21.5	36.2	17.9	23.8 f**
0-4	18.0	37.4	55.4	22.3	33.3 ef
0-8	22.0	46.0	54.9	27.8	37.7 def
0-12	19.3	58.5	83.2	27.8	47.2 a-e
5-0	26.7	36.7	56.9	24.0	36.1 def
5-4	23.5	47.6	69.3	17.8	39.5 c-f
5-8	23.5	48.3	72.2	29.5	43.4 b-e
5-12	31.2	59.2	61.0	40.9	48.1 a-e
10-0	25.6	47.8	119.8	30.1	55.8 abc
10-4	35.4	53.4	93.5	40.3	55.6 abc
10-8	35.8	63.7	98.0	57.4	63.7 a
10-12	28.6	51.3	72.2	49.3	50.4 a-e
15-0	19.7	42.5	140.1	32.9	58.8 ab
15-4	27.8	68.7	107.8	38.9	60.8 ab
15-8	35.0	66.0	94.1	40.2	58.8 ab
15-12	27.1	53.3	80.6	52.0	53.2 a-d
Mean	26.2 D**	50.1 B	80.9 A	34.3 C	47.9

**$P < 0.01$, CV(%): 25.6

Considering the crude protein yields of the years, the greatest value (80.9 kg da^{-1}) was obtained in 2016 and the lowest value (26.2 kg da^{-1}) was obtained in 2014. As it was in green herbage and dry herbage yields, the lowest crude protein yield was observed in the first year of the experiments, crude protein yields then systematically increased in the second and third year and decreased again the fourth year. Since crude

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protein yield is obtained by multiplying dry herbage yield with crude protein ratio, crude protein yields were higher at doses and years with greater dry herbage yields.

As compared to control plots, nitrogen and phosphorus-treated plots had greater crude protein yields (*Fig. 4*). However, only 10-0 kg da^{-1}, 15-0 kg da^{-1}, 5-12 kg da^{-1}, 10-4 kg da^{-1}, 10-8 kg da^{-1}, 10-12 kg da^{-1}, 15-4 kg da^{-1}, 15-8 kg da^{-1} and 15-12 kg da^{-1} nitrogen and phosphorus treatments had crude protein yields greater than the general average (47.9 kg da^{-1}).

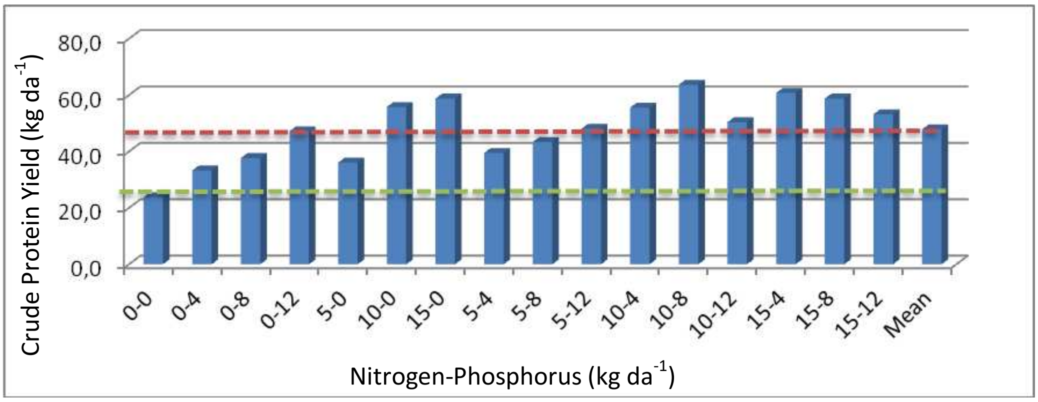

***Figure 4.** The crude protein yield of the pasture parcels (kg da^{-1})*

ADF and NDF ratios (%)

ADF ratios and averages of pasture plots with different nitrogen and phosphorus treatments are provided in *Table 5* and NDF ratios and averages are provided in *Table 6*. The effects of nitrogen and phosphorus treatments on ADF and NDF ratios were not found to be significant. However, the differences in ADF and NDF ratios of the years were found to be significant (1%).

***Table 5.** ADF ratios and averages of the pasture parcels (%)*

Azot-Fosfor	2014	2015	2016	2017	Mean
0-0	38.5	32.6	31.5	34.8	34.4^{NS}
0-4	34.3	35.8	30.8	35.2	34.0
0-8	31.8	35.7	33.6	36.7	34.5
0-12	34.3	31.5	31.1	37.7	33.7
5-0	38.4	33.0	30.8	33.8	34.0
5-4	37.1	31.2	31.3	36.1	33.9
5-8	36.7	33.3	31.0	36.9	34.5
5-12	36.6	34.4	30.0	35.1	34.0
10-0	35.9	32.8	27.4	30.9	31.7
10-4	37.0	30.1	27.4	35.4	32.5
10-8	39.3	27.9	29.3	36.8	33.3
10-12	37.8	31.7	27.3	37.1	33.5
15-0	37.3	32.1	26.9	32.9	32.3
15-4	36.6	30.4	26.5	35.2	32.2
15-8	38.1	31.3	26.9	33.9	32.6
15-12	38.5	30.5	25.3	34.3	32.1
Mean	36.8 A**	32.1 C	29.2 D	35.2 B	33.3

NS: Non significant, **) $P < 0.01$, CV(%): 7.74

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ADF ratios varied between 31.7-34.5% with an average value of 33.3% and NDF ratios varied between 53.3-57.8% with an average value of 56.3%. The greatest ADF and NDF ratios were observed in the first year, the ratios systematically decreased in the second and third year and both ADF and NDF ratios increased again in the fourth year.

Table 6. *NDF ratios and averages of the pasture parcels (%)*

Azot-Fosfor	2014	2015	2016	2017	Mean
0-0	59.4	55.4	57.2	56.9	57.2^{NS}
0-4	59.7	57.8	45.7	57.9	55.3
0-8	62.5	58.5	38.1	60.0	54.8
0-12	56.1	53.9	41.9	61.1	53.3
5-0	59.2	56.9	60.4	54.7	57.8
5-4	59.9	57.4	52.8	57.3	56.8
5-8	61.5	55.4	53.3	61.0	57.8
5-12	59.1	57.6	51.4	57.6	56.4
10-0	56.4	57.5	58.1	50.9	55.7
10-4	58.3	52.6	57.0	59.3	56.8
10-8	59.7	49.9	59.4	60.5	57.4
10-12	57.9	55.2	55.4	59.8	57.1
15-0	55.1	53.2	59.1	51.2	54.7
15-4	56.7	54.9	54.0	56.4	55.5
15-8	57.0	56.3	61.3	56.2	57.7
15-12	55.0	53.8	59.0	56.1	55.9
Mean	58.3 A**	55.4 BC	54.0 C	57.3 AB	56.3

NS: Non significant, **P < 0.01, CV(%): 9.07

Relative feed value

Relative feed values (RFV) and averages of the pasture plots treated with different nitrogen and phosphorus doses are provided in *Table 7*. The effects of different nitrogen and phosphorus treatments on relative feed values were not found to be significant, but the differences in relative feed values of the years were significant (1%). Relative feed values varied between 100.7-112.6 with an average value of 105.3.

With regard to ADF and NDF ratios of the years, it was observed that ADF and NDF ratios systematically decreased, thus relative feed values increased in the first three years. However, in the fourth year, ADF and NDF ratios started to increase, thus relative feed values get into decreasing trend again.

Discussion

Fertilizer doses increased weight-based ratios of the legumes in botanical composition. However, legume ratios decreased in 2014 and 2017 with lower precipitation levels than the other years. Phosphorus fertilizers were reported to increase legume ratios in botanical composition (Hatipoglu et al., 2001; Polat et al., 2007; Kacorzyk and Glab, 2017). However, phosphorus fertilization was recommended for pastures with less than 10% legume ratio in botanical composition (Altin et al., 2005).

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Since the legume ratio in present botanical composition was 2.33%, phosphorus fertilization was not necessary for these pasture plots. Phosphorus supplementation not only meets the phosphorus needs of the plants, but also improves nitrogen efficiency (Black, 1968 and Whitehead, 1995). Therefore, phosphorus fertilizers to be made to enrich the botanical composition should be applied together with nitrogenous fertilizers.

Table 7. *Relative feed values and averages of the pasture parcels*

N-P	2014	2015	2016	2017	Mean
0-0	92.2	106.7	105.5	101.4	101.5^{NS}
0-4	97.0	98.6	135.6	100.5	107.9
0-8	95.5	97.9	154.7	93.6	110.4
0-12	104.1	112.5	143.4	90.6	112.6
5-0	93.0	103.7	100.4	106.6	101.0
5-4	93.2	104.8	114.0	98.8	102.7
5-8	91.2	105.9	113.6	92.0	100.7
5-12	95.1	100.9	119.1	99.8	103.7
10-0	100.8	102.5	108.1	119.1	107.6
10-4	95.8	116.2	111.2	96.2	104.9
10-8	90.9	127.5	103.4	92.6	103.6
10-12	95.6	108.3	114.0	93.8	102.9
15-0	101.0	111.9	107.1	115.1	108.8
15-4	99.1	110.6	121.7	101.5	108.2
15-8	96.7	106.7	103.3	103.6	102.6
15-12	100.2	113.0	109.2	103.4	106.4
Mean	96.3 C**	108.0 B	116.5 A	100.5 C	105.3

NS: Non significant, **$P < 0.01$, CV(%): 11.78

With the fertilizer treatments, green and dry herbage yields increased from the first year, increases went on in the second and third year. However, in the fourth year, both green and dry herbage yields started to decrease again. There are two reasons of these trends. The first one was the lower precipitations of the first and the forth years than the other years. The second one was limited yield increase potential of fertilizers. It is normal to expect a yield increase with fertilizers, but such an increase will not be infinite. Yields reach to a peak value and then a stable yield levels expected. In present study, yields reached to a peak value in the third year, but a table peak levels were not attained in the fourth year because of lower precipitations. Kacorzyk and Glab (2017) carried out a study over the pastures for 10 years and indicated significant relationships between climate factors (temperature and precipitation) and yield parameters and reported lower yields in years with lower temperature and precipitation levels.

It was reported in previous studies that nitrogen and phosphorus fertilizations increased herbage yields of the pastures (Polat et al., 2007), nitrogen treatments alone increased dry matter yields (Aydin and Uzun, 2005; Balabanli et al., 2010; Budakli and Carpici, 2011), nitrogen treatments increased herbage yields of a pasture (with an average yields of 151.5 kg da^{-1}) three folds (Celik et al., 2001). It was reported in previous studies that fertilizers increased dry herbage yield of a pasture from 166.3 kg da^{-1} to 386.4 kg da^{-1} (Hatipoglu et al., 2005), increased green herbage yields from

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538.5 kg da^{-1} to 1228.5 kg da^{-1} and dry herbage yields from 337.6 kg da^{-1} to 808.0 kg da^{-1} (Gur, 2007), increased green herbage yields by 189.5% and dry herbage yields by 163.3% (Altin et al., 2010).

The greatest yields in present study were obtained from 10 kg da^{-1} nitrogen and 8 kg da^{-1} phosphorus treatments. Similarly, Cinar et al. (2005) in a tree-year study reported the greatest yield levels for 10 kg da^{-1} nitrogen and 5 kg da^{-1} phosphorus treatments, Hatipoglu et al. (2005) and Dasci et al. (2009) recommended 10 kg da^{-1} nitrogen treatments. Altin et al. (2005) recommended 7.5-10 kg da^{-1} nitrogen and 5-10 kg da^{-1} phosphorus treatments for high-altitude Eastern Anatolia pastures. Present findings comply with all those earlier ones.

The crude protein yield of control (0-0 kg da^{-1}) plot (23.8 kg da^{-1}) increased to 47.9 kg da^{-1} with fertilizer treatments. Since crude protein yield is obtained by multiplying dry herbage yield with crude protein ratio, the variations in crude protein yields were similar with the variations in dry herbage yields. The crude protein yields were almost doubled (101.3%) with fertilizer treatments. It was reported in previous studies that nitrogenous fertilizers increased crude protein yields (Andic et al., 2001; Polat et al., 2007; Balabanli et al., 2010; Budakli Carpici, 2011).

It was reported in previous studies that phosphorus fertilizers did not have significant effects on ADF and NDF ratios (Budakli Carpici, 2011; Budakli Carpici et al., 2012), nitrogenous fertilizers combined with phosphorus and potassium reduced ADF and NDF ratios (Balabanli et al., 2010) and increasing nitrogen doses decreased ADF ratios of the herbage (Budakli Carpici, 2011). In present study, effects of fertilizer treatments on ADF, NDF ratios and RFV were not found to be significant. Therefore, great variations in ADF, NDF ratios and RFV should not be expected from one-year fertilization plans. Thusly, Heady and Child (1994) reported that fertilizers did not improved chemical composition of the herbage, but increased freshness and leaf ratios and stay-green durations of the herbage. However, multi-year studies may reveal significant variations in ADF, NDF ratios and RFV of pasture herbage.

Conclusion

Present nitrogen and phosphorus treatments increased legume ratio in botanical composition, and also systematically increased green herbage yields, dry herbage yields, crude protein yields and relative feed values and reduced ADF and NDF ratios during the initial three years. There was a direct relationship between the precipitations and herbage yields, the lower values were observed in 2014 and 2017 with lower precipitations than the other years.

The effects of nitrogen and phosphorus doses on ADF, NDF ratios and relative feed values of the years were found to be significant, however, the effects of fertilizer treatments with the same year were not found to be significant. Therefore, significant changes in ADF, NDF ratios and relative feed values should not be expected from one-year fertilization programs.

Nitrogen and phosphorus treatments generally yielded greater values than the control plots. However, cost-benefit analyses should be performed while preparing fertilization programs and the doses yielding greater values than the general averages should be selected.

In present study, the greatest green herbage yield, dry herbage yield and crude protein yield were obtained from 10 kg nitrogen and 8 kg phosphorus treatments.

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Therefore, this combination (10-8 kg da^{-1} N-P) could be recommended for fertilization programs to be applied over the pastures of Eastern Anatolia region or over the pastures with similar ecological conditions.

The effects of applied nitrogen and phosphorus doses on plant nutrients and soil structure were not investigated in this study. Therefore, it is suggested to investigate the effects of recommended 10 kg da^{-1} nitrogen and 8 kg da^{-1} phosphorus doses on the plant nutrients and the soil structure in the future.

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ROOTSTOCK INFLUENCES ON SEASONAL CHANGES IN LEAF PHYSIOLOGY AND FRUIT QUALITY OF RIO RED GRAPEFRUIT VARIETY

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Abstract. Citrus is a conventionally produced fruit crop in extensive agricultural areas in Mediterranean-type agroecosystems. The use of rootstocks for citrus fruits is necessary for profitable production under some limiting factors, such as climactic factors, bad soil conditions, and diseases. In addition, the use of the citrus rootstocks provides a large number of choices to growers to increase fruit quality and yield, obtain early fruiting, uniform cropping and high-density planting, avoid juvenility, and control tree size. The aim of the present study was to evaluate the effects of several citrus rootstocks on the fruit yield, quality, physiological changes in leaves and leaf mineral composition of Rio Red grapefruit. Thus, seasonal changes in the leaf chlorophyll concentration, PSII efficiency (Fv / Fm), stomatal conductance (g_S), leaf temperature, leaf mineral nutrient, fruit yield and fruit quality traits of the Rio Red grapefruit variety grafted onto six commonly used rootstocks (Carrizo citrange, citremon, sour orange, Swingle citrumelo, Troyer citrange and Volkameriana) in citriculture were evaluated. The physiological responses of fully expanded young leaves to rootstocks were significantly affected by seasonal changes. Two-way ANOVA indicated significant main effects of rootstock and season and their interaction ($p \leq 0.05$) on the leaf Chl concentration. Leaf Chl concentrations were lowest in April for all rootstocks. The PSII efficiency slightly decreased in the leaves of Rio Red grafted onto Volkameriana in February. The rootstocks significantly affected seasonal changes in the leaf stomatal conductance. The highest g_S was recorded in October in the leaves of a Rio Red variety grafted onto the Volkameriana rootstock. Significant rootstock effects on leaf Ca, Mg, Mn, Zn and Cu concentrations were observed in the Rio Red variety. The highest leaf Zn concentration (ppm) was recorded in plants on Volkameriana, whereas the lowest Zn concentrations were recorded in plants on Troyer citrange. There were no significant rootstock effects on leaf N, P, K and Fe concentrations. The rootstocks significantly affected the fruit yield of the Rio Red variety in the 2013 and 2014 harvest years. The highest fruit yield was observed in Rio Red grafted onto Carrizo citrange in 2013, whereas it was highest in plants on Troyer citrange in 2014. The fruit weight (g), fruit diameter (mm), total soluble solids (%), juice content (%) and juice color (hue^o) of Rio Red grapefruit were significantly affected by the rootstocks.
Keywords: *stomatal conductance, chlorophyll concentration, fruit quality, fruit juice color*

Introduction

The citrus growing areas of Turkey are situated in the northern hemisphere of the citrus belt. Turkey has very suitable ecological conditions and a high potential for citrus production; in fact, 4.293.007 t of citrus fruit was produced in Turkey in 2016 (TUIK, 2017). The eastern Mediterranean Region produces 80% of Turkey's total citrus fruit and 99% of its total grapefruit. In recent years, there has been a large increase in the exportation of citrus fruits, especially grapefruit. For example, Turkey produced 253.120 t of grapefruit in 2016 (TUIK, 2017) and exported 183.329 t of grapefruit in the

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same year (AKIB, 2017). Star Ruby is the dominant grapefruit variety of Turkey. It is followed Rio Red, which is the latest variety and was derived from ‘Redblush’ by bud irradiation.

Rootstocks have a substantial role in the development of the citrus industry. It is necessary to use rootstocks for citrus fruits to have profitable production under some limiting factors, such as climate, bad soil conditions, and diseases. In addition to these factors, the use of citrus rootstocks provides a large amount of choices to growers, enabling growers to, among other things, increase fruit quality and yield, obtain early fruiting and uniform cropping, avoid juvenility, control the tree size and perform high-density planting (Tuzcu et al., 2005). Choosing a rootstock is an important decision, and local climatic and soil conditions are important factors in rootstock selection. Although any citrus variety can be used as a rootstock, some of them are better suited to specific conditions than the others (Davies and Albrigo, 1994; Lawrence and Bridges, 1974).

Citrus rootstocks have a large impact on scion growth, fruit quality and yield (Castle, 1987; Georgiou, 2002; Forner-Giner et al., 2003; Castle et al., 2009; Hussain et al., 2013). In addition, scion behavior depends in part on the rootstock-induced effects on leaf gas exchange (González-Mas et al., 2009) and chlorophyll content (Garci-Sanchez et al., 2002). The influence of rootstocks on scion photosynthetic capacity may play a key role in citrus plant performances in terms of vigor, crop load, and fruit characteristics and should be considered (Jover, 2012). In the grafted tree, rootstock type affects many traits such as leaf mineral elements (Jr et al., 2003; Toplu et al., 2008). Rootstocks directly affect the ability of plants to uptake water and nutrients from soil. Plant nutrient concentrations in scion cultivars may differ, even though they are grown under the same conditions. For this reason, it is important to determine the effects of rootstocks on plant nutrient statuses to optimize fertilization programs.

Sour orange is the main rootstock (approximately 85%) used in citrus growing in Turkey, because of the high soil clay content and high pH (causing to iron, zinc and manganese chlorosis) of many soils in the country. Trees on sour orange produce excellent quality fruit but are susceptible to citrus tristeza virus (CTV) and poor compatibility with some citrus cultivars; in some cases, these trees produce low fruit yields compared to trees with other rootstocks (Castle et al., 2010; Shafieizargar et al., 2012). To address these problems, researchers and citrus growers have made an effort to look for alternative rootstocks. In the selection of a suitable rootstock, its adaptability to soil conditions and interactive effects with the scion cultivar have to be carefully considered (Shafieizargar et al., 2012). Given that rootstocks are unlike genotypes and modify growth, fruit production, photosynthetic capacity and leaf mineral composition, the present study aimed to determine the effect of several citrus rootstocks on the fruit yield, quality and leaf mineral composition of Rio Red grapefruit. In addition, the rootstock effects on the photosynthetic performance of the scion were assessed based on leaf chlorophyll concentration (Chl), chlorophyll fluorescence, stoma conductance and gas exchange measurements.

Materials and methods

Plant material and growing conditions

‘Rio Red’ grapefruit which is a normal appearing grapefruit (shape and rind texture) variety with attractive dark pink flesh was grafted on the following rootstocks: local sour orange (*Citrus aurantium* L.), ‘Troyer’ and ‘Carrizo’ citranges (*Citrus sinensis*

Osb. × *Poncirus trifoliata* (L.) Raf.), Volkameriana (*Citrus volkameriana* Tan. and Pasq.), Swingle citrumelo (*Citrus paradisi* Macf. x *Poncirus trifoliata*), and Citremon (*Citrus limon* x *Poncirus trifoliata).* The grafted trees were planted in 1997 with 8 × 8 m spacing at the Research Station of Cukurova University, Agricultural Faculty, Citrus Experiment Station, Adana (Latitude, 35°23' N; Longitude, 36°50' E, altitude 27 m), Turkey. A completely randomized experimental design was used, with five replicates for each combination. In the experimental area, the soil was a clay-loam (57% clay, 21% silt, and 22% sand, containing 12% CaCO3), and the soil pH was in the range of 7.39-7.46 at a depth of 0-90 cm and considered slightly alkaline. The salt content of the soil was 0.22 EC (mmhos cm-1). The area has a mean maximum and a mean minimum temperature of 26 and 14.5 °C, respectively, and an average annual rainfall of 465 mm (*Fig. 1*). The trees were irrigated weekly from May to October using drip irrigation. Nitrogen (N) was applied as Ammonium nitrate at a rate of 1.5 kg N tree^{-1} (2/3 in mid-February and 1/3 in mid-May), phosphorus (P) was applied as triple superphosphate at a rate of 1 kg P tree^{-1} (December) and potassium was applied as potassium nitrate (K) at a rate of 1 kg K tree^{-1} (January).

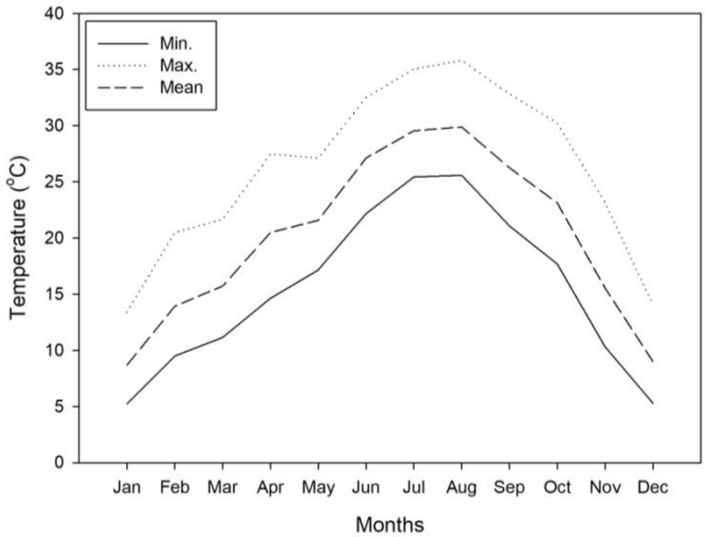

Figure 1. *Minimum, maximum and mean temperature data of the experimental field in 2016*

Fruit yield and quality

Rio Red grapefruit is mid to late-season in maturity with juicy and well pigmented flesh. The effects of different rootstocks on fruit yield were evaluated in 2013 and 2015. Each year, the fruit yield of each tree was determined during the harvesting period. Fruits were harvested at the optimum harvest time (at the end of December or at the beginning of January). The yield per tree (kg/tree) was obtained by weighing the harvested fruit.

A random sample of 25 grapefruits was evaluated for fruit quality analysis. The fruit samples were weighed, the fruit diameter at the equator was measured with a digital caliper, and the rind thickness was measured after cutting the fruit in half with a digital caliper (Mitutoyo CD-15CPX, Mitutoyo America Corporation, USA). The fruit samples were weighed and juiced using a standard juicer; then, the juice was weighed and expressed as a percentage of the total fruit weight. The total soluble solids (TSS) values

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were determined with a portable refractometer (FG-103/113) using a few drops of juice. The total acidity (TA) of the juice was determined by titrating 5 ml of the juice sample with 0.1 N sodium hydroxide (NaOH) and phenolphthalein as the indicator. Fruit juice color determinations were made at 25 ± 1 °C using a Hunterlab Colorflex (Hunterlab, Reston, Virginia, USA). This spectrophotometer uses an illuminant D65 and a 10° observer as references. A sample cup for the reflectance measurements was used (5.9 cm internal diameter × 3.8 cm height) with a path length of light of 10 mm. The results were expressed as the Hue angle [hab = arc tg (b*/a*)], which is determined as starting at the +a* axis and is expressed in degrees; 0% is +a* (red), 90% is +b* (yellow), 180% is -a* (green), and 270% is -b* (blue).

Leaf chlorophyll concentration and photosystem II efficiency

The leaf chlorophyll concentration was estimated using a portable SPAD meter (Minolta, Japan), and the chlorophyll fluorescence parameter ((*Fv' / Fm'*) = quantum yield in light-adapted leaves) was measured with a portable fluorometer (FluorPen FP100, Photon System Instruments Ltd, Drasov, Czech Republic). The evaluations of the chlorophyll concentration and fluorescence measurements were taken in six periods between 2015 and 2016. The same leaves were used to estimate the leaf chlorophyll (Chl) concentrations and PSII efficiency, based on the quantum yield of light-adapted leaves (*Fv' / Fm'*), The chlorophyll concentration and maximum chlorophyll fluorescence efficiency readings for the light-adapted leaves were measured on 10 fully expanded young leaves (third and fourth leaves from the shoot apex) of each replicate at the four chosen time points. Measurements were performed before midday (08^{00}-11^{00} h).

Leaf stomatal conductance measurements (g_s) and leaf temperature (°C)

After the leaf chlorophyll concentration and fluorescence measurements, the same leaves were analyzed by a portable SC-1 leaf porometer (Meter Group, Inc. USA) to estimate leaf stomatal conductance (mmol $m^{-2}s^{-1}$) and leaf temperature (°C). The stomatal conductance evaluations were conducted in five periods between 2015 and 2016. The stomatal conductance efficiency of leaves in the light-adapted stage was measured on 10 fully expanded young leaves (third and fourth leaves from the shoot apex) from each replicate at the four chosen time points (*Fig. 2*). Measurements were performed before midday (08^{00}-11^{00} h).

Figure 2. *A general view of the examined orchard and stomatal conductance measurements by porometer*

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Leaf mineral content

Leaf samples were taken from the fruitless shoots in autumn for the analysis of plant nutri-elements in 2015, according to Chapman (1960). The leaves were washed in a detergent solution, rinsed several times in distilled water, and then dried at 70 °C for 48 h using a thermoventilated oven. The dried leaves were ground (<0.5 mm) and ashed in a muffle furnace at 550 °C for 8 h. The ash samples were digested in HNO_3-$HClO_4$ (3:1 v/v) and filtered through a blue band paper filter (Kacar, 1972). In the extract solutions, sodium (Na), potassium (K), magnesium (Mg), iron (Fe), manganese (Mn) and copper (Cu) concentrations were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Varian Liberty Series II), and the concentrations of phosphorus (P) were determined by UV-VIS spectrophotometry (Barton, 1948). The leaf total nitrogen (N) content was determined by the semi-micro Kjeldahl method (Lees, 1971).

Statistical analysis

Data were subjected to two-way analysis of variance (ANOVA), and significant differences between means were evaluated using Tukey's multiple range test at $p \leq 0.05$ and $p \leq 0.01$. All statistical analyses were performed by using SAS v9.00 statistics software procedures (SAS, 2006), and SigmaPlot® version 11.00 was used for the data presentation.

Results

Fruit yield and quality

Rootstocks significantly affected the cumulative yield. The highest cumulative yield of the Rio Red grapefruit in the three-year period was obtained from the trees on Carrizo and Troyer citrange, followed by those on Swingle citrumelo. Trees on Citremon, sour orange and *Volkameriana* had the lowest cumulative yield (*Fig. 3*). There were statistically significant differences in the fruit yield per tree between rootstocks, except for in 2015 (*Fig. 3*).

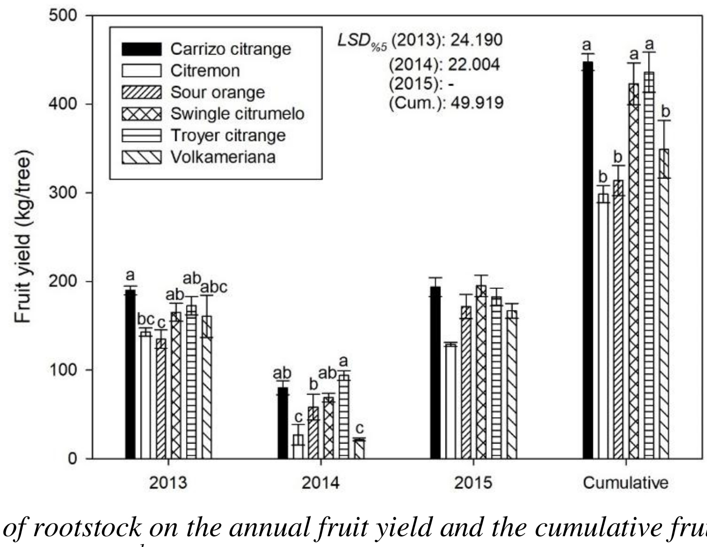

Figure 3. *Effect of rootstock on the annual fruit yield and the cumulative fruit yield over three consecutive years (kg tree^{-1}) of 'Rio Red' grapefruit. Error bars represent ± standard error*

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In 2013, the highest yield was obtained from trees on Carrizo citranges, followed by trees on Troyer citrange and Swingle citrumelo. In 2014, the highest yield was obtained from trees on Troyer citrange, followed by trees on Carrizo citrange and Swingle citrumelo. In 2013, the lowest yield was obtained from trees on sour orange, followed by those on Citremon; in 2014, the lowest yield was obtained from trees on Volkameriana and Citremon, followed by those on Sour orange.

According to the results obtained from this study, Carrizo and Troyer citranges followed by Swingle citrumelo generally had the highest fruit yields during the three-year study period. The lowest yield was found for the trees on Citremon (*Fig. 3*).

The present study showed rootstocks had a significant effect ($p \leq 0.05$) on the fruit weight, fruit diameter, TSS and juice content of ‘Rio Red’ grapefruit (*Table 1*). The effects of rootstocks on the rind thickness, TA and TSS/TA ratio were not statistically significant, and all of the rootstocks gave similar values (*Table 1*). Fruits from trees on sour orange were significantly heavier than those on the other rootstocks, followed by fruits from trees on Swingle citrumelo and Troyer citrange. The lightest fruits were obtained from the trees on Citremon. The fruits from the trees on Troyer citrange had the highest fruit diameters. Trees on Citremon produced fruit with a small diameter. Rootstock had a significant effect on the total soluble solids (TSS). The highest TSS was detected in the fruits from the trees on *Swingle citrumelo* rootstock and the lowest TSS in the juice was obtained from the trees on Volkameriana. There were significant differences among the juice contents of fruits with different rootstocks. The juice content was the highest for fruits from trees on *Swingle citrumelo* and the lowest for fruits on other rootstocks.

Table 1. *Effects of rootstocks on the fruit quality parameters of ‘Rio Red’ grapefruit*

Rootstock	Fruit weight (g)	Fruit Diameter (mm)	Rind thickness (mm)	TSS (%)	TA (%)	TSS/TA	Juice content (%)
Carrizo	354.35 bcd	93.30 ab	6.76	9.50 ab	2.70	3.54	33.95 b
Citremon	335.15 d	90.85 b	6.80	9.58 ab	2.51	3.85	30.29 b
Sour orange	445.60 a	99.14 ab	7.23	8.93 b	2.47	3.67	29.73 b
Swingle	418.40 ab	98.36 ab	6.85	9.95 a	2.69	3.71	42.56 a
Troyer	415.10 abc	101.94 a	7.24	9.40 ab	2.64	3.56	31.84 b
Volkameriana	344.47 cd	94.98 ab	7.70	7.87 c	2.24	3.52	31.73 b
Prob>F	*0.0155*	*0.0362*	*0.6066*	*0.0062*	*0.1403*	*0.8517*	*0.0374*
LSD%5	*62.369*	*8.730*	-	*0.904*	-	-	*7.165*

The fruit juice color was significantly affected by rootstock ($p \leq 0.05$) (*Fig. 2*), and the juice hue^{o} values ranged between 43.11 and 51.84. The juice from trees on Carrizo citrange had the lowest hue^{o} (most red), followed by the juice from trees on Swingle citrumelo. The highest hue^{o} values were obtained from the trees on Sour orange and Volkameriana (*Fig. 4*).

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Leaf chlorophyll concentration and fluorescence measurements (PSII efficiency)

The SPAD measurements were used as an estimate of leaf chlorophyll concentration because there is a positive linear relationship between these two parameters ($r^2 > 0.8$, Jifon et al., 2005). Two-way analysis of variance (ANOVA) indicated a significant main effect of rootstocks, sample date and their interaction ($p \leq 0.05$) on the Chl concentration (*Table 2*).

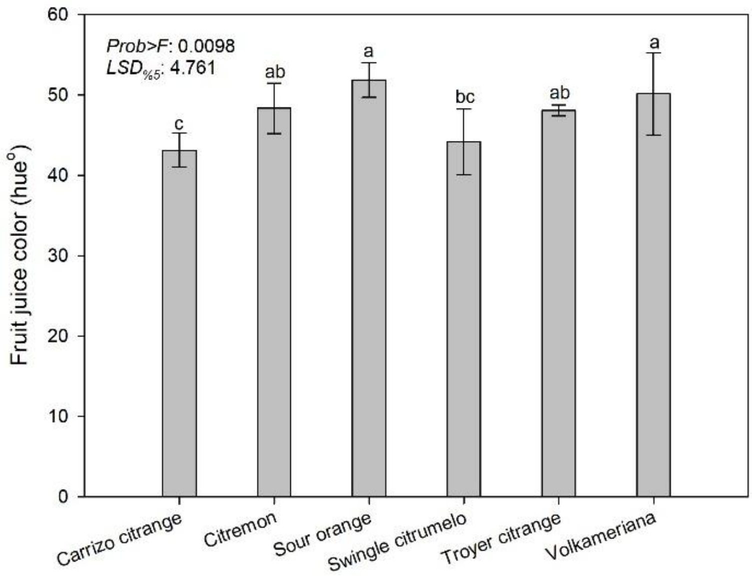

***Figure 4.** Effects of different rootstocks on the fruit juice color of 'Rio Red' grapefruit. Error bars represent ± standard error*

***Table 2.** Results of two-way analysis of variance (ANOVA) of the effects of rootstock (R) and season (S) and their interaction (R × S) for the dependent variables considered. Numbers represent F values. *p < 0.05*

Dependent variable	Independent variable		
	S	**R**	**S x R**
SPAD	296.73*	5.17*	1.84*
Fv'/Fm'	134.14*	4.29*	3.90*
g_s	250.27*	14.27*	6.80*
Leaf temperature	196.23*	4.08*	10.33*

As shown in *Figure 5a,* in the present study, the leaf chlorophyll concentration ranged between 36.20 and 72.25. Rootstocks significantly affected the leaf chlorophyll (Chl) concentration. The scion leaves of shoots grafted onto Troyer citrange and Volkameriana had the lowest Chl concentrations, whereas the leaves of shoots budded on to Citremon and Swingle citrumelo had the highest Chl concentration for nearly all periods. In terms of Chl measurements, statistically significant differences were found between the measurement periods. The lowest Chl was observed in April 2015 and 2016. The highest Chl was observed in February and October every year. According to

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the interaction between the rootstocks and the sampling date, Troyer citrange has the lowest value of Chl in all periods (*Fig. 5a*).

At the end of the experiment, the PS II efficiency ranged between 0.503 and 0.737. Two-way analysis of variance (ANOVA) indicated a significant main effect of rootstocks and sampling date and their interaction ($p \leq 0.05$) on the PS II efficiency (*Table 2*). Rootstocks significantly affected the PS II efficiency of Rio Red grapefruit. The maximum quantum yield of PSII was the smallest in the leaves of shoots grafted onto Volkameriana and Troyer citrange; the highest maximum quantum yield of PSII was observed in the leaves of shoots grafted onto Sour orange and Citremon (*Fig. 5b*). In terms of measurement period and the maximum quantum yield of PSII, in 2015 and 2016, the highest values were in July and October, followed by those in April. The smallest values were observed in February 2015 and 2016.

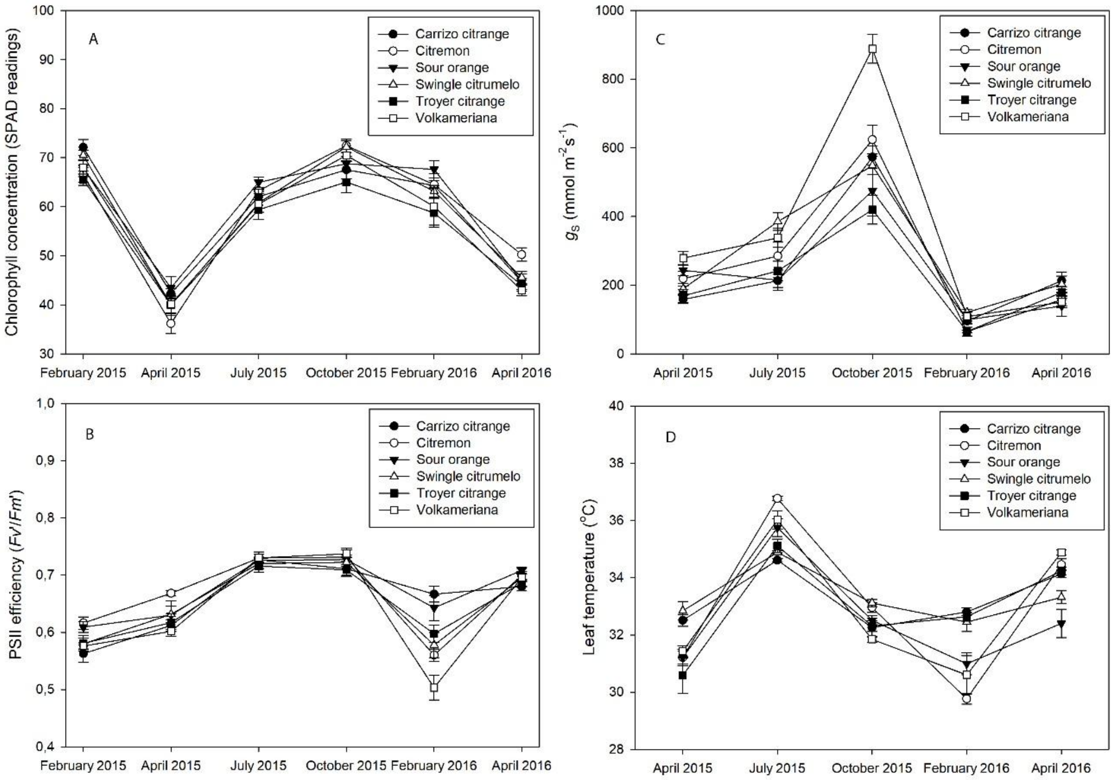

Figure 5. *Rootstock effects on the Chlorophyll concentration (A), PSII efficiency (B), stomatal conductance (C), and leaf temperature (D) of 'Rio Red' grapefruit. Error bars represent ± standard error*

Leaf stomatal conductance measurements (g_s) and leaf temperature (^oC)

As shown in *Table 2* in the present study, two-way analysis of variance (ANOVA) indicated a significant main effect of rootstocks and sampling date and their interaction ($p \leq 0.05$) on the leaf stomatal conductance and leaf temperature (°C). There were also significant differences among rootstocks in terms of leaf stomatal conductance (g_S). The highest leaf stomatal conductance values were recorded in the trees on Volkameriana in almost all measurement periods. The lowest g_S values were recorded in the leaves of shoots on Troyer citrange, followed by those in the leaves of shoots on Carrizo. In terms of measurement period, the highest *gs* was recorded in October 2015; the lowest g_S was recorded in February 2016, followed by April 2016. According to the interaction between the rootstocks and the sampling date, Troyer citrange had the lowest *gs* value in all periods (*Fig. 5c*). When all the measurement periods were examined, a decrease in leaf temperature due to the decrease in air temperature was clearly identified in the leaves of the trees grafted onto the Volkameriana and Citremon rootstocks. Decreases in stoma conductance were observed during periods when leaf temperature increased (*Fig. 5d*).

Leaf nutrients concentration

Leaf macro nutrients

The impact of rootstocks on the macronutrient concentrations of Rio Red leaves were significant, except for the N, P and K values (*Table 3*). There was no significant difference between the leaf N-K contents and rootstocks, but high N and K contents in the leaves of trees grafted onto the Volkameriana rootstock were found. The Ca and Mg contents in the leaves were affected by rootstocks. The leaf Ca content was significantly highest in trees on sour orange, while it was the lowest in trees on Volkameriana, followed by trees on Troyer citrange. The magnesium (Mg) contents of the trees on the Troyer and Carrizo citranges were significantly higher than those of trees on all other rootstocks, while the minimum Mg contents were recorded in trees on Volkameriana and Swingle citrumelo.

Table 3. *Rootstock effects on the leaf mineral nutrients of 'Rio Red' grapefruit*

Rootstock	%N	% P	% K	% Ca	% Mg	ppm Fe	ppm Mn	ppm Zn	ppm Cu
Carrizo	2.42	0.11	0.74	5.13 ab	0.34 a	85.70	46.10 cd	24.86 bc	13.82 a
Citrumelo	2.50	0.11	0.88	5.03 ab	0.32 c	71.54	48.53 bc	26.12 b	11.35 ab
Sour orange	2.56	0.10	0.76	5.35 a	0.25 b	73.17	51.17 ab	25.27 bc	12.67 a
Swingle	2.46	0.10	0.81	4.96 ab	0.23 c	74.22	49.32 b	26.05 b	13.78 a
Troyer	2.58	0.11	0.85	4.62 bc	0.35 a	70.36	44.82 d	24.47 c	13.10 a
Volkameriana	2.73	0.11	1.00	4.16 c	0.21 c	77.04	53.47 a	28.76 a	8.37 b
Prob>F	*0.6518*	*0.1466*	*0.1477*	*0.0244*	*0.0001*	*0.6620*	*0.0002*	*0.0005*	*0.0230*
LSD%5	-	-	-	*0.560*	*0.022*	-	*2.786*	*1.381*	*2.781*

Leaf micro nutrients

The concentration of micro-nutrients in the leaves of the Rio Red grapefruit cultivar was also significantly affected by rootstock-scion combinations, except for Fe (*Table 3*). The Rio Red/Volkameriana combination accumulated the highest scion leaf Mn and Zn concentrations, but the lowest concentrations of these micronutrients were recorded in the Rio Red/Troyer citrange combination. The leaf Cu content was significantly highest in trees on the Carrizo and Troyer citrange, Swingle citrumelo and sour orange rootstocks, while it was the lowest in trees on the Volkameriana rootstock, followed by the Citremon rootstock.

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Discussion

Yield is the leading agronomic property for producers (Koepke and Dhingra, 2013) Most horticultural characters, such as tree growth, yield and quality, are influenced by rootstocks (Davies and Albrigo, 1994). In this study, rootstocks induced significant changes in fruit yield and some quality parameters. The highest cumulative yield of the 'Rio Red' grapefruit in the study period of three consecutive years (2013, 2014 and 2015 seasons) was obtained from the trees on the Carrizo and Troyer citranges and the Swingle citrumelo. Trees on the Citremon, Sour orange and Volkameriana rootstocks had the lowest cumulative yield. Previous studies have found that Carrizo citrange has positive effects on the fruit yield of Redblush and Marsh seedless grapefruits (Fallahi, 1992; Yalcın and Hızal, 1994; Tuzcu ve Toplu, 1999). In addition, in their study of Marsh seedless grapefruits, Mehrotra et al. (1999) reported that the Carrizo citrange rootstock had positive effects on yield. Despite these results, Ramin and Alirezanezhad (2005) reported that Volkameriana was the most productive rootstock for Marsh seedless and Redblush grapefruit. Volkameriana is a productive rootstock, and trees on Volkameriana may show high productivity. In this study, the low yield of trees on Volkameriana was probably due to the extremely low winter temperatures in the 2014 and 2015 seasons. The results obtained for sour orange productivity are in agreement with those of Louzada et al. (2008), who reported that 'Rio Red' trees on sour orange had the lowest yields compared to the other rootstocks evaluated, except for Goutou sour orange. In addition, Sharma et al. (2016) reported that sour orange was a low-yield rootstock for the Redblush grapefruit variety. Yield is controlled by rootstocks in fruit crops (Bassal, 2009) and the rootstocks induced changes in yield depending on the crop and type of rootstocks (Whiting et al., 2005). Based on the results of the current study on Rio Red grapefruit scions, Carrizo and Troyer citranges are very promising alternative rootstocks.

Fruit size is a valuable trait for both producers and consumers. Fruit size is one of the most important factors affecting the marketability of fresh citrus fruits, and medium- to large-sized fruits contribute maximum consumer returns in fresh markets. The present study showed rootstocks had significant effects on the fruit weight. The heaviest fruits were obtained from the trees on Sour orange, followed by the trees on Swingle citrumelo and Troyer citrange, while the Citremon rootstock produced the lightest fruit. Fruit diameter was significantly affected by rootstocks, and fruits from the trees on Troyer citrange had the highest fruit diameters. The trees on Citremon produced fruit with a small diameter. The results obtained in the present study are in agreement with those described in a previous report by Sharma et al. (2016), who studied the effects of rootstocks on the fruit quality of Marsh seedless grapefruit and indicated that Marsh seedless on Troyer citrange produced the heaviest fruit. Similar results have been reported for Redblush (Fallahi et al., 1989; Yalcin and Hizal; 1994), Marsh seedless (Mehrotra et al., 1999), and Lane Late (Emmanouilidou and Kyriacou, 2017), for which the highest fruit weights were recorded from trees on Carrizo and Troyer citranges. In their study of Marsh seedless grapefruit, Economides and Gregoriou (1993) concluded that the largest and heaviest fruits were produced by trees on the rough lemon group, Palestine sweet lime, Rangpur lime, Carrizo citrange and *C. volkameriana*. The TSS contents were higher in fruits from trees on Swingle and lower in fruits from trees on Volkameriana, followed by sour orange. The juice content per unit weight was higher for trees on Swingle citrumelo and lower for those on *C. volkameriana*. These results are in agreement with those of previous work, in which the fruits with the highest TSS contents were from trees on Swingle citrumelo (Economides and Gregoriou, 1993; McCollum et al., 2002), and the lowest TSS contents were from trees on Volkameriana (Fallahi and Rodney, 1992; Stuchi et al., 2002; Ramin and Alirezanezhad, 2005; Emmanouilidou and Kyriacou, 2017). Variations in juice content due to rootstocks have also been reported in citrus (Economides and Gregoriou, 1993; Sharma et al., 2016). Fruit quality characters are important variables, as grapefruit is grown for fresh consumption and processed for its deep red coloration (Castle, 2012), which is highly influenced by the rootstock (Forner-Giner et al., 2003). Carrizo citrange and Swingle citrumelo produced fruit with more color, and the lowest color values were obtained from the trees on Sour orange and Volkameriana. Other researchers have also reported (Tuzcu et al., 1999; García-Sánchez et al., 2006; Bassal, 2009) that the fruits with the best color values were obtained from trees grafted on citranges.

Chlorophyll fluorescence is very useful to study the effects of environmental stresses on photosynthesis in plants (Bron et al., 2004). The maximum quantum yield of PSII was the smallest in the leaves of shoots grafted onto Volkameriana and Troyer citrange. Pestana et al. (2001) indicated that the chlorophyll fluorescence parameters of 'Newhall' orange grafted on 'Troyer' citrange slightly decreased under Fe-deprived conditions. González-Mas et al. (2009) reported clear decreases in the leaf chlorophyll fluorescence parameters of Navelina orange trees grafted onto Carrizo citrange growing in calcareous soil. In addition, Pestana et al. (2001) indicated that the chlorophyll fluorescence parameters of Newhall orange grafted onto Troyer citrange were slightly decreased under Fe-deprived conditions.

Our results demonstrate that rootstocks significantly affected leaf chlorophyll (Chl) concentrations. The scion leaves of shoots grafted onto Troyer citrange and Volkameriana had the lowest Chl concentrations, whereas the leaves of shoots budded onto Citremon and Swingle citrumelo had the highest Chl concentrations for nearly all periods. The results obtained in the present study are in agreement with those described in a previous report by Aboutalebi et al. (2012), who reported that rootstocks had significant effects on the leaf chlorophyll content of Valencia orange. In addition, González-Mas et al. (2009) indicated that the leaf Chl concentration of Navelina orange grafted on Carrizo citrange and grown in the calcareous soils of the Mediterranean region was lower than those of the other rootstocks evaluated. Considering the soil of the study area had 12%$CaCO_3$ and a slightly high pH level, the decreases in the leaf Chl concentrations of Rio Red grafted onto Troyer citranges are in agreement with the results of González-Mas et al. (2009).

There were also significant differences between the rootstocks in terms of leaf stomatal conductance (g_s). The highest the g_s value was recorded in the trees grafted onto Volkameriana; the lowest g_S values were recorded in the leaves of shoots on 'Troyer' citrange followed by the leaves of shoots on Carrizo. Chouliaras et al. (2004) indicated that gs was significantly reduced in two orange cultivars on Swingle citrumelo. Similarly, Incesu et al. (2015) reported that 'Valencia' scion grafted on 'Swingle' citrumelo had a significantly lower stomatal conductance (mol m^{-2} s^{-1}) that scion grafted onto other rootstocks grown in calcareous soil. Fe deficiency has a noticeable effect on the g_s of leaves because of the importance of Fe in many enzyme systems and in energy transfer during photosynthesis. Trees that were not affected by the calcareous soil had higher chlorophyll contents and photosynthesis values, which were associated with increased g_s values, as previously observed (Flexas et al., 2001; Afrousheh et al., 2010). Regarding the measurement periods, g_s values decreased in

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periods when the leaf temperature became too high (>35 °C). The highest response of stoma conductance to changes in leaf temperature was detected for the Citremon and Volkameriana rootstocks. This may be due to the lemon hybridization of both rootstocks. In citrus plants, the optimum temperature for g_s is ≈ 30 °C (Khari and Hall, 1976). In addition, Ribeiro et al. (2004) indicated that the g_s values of sweet orange seedlings decreased as leaf temperature increased.

Rootstock had a meaningful impact on nutrient absorption, and the nutrient uptake efficiency varied with rootstock (Parvaneh et al., 2011). Taylor and Dimsey (1993) indicated that rootstock type and scion cultivar had different effects on the mineral element concentrations of scion leaves. In our experiment, there was no significant difference between the leaf N-K contents and rootstocks, but considerably high N and K contents in the leaves of the trees grafted onto the Volkameriana rootstock were observed. Barakat et al. (2013) reported that Volkamer lemon generally produced significantly higher N, P, K and Mg contents compared to sour orange. Georgiou (2000) showed that P and K in Nova mandarin leaves were induced by the Volkamer lemon rootstock. Similar results have been detected for Ca in previous studies, and higher Ca contents have been reported for trees on sour orange than for trees on other rootstocks (Toplu et al., 2008; Barakat et al., 2013; Dubey and Sharma, 2016). Citrange rootstocks produced higher concentrations of leaf Mg. These findings are in agreement with those of Taylor and Dimsey (1993) in Valencia and Navel oranges, Toplu et al. (2008) in Rhode Red Valencia and Valencia late, and Dubey and Sharma (2016) in Kagzi Kalan lemon.

Rootstocks significantly influenced the leaf Mn, Zn and Cu contents. The lower foliar Mn in trees on Troyer citrange reported by Iyengar et al. (1982), Toplu et al., (2008), Dubey and Sharma, (2016) is also supported by our results. Similar to previous studies, trees on Volkameriana had sufficient levels of leaf Zn, which were significantly higher than those in trees on Troyer citrange (Platt, 1981; Fallahi and Rodney, 1992). When compared to the budding of trees on Volkameriana, the budding of trees on citranges and other trifoliate orange hybrids accumulated the highest leaf Cu contents in Rio Red. This result is compatible with the findings of Fallahi and Rodney (1992). Differences in leaf nutrient concentrations might be due to the alteration of root morphology and hydraulic conductance due to scion morphology and physiology (Save et al., 2000; Sharma et al., 2016).

Conclusion

In this study, we evaluated the influences of rootstock on the fruit yield, quality, leaf mineral composition and photosynthetic performances of ‘Rio Red’, which is an economically important grapefruit cultivar in Turkey. The results indicated that rootstock had significant effects on the evaluated variables. Fruit yield and quality were affected by the use of rootstocks. Carrizo and Troyer citrange and Swingle citrumelo rootstocks achieved the highest cumulative yields and improved the quality of fruits. The fruits with the lowest quality were obtained from trees on Volkameriana. In addition, fruit juice color is an important variable because grapefruit is grown for fresh consumption and processed because of its deep red color. Carrizo citrange produced fruit with more color than the fruit of other rootstocks. Rootstocks had different abilities to utilize plant nutrient elements; considering the yield, fruit quality and mineral nutrient uptake, Carrizo and Troyer citranges are suitable rootstocks for Rio Red

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grapefruit grown under Mediterranean region conditions. Based on the chlorophyll content, gas exchange and the chlorophyll fluorescence parameters, “Rio Red” scion grafted onto the ‘Troyer’ and Carrizo citranges were affected by calcareous soil. On the other hand, trees on the sour orange rootstocks were shown to perform much better in calcareous soil. Considering its overall performance and its CTV resistance, Carrizo is a promising rootstock alternative to Sour orange, particularly in calcareous soils.

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ACCUMULATION OF TRACE METALS IN MANGROVE PLANT *SONERATIA CASEORALIS* IN SONGKHLA LAKE, THAILAND

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Abstract. Mangrove sediment and mangrove plant *Soneratia caseolaris* in Songkhla Lake, Thailand have been increasingly threatened by trace metal pollution. Mangroves receive trace metal pollution from upstream areas and the sea. However, little is known about the mangrove plant's capacity to uptake and store trace metals. In this study, the concentrations of As, Cd, Pb and Zn in mangrove plant parts (leaf, root, and bark) of *Sonneratia caseolaris* and sediments were determined. Sediment and plant samples were digested using total metal concentration procedure and ICPMS techniques. It was found that the maximum values of total concentrations of As in mangrove sediments were classified as heavily polluted according to USEPA. The highest concentrations of As and Zn were found to be in bark whereas Cd and Pb in the root. The fractions of all the metals were measured using BCR techniques and their results revealed that the bioavailability fraction was greater than the residual fraction. The order of the amounts of trace metals present in the fractions was as follows; As: Oxidizable > Reducible > Residual > Exchangeable and the order of Cd: Exchangeable > Reducible > Oxidizable > Residual. Both BCF and TF values showed limited accumulation of these elements in their aboveground parts and thus presented a low food chain hazard except at Phawong and U-Taphao canals.
Keywords: *BCR techniques, coastal lagoon, BCF, TF, food chain*

Introduction

Mangrove forest is a group of trees and shrubs and commonly found in coastal areas. Mangrove is like a connection place between land and sea and important to the ecosystem especially to the aquatic population. Mangroves are an important food source of aquatic fauna in the tidal areas. In addition, the elongated root system makes it possible to trap sediment and trap pollutants such as trace metals. Generally, mangrove increases metal accumulation in sediments by modifying the soil acidity, redox potential, organic contents and salinity (Zhou et al., 2010; Sekomo et al., 2011) and subsequently reduces metal exposure to adjacent aquatic environment (Nath et al., 2013). Active uptake requires metabolic energy and takes place against a chemical gradient. The rate of heavy metals uptake is corresponding with its available pool at the root surface (Lanno et al., 2004). For example, when As, Cd and Pb are present in soluble forms in pore water, plant roots are able to take up great amounts of these metals. The uptake rates increase as concentration in the solutions increase. The plant uptake is also affected by temperature. According to Pendias and Pendias (2001), a higher ambient temperature influences a greater uptake of trace metals by plants.

It is widely recognized that the adverse impact of heavy metals does not simply depend on their concentrations but critically on their bioavailable fraction of the total metal

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concentration in sediment (Kim et al., 2015; Tokalioglu et al., 2000). Toxicological bioavailability is defined as a bioaccumulation effect of heavy metals within plants. Among various pollutants, trace metals with persistence, non-biodegradation, toxicity and bioavailability pose a major threat to the mangrove biodiversity and human health (Liu et al., 2014). Mangroves have the capacity to contain trace metals by accumulating them in their sediments and plant tissues (MacFarlane et al., 2003). Accumulation of these metals also can cause potential problems to animals and human through food chain interaction (Hosani and Anouti, 2014).

Soneratia caseolaris is one of the mangrove trees found in Songkhla lake, Thailand and a lead or pioneer species. In the Outer Section of Songkhla Lake, *Soneratia caseolaris* is generally found at the mouth of the Phawong canal and U-Taphao canal and Kuannieng District, the Middle Section of Songkhla Lake is found in Kukhut non hunting areas as well. In the Songkhla Lake, there were several studies on trace metal contamination in the mangrove sediment but little is known about heavy metal uptake by mangrove plant (*Sonneratia caseolaris*). Therefore, the aims of this study were to determine the bioavailability of As, Cd, Pb and Zn in mangrove sediments of the Songkhla Lake using modified European Community Bureau of Reference (BCR) sequential extraction procedure and to analyze the accumulation of trace metals (As, Cd, Pb and Zn) in mangrove sediment and mangrove plant parts (leaves, root, bark) of *Sonneratia caseolaris.*

Material and method

Study area

The Songkhla Lake is a shallow coastal lagoon located in the southern part of Thailand and occupies approximately 1,042 km^2 formed by an interaction of land and ocean processes over geological time (Sompongchaiyakul and Sirinawin, 2007). The lake is divided into 4 sections; Thale Noi, Inner Section, Middle Section and Outer Section. The salinity ranges from fresh water in Thale Noi to saline water in the Outer Section. The Outer Section is connected to the Gulf of Thailand through a deep and narrow outlet at Songkhla city. The system receives runoff and wastewater from the surrounding watershed. The runoff carries sediment onto the lakes, which will be transported through the lake by the general movement of water towards the Gulf of Thailand. According to the EmSong Project (1998), the average residence time of the water mass in the Inner, Middle and Outer Lakes is 55, 28 and 15 days respectively. The average sediment accumulation rate of in the lake is 2.5-3.5 mm/year and at some places it is higher than 15 mm/year (Sojisuporn, 2005). The average depth of Inner, Middle and Outer Lakes is 2, 1.5 and 1.5 meters, respectively. The lake is facing problems of environmental degradation due to urban expansion and industrialization. Sources of pollutants to the lake system include municipal wastes from Hat Yai and Songkhla cities, industrial wastes mainly related to the rubber industries, seafood processing industries, mining activities and pollution from boats in Songkhla harbour (Sompongchayakul and Sirinawin, 2007; Pradit et al., 2010).

Sample collection and preparation

The sediment cores were collected from 4 stations (*Fig. 1*) which were located in the Middle Section (station 1: Kukhut non hunting area) and in the Outer Section (station 2: Kuannieng, station 3: Phawong canal and station 4: U-Taphao canal). The selected station

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based on the appearance of *Soneratia caseolaris* was found in the Middle Section (Kukhut non hunting area; station 1) and in the Outer Section of the lake (Kuannieng, Phawong and U-Taphao canals). The survey was carried out in November, 2016.

The surface of mangrove sediment (0-10 cm) was taken from the active root zone using a Plexiglas tube (5 cm diameter and 30 cm length) by pushing into the bottom sediment with 3 replicates for each station. After that the sediment cores were sectioned 5 cm (1 core got 2 pieces) and then packed into a plastic zip lock, labeled and put in to a plastic container before being transported to the laboratory.

Mangrove plant*; Sonneratia caseolaris* samples consisted of root, bark and leaf. The leaves (old leave = yellow colour, mature leave = green colour and young leaves) were collected by hand pick (in total about 30 leaves per stations) whereas roots (about 10 roots per station) and barks (about 5 pieces; each piece about 3 × 5 cm) were collected using a ceramic knife. The plant samples were stored in clean plastic bags. The 200 g wet sample (plant and sediment) of each station was put on tray and then oven dried at 50 °C, homogenized and grinded in a mortar and sieved through a 1 mm nylon sieve and then stored in plastic zip locked bags with label and kept in desiccators prior to further analysis.

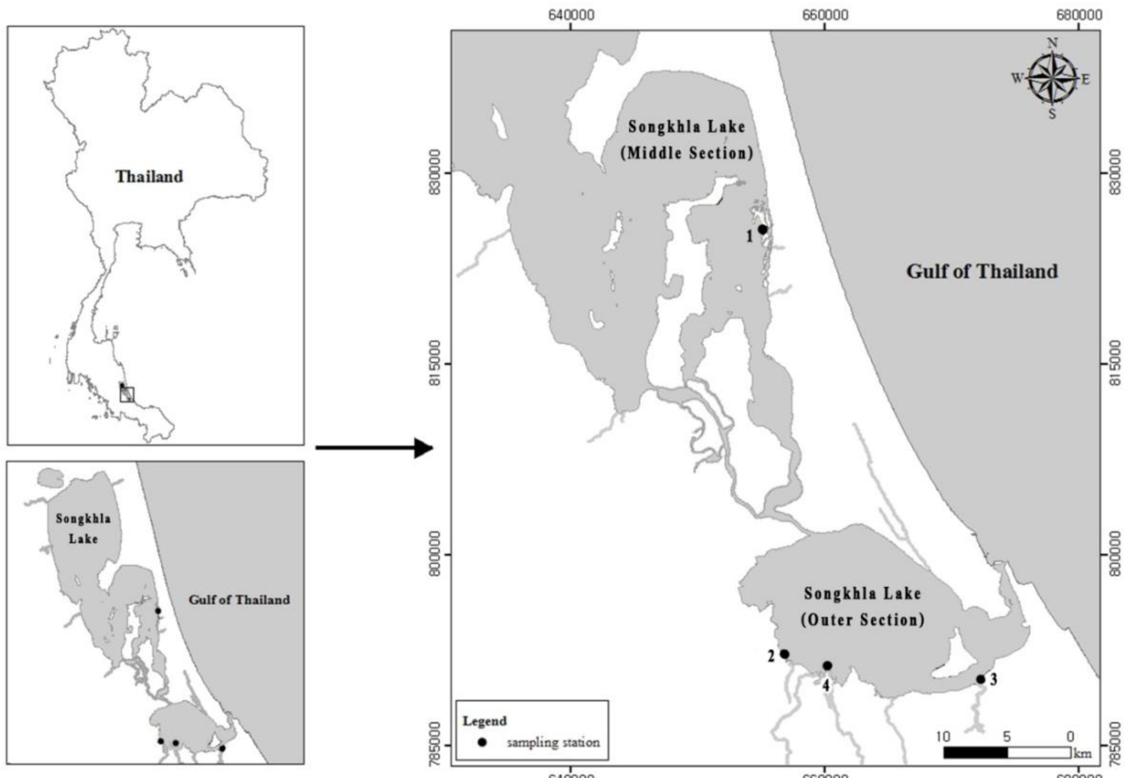

***Figure 1.** Map of sampling station in Songkhla Lake (station 1: Kukhut non hunting area; station 2: Kuannieng; station 3: Phawong canal; station 4: U-Taphao canal)*

Sample analysis

The bulk sediment samples (about 0.1 g) were totally digested in an acid mixture (5 ml HCl: 8 ml HNO_3: 2 ml HF) and analyzed for trace metals, according to the published methodologies of Noriki et al. (1980) Chojnacka et al. (2004) and Loring and Rantala (1992), with some modifications (the Teflon vessels were heated in an oven instead of a

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microwave until complete digestion of the sample occurred). The amounts of trace metals (As, Cd, Pb and Zn) were measured by ICP-MS (Perkin Elmer Elan 9000). The sediment certified reference material; SRM 1646/estuarine sediment was similarly analyzed to validate the accuracy of the analytical procedure .The analytical values were within 90 %of the certified values, which demonstrated the validity of the methods applied. Plant sample was digested in a closed teflon vessel. Sample of 0.5 g was dissolved in 5 ml of HNO_3 (Chojnacka et al., 2004). Concentrations of As, Cd, Pb and Zn in digested samples were then determined by Inductively Coupled Plasma Mass Spectrometry (ICPMS). Standard reference material for plant (Peach Leaves: NIST 1547) was digested following the same procedure and analyzed for metal concentrations to validate accuracy and precision of the analysis.

Sequential extraction of mangrove sediment was performed using the revised BCR protocol (Rauret et al., 1999) briefly describes as follows:

Step 1 (Fraction 1: Exchangeable and acid soluble fractions): 40 ml of 0.11 mol/l acetic acid was added to 1 g of the air-dried sediment sample in a 50 ml polyethylene centrifuge tube. The tube was shaken for 16 h at room temperature at a speed of 23 ± 1 rpm. The extract was separated from the solid residue by centrifugation (3000 rpm for 20 min), decanted into a polyethylene bottle and stored at 4 °C. The residue was washed by shaking with 20 ml of distilled water for 15 min and centrifuging after which the supernatant was discarded.

Step 2 (Fraction 2: Reducible fraction): 40 ml of 0.5 mol/l hydroxylamine hydrochloride (adjusted to pH 1.5 by addition of a fixed amount of HNO_3) was added to the residue from step 1, and the extraction performed as in step 1 above.

Step 3 (Fraction 3: Oxidizable fraction): 10 ml of 8.8 mol/l hydrogen peroxide was added in aliquots to the residue from step 2. The vessel was covered loosely and the contents were digested at room temperature for 1 h with occasional agitation. It was then placed in a water bath and digested at 85 °C until the volume was reduced to less than 3 ml. Another 10 ml of the hydrogen peroxide was added, and further heated to near dryness. Thereafter, 50 ml of 1.0 mol/l ammonium acetate (adjusted to pH 2 with HNO_3) was added, and the extraction was performed as in the previous steps.

Step 4 (Fraction 4: Residual fraction): The residue from step 3 was transferred into a suitable vessel and the metal content was determined by microwave-assisted digestion with *aqua regia* .This step was for analyzing the metals content in their primary and secondary minerals.

The trace element (As, Cd and Pb) content of each fraction was analyzed using ICP-MS model Perkin Elmer Elan 9000.

Data analysis and statistical

Two comparative measures were chosen to assess the trace metals uptake and distribution within the plant. Bioconcentration factor (BCF) and translocation factor (TF) was calculated using the following equations:

$$\mathrm{BCF} = [\text{plant tissue}] \,/\, [\text{sediment}]$$

$$\mathrm{TF} = [\text{shoot}] \,/\, [\text{root}] \quad (\mathrm{TF} > 1\text{: high mobility})$$

where [ ] is metal concentration in μg/g.

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In order to evaluate the degree of trace metal amount in the mangrove sediments of mangrove ecosystem, ecological risk assessment was conducted using the Håkanson, ecological risk index (R_I) (*Eqs. 1* and *2;* Håkanson, 1980).

$$R_I = \sum Er^i = \sum Tr^i Cf^i \qquad \text{(Eq.1)}$$

$$Cf^i = C_o^i / C_n^i \qquad \text{(Eq.2)}$$

where:
R_I = the sum of all risk factors for heavy metals in sediments
Er^i = the monomial potential ecological risk factor
Tr^i = the toxicity coefficient which represents the toxic response factor for a given metal. The value of Tr for As, Cd, Pb and Zn is 10, 30, 5 and 1 respectively
Cf^i = the contamination factor
Co^i = the concentration of metal in the sediment of mangrove ecosystem
Cn^i = the background value of heavy mental in coastal sediment

Statistic analysis

The data analysis using ANOVA to detect if any significant difference in mean exited between the mangrove sediment and plant parts. Duncan's new multiple range test (DMRT) was used to describe the differences between the variables (sediment and plant parts). Significance was set at 95% confidence level. Correlation was used to clarify the relationship between sediment fractions and metal accumulation in plant parts.

Results

Sediment characteristic in mangrove sediment

The metal concentrations in sediments from of Songkhla Lake (*Table 1*) ranged from 20.4 (station 1) - 50.5 (station 2) µg/g dry weight for As, 0.006 (station 4) -0.182 (station 3) µg/g dry weight, for Cd, 48.8 (station 4) -78.8 (station 1) µg/g dry weight, for Pb and 48.6 (station 4) -126.6 (station 1) µg/g dry weight, for Zn. The amounts of As, Zn and Pb were highest at Kuannieng (station 2) whereas Cd was highest at Phawong canal (station 3). The amounts of Zn, Cd and Pb were lowest at mouth of U-Taphao canal (station 4) whereas As was lowest at Kukhut (station 1). Besides that the results of the selected physical properties of sediment and water samples were summarized in *Table 1*. The pH in water ranged from 7.03 – 7.85 and in sediment ranged from 6.71-7.53. Salinity ranged between 8 ppt (station1) – 20 ppt (station 2).

Table 1. *Trace metals, physical properties of sediment and water in Songkhla Lake*

Station	pH water	Salinity (ppt)	pH sed	Eh (mV)	As	Zn	Cd	Pb
					µg/g dry weight (in sed.)			
St.1	7.33	8	7.53	-292	20.35	86.5	0.0759	59.8
St.2	7.85	20	6.71	-63	50.5	126.6	0.0984	78.8
St. 3	7.42	17	7.4	-149	28.7	102.1	0.1818	51.3
St.4	7.03	10	6.99	-79	31.7	48.6	0.0061	48.8

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Accumulation of trace metals in Sonneratia caseolaris

Accumulation of trace metals (As, Cd, Pb and Zn) in *Sonneratia caseolaris* is shown in *Table 2* and summarized as below:

As: The amount of As ranged from 0.20-4.72 µg/g dry weight. It was found that the concentration of As was highest in the barks (4.72 µg/g dry weight) at U-Taphao Canal (station 4), followed by roots (1.51 µg/g dry weight) and old leaves (1.03 µg/g dry weight).

Cd: The amount of Cd ranged from 0.003-0.041 µg/g dry weight. The result revealed that the concentration of Cd was highest in root (0.041 µg/g dry wt.) at Phawong canal (station 3), followed by old leaves (0.033 µg/g dry weight), green leaves (0.029 µg/g dry weight) and barks (0.029 µg/g dry weight).

Pb: The amount of Pb ranged from 0.18-6.76 µg/g dry weight. It was found that the concentration of Pb was highest in the root (6.76 µg/g dry wt.) at U-Taphao canal (station 4), followed by bark (4.12 µg/g dry weight) and old leaves (2.74 µg/g dry weight).

Zn: The amount of Zn ranged from 2.66-35.80 µg/g dry weight. The result showed that concentration of Zn was highest in the bark (35.80 µg/g dry wt.) at Kuannieng (station 2), followed by young leaves (17.10 µg/g dry weight) and root (16.58 µg/g dry weight).

Overall the amounts of trace metals were in order Zn > Pb > As > Cd.

Table 2. *Trace metals content in different parts of Sonneratia caseolaris of Songkhla Lake*

Station	Plant part	Zn	As	Cd	Pb
		(µg/g dry weight)			
1	Root	16.16	0.83	0.021	1.39
1	Bark	9.90	0.20	0.029	0.94
1	Leaf_Y	17.10	0.26	0.009	0.28
1	Leaf_M	12.98	0.29	0.014	0.48
1	Leaf_O	12.08	0.70	0.022	0.73
2	Root	24.40	1.51	0.027	1.85
2	Bark	35.80	0.79	0.026	2.08
2	Leaf_Y	4.14	0.04	0.006	0.18
2	Leaf_M	11.10	0.36	0.029	1.12
2	Leaf_O	10.44	0.57	0.033	2.74
3	Root	16.58	0.89	0.041	2.12
3	Bark	2.66	0.28	0.010	0.43
3	Leaf_Y	11.20	0.26	0.004	0.27
3	Leaf_M	5.36	0.53	0.006	0.44
3	Leaf_O	2.84	0.95	0.007	0.32
4	Root	5.88	0.41	0.003	6.76
4	Bark	8.04	4.72	0.019	4.12
4	Leaf_Y	7.60	0.37	0.004	0.21
4	Leaf_M	4.80	0.72	0.005	0.26
4	Leaf_O	4.76	1.03	0.006	0.70

Remarks: Y = young leave, M = mature leave (green colour), O = old leave (yellow colour)

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Trace metal speciation in mangrove sediment

The speciation of As, Cd and Pb in the mangrove sediment samples from the Songkhla Lake using the BCR protocol were presented in *Figure 2*.

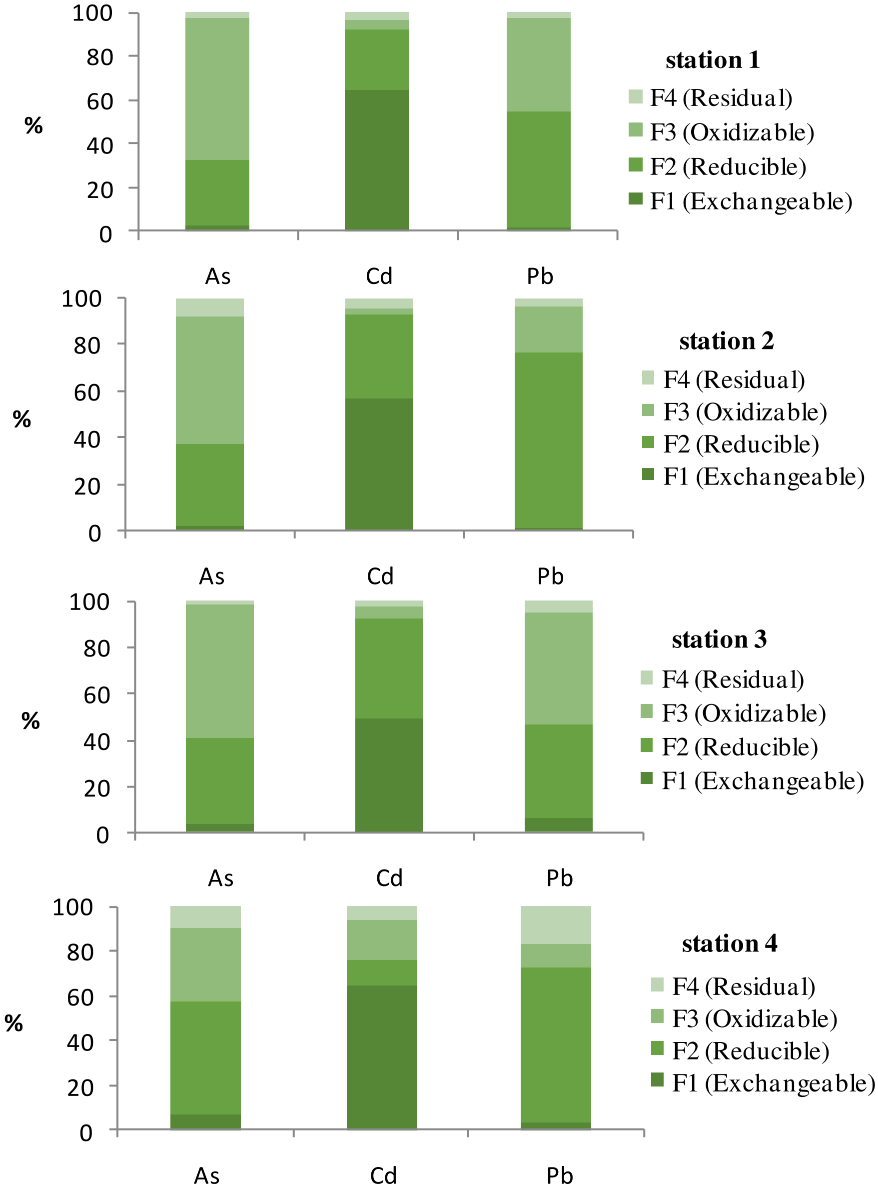

Figure 2. *The speciation distribution of As, Cd and Pb in mangrove sediments in Songkhla Lake, Southern Thailand*

The results of the sequential extraction for overall stations were as follows:

Exchangeable and acid soluble fraction (F1): Cd (49.58% - 64.33%) was found dominant in the acid-soluble fraction (F1), associated with exchangeable cations and carbonates for all the stations.

Reducible Fraction (F2): Pb (40.89% - 74.81%) was found dominant in F2 in all the stations except station 3 .This fraction is readily available with medium mobility and may be released into the environment under unstable anoxic conditions.

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Oxidizable fraction (F3): As (32.47% - 64.76%) was found dominant in F3 at all the stations except station 4 .Metals bound to organic matter are reasonable stable in nature however under strong oxidizing conditions such as currents, dredging, flooding and tides the organic matter can be degraded, hence leading to a release of heavy metals bound to this component (Sarkar et al., 2014; MacFarlane and Burchett, 2000).

Residual fraction (F4): Pb (2.60% - 16.49%) was found highest in F4. The residual solid normally consists of mainly primary and secondary minerals that retained heavy metals within their crystal structure. They are regarded as immobile and unavailable fraction.

As: Oxidizable (F3) > Reducible (F2) > Residual (F4) > Exchangeable (F1)
Cd :Exchangeable (F1) > Reducible (F2) > Oxidizable (F3) > Residual (F4)
Pb: Reducible (F2) > Oxidizable (F3) > Residual (F4) > Exchangeable (F1)

Bioaccumulation factors (BAF) and transfer factors (TF)

The bioconcentration factor (BCF) of *Sonneratia caseolaris* in different plant parts was varied as As>Cd>Zn> Pb. The BAF of Zn, As, Cd and Pb was shown in *Table 3*. The bioconcentration factor (BCF) was always below 1 for all the trace metals and all the stations except As (1.06) in old leave at Phawong canal (station 3) and Cd in old leave (1.02) at the U-Taphao canal (station 4). These results suggested that *Sonneratia caseolaris* showed the limited uptake of Zn and Pb (BCF < 1) but surprisingly not for As and Cd which BCF > 1 in old leave was found in Phawongcanal (station 3) and U-Taphao canal (station 4).

Table 3. *Bioconcentration factor (BCF) of Zn, As, Cd and Pb in Sonneratia caseolaris of Songkhla Lake*

Station	Plant part	Zn	As	Cd	Pb
1	Root	0.19	0.04	0.27	0.02
1	Bark	0.11	0.01	0.38	0.02
1	Leaf_Y	0.20	0.01	0.11	0.00
1	Leaf_M	0.15	0.01	0.19	0.01
1	Leaf_O	0.14	0.03	0.28	0.01
2	Root	0.19	0.03	0.27	0.02
2	Bark	0.28	0.02	0.26	0.03
2	Leaf_Y	0.03	0.00	0.06	0.00
2	Leaf_M	0.09	0.01	0.29	0.01
2	Leaf_O	0.08	0.01	0.34	0.03
3	Root	0.16	0.03	0.23	0.04
3	Bark	0.03	0.01	0.06	0.01
3	Leaf_Y	0.11	0.01	0.02	0.01
3	Leaf_M	0.05	0.02	0.03	0.01
3	Leaf_O	0.17	1.06	0.16	0.15
4	Root	0.12	0.01	0.42	0.14
4	Bark	0.17	0.01	3.19	0.08
4	Leaf_Y	0.16	0.01	0.70	0.00
4	Leaf_M	0.10	0.02	0.74	0.01
4	Leaf_O	0.10	0.03	1.02	0.01

Y = young leave, M = mature leave (green colour), O = old leave (yellow colour)

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The translocation factor (TF) of *Sonneratia caseolaris* in different plant parts varied as Cd >As>Zn> Pb (*Table 4*). The TF value of Zn ranged 0.16 -1.47 µg/g dry weight; As ranged 0.02-2.55 µg/g dry weight; Cd ranged 0.10-7.60 µg/g dry weight; Pb ranged 0.03-1.48 µg/g dry weight. The TF of these metals was above 1 at some stations and below 1 at other stations. At U-Taphao canal (station 4) TF value was above 1 for Zn, As, and Cd. TF value of As and Cd in old leave was greater than one at station 4 and 1. TF value of Pb in old leave was greater than one at station 2. TF value in bark was greater than 1 for Zn at station 1 and 4; As at station 4; Cd at station 1 and 4; Pb at station 2. Thus the result revealed that this plant species was effectively transferring Cd to the aerial parts. The TF of Pb was always below 1 (except station 2 in bark and station 3 in old leave), which might be described as having moderate mobility for this metal.

Table 4. *Translocation factor (TF) for mangrove plant parts*

Station	Plant part	Zn	As	Cd	Pb
1	Bark	0.61	0.24	1.39	0.68
1	Leaf_Y	1.06	0.31	0.42	0.20
1	Leaf_M	0.80	0.35	0.70	0.35
1	Leaf_O	0.75	0.85	1.05	0.53
2	Bark	1.47	0.52	0.96	1.12
2	Leaf_Y	0.17	0.02	0.22	0.10
2	Leaf_M	0.45	0.24	1.08	0.60
2	Leaf_O	0.43	0.37	1.23	1.48
3	Bark	0.16	0.31	0.24	0.20
3	Leaf_Y	0.68	0.29	0.10	0.13
3	Leaf_M	0.32	0.60	0.14	0.21
3	Leaf_O	0.17	1.06	0.16	0.15
4	Bark	1.37	1.16	7.60	0.61
4	Leaf_Y	1.29	0.90	1.67	0.03
4	Leaf_M	0.82	1.78	1.76	0.04
4	Leaf_O	0.81	2.55	2.42	0.10

Y = young leave, M = mature leave (green colour), O = old leave (yellow colour)

Ecological risk index of heavy metals in mangrove sediments of different areas

The potential ecological risk index of single element E^i_r shown in *Table 5* indicated that As and Zn were classified as moderate level whereas Cd and Pb were classified as low level. As shown in *Table 5*, the ecological risk indexes (R_I) of trace metals in the mangrove sediments from different part of Songkhla Lake showed that Kuannieng (station 2) and Phawong canal (station 3) were classified as moderate level whereas Kukhut nonhunting area (station 1) and U-Taphao canal (station 4) were classified as low level.

Comparison of trace metal concentration with Sediment Quality Guidelines (SQGs)

Several sediment quality guidelines (SQGs) for the assessment of sediment quality using chemical and biological effect databases have been established. These SQGs are

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summarized in *Table 6*. NOAA presents ERL (effects range low) and ERM (effects range mean) guidelines for estuarine and marine environments which represent the 10th and 50th percentiles of adverse biological effects (NOAA, 1999). The MacDonald et al. (2000) SQGs for freshwater environments have a lower TEC (threshold effects concentration) and an upper PEC (probable effect concentration) at which toxicity to bottom dwelling organisms are predicted to be unlikely and probable, respectively. The US-EPA has also made classifications (non-polluted, moderately polluted, heavily polluted based on toxicity tests (Baudo et al., 1990; Filgueiras et al., 2004). Regional background values assessed from core sediment samples were reported by Choi et al. (2008) and offshore sediments in the Gulf of Thailand by Shazili et al. (1999). Background levels varied from 55-115 µg/ g dry weitht for Zn, from 5-9 µg/g dry weight for As, from 0.03-0.2 µg/g dry weight for Cd, from 15-30 µg/g dry weight for Pb.

Table 5. *Ecological risk index of trace metals in mangrove sediments of Songkhla Lake*

E_r^i	Potential ecological risk for single regulator	R_I	Ecological risk for all factors
<40	Low	$R_I < 95$	Low (L)
$40 \leq E_r^i \leq 80$	**Moderate**	$95 \leq R_I \leq 190$	Moderate (M)
$80 \leq E_r^i \leq 160$	Considerable	$190 \leq R_I < 380$	Considerable (C)
$160 \leq E_r^i \leq 320$	High	$R_I \leq 380$	Very high (V)
$E_r^i \geq 320$	Very high		

Sites	E_r^i				R_I
	Zn	As	Cd	Pb	
1 Kukhut	**44.0**	29.1	3.2	2.7	78.9 (L)
2 Kuannieng	**64.4**	**72.1**	4.1	3.5	144.1(M)
3 Phawong canal	**51.9**	**41.0**	7.6	2.3	102.8 (M)
4 U-Taphaocanal	24.7	**45.3**	0.3	2.2	72.4 (L)

Table 6. *Comparison the metal concentration (µg/g dry weight) and the Sediment Quality Guidelines (SQGs)*

Element	**US EPA**			**NOAA**		**MacDonald et. al. (2000)**		**This study**
	Non polluted	**Moderately polluted**	**Heavily polluted**	**ERL**	**ERM**	**TEC**	**PEC**	
Pb	<40	40-60	>60	46.7	218	36	130	48.8-78.8
As	<3	3-8	>8	8.2	70	9.8	33	20.35-50.5
Cd			>6	1.2	9.6	0.99	5	0.0061-0.1818
Zn	<90	90-200	>200	150	410	120	460	48.6-126.6

TEC: threshold effect concentration, PEC: probable effect concentration, ERL: effects range low, ERM: effects range mean

Sediment quality guidelines (SQGs) were recognized as appropriate thresholds to reveal adverse effects of heavy metals in sediments for plants, animals and human health. SQGs were applied to our study to determine the overall pollution status in sediments of Songkhla Lake. The amount of As and Pb was above the background value. Our study showed that highest amount of As at Kuannieng (station 2) of

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Songkhla Lake exceeded the ERL value; TEC value was classified as heavily polluted probably affected the health of plants and organisms. Pb levels from both sites exceeded the ERL and TEC value and were regarded as heavily polluted at Kuannieng (station 2) and classified as moderately polluted for stations 1, 3, and 4. Cd level was classified as lower than ERL and TEC. Zn was classified as moderately polluted and exceeded TEC value.

The comparison of trace metals in mangrove sediment and plant parts

Our result revealed that the total concentration of all metals (As, Zn, Pb, Cd) in mangrove sediment no statiscally significant difference among the stations ($p > 0.05$). For the comparison of the trace metal concentrations between plant parts of *Soneratia caseolaris*, all the values show variations among the stations. The post hoc Duncan-Test showed no significantly different accumulation in barks ($p > 0.05$) whereas roots and leaves were significantly different ($p < 0.05$). The comparison of the metal contamination in the different sediment fractions revealed the low metal concentration in residual fraction and high in bioavailability fractions. The post hoc Duncan-Test showed among the stations showed no significant difference in residual fraction ($p > 0.05$) whereas the other fractions (Exchangeable, Reducible and Oxidizble) were significantly different ($p < 0.05$).

Correlation between fractionation of heavy metals in sediment and accumulation in plant parts

The accumulation in various parts of *Soneratia caseoralis* was investigated to determine the relationship between the fractionation and plant's uptake as showed in *Table 7*. Fraction 1 (Exchangeable) was significantly negative correlated with accumulation in leaves (young, green, old). The result indicated that Fraction 2 (Reducible) was showed positive correlation with root ($r = 0.634$, $p < 0.05$), bark ($r = 0.682$, $p < 0.05$) leaves (Y: $r = 0.678$, $p < 0.05$; O: $r = 0.712$, $p < 0.05$). Higher Fraction 2 resulted in higher plant uptake. Fraction 3 (oxidizable) had moderate correlation ($r = 0.585$, $p < 0.05$) and Fraction 4 (Residual) had high correlation with plant uptake in root ($r = 0.749$, $p < 0.05$) and bark ($r = 0.776$, $p < 0.05$). High correlation was found between young leave and green leave ($r = 0.661$, $p < 0.05$) and between green leave and old leave ($r = 0.933$, $p < 0.05$).

Table 7. *Correlation between fractionation of trace metals in sediment and accumulation in plant parts*

	Fraction1	Fraction2	Fraction3	Fraction4	Root	Bark	Leaf_Y	Leaf_M	Leaf_O
Fraction1	1								
Fraction2	-.590'								
Fraction3	-.711"	-.131							
Fraction4	-.228	.388	-.208						
Root	-.515	.634'	-.005	.749"					
Bark	-.480	.682'	-.089	.776"	.573				
Leaf_Y	-.798"	.458	.585'	.143	.313	.556			
Leaf_M	-.742"	.678'	.368	.031	.197	.506	.661'		
Leaf_O	-.627'	.712"	.201	.037	.249	.454	.470	.933"	1

Y = young leave, M = mature leave (green colour), O = old leave (yellow colour)
'Correlation is significant at the 0.05 level. "Correlation is significant at the 0.01 level

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Discussion

In this study, the samples were collected during the heavy rainy season and therefore several large freshwater bodies flowed into the lake and lessened the salinity of the lake water. However, the metal concentrations in the collected mangrove sediment samples in our study area as compared to the sediment quality guideline revealed that the As concentration was still higher than that of the quality guideline by 2-4 times and classified as heavily polluted. Comparison of our data regarding the metal concentrations (As, Cd, Pb and Zn) with the previous data from the other studies of Songkhla lake as well as other areas in the world was illustrated in *Table 8*. The maximum values of As and Pb at the Outer Section of Songkhla Lake previously reported by Pradit et al. (2010, 2013) were considerably lower than those of this study. Especially, As levels in Songkhla Lake were much higher than those found in Pattani bay (Pradit et al., 2016), Seitu Wetland (Pradit et al., 2016), Sundarban Wetland (Chowdhury et al., 2015) and at Kelantan Delta reported by Baruddin (2017). This result is certainly supported by Sompongchaiyakul and Sirinawin (2007), Pradit et al. (2010, 2013); who reported that concentrations of As, and Pb were ascending owing to rapid urban and industrial expansion. Outer Section of Songkhla Lake receives municipal wastes from two large and rapidly expanding cities of Songkhla and Hatyai as well as agricultural and industrial discharges transported by U-Taphao canal (Sompongchaiyakul and Sirinawin, 2007). The mean concentration values of As and Pb of this study were similar to the result of Pradit et al. (2010) but higher than those of Pradit et al. (2013). It is well accepted that the mangrove area is considered as a sink of trace metals and therefore the mangrove sediment samples of this study possesses the higher amounts of trace metals than those of the lake sediment reported by Pradit et al. (2013). Although the total concentration of metal is an appropriate indicator of contamination assessment, it does not provide enough information about bioavailability and toxicity of heavy metals (Zhong et al., 2011).

Chemical fractionation differentiates metals of natural origin from those derived from anthropogenic sources. The bioavailability fractions (exchangeable, oxidizable and reducible) of As, Cd and Pb in mangrove sediments of this study were greater than 90%whereas the residual fraction of these elements was very low. Thus, it was probably concluded that mangrove area in this study had high bioavailability. This may suggest that there was considerable anthropogenic input to the Songkhla Lake as compared to the low bioavailability of As (about 30%) reported by Baruddin et al. (2017). This was probably caused by different mangrove plant types. Our sampling area was *S. caseolaris* habitat whereas Baruddin et al. (2017) was *R. mucronata.*

In our study Pb seemedto be accumulated in plant parts. This was concordant with the study of Toledo-Bruno et al. (2016) in Mangrove forest reserve in Mindanao, Philippines discovering of *Sonneratia alba* being Pb-hyperaccumulator. Our study Pb was highest in root and well agreed with the study of Nazli and Hashim (2010).

Plants may be passive receptors of heavy metals, but they also exert control over uptake or rejection of some elements by appropriate physiological reactions. To illustrate this point, based on *Table 3,* the BAF values for all the studied heavy metals were always less than 1, which indicated that *Soneratia caseolaris* exhibited restricted metal sediment-root uptake for non-essential heavy metals similar to BAF of *R. mucronata* in the Outer Section of Songkhla Lake (Barrudin et al., 2017)

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Table 8. *Trace metals concentrations (µg/g dry wt) in sediments of Songkhla Lake and at other sites*

	As	Cd	Pb	Zn	References
Songkhla lake	20.4-50.5	0.006-0.182	48.8-78.8	48.6-126.6	This study
Songkhla Lake	5.1-25.7	-	-		Sompongchaiyakul and Sirinawin, 2007
Songkhla Lake	0.8 -70.7	0.1-2.4	8.2-131	5.4-561.6	Pradit et al., 2010
Songkhla Lake	20-22.0	0.21-0.28	31-35		Pradit et al., 2013
Songkhla Lake	20.7		55		Baruddin et al., 2017
Futian, China	152.4		70.7		Li et al., 2016
Pattani Bay, Thailand	2.98-9.34	0.00-0.02	2.40-11.48	0.01-7.06	Pradit et al., 2016
Seitu Wetland, Malaysia	0.89-3.52	0	0.63-1.56	0.50-2.30	Pradit et al., 2016
Kelantan Delta, Malaysia	18.28-34.95		31.30-75.84		Baruddin et al., 2017
Sundarban Wetland, India	3.22-4.41	0.19-0.22	11.6-20	32.51-36.33	Chowdhury et al., 2015
Background value	7	0.12	22.5	56	Choi et al., 2008
Heavily polluted	>8	>6	>60	>200	US EPA
Moderately polluted	3-8		<3	90-200	US EPA

Conclusion

The anthropogenic inputs of As and Pb were high in the mangrove sediments of the lake especially at Kuannieng (station 2). As and Zn were found highest concentration in bark whereas Cd and Pb were found highest concentration in root. The BCF and TF values proposed that *Sonneratia caseolaris* showed limited (BCF < 1, and TF > 1) accumulation of As, Cd, Pb and Zn in their aboveground parts and thus presented a low food chain hazard except As and Cd at Phawong canal (station 3) and U-Taphao canal (station 4). The potential ecological risk index of single element indicated that As was classified as moderate risk level. The concentration of As should be concerned and regularly monitored.

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EFFECT OF PIX (MEPIQUAT CHLORIDE) ON THE SALINITY RESISTANCE OF NEW CULTIVARS OF COTTON (*GOSSYPIUM HIRSUTUM* L.) IN THE SEEDLING STAGE

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Abstract. In modern agriculture, mepiquat chloride (pix) as a synthetic growth regulator, is considered as an important management tool for reducing stress damage in Cotton seed. In the present study, the effects of the growth regulator, pix were investigated on the physiological and biochemical characteristics in new cultivars of cotton in response to salinity stress in Golestan province, Iran. The experiment was carried out in a completely randomized design enriched with 10 gr. L^{-1} Pix and 15 ds.m^{-1} salt (sodium chloride) in Petri dish with three cultivars (*Latif*, *Kashmar* and *Sahel*). The physiological and biochemical parameters of seedlings were measured after 4 days. The use of Pix in saline conditions resulted in a significant increase in germination percentage, radicle length, dry and fresh weights, phenolic compounds, as well as proline and glycine betaine content in the *Latif* and *Kashmar* cultivars ($p \leq 0.05$). The amount of Na^+ ion absorption was lower in *Kashmar* and *Latif* cultivars compared with the *Sahel* cultivar while the amount of K^+ and Ca^{2+} ions absorption was increased in the two mentioned cultivars compared to the Sahel cultivar. This study showed that the effects of stress were reduced after treating the seeds with Pix, especially in the *Kashmar* and *Latif* cultivars during the seedling stage.
Keywords: *salt stress, mepiquat chloride, synthetic growth regulator, enzyme activity, ion accumulation, osmotic regulator, Malvaceae*

Introduction

Cotton (*Gossypium spp.*) is a flowering plant which belongs to the Malvaceae family. Today, it has gained importance both economically and commercially with regard to the production of fibers, raw material for textiles, the oil industry, food (Ozarslan, 2002) and various other materials for consumption. For a long time, salinity has been one of the major environmental problems faced by humankind (Jaleel et al., 2009) and is the basic limiting factor for production in arid and semi-arid inlands (Munns et al., 2002; Ashraf and Foolad, 2005). Like many areas of the world, Iran has a hot and dry climate and more than half of its cultivation lands has been transformed into saline and sodic soils (Jamali et al., 2015). Salinity stress affects the physiological and bio-chemical features of the plant and disrupting the osmotic balance (Kook et al., 2009) can lead to severely decreased plant growth and reduced amount of production (Koca et al., 2007).

Salinity stress and increasing Na^+ concentration cause a decrease in essential absorption including K^+ and calcium, and a reduction of enzyme activity and membrane structure (Demir Kaya et al., 2006; Netondo et al., 2004). An irreparable damage is caused by the plant through the production of reactive oxygen species (ROS) (Koca et al., 2007). Also, plants in saline areas are faced with water deficit in addition to the salinity stress. This affects plant metabolic reactions such as photosynthesis, respiration, and protein synthesis (Pal et al., 2004; Meloni et al., 2003). The effect of salinity is different in different periods of plant growth. Germination is the most critical stage of plant growth (Khan et al., 2002; Khan and Panda, 2008) including cotton (Weiping et al., 2009; Mer, 2000; Prado, 2000) so that germination can be prevented by increasing salinity (Sosa et al., 2005; McDonald, 2000; Godfery et al., 2004).

Each plant with more resistance can have a better growth during the later stages (Gulzar, 2001). The investigation showed that an increase in salinity caused a decrease in plant height, leaf surface, fresh and dry weight, as well as radicle and plumule length in the studied cultivars of cotton (Saranga et al., 2004; Noor et al., 2001). Even in susceptible (*Sahel*) and resistant (*Siokra*) cultivars of cotton, salinity also causes shortening in radicle and plumule length (Rezaei et al., 2005). An important effect of stress in plants is the overproduction of reactive oxygen species (ROS) in chloroplasts and mitochondria (Mittler, 2002; Masood et al., 2006).

The accumulation of secondary metabolites is one of the possible responses of plants to the changes in osmotic potential of the environment (Hasegawa et al., 2000). Plants accumulate compatible solution such as proline (Mansour et al., 2005a) and glycine betaine in the cell osmotic regulation (Rhodes and Hanson, 1993) to counteract the harmful effects of salt.

Glycine betaine is increased in plant as consistent and effective osmolytes under stressful conditions (Ashraf and Foolad, 2007), particularly under salinity stress (McNell et al., 2001). Increasing glycine betaine has been observed in response to salinity stress in spinach, cabbage, tomato, potato, Rice and sorghum (Yang et al., 2003). Also, increase in water salinity resulted in an increase in glycine betaine in two cotton cultivars (*Aria-Anubam and LRA - 5166*) (Desingh and Kanagaraj, 2007). Proline is the amino acid that is accumulated under salinity stress as active osmotic regulator; it is widely used in higher plants (Abraham et al., 2003) and causes membrane stability and affects the membrane process.

Another effect of salinity stress on plants is the disruption of important nutrient balance including K^+ and Ca^{2+} (Renault, 2005). K^+ is an essential cytoplasmic element and is considered to be an important element in saline conditions as a result of its role in regulating osmosis and the competition effect. Also, several reports have emphasized the beneficial effects of Ca^{2+} in reducing the effects of salinity and in most cases these effects are related to restoring the plasma membrane, health maintenance and cohesion in roots and shoots (Nedjimi and Daoud, 2009; Kaya et al., 2002; Tuna et al., 2007). Various methods are used to reduce salinity in cotton. These include the use of growth regulators, selection and the cultivation of resistant genotypes. Pix as a synthetic growth regulator in Cotton seed is considered as an important management tool for reducing stress damage in modern agriculture (Jost et al., 2006; Niakan et al., 2012). Pix with the common name, mepiquat chloride, is composed of 4.2% N, N-dimethylpiperidinium chloride and a multi-component composition of Ammonia (it inhibits the synthesis of Gibberellic acid) (Stewart, 2005). The colonization of seeds with Pix before planting is an appropriate method of reducing salinity which can increase the length of roots with changes in seed physiology and impressing cell wall in the early stages of germination. Studies have shown that a significantly higher seed germination was observed in seeds treated with Pix compared to the control (Niakan et al., 2012).

It has been found that plants primed with Pix are shorter, denser and darker than plants that are not stained with it (Stewart, 2005). The use of Pix leads to the absorption of more phosphorus and nitrogen (Oosterhuis and Robertson, 2000) and the concentration of

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magnesium, phosphorus, and nitrogen in the root is more compared to the control group (Osterhuis and Robertson, 2000; Zhang et al., 2001). The literature review revealed that the use of Pix at high concentrations of salt resulted in improved germination and an increase in physiological characteristics such as root length, fresh and dry weight and increased biochemical properties such as catalase activity and the amount of phenol (Alishah et al., 2012).

The objective of this study was to investigate the effect of Pix (mepiquat chloride) on the salinity resistance of the new cultivars of cotton (*Gossypium hirsutum* L.) in Gorgan area, Golestan province, Iran.

Materials and methods

Plant material

Delinted cottonseed (*Gossypium hirsutum* L.) from three cultivars (*Kashmir*, *Latif* and *Sahel*) was prepared in the Cotton Research Center of Iran, Gorgan city, Golestan Province, (36° 50' North and 54° 26' East) which is located in the North East of Iran.

The experimental design was completely randomized with priming Pix (10%) and salt (15 $ds.m^{-1}$) in the Petri dish with three mentioned cultivars (*Latif*, *Kashmar* and *Sahel*) at the germination stage. In addition, four treatments (Pix, salinity, Pix and salinity and control) were studied with four replications from three cultivars in 48 Petri dishes.

Sahel cultivar was considered as control (grown in northern areas and most areas of Iran) and two new cultivars of *Latif* and *Kashmar* were used as treatments that were developed and recorded in the year 2015 in the Cotton Research Institute of Iran through crop breeding. Cotton seeds priming was performed with10 gr. L^{-1} (gram/liter) Pix for 5 h at 25 °C. Thereafter, 3 milliliters (ml) of distilled water was added to the control and 3 ml of water soluble salt (sodium chloride) (EC: 15 $ds.m^{-1}$) was added to salt treatment and the seeds were covered with a filter paper, and were placed in a germinator for 4 days at 27 °C. Moreover, the following physiological and biochemical parameters were measured: percentage of germination, root length, fresh and dry weight, the phenolic compounds, glycine betaine, proline, the concentration of Na^+ ions, K^+, and calcium.

Assessment of germination percentage: The germination of seeds was calculated after 4 days by *Equation 1*. It should be noted that seeds with radicle length of 1 mm or more were considered as germinated seeds (Weston et al., 2004).

$$PG = 100\ n/N \qquad \text{(Eq.1)}$$

where N is the total number of seeds, n is the number of germinated seeds and PG is the Percentage of Germination.

Measuring the length of radicle seedlings: The radicle length of the seedling (mm) was measured (4 days) after the initial radicle emergence.

Measurement of the fresh and dry weight of seedlings: Seedlings from each treatment (4 repetitions) were randomly selected and their fresh weight was measured using a Digital Scale (gram). To determine the dry weight, seedlings from each treatment (4 repetitions) were placed in the oven at 90 °C for 24 h, followed by weighing.

Assessment of proline (Bates et al., 1973): 0.2 gr of frozen seedlings was homogenized in 3% sulfosalicylic acid, and the homogenate was centrifuged at 10000 rpm (revolutions per minute). The supernatant was used for the estimation of proline content. The reaction mixture (2 ml acid ninhydrin and 2 ml of glacial acetic acid) was heated at 100 °C for 1 h.

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The reaction mixture was extracted with 4 ml of toluene and absorbance was read at 520 nm.

Phenolic compounds assay (Matta and Giai, 1969): The samples were boiled in 10 ml alcohol 80% for 15 min and then, centrifuged for 15 min at 3000 *g*. 5 ml of diluted foline (1:3) and 10 ml of saturation Na_2CO_3 were added to 5 ml of this solution. Samples were incubated at 25 °C for 10 min and then, centrifuged for 15 min at 4000 *g*. Supernatant absorption was determined at 640 nm. The standard curve of catechol was used to determine the content of phenolic compounds (milligram / gram dry weight: mg. g^{-1} FW).

Measurement of glycine betaine content (Sairam et al., 2004): 0.5 gr dried powder of seeds was distilled in 20 ml water and shaken at 25 °C. The samples were filtered by filter paper and diluted with an equal ratio of sulfuric acid 2 N. 2 ml was separated and mixed with 8.0 ml of iodide - cold iodine K^+ (Lugols). The solution was placed in a refrigerator for 16 h and was centrifuged at 3000 rpm for 30 min. 1 ml of the upper phase was separated and dissolved with 9 ml of 2,1-dichloroethane. The samples were vertex and after 2.5 h, its absorption was read at 365 nm and then, the glycine betaine content (micromol / gram dry weight: µmol/g DW) was calculated using *Equation 2*.

$$C = \text{Abs} \times 0.00017 + 0.014636 \qquad \text{(Eq.2)}$$

Measurement of Na^+ and K^+ (Williams and Twine, 1960): 1 gr of fresh material was placed in the oven at 80 °C for 48 h. Thereafter, it was put in an electric furnace at 600 °C for 6 h. Subsequently, after weighting, the ashes was dissolved in 1 ml of hydrochloric acid 6 N, using a stirrer. Next, the samples were filtered and diluted with distilled water to a volume of 50 cc. Then, the amount of Na^+ and K^+ were determined using a flame photometer and a standard curve (gram / gram dry weight: g. g^{-1} DW).

Measurement of Ca^{2+} ions (Manteghi et al., 1987): 10 ml mineral extraction plant was poured into Erlenmeyer flasks and then, 2 ml NaOH 4 normal was added and 3.0 gr Murexide powder was added. Then, the titration was performed using EDTA 0.01 normal to change its color to purple. This process was performed in the presence of calcium chloride solution as control. The value of Ca^{2+} as gram per gram of dry matter was calculated using the formula (g. g^{-1} DW).

Statistical analysis

The data was represented as mean ± standard deviation (SD). The experiment was conducted using a completely randomized design with Four replicates (n = 4) having twenty seeds of each replicate. The data were statistically treated by one-way analysis of variance (ANOVA) and the mean values were compared using Duncan's test at $P < 0.05$ to establish significant differences between each treatment and control.

Results

Pix, salinity effect and their influence on the germination percentage, radicle length, fresh and dry weight

The results showed that germination percentage and radicle length were reduced under salinity stress in three cultivars compared with the control. Also, the use of Pix resulted in a significant increase in germination percentage compared with the control in all three

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cultivars and among them, the rate of increase of *Latif* and *Kashmar* cultivars was more than that of the *Sahel* cultivar (*Fig. 1*).

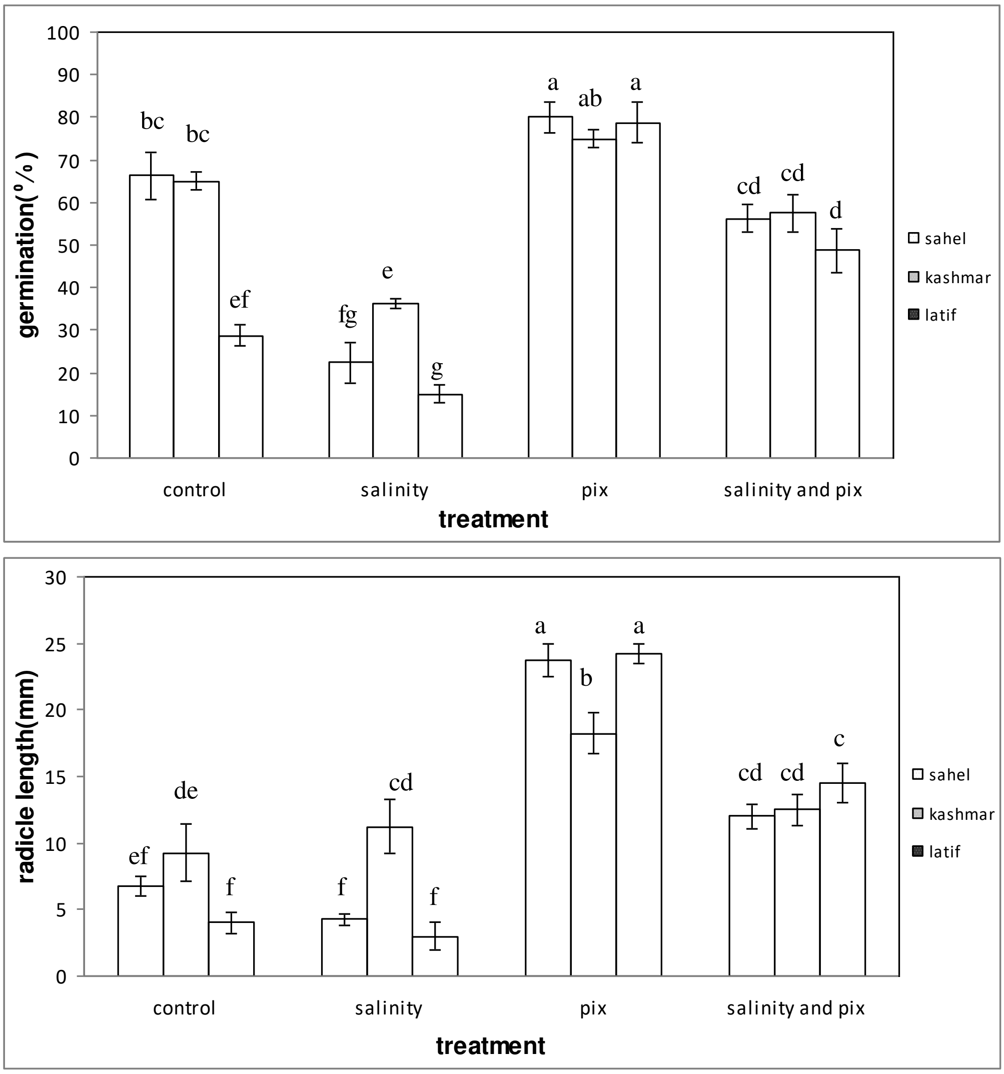

Figure 1. *Effect of Pix, salinity and their interaction on the percentage of germination and radicle length. *Average with the same letters was not statistically significant (p ≥ 0.05)*

Figure 2 shows that Sahel cultivar is sensitive to salinity and its fresh weight and dry weight decreased under salt stress but the use of Pix was very useful for salt stress tolerance. On the other hand, the fresh and dry weights of *Latif* cultivar were increased under Pix treatment alone as well as Pix and salinity combination treatments.

Effect of Pix, salinity and their interaction on the amount of seed phenol in new cultivars of cotton seedlings

The results showed that the phenolic compounds in Sahel cultivar treated with Pix presented a significant increase compared with the control treatment. Also, the phenolic compounds in all the treatments were increased in Latif and Kashmar cultivars in comparison with the control (*Fig. 3*).

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Effect of Pix, salinity and their interaction on the glycine-betain content

Figure 4 shows that the use of Pix and salinity combination caused an increase in the glycine betaine content in the three cultivars. Despite the fact that the Sahel cultivar is sensitive to salt stress, the highest glycine betaine content belonged to this cultivar.

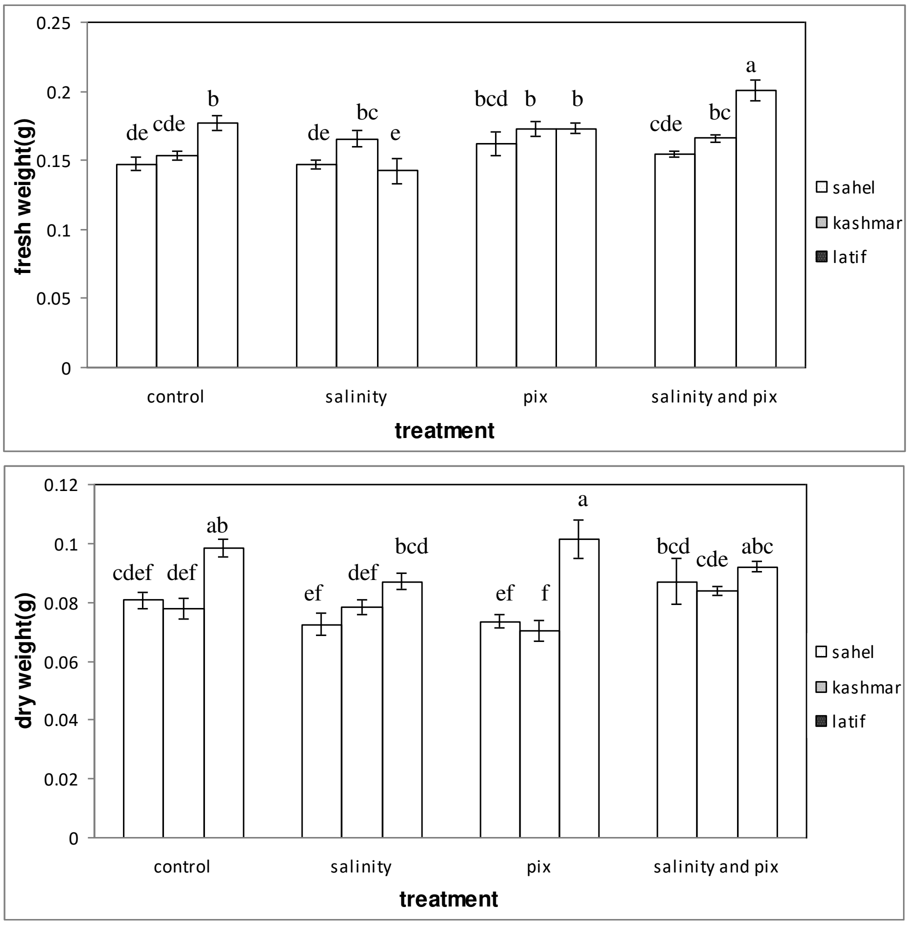

Figure 2. *Effect of Pix, salinity and their interaction on the fresh and dry weights*

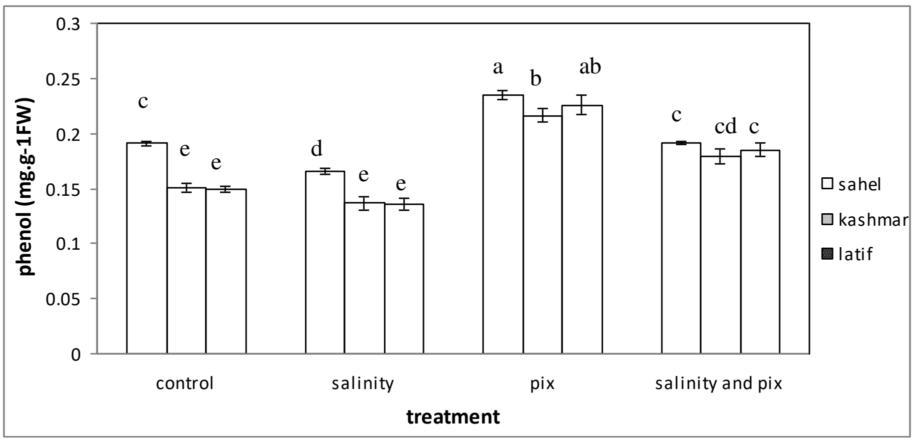

Figure 3. *Effect of Pix, salinity and their interaction on the amount in phenol of new cultivars of cotton seedlings*

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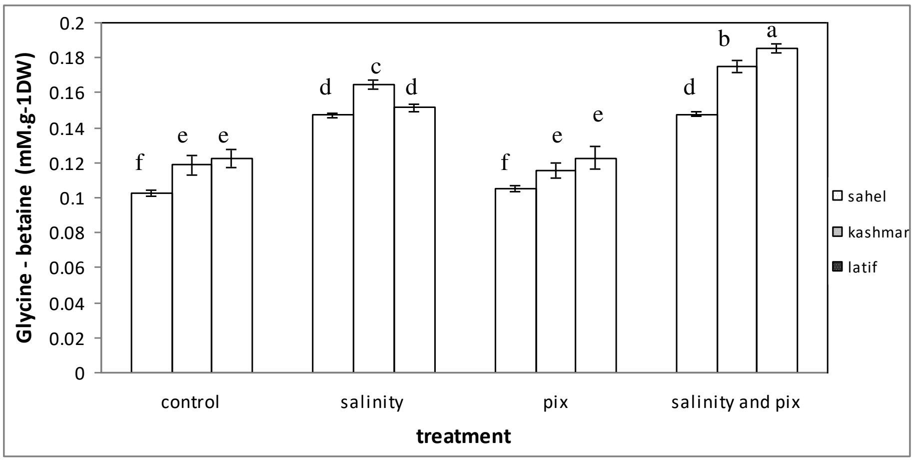

Figure 4. *Effect of Pix, salinity and their interaction on the amount of Glycine - betaine in new cultivars of cotton seedlings*

Effect of Pix, salinity and their interaction on the amount of proline

The results showed that the proline content had a significant increase in all three cultivars in the different treatments compared with the control treatment, but the proline content in cultivars of Kashmar and Latif was more compared to the sensitive cultivar of Sahel (*Fig. 5*).

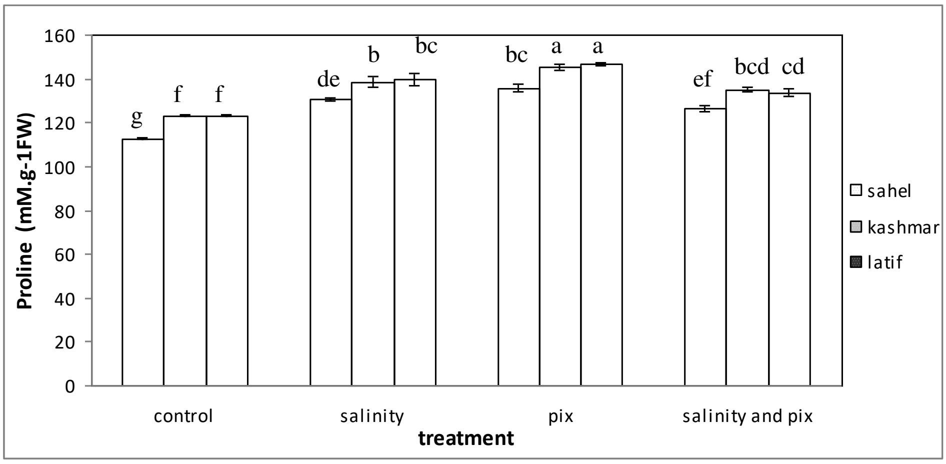

Figure 5. *The effect of Pix, salinity and their interaction on the amount of proline in new cultivars of cotton seedlings*

Effect of Pix, salinity and their interaction on the amount of Na^+ and K^+ in new cultivars of cotton seedlings

The results of this research showed that the amount of Na^+ in all three cultivars in different treatments had a significant increase compared with the control treatment. The amount of Na^+ in Latif and Kashmar cultivars was less than in the Sahel cultivar. Also, the amount of K^+ caused a significant decrease in the Sahel cultivar in all three treatments compared to the control treatment (*Figs. 6* and *7*).

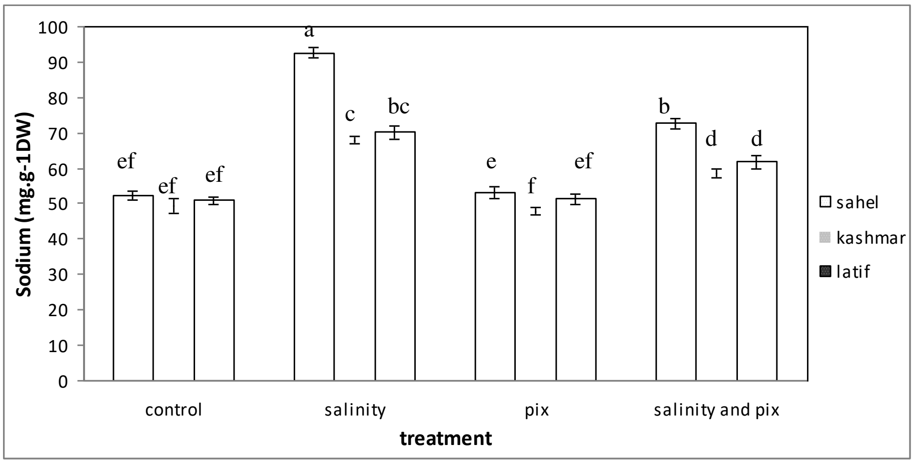

Figure 6. *The effect of Pix, salinity and their interaction on the amount of* Na^+ *of new cultivars of cotton seedlings*

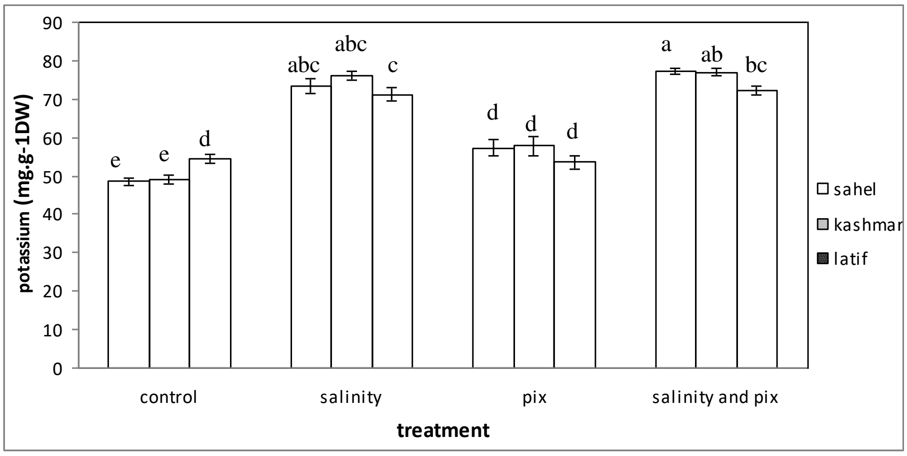

Figure 7. *The effect of Pix, salinity and their interaction on the amount of* K^+ *in new cultivars of cotton seedlings*

Effect of Pix, salinity and their interaction on the amount of Ca^{2+} *in new cultivars of cotton seedlings*

Compared to the control treatment, the results indicated that the amount of Ca^{2+} had a significant increase in all three cultivars in different treatments (*Fig. 8*).

Discussion

Previous studies showed that the length of root and stem was significantly reduced under salinity (Habib et al., 2014; Pervaiz et al., 2007; Noor et al., 2001; Ibrahim et al., 2007; Weiping et al., 2009; Rezaei et al., 2005). Moreover, the fresh and dry weight of the root and stem was reduced in cotton cultivar (Habib et al., 2014; Ibrahim et al., 2007; Saleh, 2011). In addition, the results of previous studies showed that salinity stress resulted in a decrease in germination percentage and growth parameters of cotton seedling in S*epid 2* cultivar. Also, priming with Pix improved injuries that were caused

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by salinity on these parameters (Alishah et al., 2012). The most important effect of Pix on cotton was longitudinal growth prevention (Singh, 2010). Pix can cause changes in seed physiology and affect the cell wall. Reviews on the germination percentage showed that seeds treated with Pix had significantly higher germination percentage compared with the control (Niakan et al., 2012). The findings revealed that at high concentrations of salt, the use of Pix resulted in improved germination and increased physiological characteristics including root length, as well as fresh and dry weight (Alishah et al., 2012). Regarding the obtained results, there was an increase in the dry weight of the seedlings due to an increase in the length of the radicle when Pix was used. The priming of seeds with Pix before planting can be a suitable method in cotton production that leads to increase in root length in the early stages of germination.

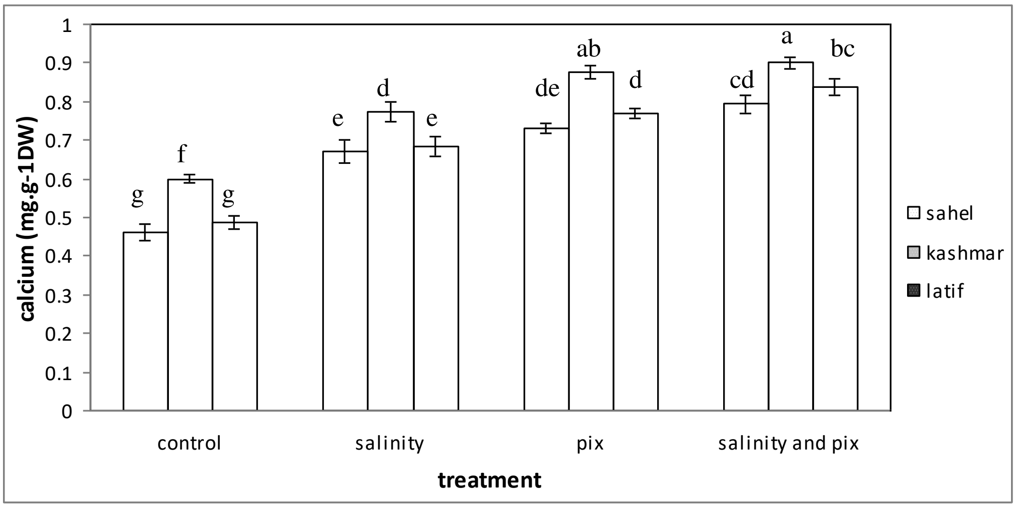

Figure 8. *The effect of Pix, salinity and their interaction on the amount of Ca^{2+} in new cultivars of cotton seedlings*

Phenolic compounds are different secondary metabolites that exist in large quantities in plant tissues until the plant can cope against reactive oxygen species, these compounds play an antioxidant role and protect plants from the oxidizing action of malicious free radicals (Grace and Logon, 2000; Zhu, 2000). Previous researches showed that salinity stress in barley seedlings leads to a decrease in phenolic compounds, flavonoids and growth (Ali and Abbas, 2003). Tamer and Abdel-al (2012) in their research findings on the content of the phenolic compounds of barley seedlings under the NaCl -salinity stress showed that the content of phenolic compounds was reduced. These findings are based on the fact that the reduction of phenolic compounds in the *Sahel* cultivar under salinity stress was consistent with the results of the present study.

The results showed that the amount of phenolic compounds increased in *Kashmar* and *Latif* Cultivars subjected to Pix and salinity along with Pix treatments. This issue indicated that seed treatment with Pix in saline or non-saline conditions caused the increase of phenolic compounds that were resistant to stress. The first step in the phenylpropanoids metabolism was deamination of L-phenylalanine of cinnamic acid by phenylalanine ammonia-lyase (PAL) (Iijima et al., 2004; Achnine et al., 2004). PAL is an enzyme intermediate between the primary and secondary metabolism of

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phenylpropanoids (Adams, 2001). Metabolites that occur as a result of PAL activity are classified as phenolic derivatives. The results of this study demonstrated that the amount of glycine - betaine increased in all three treatments in the *Sahel* cultivar compared to the control treatment.

In plants, osmotic adjustment takes place through the production of various types of compatible organic solvents and some inorganic ions. These materials may insulate the injuries of plant cells through osmotic stress by modulating and maintaining membrane fluidity and stability of proteins or enzymes (Bohnert and Jensen, 1996).

Glycine betaine is one of the most abundant compatible organic substances in plants (Weibing and Rajashekar, 1999). In many plants, such as spinach, barley, wheat and sorghum, there is an increase in glycine betaine in response to various stresses (Rhodes and Hanson, 1993; Yang et al., 2003; McNell et al., 2001; Hellman et al., 2000; Ashraf and Foolad, 2007; McNell et al., 2001; Gorham et al., 2000).

Glycine betaine is naturally accumulated in some plants whereas in others, it is not produced even under stress condition (Ashraf and Foolad, 2007). However, it appears that the moderating role of glycine betaine depends on many factors including product type, time, its application, and the environmental conditions (Makela et al., 1998). The glycine betaine concentration is changing in different species and are used as an osmotic regulator (Agastian et al., 2000).

An investigation revealed that an increase in the different levels of glycine betaine in cotton under salinity is different in some variety compared to other varieties that accumulate higher amounts of proline and glycine betaine (Desingh and Kanagaraj, 2007).

With increasing salinity in two cotton cultivars, (*Aria- Anubam and LRA - 5166*), the amount of glycine betaine increased in both cultivars (Desingh and Kanagaraj, 2007).

The results of this study showed that the amount of glycine betaine in the seedling was significantly increased as compared to the control. This combination is one of the physiological resistances against salinity in the early stages of germination.

Studies have shown that the osmotic stress injuries caused by salinity and NaCl can be reduced with the use of glycine betaine and this issue depends on the concentration and time of conducting this research (Ashraf, 2009). Interestingly, plants under salinity stress are divided into three categories of plants with resistance strategy via proline or glycine betaine or both (Larher et al., 1996). It appears that *Kashmar* and *Latif* cultivars are the apparently tolerant ones in the salinity treatment and showed increase in the glycine betaine. Hence, they could be regarded among plants that act with Resistance strategy through the accumulation of glycine betaine. Proline is an amino acid which is widely accumulated in higher plants under salinity and drought stress as an osmotic regulator (Abraham et al., 2003; Rontein et al., 2002; Rontein et al., 2002; Fahramand et al., 2014; Ashraf and Harris, 2004; Mansour et al., 2005b) and substantially contributes to osmotic cytoplasm adjustment, and to the stability of the membrane and membrane process.

The results showed that salinity increased the amount of proline in alfalfa (Parida et al., 2004), cotton (Desingh and Kanagaraj, 2007; Mirghasemi et al., 2010), as well as different ecotypes of fox berry plant in the germination stage (Nad Ali et al., 2014). Some studies have indicated that salinity stress causes the excessive accumulation of proline in the *Sayochra* cultivar of cotton and show that this cultivar follows the proline accumulation strategy in salt tolerance (Kumar, 1987). Regarding the increase in proline and glycine betaine content in *Kashmar* and *Latif* cultivars that are apparently tolerant

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in salinity treatment; therefore, an increase in these compounds in the cell relieves stress for osmotic moderation. *Kashmar* and *Latif* cultivars accumulate proline and glycine betaine that can be considered part of the plants that act through resistance strategy.

In this research, the use of Pix in saline condition caused a decrease in seedling proline content, as compared to the treatment of Pix and saline treatment. It is possible that the decrease in the amount of proline in saline conditions is as a result of the activation of the enzyme proline oxidase that has a role in the conversion of proline to glutamate. Glutamate is used in the biosynthesis of other amino acids for protein synthesis (Mundree et al., 2002).

Even at low concentrations, Na^+ ions are toxic inside the plant and limit the metabolic activity while K^+ ion is known as one of the macronutrients essential for membrane maintenance and enzyme activity. The high concentrations of Na^+ results in the reduced absorption of other nutrients into the soil (Silber bush and Ben-Asher, 2001). Na^+ ions occupy k^+ transfer channels in the plasma membrane of root cells, thereby preventing the absorption of K^+ (Tester and Davenport, 2003) and lead to reduced absorption of other essential elements (Kaymakanova and Stoeva, 2008).

A research of resistant and susceptible genotypes of cotton to salt indicated that the amount of Na^+ was significantly increased under salinity condition and resulted in a decrease in the amount of K^+ (Habib et al., 2014). The ratio of K^+ to Na^+ has been proposed as a success indicator in salt resistance. The terrestrial life of plants depends on high-affinity K^+ uptake systems and benefits from high-affinity Na^+ uptake systems. In plants, both systems have received extensive attention during recent years and a clear insight of their functions is emerging. Some plant HAK transporters mediate high-affinity K^+ uptake in yeast, mimicking K^+ uptake in roots, while other members of the same family may be K^+ transporters in the tonoplast. In parallel with the HAK transporters, some HKT transporters mediate high-affinity Na^+ uptake without cotransporting K^+. HKT transporters have two functions: (i) to take up Na^+ from the soil solution to reduce K^+ requirements when K^+ is a limiting factor, and (ii) to reduce Na^+ accumulation in leaves by both removing Na^+ from the xylem sap and loading Na^+ into the phloem sap (Rodriguez and Rubio, 2006). Although there is no data available on the presence of HAK in Cotton but perhaps, there are effective mechanisms to increase transcription of these proteins that may be due to the adjusting effect of K^+ to maintain osmotic salinity stress (Yokoi, 2002; Rodrıguez and Rubio, 2006). The results indicated that in the sensitive cultivar of *Sahel*, the number of Na^+ ions is higher in plants than in the other two cultivars, *Latif* and *Kashmar*. Therefore, there is more damage to plants. However, the Na^+ content was increased in the *Latif* and *Kashmar* cultivars as compared to the control, but this increase was less compared to the sensitive *Sahel* cultivar. Indeed, the number of ions in the plant was reduced when treatment with Pix and the amount of damage was decreased, as well. It can be said that in the sensitive cultivar of *Sahel*, K^+ was not increased but this amount was increased in *Latif* and *Kashmar* cultivars. This result is encouraging and shows that two cultivars of *Kashmar* and *Latif* were resistant to salinity and thus, the amount of K^+ was increased in order to reduce the losses of the Na^+ adsorption.

As a result of the importance of Ca^{2+} permeability and the properties of selective membranes, high levels of Na^+ to Ca^{2+} ions in tissues can cause changes in the cell activities (Ashraf, 2004; Mahmoud, 2009). Ca^{2+} is the main component of the wall and preserve the integrity of the plasma membrane and maintains coherence in roots and shoots during stress (Ashraf, 2004; Mahmoud, 2009; Ashraf et al., 2008).

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Ca^{2+} is an immobile element which remains during the growing season in older tissues, and transfers carbohydrates, facilitates the absorption of nitrogen and reduces the harmful effects of Na^{+} (Ashraf, 2008). Cell wall formation and its development is impossible without Ca^{2+} (Taiz and Zeiger, 1998). Research has shown that Ca^{2+} plays a significant role in the salinity resistance of cotton and it will reduce the toxic effect of Na^{+} with a decrease of Ca^{2+} (Cramer et al., 2002). Pix cause an increase in the concentration of Ca^{2+} ion in the tissues and K^{+} ion at the root. Pix controls the vegetative and reproductive growth by reducing the concentration of gibberellic acid in the plant and reducing the length of internodes (Havargi, 2007; Jonathan, 2006; Joseph, 2006). The results showed that the amount of Ca^{2+} in each cultivar was increased with increasing salinity compared to the control, but this increase was more in *Latif* and especially, *Kashmar* cultivars. This suggests that the mechanism of Ca^{2+} ion to reduce Na^{+} ion was used in these two cultivars under stress conditions compared to the *Sahel* cultivar. Therefore, these two cultivars, and especially *Kashmar* cultivar, are considered as the resistant cultivar to salinity.

The results of this research indicated that *Latif* and especially, *Kashmar* cultivars are considered as resistant cultivars to salt stress. The results of this study showed that growth parameters including germination percentage and radicle length in the *Latif* and especially, *Kashmar* cultivars had a significant increase compared to the sensitive cultivar of *Sahel*. Considering the amount of dry and fresh weight of these two cultivars in particular, *Latif* cultivar was higher than *Sahel* cultivar.

The number of phenolic compounds, proline and glycine betaine in these two cultivars have increased with increasing salinity compared to the control and *Sahel* cultivar. Also, in these two cultivars, the amount of Na^{+} ion absorption was lower than that of the *Sahel* cultivar while the absorption amount of K^{+} and Ca^{2+} ions increased compared to that of the *Sahel* cultivar and control treatment. In Pix treatment as well as Pix and salinity treatment, phenolic compounds and proline increased in the two cultivars of *Kashmar* and *Latif* compared to the controls and *Sahel* cultivar. In Pix and salinity treatments, the amount of proline was increased in salinity treatment and control. The Na^{+} absorption ratio in the *Latif* and *Kashmar* cultivars was decreased in salinity and Pix treatment compared to the salinity treatment. Pix and salinity treatment cause increase in uptake of K^{+} compared to salinity treatment in the *Kashmar* cultivar. Indeed, in Pix and salinity treatment, the amount of Ca^{2+} in all cultivars, especially *Kashmar* and *Latif* was higher compared to the salinity treatment. Therefore, it can be concluded that priming seeds with Pix can lead to a decrease in salinity, especially in resistant cultivars. The *Latif* cultivar and especially, *Kashmar* cultivar showed more resistance to the salinity; hence both are considered as resistant cultivars. Indeed, the priming of seed with Pix has a significant effect on increasing resistance to salinity in these cultivars.

As reported in *Figure 9,* Na^{+} in the aerial parts was reduced through priming of *Kashmar* cultivar with Pix and therefore, the number of these toxic ions decreased. Interestingly, it would occur due to the number of reasons including low absorption of Na^{+} by the roots, reflux Na^{+} from root seedlings, inhibition of Na^{+} transport to the aerial parts and the choice of K^{+} and Ca^{2+} as well as the removal of Na^{+}. Increasing the amount of glycine betaine, proline and phenolic compounds in the seedlings of *Kashmar* cultivars along with reducing the amount of Na^{+} ions in the aerial parts of plants cause less damage of the membrane system and better ion balance in these cultivars. These factors result in an increase in the germination, radicle length, fresh and

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dry weight in the seedlings of *Kashmar* cultivar and finally, had better performance than other cultivars (*Fig. 9*).

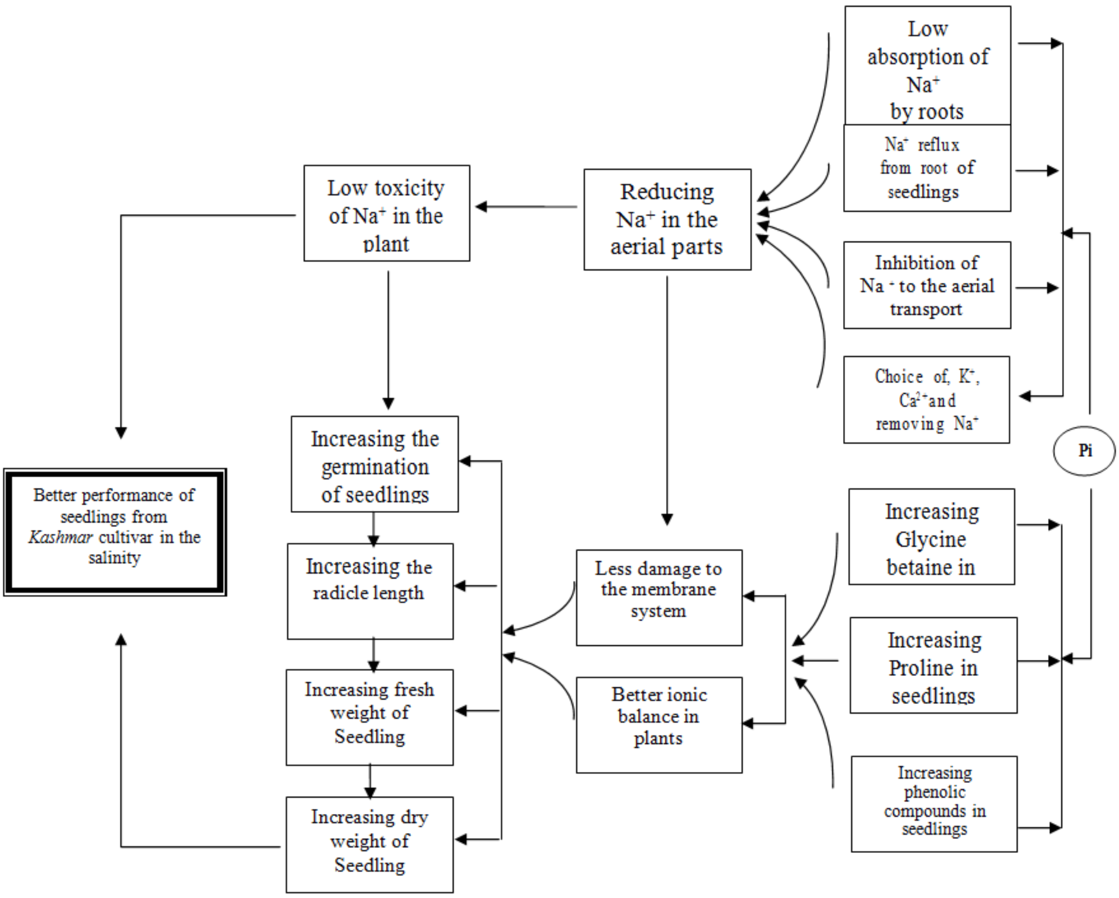

Figure 9. *The effect of Pix on the resistance of salinity in Kashmar cultivar at seedling stage*

Conclusion

The manipulation of cotton plant architecture using plant growth regulators such as Pix can be an agronomic strategy for obtaining high yields. The results indicated that priming Pix improved the physiological and biochemical parameters of cotton (especially in the Kashmar and Latif cultivars) in terms of salinity.

The NaCl stress caused changes in physiological and biochemical traits and reduced germination percentage, radicle length, dry and fresh weight, phenolic compounds, proline and glycine betaine in the Latif and Kashmar cultivars, whereas the treatment of cottonseed with mepiquat chloride in saline stress resulted in significant increase in these parameters. The amount of Na^+ in all three cultivars in different treatments had a significant increase compared to the control treatment.

The amount of Na^+ ion absorption was lower in Kashmar and Latif cultivars compared to the Sahel cultivar while the amount of K^+ and Ca^{2+} ions absorption was increased in the two mentioned cultivars compared to the Sahel cultivar.

It appears that Pix treatment in NaCl stress can be one of the tolerance mechanisms in cotton (especially in the Kashmar and Latif cultivars) which requires further research.

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Considering the fact that in the present study, cotton seed priming with Pix was used, it is recommended that this experiment be carried out with a Pix spray on the plant. Also, the morphological and genetic parameters of the plant in vegetative and reproductive stages, as well as the final yield of the plant should be investigated. Also, given the fact that in the current and traditional agriculture system, cotton is pruned to prevent it from extra growth, it is recommended that the relationship between Pix level and pruning should be investigated.

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STUDY ON OXIDATION OF PICKLE CABBAGE WASTEWATER BY SODIUM PERSULFATE

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Abstract. With activated carbon as carrier and sodium persulfate as oxidant, the preparation conditions of catalyst and the optimal reaction conditions of sodium persulfate advanced oxidation are studied. The study shows that the catalytic performance of manganese in sodium persulfate advanced oxidation treatment of pickle wastewater is the best. The optimum preparation conditions of the catalyst are as follows: at 30 °C, the carrier is soaked in 0.15 mol/L manganese nitrate solution for 2 h and calcined for 2 h at 400 °C. The optimum reaction conditions: the amount of oxidant, dosage of catalyst, reaction pH and reaction time are kept at 1 mmol/L, 10 g/L, 4 and 75 min respectively. and the reaction temperature is set to 30 °C. Under the optimal reaction conditions, the removal rate of chemical oxygen demand (COD) can reach 84%. According to the analysis of BET, FT-IR and X ray diffraction, manganese has been successfully loaded onto the carrier. Meanwhile, the activity of catalyst is affected by physical factors such as specific surface area, aperture and pore volume.
Keywords: *advanced oxidation, characterization analysis, catalyzer, reaction conditions, preparation conditions, COD*

Introduction

As a kind of flavour food, pickle has the effect of reducing blood fat and enhancing immunity (Bao et al., 2011), and it is welcomed by the majority of consumers. With the rapid expansion of the scale of production, environmental pollution caused by a large amount of salt pickle wastewater has been aggravated. Its main pollution components are plant fiber, plant amino acids, organic acids, alcohols, salts, and inorganic salts of metals such as calcium and magnesium. Therefore, they are characterized by high chemical oxygen demand (COD), high nitrogen and phosphorus and so on. The direct discharge of pickle wastewater will pollute the local soil and water, which will cause serious eutrophication of water and salinization of the soil (Zhao et al., 2011). Therefore, the effective solution to the problem of pickle wastewater has been paid more and more attention.

At present, the methods commonly used in the treatment of high salinity, high COD are biochemical method (Wang et al., 2002; Qin et al., 2006; Chen et al., 2011), membrane separation method (Jin et al., 2009; Zhao et al., 2011) and ion exchange method (Bao et al., 2010; Yuan, 2012). The ability of microorganism to treat pollutants is reduced, because of the salt in pickle water, so the effect of traditional biochemical method is not ideal. On the basis of biological method, salt tolerant microorganism has been studied in the treatment of high salt wastewater, but there are still some shortcomings such as long taming time and the fact that it is easily affected by change of salinity (Bao et al., 2010). Ion exchange and membrane separation are ideal treatment methods for high COD and high salinity wastewater, but there is also poor resistance to

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organic pollutants, frequent membrane replacement, high treatment cost and large amount of auxiliary reagents during processing, which are likely to cause extra pollution.

The persulfate advanced oxidation technology is widely used in the field of wastewater treatment, and the strong oxidation of sulfate radical ($SO_4^{-}\cdot$) is used to deal with harmful substances in water (Wang and Cheng, 2017; Zhang, 2017; Shao et al., 2017). During the treatment, $S_2O_8^{2-}$ is converted to $SO_4^{-}\cdot$ (E_0 = 2.5v-3.1v), which is equivalent to or even higher than the redox potential of HO· (E_0 = 2.7V-2.8V), resulting in the oxidation and decomposition of most organic pollutants. In this paper, persulfate advanced oxidation process was used to treat the wastewater of pickled cabbage, and the optimal preparation conditions of the catalyst are explored.

Materials and methods

Experimental reagents and instruments

Reagents and materials: sulfuric acid (AR), sodium persulfate (AR), powder activated carbon, potassium dichromate (PT), mercury sulfate (AR), ammonium ferrous sulfate (AR), silver sulfate (AR), silver nitrate (AR), ferric nitrate, zinc nitrate, manganese nitrate, nickel nitrate, copper nitrate, pH paper, distillation water, Pickle wastewater taken from the pickle plant in Xin Fan Town, the main index of wastewater is shown in *Table 1*.

Table 1. *Pickle wastewater quality indicators*

Project	Content
COD	2500~3000 mg/L
pH	4.0~6.0
Turbidity	300~400
Salt content	0.5%~2%
SS	800~1000 mg/L

Equipment and instruments: electronic balance, COD125 digestion instrument, THZ-82B gas bath constant temperature oscillator, DHG-9240A electrothermal constant temperature blower drying box, SZ-93 automatic double water distiller, SX2-5-12 electric furnace, constant temperature magnetic stirrer B11-2, Automatic multi station specific surface, micropore and mesoporous pore analyzer (BELSORP-max), X- ray diffractometer (EMPYREAN X), Fourier infrared spectrometer (Nicolet 6700).

Method of experiment and analysis

Preparation of catalyst

The activated carbon is placed in the beaker, soaked in distilled water, fully stirred and washed until the effluent is clarified, and then dried at 105 °C in the oven. A certain amount of activated carbon after pretreatment was soaked in a certain concentration of nitrate solution and placed in the air bath constant temperature oscillator for a period of time. Then filtering out, and the catalyst is made by drying and calcination.

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The impregnation conditions and calcination conditions in the process of catalyst preparation were explored through experiments #1 and #2.

(1) Experiment #1

At 30 °C, activated carbon, as a carrier, is immersed in different concentrations of ferric nitrate, zinc nitrate, manganese nitrate, nickel nitrate, copper nitrate for 2 h. The calcination temperature is 400 °C, and the calcination time is 2 h. The catalyst is prepared to deal with pickle wastewater, the reaction temperature is maintained at 30 °C, the pH value is about 5, sodium persulfate dosage was 1 mmol/L, reaction time is 2 h.

(2) Experiment #2

According to the data of Experiment 1, the best immersing solution is used and immersing time is 2 h. The calcination temperature is adjusted to 200, 300, 400, 500, 600 °C, and the calcination time is 2 h; The calcination temperature is adjusted to the best calcination temperature and the calcination time is changed from 1 to 6 h.

Method of optimizing reaction conditions

Under the condition of normal pressure, 100 ml pickle wastewater is placed in a conical bottle, then a certain amount of sodium persulfate and a certain amount of catalyst is added. Finally, the conical bottle is placed in a constant temperature reactor.

The reaction conditions, such as the amount of oxidant, the amount of catalyst, pH, reaction temperature and time, were explored and optimized through experiments #3 ~ #6.

(1) Experiment #3

The optimal conditions of catalysis are determined for the treatment of pickle wastewater based on Experiment #1 and #2. In this part, the dosage of oxidant is changed, while the other reaction conditions are kept. In other words, the reaction temperature is maintained at 30 °C, the pH value is about 5, and the reaction time is 2 h.

(2) Experiment #4

First, the dosage of oxidant is adjusted to be the best, then the dosage of the optimized catalyst is changed, while the other reaction conditions were maintained at 30 °C, the pH value is about 5, and the reaction time is 2 h.

(3) Experiment #5

First, the dosage of oxidant and catalyst are adjusted to be the best, then the pH value of the reaction system was changed while the other reaction conditions are maintained.

(4) Experiment #6

The experiment is carried out by adjusting pH, oxidant dosage, catalyst dosage to the best condition. the reaction temperature and reaction time are changed from 20°C to 45 °C and from 0min to 120 min. The treatment effect of wastewater was determined once every 15 min at different temperatures.

Analysis of results

The components of pickle wastewater is complex. The main components are plant fibre, plant amino acids, organic acids, alcohols, salts, calcium and magnesium ions etc. Therefore, in this experiment, the effect of COD removal rate was used as the evaluation index. The determination of COD was determined by potassium dichromate method (GB11914 - 89). The calculation is based on *Equation 1:*

$$COD_{Removal\ rate} = \frac{(COD_{inlet} - COD_{effuent}) \cdot 100\%}{COD_{inlet}} \quad \text{(Eq.1)}$$

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The specific surface area and pore size distribution of activated carbon carrier and catalyst are analyzed by BELSORP-max (full automatic multistation ratio surface, micropore and mesoporous pore analyzer).

The Nicolet 6700 infrared spectrometer was used to analyze the catalyst and the active carbon carrier, and the characteristic peak change mechanism of the material was analyzed by the EZOMNC software.

The crystal structure of catalyst and carrier activated carbon was determined and qualitatively analyzed by EMPYREAN X ray diffraction analyzer, and the variation of characteristic peak of the material was analyzed by JADE6.0 software.

Results and discussion

Optimization of the catalyst preparation conditions

Determination of active components and impregnation concentration

After the Experiment #1, the COD value of the effluent is measured, the results are shown in *Figure 1*.

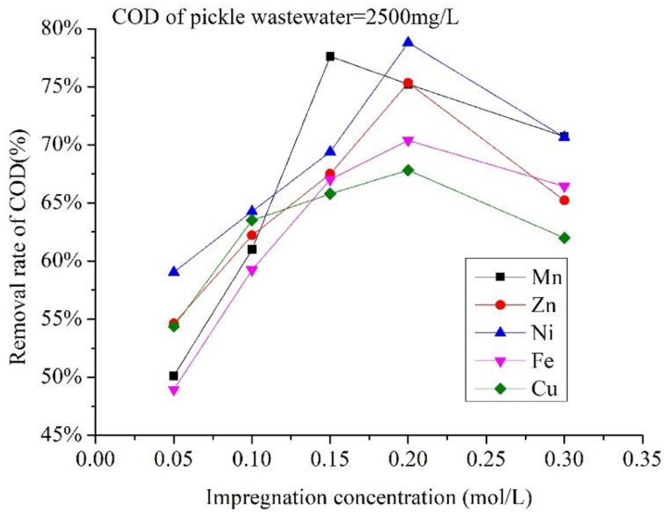

Figure 1. *Treatment effect of different active component catalysts*

As shown in *Figure 1,* the COD removal rate of all catalysts are above 35%. In contrast, the catalytic activity of activated carbon immersed 0.2 mol/L nickel nitrate is the highest, and the removal rate of COD reached 78.80%, the second highest is the catalyst which is immersed in 0.15 mol/L manganese nitrate, reaching 77.61%. This is due to the strong adsorption capacity of activated carbon. In the process of immersion, the active component of nitrate enters its pore structure. During the reaction, the generation of $SO_4^{-\cdot}$ and related chain reactions are accelerated by the transition metals in the carrier, increasing the number of $SO_4^{-\cdot}$ in the reaction system, and the rate of $SO_4^{-\cdot}$ propagation is also increased. When the immersing concentration is too large, the activity of the catalyst decreases. Because in this situation, the active components in the carrier pore will be saturated. The accumulation of active components on the surface of the carrier will lead to uneven distribution, and the active sites will be obscured. The

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efforts between the catalytic efficiency of manganese and nickel is about 1%, but the concentration of manganese nitrate solution is lower and the price is cheaper. Based on experimental data and economic considerations, 0.15 mol/L manganese nitrate is used as the immersing solution, and immersing time is 2 h.

The effect of calcination conditions on the activity of catalyst

After the Experiment #2, the effect of calcination time and calcination temperature on the activity of the catalyst were tested. The results of the test are shown in *Figure 2*(a and b).

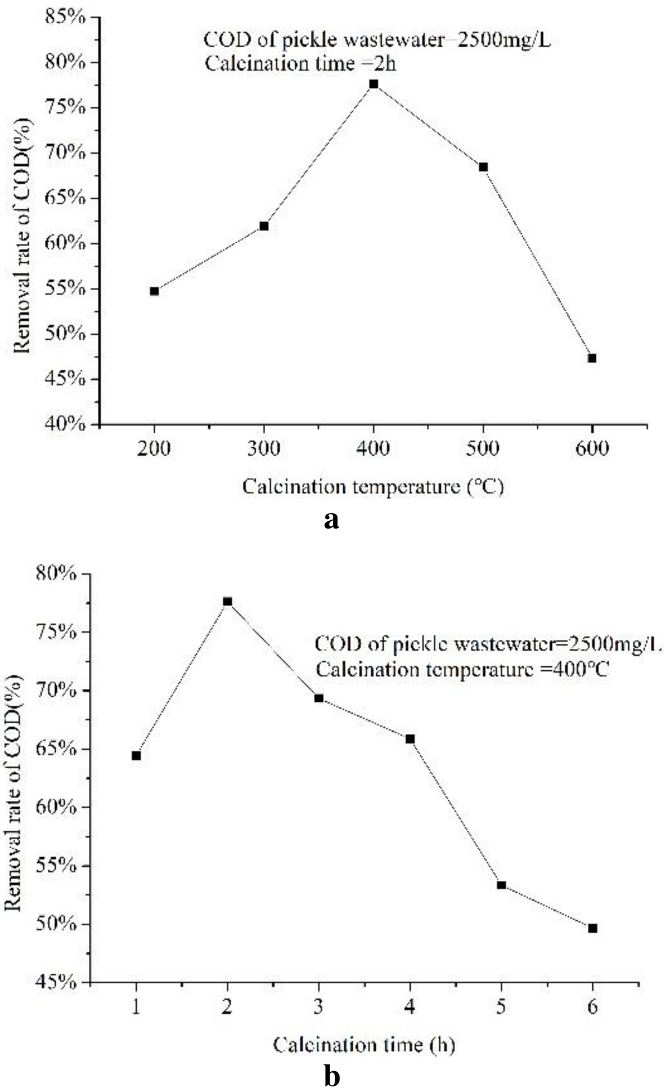

Figure 2. *The effect of calcination temperature on the activity of catalyst (a). The effect of calcination time on the activity of catalyst (b)*

As shown in *Figure 2a,* the activity of the catalyst increases with the calcination temperature when the calcination time is 2 h and the calcination temperature is changed during 200 °C to 400 °C. This is because at a lower temperature, the nitrate on the carrier is not completely decomposed, and the activity of the catalyst is increased by

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raising the calcination temperature. The effect is best at 400 °C, and the removal rate is 77.61%. When the calcination temperature continues to rise, obvious sintering phenomenon will appear, and it leads to the decrease of the specific surface area and the decrease of the activity of the catalyst. From *Figure 2b*, it reveals that the calcination time is 0 h, that is, unroasted, the treatment effect is not good. In contrast, after calcination, the activity increased significantly, This is not only due to the transformation of manganese nitrate into manganese oxide, but proper calcination can also make the surface of the carrier structure is more stable. When the calcination time is 2 h, the removal rate of COD is the largest. The activity of catalyst decreases with increasing calcination time, because the sintering phenomenon occurs, and the pore structure collapses and the specific surface area decreases.

Optimization of reaction conditions

Influence of the amount of oxidant on the treatment effect

Through the Experiment #3, the effect of the catalyst on the treatment effect was tested. The results of the test are shown in *Figure 3*.

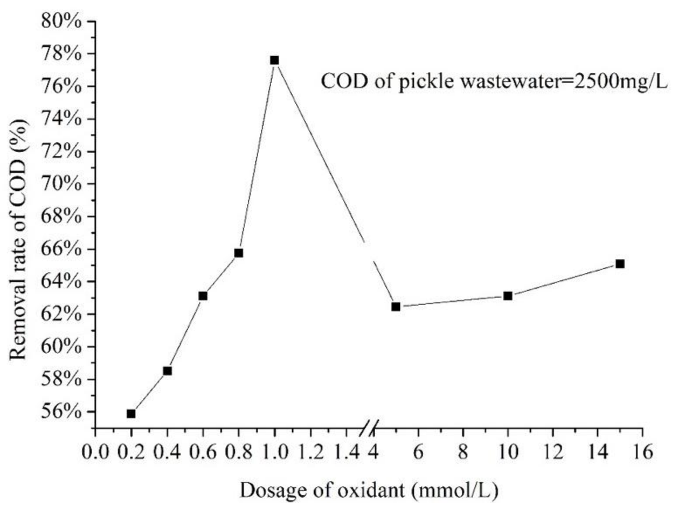

Figure 3. *The effect of the amount of oxidizing agent on the treatment effect*

As shown in *Figure 3,* with the increase of the amount of oxidant, the removal rate of COD increases first and then decreases. When the dosage is 1 mmol/L, the removal rate is 77.61%. When the dosage of sodium persulfate continues to increase, the removal rate of COD will decrease. When the sodium persulfate dosage is 5 mmol/L, the removal rate is 62.46%. The reason for this phenomenon is that with the increase of oxidants, the conversion of persulfate to $SO_4^{-\cdot}$ is accelerated under the action of catalyst, and the $SO_4^{-\cdot}$ content is increased. Its strong oxidizing property is used to decompose organic matter in the waste water and the concentration of COD is reduced. When the dosage of oxidant continues to increase, in the reaction system, a large number of $S_2O_8^{2-}$ will be quenched with $SO_4^{-\cdot}$ (Yang et al., 2010; Liang and Su, 2009), which leads to the decrease of $SO_4^{-\cdot}$ content. At the same time, $SO_4^{-\cdot}$ will also be self coupled. The oxidant utilization is declined by these two factors. Therefore, the dosage of the oxidant in the subsequent experiment was determined to be 1 mmol/L.

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Influence of catalyst dosage on treatment effect

Through the Experiment #4, the effect of the oxidant on the reaction was tested. The results of the test are shown in *Figure 4*.

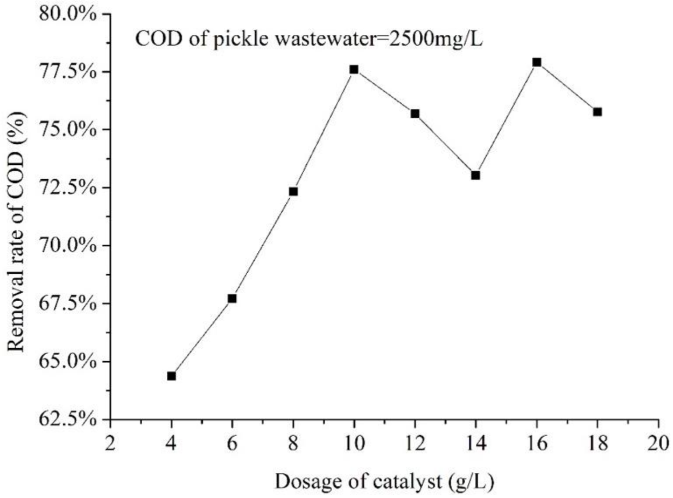

Figure 4. *The effect of catalyst dosage on treatment effect*

As shown in *Figure 4,* with the increase of the amount of catalyst, the removal rate of COD increases first and then tends to be stable. The removal rate of COD in the wastewater increases with the increase of dosage, and the removal rate is up to 77.61% when the dosage is 10 g/L. This is due to the increase of the active site with the addition of the catalyst. The contact probability of the reactant and the active site will increase. The generation of $SO_4^{-}\cdot$ and the reaction of chain reaction were accelerated, resulting in the increase of $SO_4^{-}\cdot$ content, which resulted in higher reaction efficiency and increased COD removal rate. When the dosage of catalyst is more than 10 g, the removal rate of COD always fluctuates around 75%, and the change tends to be stable. When the amount of dosage is 4~18 g/L, the removal rate of COD is all above 64%. Therefore, in the comprehensive economic consideration, the dosage of the subsequent experimental catalyst is determined to be 10 g/L.

Influence of reaction system pH on reaction effect

Through the Experiment #5, the effect of the reaction pH was tested. The results of the test are shown in *Figure 5:* As the pH value of the reaction system increases, the removal rate of COD increases first and then decreases. When pH is 2, the removal rate of COD is 72.34%, and when pH is adjusted to 4, the removal rate of COD increases to 85.81%, and then continues to increase pH value, the COD removal rate will decrease. When pH is alkaline (pH > 8), the removal rate of COD is less than 75%, and when pH is 12, the removal rate of COD is only 52.78%. When pH is medium / strong alkaline (pH > 8), the removal rate of COD is less than 75%, and when pH is 12, the removal rate of COD is only 52.78%. It can be seen that the pH reaction system has great influence on the reaction effect, and this is due to the fact that the mechanism of advanced oxidation reaction system for sodium persulfate in acidic or alkaline condition

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is completely different. When pH is acidic, the $SO_4^{-\cdot}$ in the reaction system is stable (Couttenye et al., 2002), and it can play a role in the process of oxidation.

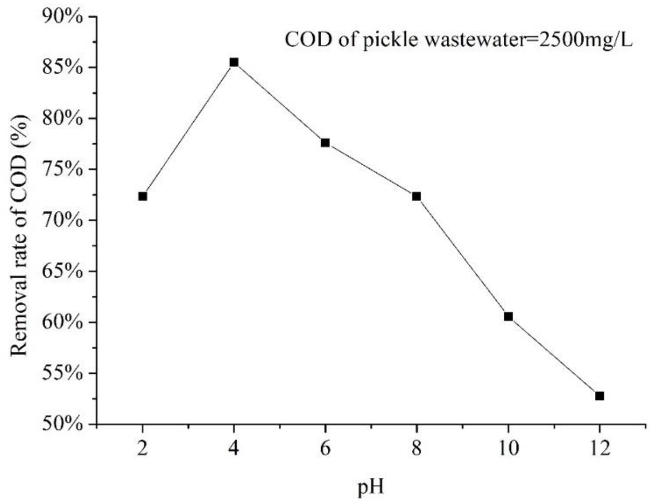

Figure 5. *The effect of reaction system pH on treatment effect*

When the reaction system is alkaline, in the reaction system, $SO_4^{-\cdot}$ Will react with H_2O and OH^-, and the chemical equations are as follows:

$$SO_{4^-}\cdot + H_2O \rightarrow HSO_{4^-} + \cdot OH$$

$$SO_{4^-}\cdot + OH^- \rightarrow SO_4^{2-} + \cdot OH$$

At this time, the hydroxyl radicals (·OH) were involved in the oxidation. However, there was a lot of Cl^- in the pickle wastewater, and Cl^- has quenching effect on ·OH, so that ·OH have been quenched before oxidized, resulting in a sharp decrease in COD removal rate.

Based on the above experimental data and analysis, when the reaction pH changes during 4 to 7, the COD removal rate is kept at a high level. In order to find the best reaction effect, the subsequent pH will be 4.

Influence of reaction temperature and reaction time on the treatment effect

The results of the Experiment #6 are shown in *Figure 6:* From the reaction time, the reaction in the first 15 min is very fast, and the COD removal rate can reach 63.69%. Then, the reaction rate will slow down from 15 to 75 min, and the reaction will tend to be stable after 75 min. From the reaction temperature, when the temperature rises from 20 °C to 30 °C, the removal rate of COD increases from about 72% to about 84%, and the reaction effect is obviously better. When the temperature is higher than 30 °C, the increase of temperature also plays a role in the improvement of the treatment effect but is not obvious, and the change tends to be stable. From the aspect of kinetics, when the temperature is increased, the mass transfer process between reactants and products in liquid phase and active sites is accelerated, which improves the reaction efficiency and increases the removal rate of COD. As the temperature continues to rise, the “forward”

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effect of temperature continues, but from the aspect of thermodynamics, the oxidation process of the organic matter and the adsorption process on the surface of the catalyst are both exothermic. From this point of view, the increase of temperature is not conducive to the oxidation and adsorption. Therefore, when the temperature is higher than a certain temperature, the removal rate increases little. Therefore, considering the energy saving and economy, the reaction time is 75 min, the optimum reaction temperature is 30 °C. From the experimental data, the oxidation process has a good tolerance for temperature.

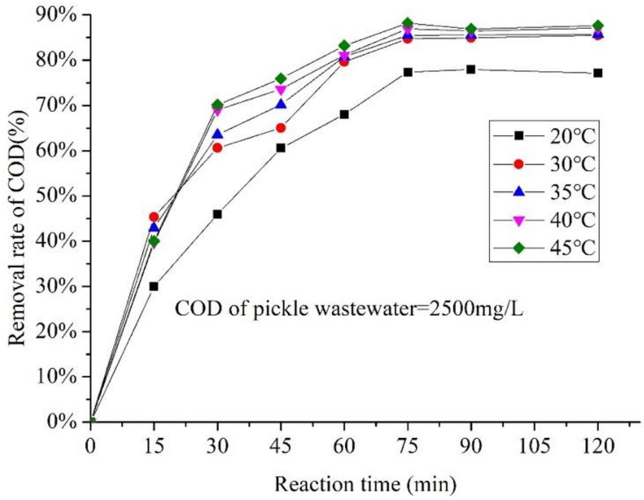

Figure 6. *The effect of reaction temperature and reaction time on the treatment effect*

Characterization and analysis of catalyst

Specific surface area/aperture analysis

In order to further explore the influence of immersion and calcination on the structure of the catalyst, the BET specific surface area of the prepared catalyst was tested. In the experiment, the liquid nitrogen was used as the adsorbent and the desorption curve is shown in *Figure 7,* and the results of the BET test are shown in *Table 2*.

As shown in *Figure 7,* the adsorption curve and the desorption curve coincide in p/p 0 < 0.4, and at p/p 0 > 0.4, the desorption curve is higher than the adsorption curve, then it is closed again at about p/p 0 = 1.0, forming a hysteresis loop. It affects the pore structure of the catalyst and is related to the network properties of the pores. According to the characteristics of this curve, the nitrogen desorption isotherm curve of the catalyst should be summed up as type IV curve, and the pore structure of the catalyst is mainly mesoporous and microporous. According to *Table 2*, it reveals that the specific surface area and pore structure of the carrier changed after impregnation and calcination. The specific surface area of the catalyst increases, the total volume of the micropores increases, but the average pore size and the mean pore volume decrease. This is due to the calcination which can open the inner pores of the active carbon, so the micropores are increased. But, after calcination, manganese nitrate becomes manganese oxide, and it blocks the partial pore structure of the carrier, resulting in the decrease of the average

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pore volume. And it also reflects the active components being successfully loaded onto the catalyst carrier.

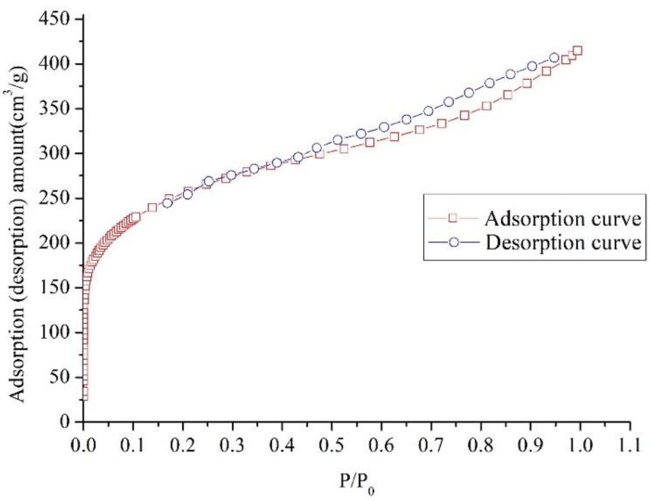

Figure 7. *Catalyst for nitrogen adsorption desorption isotherm*

Table 2. *Catalyst BET analysis*

Name	Impregnation condition	Calcination condition	Specific surface area (m^2/g)	Aperture (nm)	Pore volume (cm^3/g)	Total volume of micropores (cm^3/g)
Carrier AC	--	--	463.81	3.733	0.424	0.1874
Mn/AC catalyst	0.15 mol/L manganese nitrate, 2h	400 °C, 2 h	474.48	3.355	0.398	0.1931

Fourier infrared spectroscopic analysis

Fourier infrared spectroscopy (FT-IR) was used to analyze the changes in the chemical groups of the active carbon of the carrier before and after the load. The results of the experiment are as shown in *Figure 8*.

Figure 8 shows that the carrier activated carbon has absorption peak at 3404.64 cm^{-1}, 2921.88 cm^{-1}, 2844.86 cm^{-1}, 1618.52~1316.82 cm^{-1}, 1162.62~1029.14 cm^{-1}, 896.26 cm^{-1}, 775.70~471.78 cm^{-1}. Among them, a wider absorption peak appeared at 3404.64 cm^{-1}, and it is caused by the hydrogen bond vibration in -OH, indicating the presence of a large number of hydroxyl groups in the samples. 2921.88 cm^{-1} and 2844.86 cm^{-1} indicate the presence of phenolic group (O-H); There are several smaller absorption peaks in the 1618.52~1316.82 cm^{-1} interval, which may be related to the telescopic vibration of C=O and the presence of nitro compounds and carboxyl groups. The absorption peak in the 1162.62~1029.14 cm^{-1} interval indicates that there are also ethers (=C-O-C, C-O-C) in the activated carbon. The continuous and weak absorption peaks in the 500~1000 interval may be caused by the bending vibration of -OH and C-H. Compared with the carrier activated carbon, the overall transmittance of the Mn/AC catalyst was reduced. Among them, the hydroxyl, carboxyl and phenolic hydroxyl groups all increased. All kinds of oxygen-containing functional groups greatly enhanced the polarity of the catalyst surface and enhanced the adsorption capacity. At the same

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time, the peak of the absorption peak of the catalyst at 516 cm^{-1} and 565 cm^{-1} is due to the telescopic vibration of the Mn-O metal bond (Li, 2015a; Li, 2015b). This shows that after the carrier activated carbon impregnated, manganese is successfully loaded, and after calcination, manganese and manganese oxides are formed.

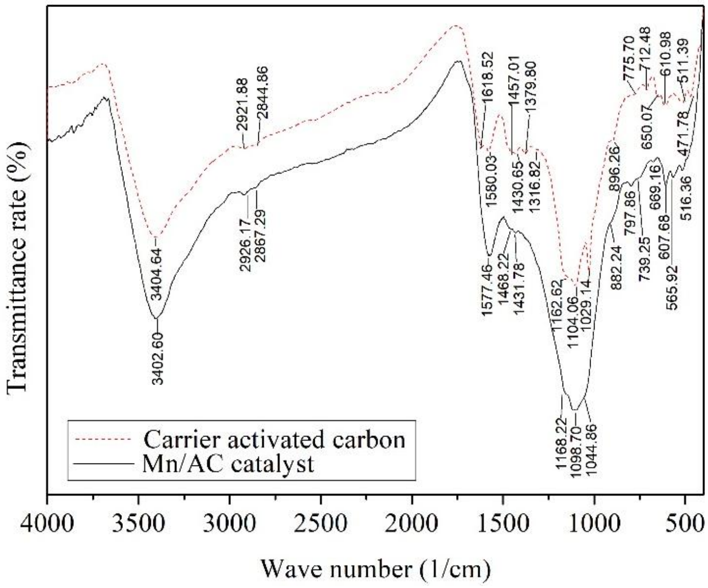

Figure 8. *Comparison of infrared spectra of active carbon of carrier before and after load*

X-ray diffraction analysis

X-ray diffraction was used to analyze the active carbon of the carrier before and after the load. By comparing the variation of the characteristic peaks, the changes in the crystals of the related material before and after the load can be found. The results of the experiment are as shown in *Figure 9*.

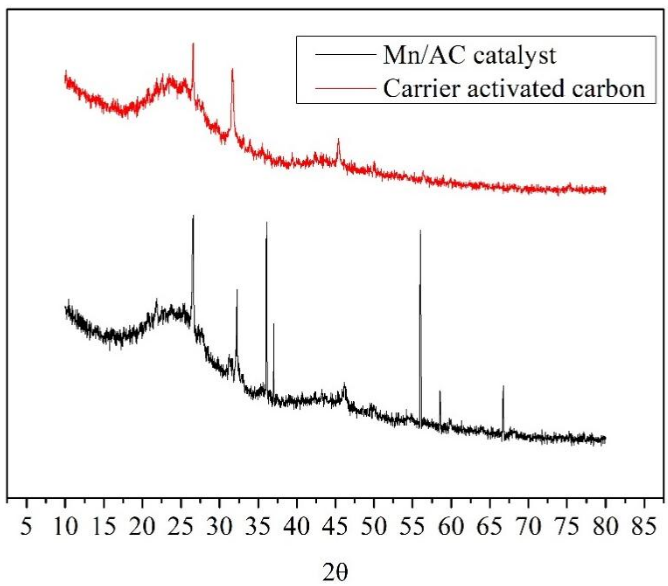

Figure 9. *The Xrd pattern of activated carbon before and after load*

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As shown in *Figure 9,* some sharp peaks of sharp diffraction are found in the carrier after treatment. The peak diffraction angle 2θ is 32.31°, 36.09°, 37.12°, 56.02°, 59.84°, 66.76°. Compared with the JCPDS standard card (30-0820), the diffraction peaks at 37.12°, 56.02°, and 66.76° indicate that MnO_2 is formed in the carrier. Compared with the JCPDS standard card (24-0734), the diffraction peaks of 2θ at 32.31°, 36.09° and 59.84° conformed to the characteristic peaks of Mn_3O_4. The characteristic peak pointed out that the manganese oxide formed the crystal, but the figure of merit (FOM) was not high, which may be related to the background peak and the sample processing conditions during the measurement. Through the comparison of the XRD diagram, it is shown that the manganese oxide has been successfully loaded with activated carbon.

Conclusion

(1) Experiments show that the manganese in sodium persulfate advanced oxidation treatment of pickle wastewater for the catalytic performance is the best. In the preparation of the catalyst, the selection of the suitable impregnation concentration can make the active component into the carrier gap and get the uniform distribution. The selection of the appropriate calcination time and temperature can efficiently convert the active component into the corresponding metal oxide, and the sintering phenomenon is controlled. Based on the experimental data, the optimum preparation conditions of the catalyst are as follows: at 30 °C, the carrier is soaked in 0.15 mol/L manganese nitrate solution for 2h and calcined for 2h at 400 °C.

(2) The prepared catalyst was used for advanced oxidation treatment of wastewater. The effects of oxidant dosage, catalyst dosage, reaction pH, reaction temperature and reaction time on the reaction effect were studied. The optimum reaction conditions: the dosage of oxidant is 1 mmol/L, and the amount of catalyst is 10 g/L, and the reaction pH is 4, and the reaction time is 75 min, and the reaction temperature is set to 30 °C. The COD can be removed 84% when the best preparation conditions of catalyst and the best reaction conditions is used in the advanced oxidation treatment of pickle wastewater.

(3) According to the BET analysis, it is found that compared with the carrier activated carbon, the specific surface area of the carrier is increased. The pore structure of the carrier material be improved by the formation of micropores. This may be the cause of the calcination. The activity of the catalyst is influenced by the specific surface area, aperture, pore volume and other physical factors.

(4) According to Fourier transform infrared spectroscopy analysis, the hydroxyl, carboxyl and phenolic hydroxyl groups on the activated carbon were increased. The oxygen functional groups greatly improve the adsorption capacity and activity of the catalyst. At the same time, the characteristic peaks of the telescopic vibration of the Mn-O metal bond were found in the catalyst. This shows that after the carrier activated carbon impregnation and calcination, the Mn has been successfully loaded.

(5) According to the X ray diffraction analysis, it was found that the catalyst showed a characteristic peak of MnO_2 and Mn_3O_4 compared with the carrier activated carbon. This shows that the carrier activated carbon has successfully loaded the manganese element, which is in accordance with the results of the FT-IR analysis.

(6) Follow up studies can focus on the following aspects: first, further study on the regeneration of the catalyst should be carried out in order to provide a basis for actual operation and economic analysis; second, in order to apply this method to practical engineering, it is necessary to further expand the scale of experiments.

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RAINFALL DISTRIBUTION AND ITS CHARACTERISTICS IN MAKKAH AL-MUKARRAMAH REGION, SAUDI ARABIA

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Abstract. Defining the characteristics of rainfall distribution is the key for many engineering applications such as road design, flood damage mitigation measures, and planning and management of water resources. Since Makkah Al-Mukarramah area is of utmost importance in Saudi Arabia, studying the characteristics of rainfall distribution is necessary. This research aims at investigating rainfall data to define the best rainfall distributions and its characteristics in the study area via rainfall frequency analysis. Different statistical analyses such as log-normal distribution, normal distribution, extreme value type I distribution, Pearson type III distribution, and log-Pearson type III distribution were preformed on more than 20 stations having annual rainfall data over ten years. The results showed the best probability distributions, and rainfall predictions at various return periods for the study area. 36% of rainfall stations follow three parameters log-normal distribution, 27% of stations follow log-Pearson type III distribution, 14% of stations follow Pearson type III distribution,9% of stations follow GEV distribution,9% of stations follow two parameters log-normal distribution and 5% follow Gumbel Type I.
Keywords: *arid and semiarid zones, frequency analysis, rainfall modelling, stochastic analysis*

Introduction

Rainfall is considered the most important factor in hydrology, and nearly most or all of the natural and unnatural activities depend on rainfall, and rainfall initiates various types of work, like water resource management, agriculture and forestry, flood protection and many other (Mahdavi et al., 2010). Predicted changes in precipitation in the future are needed for studying of the consequences of the climatic changes around the world which represent input to the hydrological cycles. And due to lack of randomization in the meteorological and hydrological data, they can be analyzed statistically using frequency data analysis of rainfall and flood. Due to these reasons, it is easy to use statistical distribution in many studies like those dealing with water structure design, water resources and watershed management and parameter estimation in the hydrological cycle (Subyani, 2011; Mashat and Basset, 2011). One of the essential things is the determination and fitting of the best distribution to the data. The analysis of frequency means relating the ultimate events magnitude to their occurrence frequency by the use of the probability distribution (Chow et al., 1988). Rainfall measurements would enhance the feasibility of flood protection works, and will also lead to successful managing of runoff water, and such data analysis will help in preventing floods and droughts (Bahrawi, 2009). It can also be applied in the management and design of most engineering works that deal with water resources like the design of reservoirs, the control of floods and drainage systems, methods to conserve soil and water. Rainfall data are needed to provide the base for all these types of design works.

Makkah Al-Mukarramah area is one of the most developed areas in Saudi Arabia. The annual average rainfall of Makkah Al-Mukarramah area exceeds 100 mm (Bahrawi et al., 2016). There is an increasing need for water for economic development. Autumn and

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winter are the major rainy seasons, and steep terrains of the study area often cause short time of concentration and extremely rapid creation of wadis during the precipitation-runoff process. It results in flash flood dangers. In order to reduce losses arising from these kinds of floods, an appropriate hydrologic design is very important. Rainfalls are not evenly distributed spatially and temporally (Mahdavi et al., 2010). Makkah Al-Mukarramah climate is affected by various air masses from Mediterranean (cyclone type) during winter season to monsoon type in summer season. The mechanisms of the movement of these air masses is explained in details in the literature (Mahdavi et al., 2010; Subyani, 2011; Mashat and Basset, 2011).

This particular research intends to study the rainfall distribution characteristics of the Makkah Al-Mukarramah area, through utilizing various statistical tests of probability distributions such as the normal distribution, log-normal distribution, extreme value type, I distribution, Pearson type III distribution, and log-Pearson type III distribution all these distributions are mentioned as suitable and practical in the literature (Kite, 1977). Rainfall gauging data from stations of Makkah Al-Mukarramah province were used to investigate the fitness of statistical distributions (*Fig. 1*). Twenty-two stations providing annual rainfall data over more than 30 years were chosen in order to execute the frequency analysis (Subyani, 2011). For determination of the best fitting distribution to maximum daily rainfall the Relative residual mean square (RRMS) is used. Shuqiu (1993) suggested in her research, that: "Nearly all rainfall stations at the same region can easily certainly not be characterized simply by only an individual probability distribution, supposing these types of stations to fit to a specific probability distribution and type a cluster." nearly all the stations in the similar regions categorized a varied cluster in agreement with their probability distributions. In case important characteristics among clusters persisted, such as a particular part of the stations being similar to rainfall characteristics and consequently fit to the exact same probability distribution, these characteristics might result from the stations of a cluster and have a special connection to a particular daily rainfall data. Subyani (2011) used annual maximum rainfalls from 8 rainfall stations in order to obtain rainfall frequency analysis. The author utilized the statistical distribution methods like Gumbel's extreme value distribution and Log Pearson Type III distribution for maximization of the daily rainfall data over 24-40 years, and he found that the best fitting for prediction of the rainfall occurrence in future is Gumbels model.

The areas with heavy annual rainfall within Saudi Arabia extend along the southwestern Red Sea Coast and North-Eastern coast and then moves over the middle region of the Saud Arabia. Hasanean and Almazroui (2015) mentioned that within Saudi Arabia, the very heavy precipitation areas were along the southwestern coast and the South West to North East inclined precipitation band, which moves over the middle region of Saudi Arabia. However, the annual average rainfall of the Makkah Al-Mukarramah area exceeds about 140 mm mean annual rainfall, generally there is a big gap between rainfalls, which usually has wide uncertainly spatially and temporally (Subyani, 2011; Shuqiu, 1993).

Materials and methods

Study area

Saudi Arabia is divided into 13 districts. Makkah Al-Mukarramah region is one of the most important districts. It has 22 recording rainfall gauges (*Fig. 1*). Makkah Al-Mukarramah region has actually a dry climate with extremely high temperature values during the day, and minimal rainfall that is inconsistent (Mahdavi et al., 2010; Subyani, 2011). There are either wadis during the entire 12 months, or the rain might just occur in the type of one or two abundant outbursts that may cause actual flooding. Data from different sources, which is usually climatological, was provided by the Ministry of Water and Electricity in Saudi Arabia. Spatially, the mountainous area has additional rainfalls compared to the regular area, and the sloping ground would definitely result from the water preservation potential of watershed to decline dramatically (Subyani, 2011). A large portion of rainfalls will become runoff and discharges quickly, causing damage to infrastructure. Types of these sources had a continuous and timely based record (*Table 1*). The rainfall data with valid documentation obtained and examined from these types of gauging stations, considerably increased in the past 20 years. Some other stations either had very few records of daily data or records with total sum of daily data.

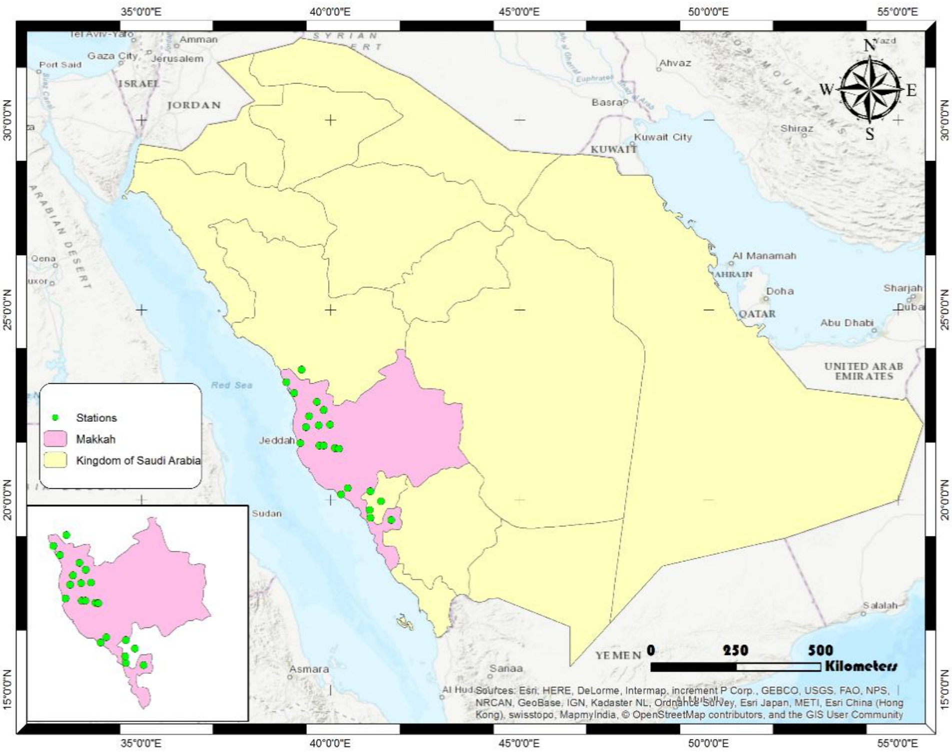

Figure 1. Stations in the Kingdom of Saudi Arabia and Makkah Al-Mukarramah Region

Data

Saudi Arabia has an arid environment characterized by extreme heat during the day, abrupt drop in temperature at night. Rainfall in the region of Makkah Al-Mukarramah is low and erratic. *Figure 2* shows the average monthly rainfall depth for the rainfall stations in the study area from (1966 to 2013). The figure shows that the spatial variation of rainfall is influenced by the topographic features. The rainfall depth increases with the elevation (which is called the orographic effect). The orographic rainfall is due to the mechanical raising of the clouds over mountain boundaries (Linsley et al., 1982). The stations near the coastal zone receive less rainfall in comparison with the stations in mountainous areas. (see e.g. station J108, J111, J134, J140 at coastal plain, and stations J113, J116, J131, J137 at the mountains). It is obvious

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from *Figure 2* that most rainfall occurs in October, November, December and January (winter season), and out of the winter season there is a lot of rainfall in March and April. May, June, July, August and September have low rainfall. There is no specific pattern spatially and temporally in every region. This behaviour is evident from *Figure 2* since in every month there is a high variation of rainfall depth in all stations.

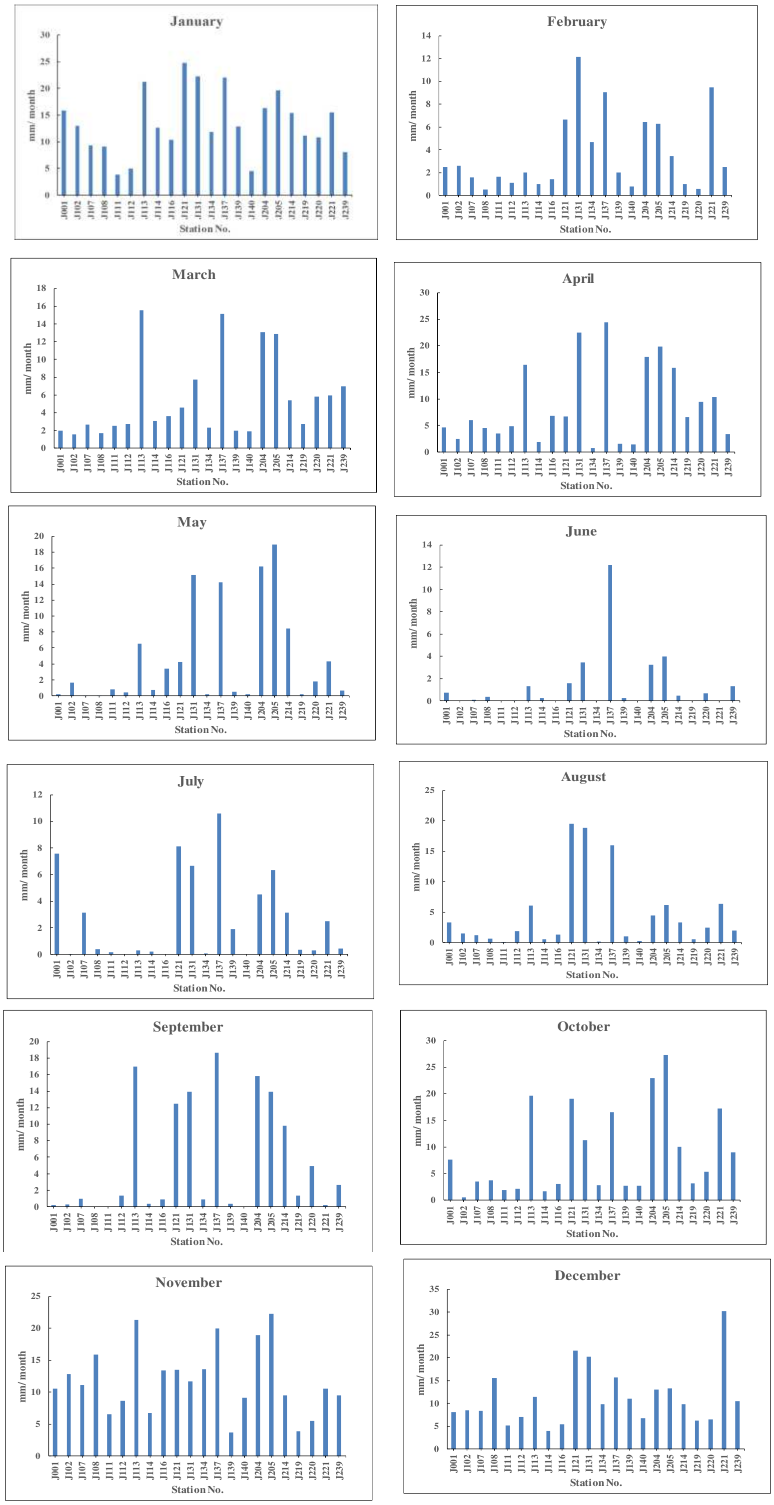

Figure 2. *Average monthly rainfall depth*

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Continuous rainfall data were collected from 1966 to 2013, from the rainfall gauge network that covers the Makkah Al-Mukarramah region. The data collected for each station includes the station number, station symbol, years of records, coordinates, and number of storms (*Table 1, Fig. 3*). Maximum daily rainfall, storms are collected from the 22 stations covering the study area (*Table 1*). *Figure 3* shows samples of time series for maximum daily rainfall data at some stations in the study area. The figures show high variations in the rainfall data over the station history. Some years show no rainfall.

Table 1. *Stations used in the current study*

Station number	Station name	Record		Number of storms	Coordinate	
		From	To		Lat. (N)	Long. (E)
J001	MUDAYLIF	1966	1998	27	19°31'47.46"	41° 3'4.32"
J102	BAHRAH	1966	2011	40	21°25'58.94"	39°42'4.45"
J107	GHUMAYQAH	1966	2012	34	20°18'59.24"	40°27'4.38"
J108	LITH	1966	2011	40	20° 8'59.29"	40°16'52.38"
J111	MASTURAH	1966	2008	38	23° 5'58.49"	38°50'4.54"
J112	UMM AL BIRAK	1964	2012	39	23°25'58.39"	39°14'4.53"
J113	FARRAIN	1966	2005	38	21°21'58.94"	40° 7'4.44"
J114	MAKKAH	1967	2013	20	21°25'58.93"	39°49'4.46"
J116	SHABAH	1966	2012	37	22°34'58.61"	39°38'4.48"
J121	HAJRAH	1966	2007	37	20°13'47.25"	41° 3'4.34"
J131	FAYJAH	1970	2012	39	19°28'11.46"	41°36'4.28"
J134	JEDDAH	1970	2012	38	21°29'58.93"	39°12'4.49"
J137	HASAN AL HABS	1966	2005	35	19°58'11.32"	41°19'52.32"
J139	WADI DDQAH	1967	2011	28	19°43'59.40"	41° 2'4.31"
J140	RABIQH	1966	2012	32	22°48'58.57"	39° 2'4.52"
J204	KURR	1966	2005	39	21°20'58.95"	40°12'4.42"
J205	MID SCARP	1966	2005	40	21°20'58.95"	40°13'4.43"
J214	MADRAKAH	1966	2006	40	21°58'58.77"	39°59'4.44"
J219	AIN AZIZIYAH	1970	2006	36	22°11'58.73"	39°25'52.49"
J220	MIDHAH	1966	2011	40	22°22'10.67"	39°49'16.47"
J221	USF AN	1971	2006	30	21°54'58.81"	39°21'4.49"
J239	BARZAH	1976	2011	32	21°57'58.79"	39°41'4.46"

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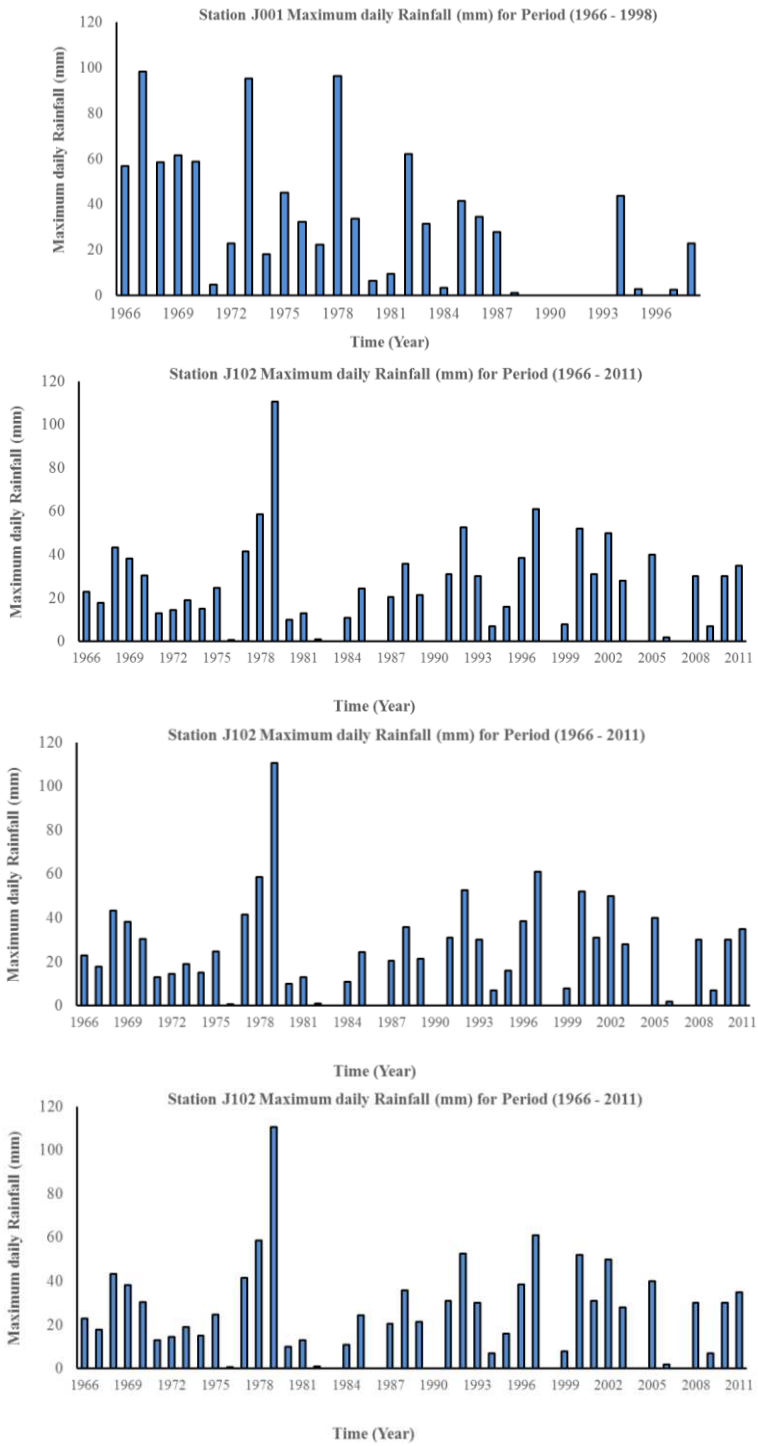

Figure 3. *Sample of maximum daily rainfall depth time series of some stations, namely J001, J102 and J108*

Research method

Frequency analysis

Hydrologic Frequency Analysis is the method used for evaluation of the probability of the hydrologic events, which are averaged out in statistical viewpoints, either greater

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than or of a specific magnitude within a certain area, that will occur within a certain period. The frequency analysis methods used in this study are as follows.

The equation (*Eq. 1*) of frequency analysis is

$$Q_T = M + K_T S \tag{Eq.1}$$

where Q_T is the hydrologic quantity (rainfall depth in the current study) with the return period T; M is the mean of hydrological data; S is the standard deviation of hydrological data; and K_T is the frequency factor, a function of the return period and probability distribution Kite (1977) has calculated K_T for the Gumbel distribution for the lognormal distribution and for the gamma distribution. The followings are short descriptions of the probability distributions used in the analysis:

Two-parameter lognormal distribution (LN2)

The lognormal distribution is used at a larger stage for the analysis of environmental extremes. The probability density function of the two-parameter lognormal distribution is calculated as *Equation 2:*

$$f(x) = \frac{1}{\sqrt{2\pi}\sigma x} \exp\{ -\frac{1}{2}(\frac{\ln x - \mu}{\sigma})^2 \} \tag{Eq.2}$$

μ is a location parameter, and σ is a scale parameter.

The magnitude of the event with return period T = 1/p can be estimated from *Equation 3:*

$$\ln x_T = \mu + \sigma Z_p \tag{Eq.3}$$

where Z_p is the standard normal variate with exceedance probability p.

Three-parameter lognormal distribution (LN3)

The three-parameter lognormal distribution which is in fact the two-parameter distribution is represented as *Equation 4:*

$$f(x) = \frac{1}{(x - x_0)\sigma\sqrt{2\pi}} \exp\{ -\frac{1}{2}(\frac{\ln(x - x_0) - \mu}{\sigma})^2 \} \tag{Eq.4}$$

μ is the location parameter and ð is the scale parameter, while X_0 controls the shape of the distribution.

The event magnitude for the return period T = 1/p is calculated according to *Equation 5:*

$$\ln(x_T - x_0) = \mu + \sigma Z_p \tag{Eq.5}$$

where z_p is the standard normal variate with exceedance probability p.

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Pearson Type III distribution (P3)

The Pearson Type III distribution is one of a large family of probability distributions developed by Pearson (Price and Harrison, 1975). Both the exponential and gamma distributions are special cases of the Pearson Type III distribution.

The distribution function is (*Eq. 6*):

$$f(x)=\frac{(x-x_0)^{\gamma-1}\exp\left\{\frac{-(x-x_0)}{\beta}\right\}}{\beta^{\gamma}\,\Gamma(\gamma)} \quad \text{(Eq.6)}$$

The X_0 which constitutes a lower bound controls locations, β controls the scale and γ controls the shape. And due to difficulty in calculating the magnitude of the event with return period T using hand calculation, the best and simplest way is to make estimations of the T-year event from the means of the sample, the standard deviation and a factor K_T (*Eq. 6*)

$$x_T=\bar{x}+\sigma K_T \quad \text{(Eq.7)}$$

The factor K_T varies with return period T and sample skewness γ, and can be read from tables (Tables 9-2 and 13-4 in Mahdavi et al., 2010, for example).

Log-Pearson Type III distribution (LP3)

Log-Pearson Type III distribution is in fact refers to Pearson Type III distribution, and it is represented as *Equation 8:*

$$f(x)=\frac{\ln(x-x_0)^{\gamma-1}\exp\left\{\frac{-\ln(x-x_0)}{\beta}\right\}}{\beta^{\gamma}\,\Gamma(\gamma)} \quad \text{(Eq.8)}$$

The X_0 which constitutes a lower bound controls location, β controls the scale and γ controls the shape. And the best and simplest way is to make estimations of the T-year event from Pearson Type III distribution.

Generalized extreme value distribution (GEV)

The generalized extreme-value (GEV) distribution was introduced by Jenkinson (1955, 1969) and Mahdavi et al. (2010) initiated the distribution based on the, Generalized Extreme Value (GEV) for annual rainfall data, and thus brought together a number of distributions into Gumbel's value, and thus the function is (*Eq. 9*):

$$F(x) = \exp\text{-}\,[1 - k(\frac{x - u}{\alpha})\,]^{1/k} \quad \text{(Eq.9)}$$

and the magnitude with Gumbel reduced variate y can be determined from *Equation 10:*

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$$x = u + \alpha\left(\frac{1 - e^{-ky}}{k}\right) \qquad \text{(Eq.10)}$$

$$where \quad u + \frac{\alpha}{k} \leq x < \infty \quad if \;\; k < 0$$

$$-\infty < x \leq u + \frac{\alpha}{k} \quad if \;\; k > 0$$

μ and α represent parameters, a location parameter and a scale parameter, and K represents the shape of the distribution. When K becomes zero the general extreme value will be reduced to extreme value 1 distribution, and when K becomes negative then the general extreme value will be type 2 without an upper limit, and when K is positive then the general extreme value becomes 3 with an upper limit of $\mu + \alpha/k$.

Results and discussions

It was indicated from the relative frequency distribution that Log Pearson type III distribution initiated the best probability distribution (Mahdavi et al., 2010). *Figure 4* shows the annual average rainfall depth of the stations in the study area. It is obvious that there is high variation between the different stations (minimum is 10 mm and maximum 45 mm) the highest annual rainfall depth is observed in station J205 and lowest rainfall station is J140. The standard deviation rainfall of the stations is shown in *Figure 5*.

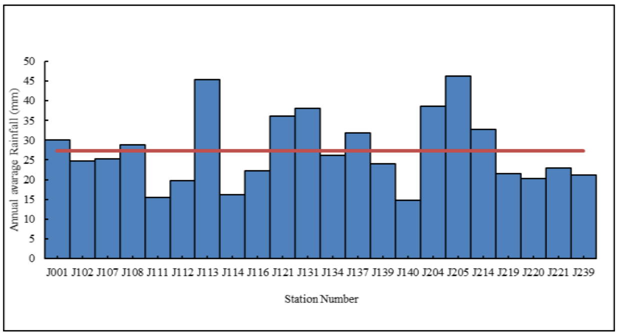

Figure 4. *Annual average rainfall depth of the stations in the study area*

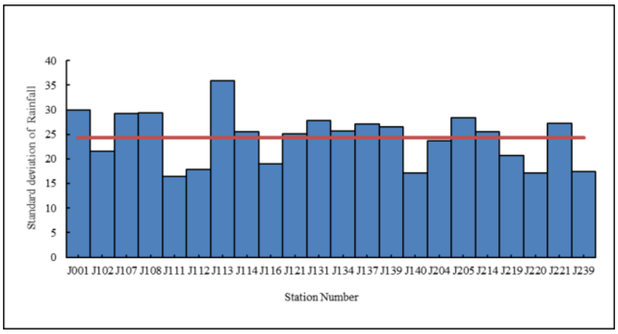

Figure 5. *Standard deviation of the rainfall depth of the stations in the study area*

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The relationship between the annual average rainfall depth and the distance from the sea shown in *Figure 6,* as the distance from the sea increases the rainfall depth increases. A trend line is made to describe the relationship. Similarly, *Figure 7* shows a relationship between the annual average rainfall depth and the elevation of the stations from the sea. As the elevation increases the rainfall depth increases. The trend line is made to describe the relationship.

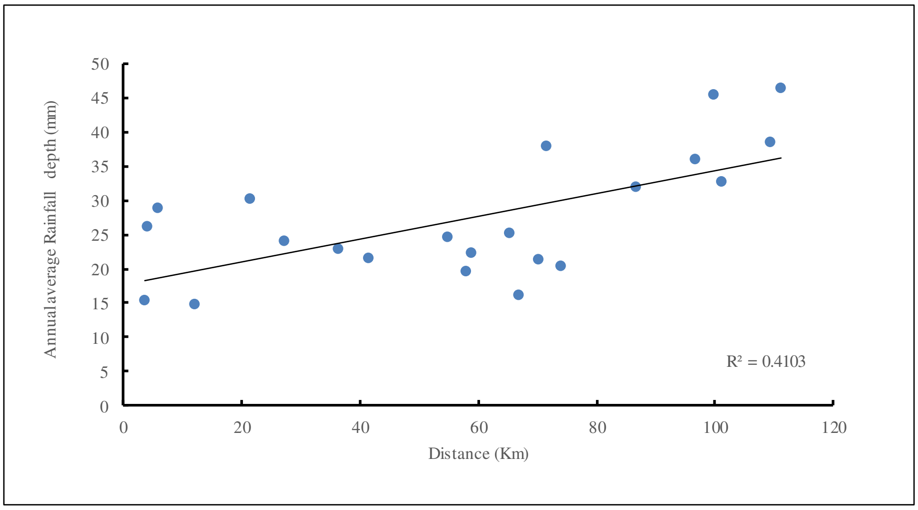

Figure 6. *Distance versus annual average rainfall depth*

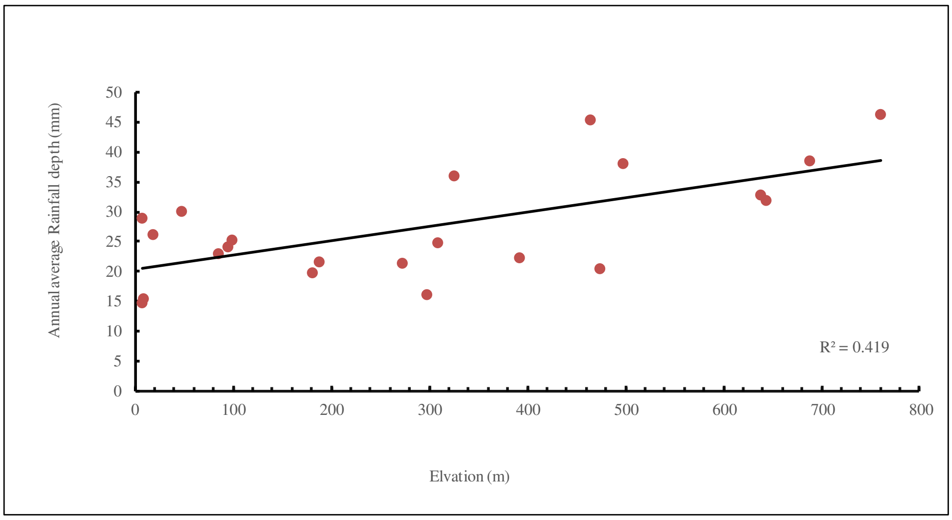

Figure 7. *Elevation versus annual average rainfall depth*

Frequency analysis

In order to determine common characteristic rainfall distribution of the study area frequency analysis is applied using various distributions, of which, three parameters log-normal distribution, two parameters log-normal distribution, log Pearson type III distribution, Pearson type III distribution, and Gumbel extreme value distribution, with rainfall data recorded through 22 stations over 30 years. Results showed that the 3 par

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log-normal distribution is the best probability distribution, occupying 36% of the total number of stations (as shown in *Figure 8a, b, c* and *Table 2*).

The best distributions were chosen dependent on the root-mean-square error measure, RMSE (Mahdavi et al., 2010). RMSE values for the different distributions explain the mean inconsistency between the predictable value and the measured one. *Table 2.* shows the best fitting distributions depending upon the minimum RMSE as given by italicization in the table. The best probability distribution is presented in *Figure 8a, b, c*.

Table 2. *Root-mean-square error (RMSE) for all stations at different distributions*

Stations	Distribution type					
	Gumbel type I	**GEV**	**Two-parameter log-normal**	**3-par log-normal**	**Pearson type III**	**Log-Pearson type III**
J001	9.49	9.78	13.13	9.43	9.96	**7.32**
J102	12.84	12.03	10.40	11.73	**9.34**	18.21
J107	20.78	19.47	**14.00**	18.71	17.84	19.61
J108	20.69	**6.23**	7.65	8.29	14.68	11.73
J111	5.39	3.07	6.28	3.68	4.19	**1.29**
J112	4.49	2.64	3.71	**2.26**	3.28	3.40
J113	14.44	12.58	**7.93**	11.99	10.81	12.94
J114	7.57	7.23	8.67	**6.44**	7.50	6.50
J116	4.50	3.92	5.78	3.77	3.77	**3.27**
J121	7.42	**7.08**	7.71	7.12	7.10	13.97
J131	10.14	7.34	7.15	**6.35**	8.02	6.47
J134	8.40	7.10	9.56	**5.39**	6.97	5.92
J137	2.32	2.38	5.73	**2.29**	3.05	3.58
J139	5.85	5.91	7.28	5.63	6.12	**4.18**
J140	5.17	5.82	6.87	5.38	5.08	**5.04**
J204	6.60	4.65	5.48	4.81	**4.64**	16.13
J205	4.66	4.96	6.78	**4.55**	4.68	23.90
J214	11.78	8.73	9.65	**8.41**	8.83	17.35
J219	8.65	7.59	10.21	**6.70**	7.87	7.13
J220	2.74	2.78	4.01	**2.62**	2.91	7.86
J221	11.20	**3.58**	4.63	4.78	8.25	6.72
J239	3.14	2.94	4.67	2.84	3.01	**1.69**

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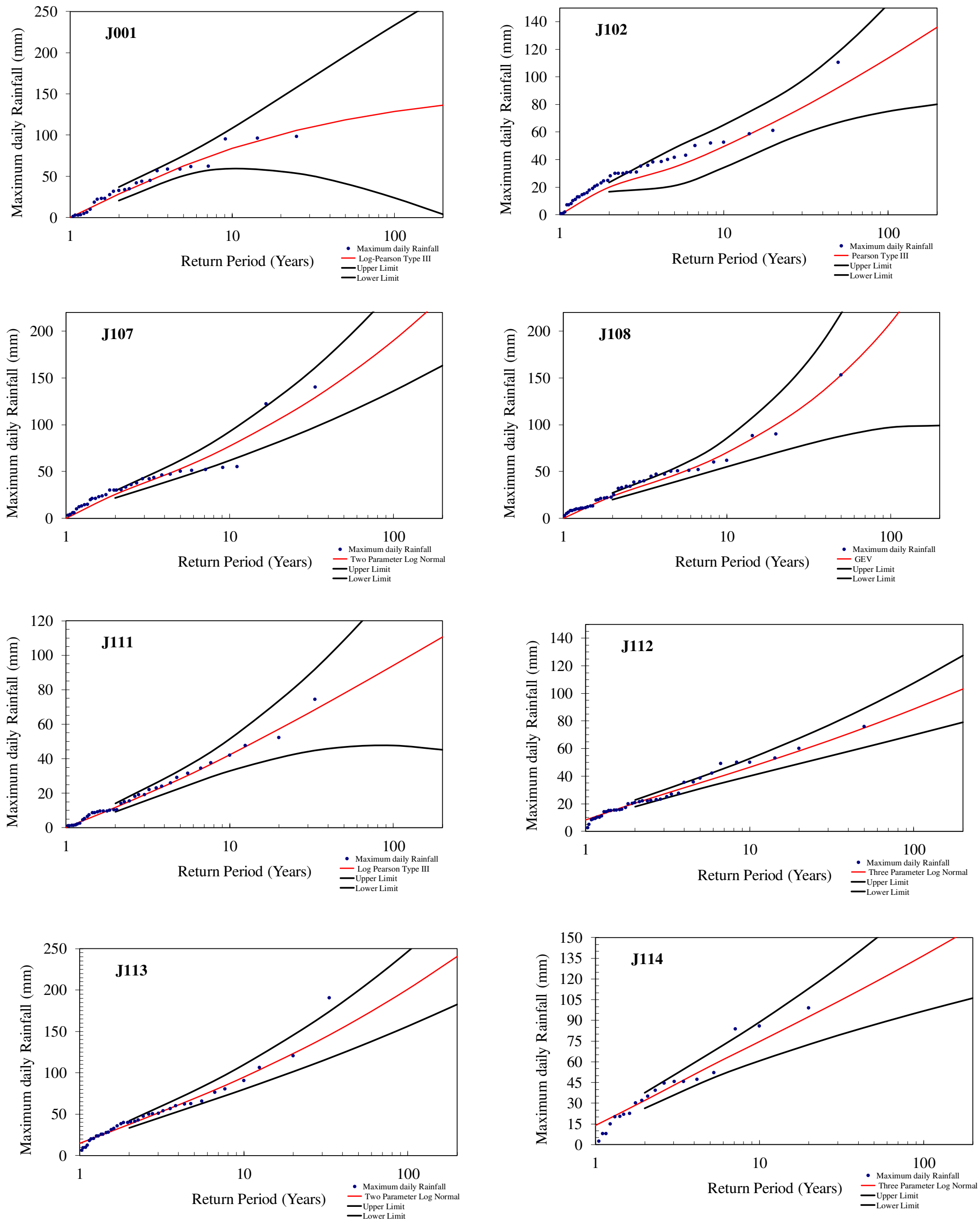
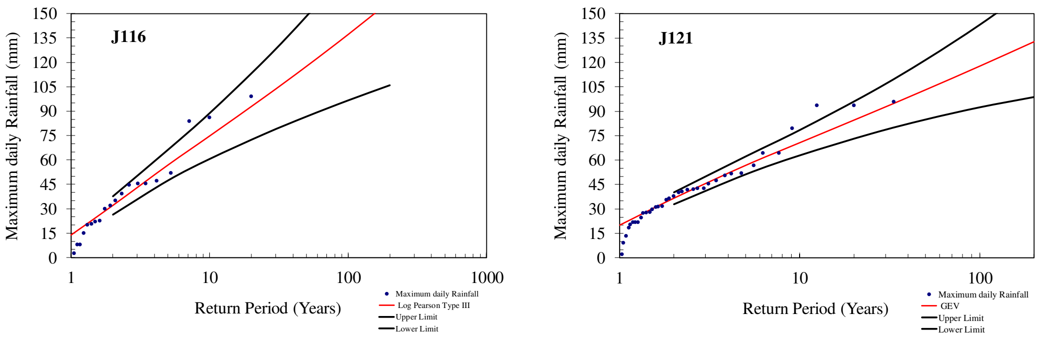


(a)



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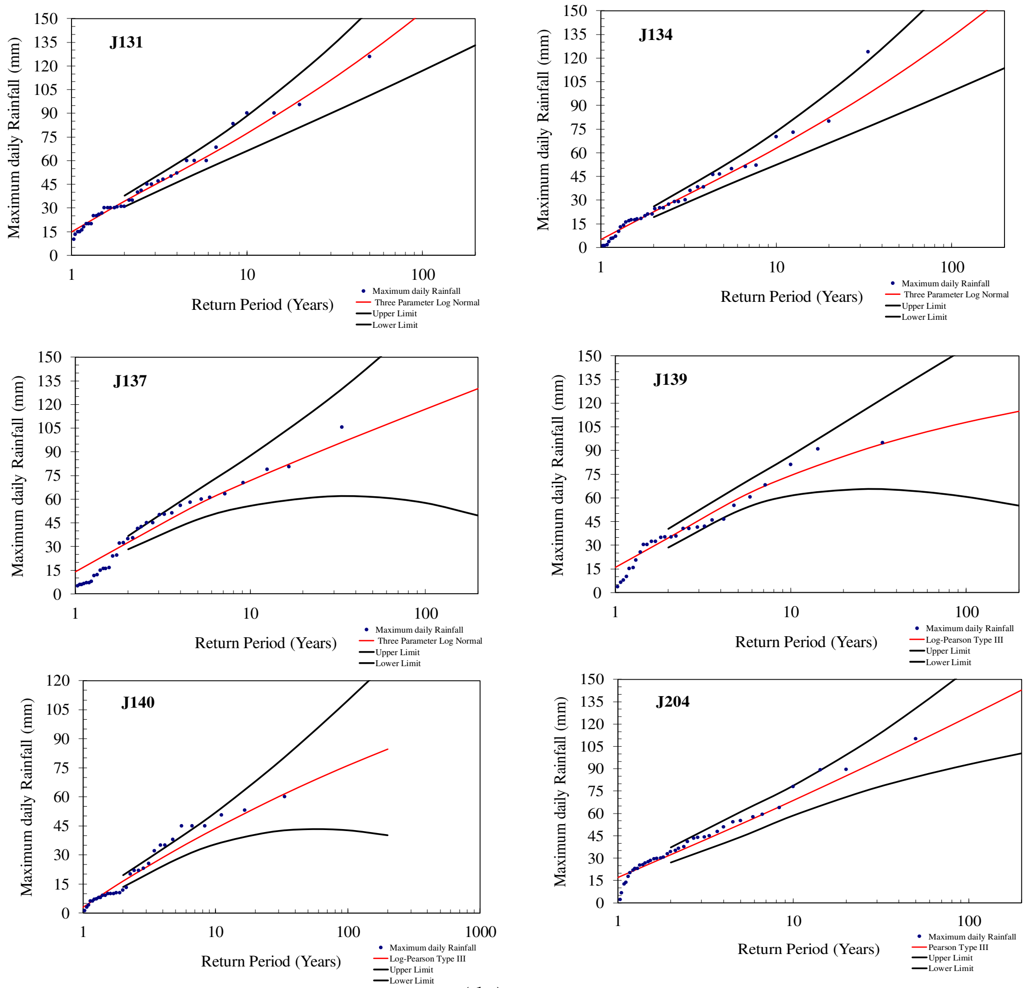
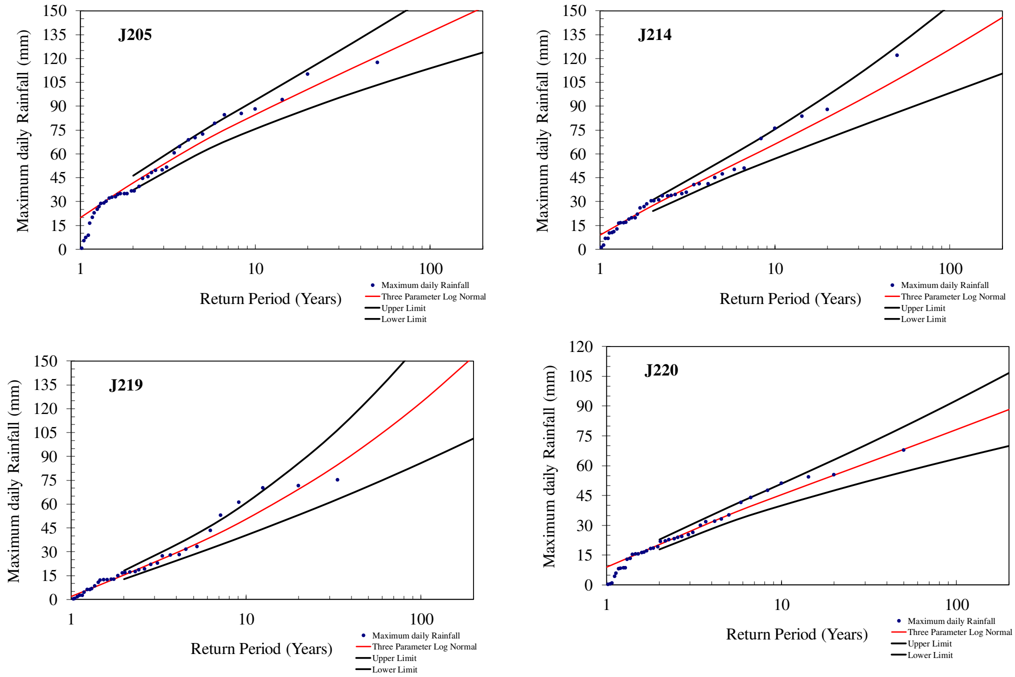


(b)



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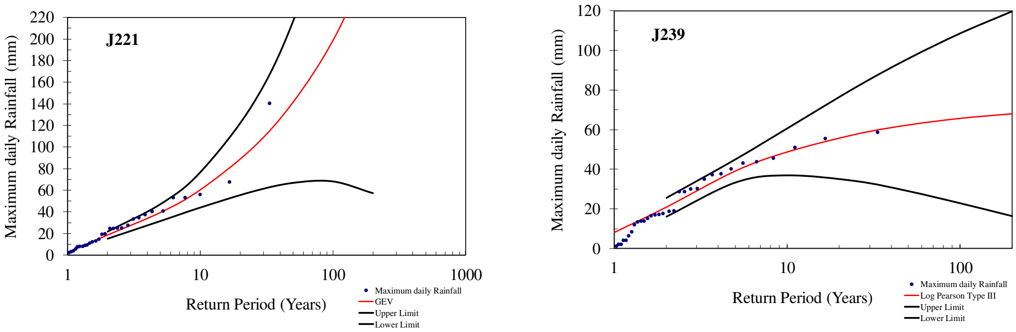

(c)

Figure 8. *(a, b, c) Rainfall depth, frequency analysis at the stations in the study area based on the best probability distribution assigned from Table 2 and the corresponding confidence interval*

Table 3 shows the maximum and minimum values of the expected rainfall depth for study area under different return periods. The table shows that the variation between the minimum, and maximum is very high depending on the location of the stations where the stations over the mountain area have high expected rainfall depth. However, stations at low plain area have low expected rainfall depth. The eastern part of the study area has no stations. Therefore, the contouring extrapolated values for these areas which need to be validated by ground stations. It is recommended to have extra rainfall stations in this area to get more reliable mapping. *Table 4* shows the percentage of each distribution of the rainfall stations. The table shows that 36% of the rainfall stations follow three parameters log-normal distribution, 27% of the stations follow log-Pearson type III distribution, 14% of the stations follow Pearson type III distribution, 9% of the stations follow GEV distribution, 9% of the stations follow two parameters log-normal distribution and 5% follow Gumbel Type I.

Table 3. *Expected maximum and minimum rainfall depth for Makkah Al-Mukarramah region at different return periods*

Return period years	Minimum rainfall depth (mm)	Maximum rainfall (mm)
5	27.08	69.17
10	42.41	94.91
25	57.47	132.98
50	62.41	165.36
100	65.76	208.85
200	68.1	282.15

Table 4. *The percentage of the best distributions over Makkah Al-Mukarramah region from all stations*

Distribution type	% per all stations
Gumbel type I	5%
GEV	9%
Two-parameter log-normal	9%
Three parameters log-normal	36%
Pearson type III	14%
Log-pearson type III	27%

Moreover, *Figure 9* displays a color contour map of the expected rainfall depth at different return periods from 2 to 200 years over Makkah Al-Mukarramah region. The maps show the locations of expected high and low rainfall depth in the region. This can be useful in the flood analysis in these areas. Locations of maximum rainfall have a high risk of flooding while locations of minimum rainfall have a low risk of flooding.

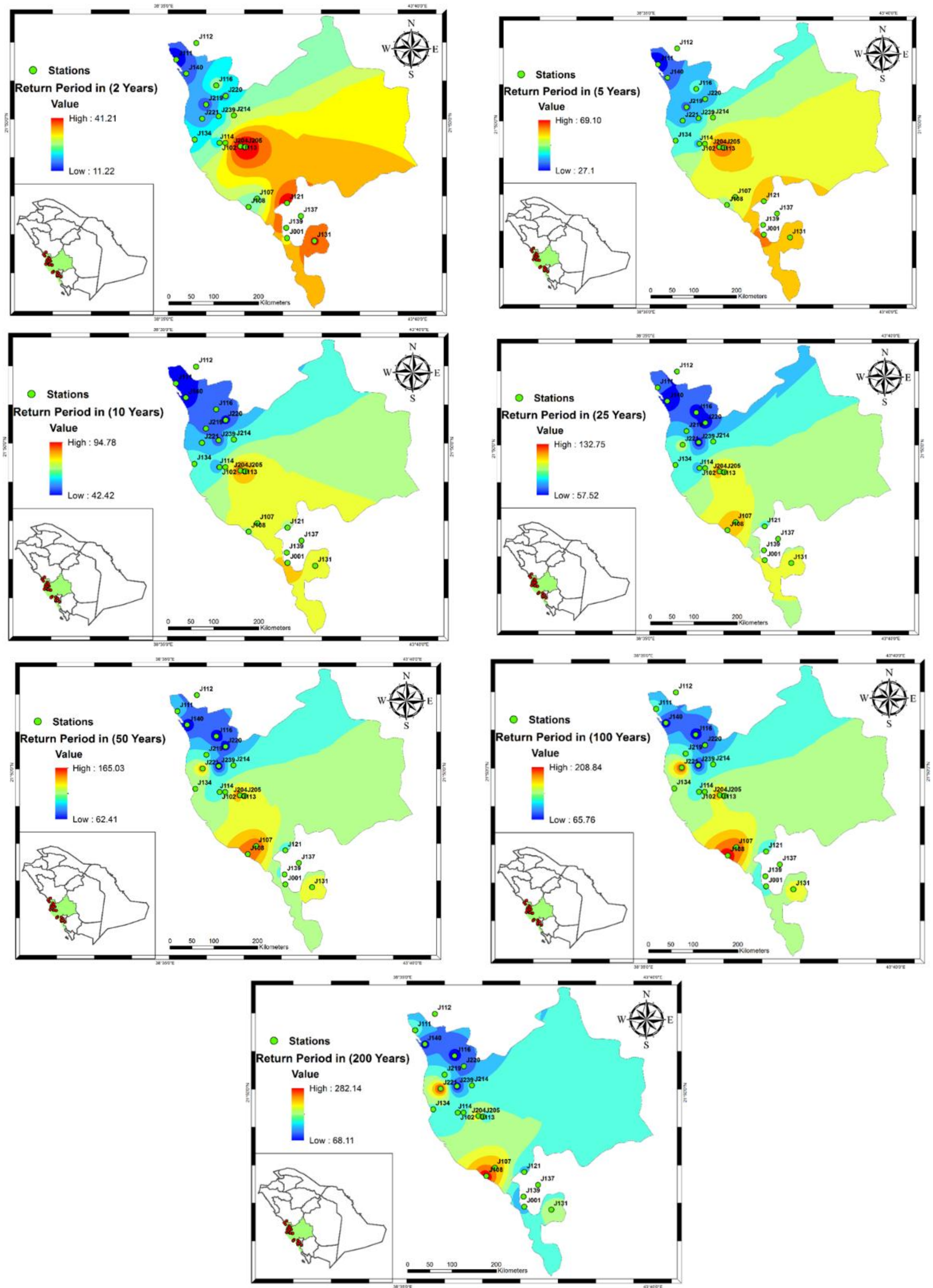

Figure 9. *Mapping rainfall depth for different return periods (5, 10, 25, 50, 100, 200 years) over Makkah Al-Mukarramah region*

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Conclusion

From the results of five frequency distributions applied in this study, it is suggested that the best frequency distribution obtained for Makkah Al-Mukarramah area is the three Parameter Log-Normal distribution, which occupies 36% of the total number of stations, followed by the log-Pearson Type III and Pearson type III distribution which accounts for 27% and 14% of the total station number, respectively. From the results of the frequency analysis, three Parameter Log-Normal distribution is the primary distribution pattern for this study site. By increasing the number of years and data and employing different methods for the analysis, it is believed that more accurate results could be obtained. Outcomes revealed that the three Parameter Log-Normal distribution, conducted the best in the probability distribution, fitting 36% of the total station data.

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LAND USE AND LAND COVER CHANGE IN CHINA'S LOESS PLATEAU: THE IMPACTS OF CLIMATE CHANGE, URBAN EXPANSION AND GRAIN FOR GREEN PROJECT IMPLEMENTATION

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Abstract. This paper used data on land use, normalized difference vegetation index, digital elevation model, temperature and precipitation, and urban distribution to analyse the impacts of climate change, urban expansion and the Grain for Green project implementation on land use and land cover change in the Loess Plateau between 1980 and 2015 based on a single factor and comprehensive factor analysis. The aim of this paper is to explore the spatial expression of human activities under the influence of urban expansion, climate change, and the implementation of the Grain for Green project. The results indicated that climate change dominated among the influencing factors of land use and land cover change in the Loess Plateau, accounting for 93.65% of the total, with a surface-like distribution. It was followed by urban expansion, accounting for 5.46% of the total, with a point-like distribution. The impact of the Grain for Green project implementation accounted for 0.64%, with a patchy distribution. However, due to the low resolution, the quantitative assessment results regarding the role of the Grain for Green project implementation was lower, while for climate change and urban expansion, results were higher.
Keywords: *NDVI, temperature, precipitation, correlation coefficient, buffer analysis*

Introduction

Land use and land cover change (LUCC) is an important component of global environmental change research and was one of the core programs for the International Geosphere-Biosphere Programme (IGBP) and International Human Dimensions Programme (IHDP) joint research between 1994 to 2005 (Liu et al., 2010). The Global land project (GLP) replaced the LUCC research program and became the global land system research program during 2005-2015. It inherited the content of the LUCC program and now focuses more on land system dynamics, change results and integrated sustainability analysis and simulation (Milne et al., 2009). Through preliminary LUCC studies, scholars have confirmed the role of LUCC in the earth surface system, and LUCC has become important data and background information for any land-related research (Liu et al., 2014a).

The main influencing factors of the LUCC process are climatic factors and human activities. Climatic factors affect LUCC process mainly through the influence of land surface vegetation, and human activities mainly impact LUCC by affecting land use on

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the earth's surface (Chen et al., 2014; Song et al., 2014). In terms of climatic factors, arid and semi-arid regions, ecologically fragile and sensitive areas are affected by climate change and dramatic changes in LUCC (Guan et al., 2015; Li et al., 2015a; Lu et al., 2016), especially with changes in precipitation and increases in temperature that increase the amount of glacial meltwater, which leads to greater water volume and improved vegetation growth conditions. These changes would promote the development of regional LUCC in a more sustainable direction, which is conducive to improved regional ecological and environmental conditions (Niu et al., 2013; Wang and Wang, 2013; Liu et al., 2014b). Human activities include agricultural reclamation, urban expansion, ecological construction and the implementation of environmental protection policies. The primary tropical rainforest areas in the world face degradation of surface vegetation caused by agricultural reclamation. Tropical rainforests and ecosystems have been severely damaged by this, which has had a serious negative effect on global environmental change (Malhi et al., 2014; Chen et al., 2016). In the arid region of northwestern China, Xinjiang also faces a large amount of land reclamation. Despite the increase in the amount of water in the region caused by global warming, reclamation of and extraction of surface water and groundwater in large quantities require the supervision and scientific management of government departments, in order to prevent terrestrial salinization and water scarcity caused by over-consumption (Feng et al., 2016; Wei et al., 2017). In terms of urban expansion, in countries that have experienced centuries of industrialization such as Europe and the United States, the pace of urban expansion has been effectively kept in check and even halted (Theobald, 2005; Seto et al., 2012). In the emerging economies of the world, such as China with its rapid economic development, the process of urbanization is accelerating, orderly and disorderly urban expansion has had a significant impact on the earth's surface and LUCC. Various types of land use and land cover in the area around the city are gradually replaced by urban lands, which leads to the simplification and homogenization of LUCC structure and the gradual loss of spatial heterogeneity in specific areas (Li et al., 2013; Chuai et al., 2015; Wang et al., 2016). With regard to the implementation of the government's ecological construction and protection policies, China has made a great deal of effort. It has successively implemented the construction of the "Three Norths" shelterbelt, closing hillsides for afforestation and prohibiting grazing activities, as well as the implementing the Grain for Green project (GGP) to restore farmlands to forest land and grassland (Li et al., 2015b; Jiang et al., 2016; Li et al., 2016). The GGP has a wide scope of work, a large resident population, significant funds and positive environmental impacts. The vegetation on the land surface has been restored and improved, and the vegetation coverage has continuously increased (Deng et al., 2014; Liu et al., 2017). The first round of GGP was started in 2000 and completed in 2008, and the second round was implemented in 2015. Through subsidies, the implementation of GGP has promoted farmers to voluntarily give up the cultivation of farmland in steeper regions.

The Loess Plateau is a major environmentally fragile and sensitive area in China and also a hotspot for research. The impacts of climate change, urban expansion and the implementation of the GGP on LUCC are well known in the region (Su and Fu, 2013; Chen et al., 2015; Xie et al., 2016). However, most studies have focused on one or two factors, while the quantitative study of three factors has not been done. This paper analyzes and quantitatively evaluates the impacts of climate change, urban expansion and the implementation of the GGP on LUCC in the Loess Plateau, using data on land

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use, normalized difference vegetation index (NDVI), digital elevation model (DEM), temperature, precipitation and urban distribution from 1980 to 2015. It provides scientific support for the protection and utilization of the environmental resources of the Loess Plateau.

Materials and methods

Study area

The Loess Plateau is located in the centre and western region of China, east of the Taihang Mountains, west Wuqiaoling Mountains and Riyue Mountains, and south of the Qinling Mountains, north of the Great Wall. The geographical coordinates are 100°50′-114°12′E and 33°40′-41°18′N, and it encompasses the provinces or autonomous regions of Qinghai, Gansu, Ningxiang, Inner Mongolia, Shaanxi, Shanxi and Henan, with a total area of 63.2×104 km^2. The area mainly has a temperate continental monsoon climate, with cold and dry winters and hot and rainy summers. The average annual precipitation is about 460 mm, which decreases in a southeast-northwest direction. The southeast is a semi-humid zone, the middle is a semi-arid zone, and the northwest is an arid zone. The Loess Plateau is between 85-5210 m above sea level, rising from east to west and is the largest loess accumulation area in the world (*Fig. 1a*).

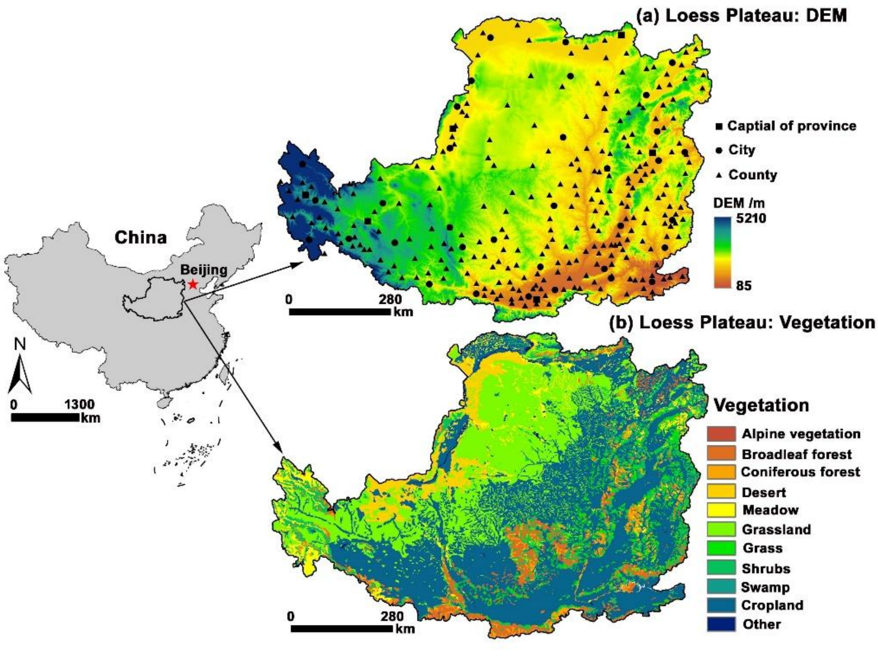

Figure 1. *Digital elevation model and vegetation type distribution in the Loess Plateau*

Loess is an Aeolian product with a loose texture and strong erodibility. In summer, concentrated precipitation and complex terrain are the important factors influencing soil water erosion in the middle and eastern parts, while the northwestern part of the plateau experiences severe soil wind erosion. Vegetation and water distribution are closely related and have a southeast-northwest distribution, mainly including coniferous forests, broadleaf forests, shrub lands, deserts, grasslands grass, meadows, swamps, alpine vegetation and cropland (CVEB of CAS, 2001) (*Fig. 1b*). The Loess Plateau is located

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in the middle of the Yellow River basin. The rivers in the territory include the Huangshui, Qingshui, Kuye, Wuding, Yanhe, Beiluo, Jinghe, Weihe and Fenhe Rivers. The Loess Plateau has a long history of development and is one of the more densely populated areas in China, which means that the original vegetation on the surface has been greatly damaged and reclaimed for cultivated lands. China's reform and opening up, rapid economic development led to a heavy urban expansion in the region. In addition, climate change, especially temperature rise and increased frequency of extreme precipitation, has caused changes in the surface vegetation in the area, while GGP has also impacted land use and land cover in the Loess Plateau. Scientific analysis of this process can provide a basis for the adjustment and restriction of human activities within the Loess Plateau.

Data

The data used in this study included: (1) land use raster data for China with a spatial resolution of 1km in 1980, 1990, 2000 and 2015, in which land use was divided into six types: cultivated land, forest land, grassland, water body, construction land and unutilized land. (2) China's annual average temperature and precipitation raster data had a spatial resolution of 1km from 1980 to 2015. The data was generated by interpolating daily observation data from more than 2400 weather stations throughout the country using ANUSPLINE software. (3) An SRTM (Shuttle Radar Topography Mission) digital elevation model (DEM) had a spatial resolution of 90m. (4) Vector boundaries of the Loess Plateau and point data for provincial capitals, cities and counties were also included. These datasets were provided by Data Center for Resources and Environmental Sciences, Chinese Academy of Sciences (RESDC) (http://www.resdc.cn). (5) Third generation Global Inventory Monitoring and Modelling System NDVI from Advanced Very High-Resolution Radiometer sensors (GIMMS AVHRR NDVI3g) data from 1982 to 2015 with a spatial resolution of 0.05° were downloaded from http://ecocast.arc.nasa.gov/data/pub/gimms/3g.v1/.

Data from (1), (2) and (5) above were used to obtain land use, temperature and precipitation; and NDVI in the Loess Plateau was extracted in ArcGIS software based on the vector boundaries. The NDVI and DEM data were resampled to 1 km resolution.

Methods

Single factor analysis

(1) Vegetation NDVI trends and *F* test

The trend analysis used a linear regression model to analyze the temporal and spatial change process and the possible future change direction of NDVI in the Loess Plateau, as well as to reflect the linear change trend and spatial distribution in the study period. It is calculated as follows (Xu, 2014):

$$Y = aX + b \tag{Eq.1}$$

$$a = \frac{\sum_{i=1}^{n} x_i y_i - n\bar{x}\bar{y}}{\sum_{i=1}^{n} x_i^2 - n\bar{x}^2} \tag{Eq.2}$$

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$$b = \bar{y} - a\bar{x} \tag{Eq.3}$$

where: Y is the NDVI value or spatial distribution data; X is the year 1982-2015; a is a coefficient; b is a constant; $\bar{x}$ and $\bar{y}$ are the average values of X and Y. Positive or negative values of a reflect a linear increase or decrease of vegetation NDVI.

For the linear regression model using the F test for significance, the formula is as follows (Xu, 2014):

$$F = U / [Q / (n-2)] \tag{Eq.4}$$

where: $Q = \sum_{i=1}^{n}(y_i - \hat{y}_i)^2$, $U = \sum_{i=1}^{n}(\hat{y}_i - \bar{y})^2$; $\hat{y}$ is the fitted value of y calculated using values for a, b and x; and the other parameters are the same as in the previous formula. In an F test threshold table, when α = 0.05, the critical value is 4.15. This means that a linear trend is significant when $F > 4.15$, and not significant otherwise.

(2) Impact analysis of climate change on NDVI

The correlation coefficient was used to identify the relationship between climate change and vegetation change, and the formula is as follows (Xu, 2014):

$$r = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}} \tag{Eq.5}$$

where: x and y are NDVI and temperature and precipitation, respectively. The r value ranges between [-1, 1], where r = 0 means that the two variables are irrelevant. The closer the value of r is to -1, the stronger the negative correlation. The closer it is to 1, the stronger the positive correlation. In the correlation coefficient threshold table, when α = 0.05, the critical value of r is 0.3494. That is, a correlation coefficient value of $|r|>0.3494$ is significant, and not significant otherwise.

(3) Impact analysis of urban expansion on LUCC

The impacts of urban expansion on LUCC were mainly concentrated in areas near cities and decreased with distance from cities. A buffer analysis method was used to generate a 30 km buffer around the provincial capitals and cities at intervals of 2 km. Counties adopted 1 km intervals within 10 km and 2 km intervals in the range of 10 km to 20 km. The average NDVI values and changes were analyzed within the buffer zones from 1982 to 2015. The limits of urban expansion affecting vegetation were identified, and that boundary was used as the criterion for the impact of urban expansion on LUCC process, after which the relative change rate of land use within and outside the boundary was calculated. The formula is as follows:

$$Area\_avg_{blct} = \frac{A_{blct}}{Urban_{nc}} \tag{Eq.6}$$

$$RC = \left(\frac{Areaavg_{blc(t+1)}}{SArea_{b(t+1)}} - \frac{Area\_avg_{blct}}{SArea_{bt}}\right) \times 100\% \tag{Eq.7}$$

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where: RC is the relative change rate, %; $Area\_avg_{blct}$ and $Area\_avg_{blc(t+1)}$ represent the average area of l land use type within buffer b of city c (or provincial capital/county) at time t and t+1, respectively, km^2; $SArea_{bt}$ denotes the total area of all land use types in buffer b at time t, km^2; A_{blct} represents the area of l land use type in buffer b of city c (or provincial capital/county) at time t, km^2; $Urban_{nc}$ represents the number of city c (or provincial capital/county).

(4) Impact analysis of GGP on LUCC

The implementation of GGP was mainly aimed at areas with slope>=25°, and the influence on LUCC as also considered to be mainly distributed in the area of >=25°. Statistics on land use and NDVI changes in the region of >=25° in the Loess Plateau before and after 2000 were used to determine the impact of GGP on LUCC. Land use statistics were calculated using the change rate method:

$$R_{tsl} = \frac{Area_{tsl}}{SumA_{tl}} \times 100\% \quad \text{(Eq.8)}$$

$$CR = R_{(t+1)sl} - R_{tsl} \quad \text{(Eq.9)}$$

where: CR is the change rate, %; R_{tsl} and $R_{(t+1)sl}$ represent the percentage of land use type l on slope s at time t and $t+1$, respectively, %; $Area_{tsl}$ denotes the area of land use type l on slope s at time t, km^2; $SumA_{tl}$ denotes the area of land use type l at time t, km^2.

Comprehensive factor analysis

Based on the single factor analysis above, the change in LUCC in the Loess Plateau from 1980 to 2015 and the impacts of climate change, urban expansion, and the implementation of GGP on LUCC were obtained. However, it was still necessary to quantitatively determine the role of each factor in the LUCC process and their spatial distributions through comprehensive mapping of multiple influencing factors. This analysis involved land use, F test results for the NDVI change process, and correlation coefficients between NDVI and temperature and precipitation. It also involved analysis results for the impact of urban expansion and the implementation of GGP. The calculated method was as follows:

(1) Land use (*LD*): Based on the land use classification of the Loess Plateau in 2015, the values of construction land, cultivated land, and water body were assigned to 4, 3, 1, respectively, and the values of forest land, grassland, and unutilized land were assigned to 2.

(2) F test results of the NDVI change process (*FT*): The values for significant and not significant decreases were assigned to 4 and 3, respectively, and the respective values for not significant and significant increases were assigned to 2 and 1.

(3) Correlation coefficients between NDVI and temperature (*TE*) and precipitation (*PE*): The values for significant and not significant negative correlation were assigned to 4 and 3, respectively, and positive and significant positive correlation values were assigned to 2 and 1, respectively.

(4) The impact of urban expansion (*UP*): Within the range of urban expansion for the provincial capitals, cities, and counties in 2015, the value of construction land was assigned to 4, farmland, forest land, grassland, water body and unutilized land were assigned to 3, and values outside the range were assigned to 2.

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(5) The impact of GGP (*IGGP*): The value of construction land in the region with slope >= 25° in 2015 was assigned to 4. The value of cultivated land converted into forest land and grassland between 2000 and 2015 was assigned to 3. The value of cultivated land in the region with slope >= 25° in 2015 was assigned to 2. All other values were assigned to 1.

(6) Comprehensive evaluation results: The formula was as follows:

$$CE = LD \times 100000 + FT \times 10000 + TE \times 1000 + PE \times 100 + UP \times 10 + IGGP \quad \text{(Eq.10)}$$

where:

CE is the comprehensive evaluation of the results.

CE ranged between 111111 and 444444 and was composed of six digits. From left to right, the first digit indicated land use, the second indicated the *F* test result for NDVI change, the third indicated the correlation between NDVI and temperature, the fourth indicated the correlation between NDVI and precipitation, the fifth indicated urban expansion, and the sixth indicated GGP. The larger the value of *CE*, the greater the impact of urban expansion was, while the smaller the value, the less affected the area was by human activities and the greater the impact of climate change was.

The specific classification method was as follows, according to the area statistics for each type and spatial mapping:

(1) If the six digits of the CE contained the number 4, it was divided into the area impacted by urban expansion.

(2) If the six digits of the CE contained the number 1 but did not contain the number 4, and the 6th number was not 3, it was divided into the area impacted by climate change.

(3) If the 6th digit of the CE was 3, it was divided into the area impacted by GGP implementation.

(4) The parts not assigned to the above areas were divided into the area impacted by other factors.

Results

The LUCC process in the Loess Plateau

Land use change process

The statistics for land use in the Loess Plateau showed that (*Table 1*) the land use structure generally remained stable with grassland, cultivated land, and forest land dominating between 1980 and 2015 and accounting for 41.80%, 32.34%, and 15.02% of the total area, respectively. However, grassland and cultivated land decreased by 0.57% and 0.59%, respectively, while forest land increased by 0.41% over the study period. In addition, the largest increase was in construction land at 1.08%.

The amount and rate of land use change were different at different periods. Between 1980 and 2015, the area of cultivated land, grassland, water body, and unutilized land decreased greatly, while the area of forest land and construction land increased greatly. Due to the large area of the Loess Plateau, although the area changed was large, it made up a relatively small proportion of the total area. The decrease in grassland was the largest at 5.45%, and the increase in construction land was the highest at 51.54%. Between 1980 and 2000, the area of forest land, grassland, water body, and unutilized

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land decreased, and the area of cultivated land and construction land increased. During this period the decrease in water body was the largest at 9.00%, and the increase in construction land was highest at 11.97%. Between 2000 and 2015, the area of farmland, grassland, and unutilized land decreased greatly, while the area of forest land, water body and construction land greatly increased. During this period, the decrease in the cultivated land was the largest at 2.74%, and the increase in construction land was the highest at 35.34%.

Table 1. *Area and change rate of land use type between 1980 and 2015*

Type	Year	Farmland	Forest land	Grassland	Waterbody	Construction land	Unutilized land
Area (km^2) / percentage (%)	1980	205530/32.51	94959/15.02	264254/41.80	9718/1.54	13229/2.09	44562/7.05
	1990	206544/32.68	94776/14.99	263861/41.74	8904/1.41	13558/2.14	44452/7.03
	2000	207497/32.82	94909/15.01	262452/41.51	8843/1.40	14812/2.34	43735/6.92
	2015	201820/31.92	97535/15.43	260661/41.23	9188/1.45	20047/3.17	42998/6.80
Amount (km^2) and rate (%) of change	1980-2000	1967/0.96	-50/-0.05	-1802/-0.68	-875/-9.00	1583/11.97	-827/-1.86
	2000-2015	-5677/-2.74	2626/2.77	-1791/-0.68	345/3.90	5235/35.34	-737/-1.69
	1980-2015	-3710/-1.81	2576/2.71	-3593/-1.36	-530/-5.45	6818/51.54	-1564/-3.51
Amount (km^2/a) and rate (%/a) of annual change	1980-2000	98.4/0.05	-2.5/-0.003	-90.1/-0.03	-43.8/-0.45	79.2/0.60	-41.4/-0.09
	2000-2015	-378.5/-0.18	175.1/0.18	-119.4/-0.05	23.0/0.26	349.0/2.36	-49.1/-0.11
	1980-2015	-106.0/-0.05	73.6/0.08	-102.7/-0.04	-15.1/-0.16	194.8/1.47	-44.7/-0.10

Between 1980 and 2015, the annual reduction in cultivated land and grassland was the largest, with the largest increase in construction land. The rate of annual change in grassland was the largest decrease and change in construction land was the largest increase. Between 1980 and 2000, the annual increase in cultivated land and construction land was the largest, and the annual decrease in grassland and the unutilized land was the largest. The largest decrease in the rate of annual change was in the waterbody, and the largest increase in the rate of annual change was in construction land. Between 2000 and 2015, the annual reduction in farmland was the largest, and the largest increase was in construction land. The rate of annual change in the cultivated land was the largest decrease, and the largest increase was in construction land during 2000-2015.

The above analysis showed that the land use structure of the Loess Plateau was stable between 1980 and 2015, but overall the area of cultivated land and grassland decreased, and the area of construction land and forest land increased. The area of farmland increased prior to 2000 and decreased after 2000, and the decrease was 2.89 times greater than the increase. The area of forest land change was opposite than that of farmland, where the area of increase was 52.52 times the area of decrease. Grassland area continued to decrease, and construction land continued to increase during 1980-2000 and 2000-2015. The change in farmland and forest land was clearly impacted by the implementation of GGP. The increase in construction land was affected by urban expansion caused by rapid social and economic development in the region. As the area of grassland continued to decrease, it may have been related to cultivated land occupied prior to 2000. However, the subsequent reduction was not consistent with the area increase caused by GGP implementation.

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NDVI change process

Equations 1, 2, 3, and *4* were used to obtain the spatial and temporal change rate of NDVI and *F* test results (*Fig. 2*). From 1982 to 2015, the NDVI in the Loess Plateau increased significantly and ($y = 0.002x - 3.824$, $R^2 = 0.653$, $P < 0.001$) with a linear growth rate of 0.02/10a. However, it could be divided into two stages: before and after 2000. Both stages showed a significant linear increase; however, the growth rate of the first stage was slower ($y = 0.001x - 1.797$, $R^2 = 0.246$, $P < 0.05$), and the linear growth rate was 0.01/10a. In the latter stage, the growth rate was faster ($y = 0.005x - 10.359$, $R^2 = 0.840$, $P < 0.001$), and the linear growth rate was 0.05/10a, which was five times the growth rate of the previous stage (*Fig. 2a*).

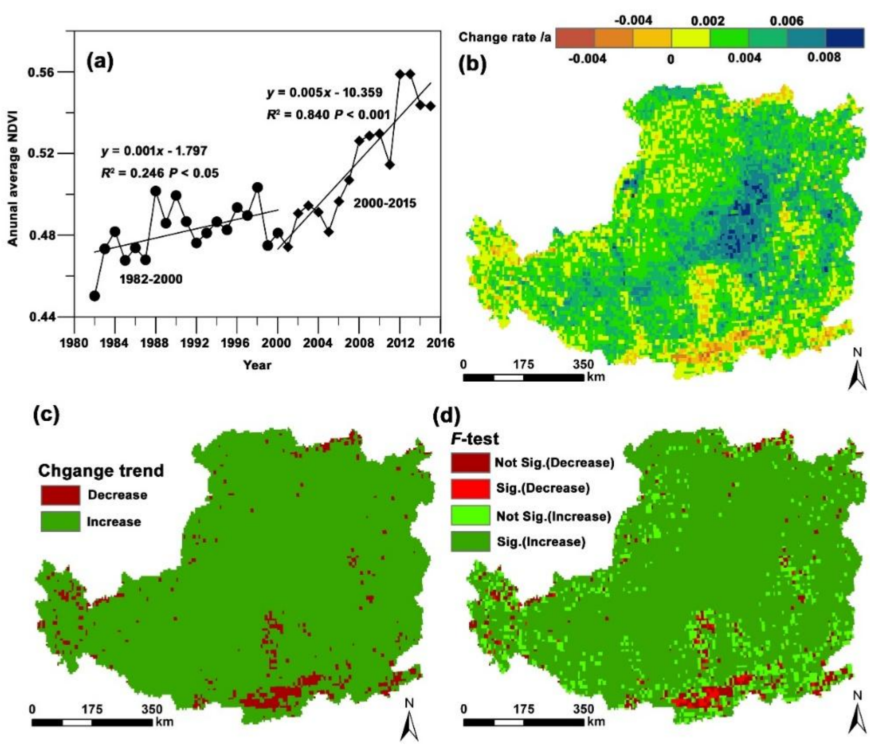

Figure 2. *Spatial and temporal distribution of NDVI change. (a) Temporal change process; (b) change rate; (c) change trend; (d) F test results of change trend*

The linear change rate and change trend of NDVI indicated that (*Fig. 2b* and *c*): the linear growth trend in the Loess Plateau from 1982 to 2015 accounted for 94.86%, while the decreasing trend only accounted for 5.14%. In the growth trend, the change rate was mostly concentrated between 0-0.006/a, accounting for 87.51%, of which 0.002-0.004/a accounted for 40.68%. The spatial growth rate and trend were widely distributed, the growth rate was greater with the land closer to the centre part of the Loess Plateau, while smaller with the land farther from the centre. Where the trend was decreasing, the areas with negative change rates were mainly located in the marginal zone, especially in the southern part of the study area, and the distribution around the western, northern and eastern areas was scattered.

The *F* test results for NDVI change indicated that (*Fig. 2d*), there was significant growth in the Loess Plateau from 1982 to 2015 that accounted for 83.82% of the total

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area, while not significant growth accounted for 11.04%, and was mainly in the marginal areas of the Loess Plateau. Areas with significant and not significant reduction trend distributions were small, accounting for only 0.93% and 4.21%, respectively, and were concentrated in the southern area, scattered around the eastern, western, and northern margins of the region.

The above analysis indicated that the NDVI of the Loess Plateau generally increased linearly from 1982 to 2015, during which time the NDVI growth rate after 2000 was affected by the implementation of GGP and was five times faster than before 2000. The NDVI of the Loess Plateau was dominated by a significant growth trend. The closer to the middle of the region, the higher the growth rate, and vice versa. The decreased trend was concentrated in the southern part of the region, scattered around the eastern, western, and northern margins.

The impact of climate change on NDVI

The LUCC process is made up of two parts: land use and land cover change. The former is mainly affected by human activities and reflects the macroeconomic pattern of human activities. The latter is affected not only by human activities but also by climate change. Human activities affect land cover by changing the land use, while climate change changes the hydrothermal conditions to affect the distribution of surface vegetation. The land cover change in this paper was represented by NDVI.

From 1961 to 2014, the temperature in the Loess Plateau increased, while the precipitation fluctuate decreased (Yan, 2015), and the frequency of precipitation intensity and extreme precipitation increased (Wang et al., 2014a, b). *Equation 5* was used to obtain the correlation between NDVI and both temperature and precipitation (*Fig. 3*). It shows that there were clear correlations between NDVI and annual temperature and precipitation in the Loess Plateau from 1982 to 2015 (*Fig. 3*). The correlation between NDVI and annual temperature was positive, accounting for 82.22% of the total, with a significant positive correlation area accounting for 25.91% of that and a significant negative correlation area accounting for only 1.77%. The positive and significant positive correlation distribution was consistent with the change trend (*Fig. 2c*), and mainly distributed in the centre of the region. The significant positive correlations were found in the centre and northern parts of the region and in the southern part of the region from west to east. The negative and significantly negative correlations were concentrated in the southern, western, and southwestern parts of the region.

From 1982 to 2015, the positive and significantly positive correlations between NDVI and precipitation accounted for 84.84%, and the significantly positive correlation area accounted for 23.63%, while the significant negative correlation area accounted for only 0.44%. In terms of spatial distribution, positive and significantly positive correlations were concentrated in a large area of the centre region. Significantly positive correlations were mainly located in the north, northwest, and southwest, while negative correlations were mainly distributed in the southeast, southwest, and northwest, as well as the centre regions with a scattered distribution.

The above analysis indicated that vegetation changes in most parts of the Loess Plateau from 1982 to 2015 were affected by temperature and precipitation, and the correlations with temperature and precipitation were mainly positive, in which significantly positive correlations were greater than 20%. It showed that climate change had the dominant effect on vegetation change in the Loess Plateau. However, human

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activities in the Loess Plateau were intense, and the changes in vegetation in some areas were completely dominated by human activities.

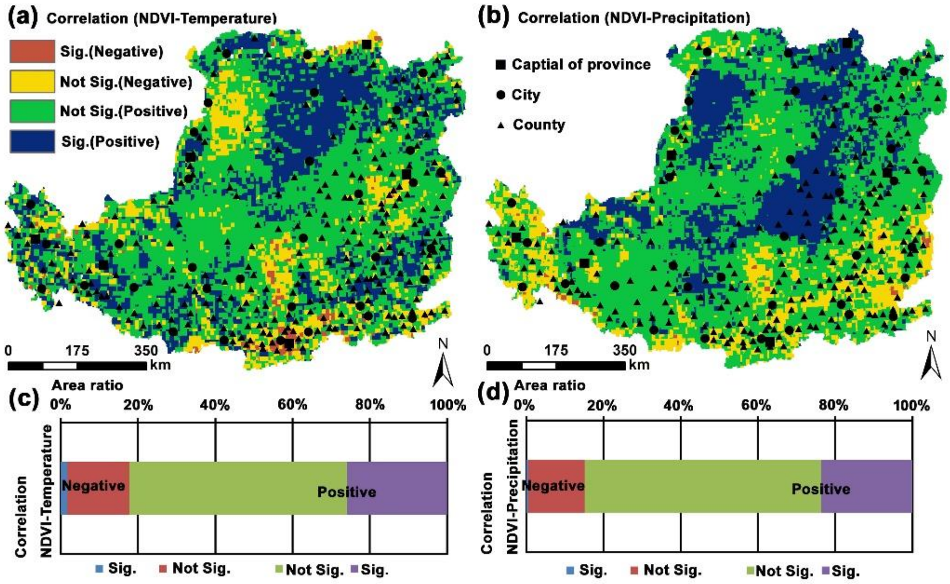

Figure 3. *Distribution of correlations and area ratios between NDVI and temperature and precipitation. (a) Correlation between NDVI and temperature; (b) correlation between NDVI and precipitation; (c) statistical results of correlation between NDVI and temperature; (d) statistical results of correlation between NDVI and precipitation*

The impact of urban expansion on LUCC

The impact of urban expansion on LUCC process was analyzed from two aspects, namely the process of NDVI change and the process of land use change. The average NDVI from 1982 to 2015 in different buffer zones was calculated in provincial capitals, cities and counties, respectively (*Fig. 4a, c* and *e*). By determining the buffer distance corresponding to the inflection point of the average NDVI value, the relative change rates for land use types between 1982-2015 before and after the inflection point were calculated according to *Equations 6* and *7* (*Fig. 4b, d,* and *f*).

Urban expansion was one of the most important characteristics of the current LUCC process. Because the administrative level of the city directly affects urban expansion, the cities of the Loess Plateau were divided into three categories: provincial capitals, cities, and counties.

Within the 30 km buffer zone of the provincial capitals, the inflection point for average NDVI change occurred at 18 km. NDVI had rapid and significant linear growth before the inflection point and stabilized after the inflection point (*Fig. 4a*). From 1982 to 2015, there were clear differences in land use change within the 18 km and 18-30 km buffer zones of the provincial capitals. For the former, the decrease in farmland and the increase in construction land were higher than those in the latter by 9.9 and 3.6 times, respectively (*Fig. 4b*). 18 km can, therefore, be used as the urban expansion boundary for provincial capitals in the Loess Plateau.

Within the 30 km buffer zone, the average NDVI inflection point occurred at 14 km, and the buffer was divided into 0-14 km and 14-30 km. The average NDVI value had a

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significant linear increase, but the linear growth of the former is 5.6 times than the latter (*Fig. 4c*). From 1982 to 2015, land use in the 14 km and 14-30 km buffer zones of cities also had clear differences, where cultivated land was reduced, and construction land increased in the former by 3.6 times, and the same value was only 2.7 times in the latter (*Fig. 4d*). This demonstrated that 14 km could be used as the urban expansion boundary of cities in the Loess Plateau.

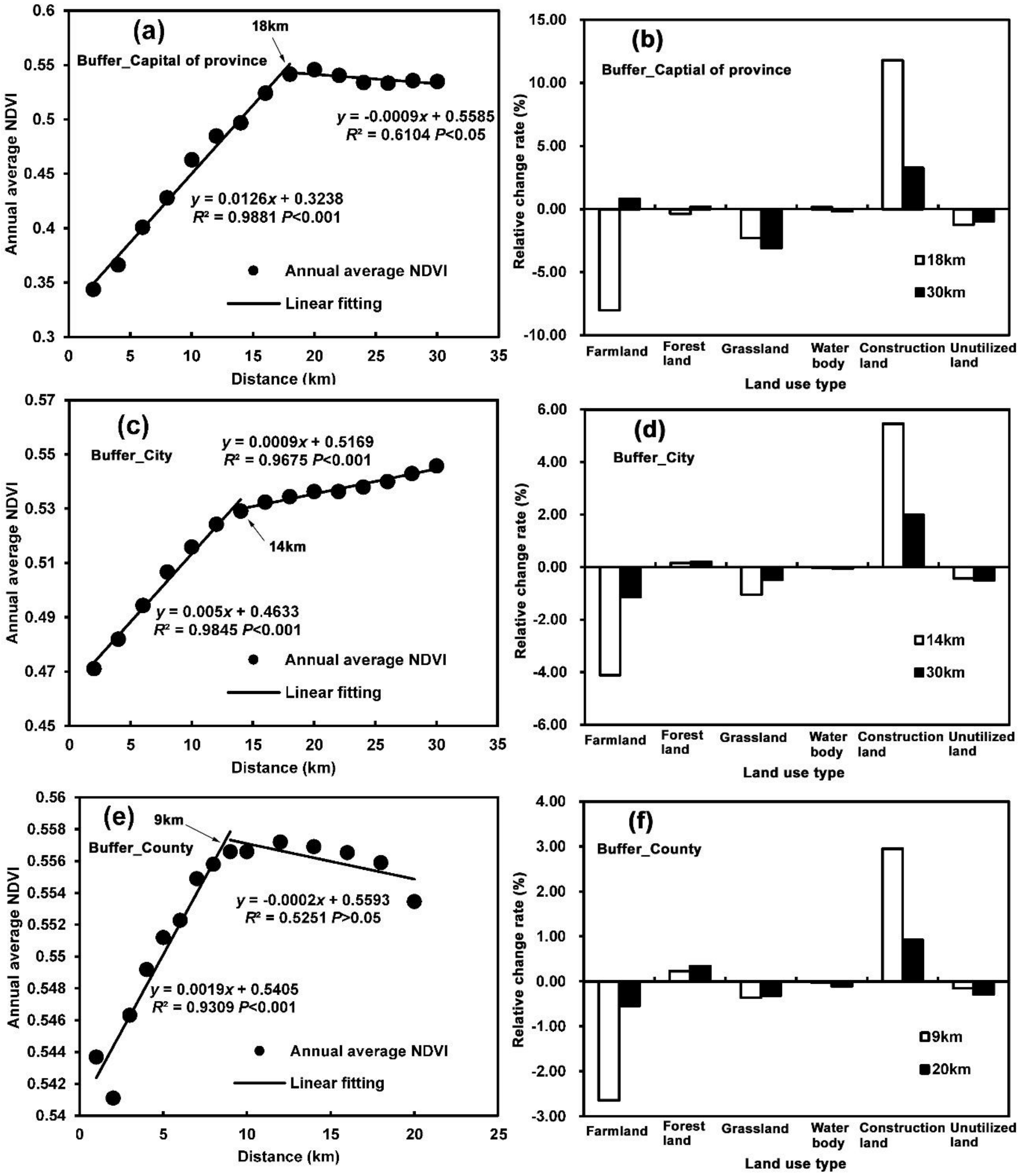

Figure 4. *Annual average NDVI changes (1982-2015) and land use change ratios (1980-2015) for different administrative buffers. (a) Average NDVI change in provincial capitals from 1982 to 2015; (b) relative change rate in land use in provincial capitals from 1980 to 2015; (c) average NDVI change in cities from 1982 to 2015; (d) relative change rate of land use in cities from 1980 to 2015; (e) average NDVI change in counties capitals from 1982 to 2015; (f) relative change rates of land use in counties from 1980 to 2015*

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Within the 20 km buffer zone, the NDVI inflection point appeared at 9 km. Prior to that, there was a significant linear NDVI growth, and the change rate in the 0-9 km buffer was higher than in 9-30 km (*Fig. 4e*). From 1982 to 2015, there was an obvious difference in land use between 9 km and 9-30 km buffer zone in counties, where farmland decreased, and construction increased in the former by 4.8 times and increased 3.2 times in the latter (*Fig. 4f*). That indicated that 9 km could be used as the urban expansion boundary of counties in the Loess Plateau.

The above analysis indicated that the urban expansion boundaries for provincial capitals, cities, and counties in the Loess Plateau were 18 km, 14 km and 9 km, respectively. Within the boundaries, the rapid reduction of cultivated land and the rapid increase of construction land were observed, while outside the boundary the reduction in cultivated land and the increase in construction land were far below the limit.

The impact of GGP on LUCC

Due to its fragile environment, the Loess Plateau is a key region for the implementation of the GGP, which focused on areas with slope >= 25°. The percentage of each land use type across the total region, annual average NDVI values for the Loess Plateau from 1980 (1982) to 2015, and accompanying statistics are calculated according to *Equations 8* and *9* (*Fig. 5*).

Taking the time of GGP implementation as the dividing point, the study period was divided into 1980 (1982)-2000 and 2000-2015. Between 1980 and 2015, the proportion of cultivated land to total cultivated land in the Loess Plateau >= 25° increased slightly, and the proportion of forest land and construction land decreased, while grassland increased (*Fig. 5a*). Correspondingly, the annual average NDVI value in the area >=25°from 1982 to 2015, was generally higher than the area <25°; however, the linear increase in the area >=25° was not significant, while the linear increase in the area <25° was significant (*Fig. 5b*). This indicated that the higher slopes limited human activities and maintained better vegetation conditions, while lower slope vegetation was also improved. Between 2000 and 2015, the GGP was implemented, and the proportion of cultivated land >=25° of the Loess Plateau decreased (*Fig. 5c*). The NDVI in >=25° also showed a faster increase from 2000 to 2015 than from 1982 to 2015; moreover, and the linear growth rate also exceeded the area of <25° (*Fig. 5d*). This showed that the implementation of the GGP greatly promoted the improvement of vegetation in the higher gradient.

Between 1980 and 2000, on the whole, the proportion of farmland decreased, and the proportion of grassland increased in the area of >=25° (*Fig. 5e*). At the same time, both areas of slope >=25° and slope <25° had a significant linear increase in annual NDVI from 1982 to 2015, and the linear growth rate was similar. However, the linear growth rate in the area of >=25° was higher than that of <25°, which directly reflected the process of land use and land cover change in high slope areas driven by policy (*Fig. 5f*).

Quantitative assessment of LUCC process driving factors

According to *Equation 10,* the impacts of climate change, urban expansion, and GGP implementation on LUCC was assessed quantitatively using comprehensive methods in the Loess Plateau between 1980 and 2015, based on the results of land use change, *F* test results for NDVI change trends, and the correlations between NDVI and

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temperature and precipitation, as well as urban expansion and GGP implementation (*Fig. 6*).

It indicated that among the influencing factors of LUCC, climate change was the main factor and accounted for 93.65%, followed by urban expansion (accounting for 5.46%), and the implementation of GGP (accounting for 0.64%) in the Loess Plateau between 1980 and 2015 (*Fig. 6*). The role of climate change on the LUCC process was characterized by a wide range of impacts, and areas that could not be covered by human activities, and agricultural production activities, which were still controlled by climate factors. The impacts of climate change on LUCC process had surface-like characteristics.

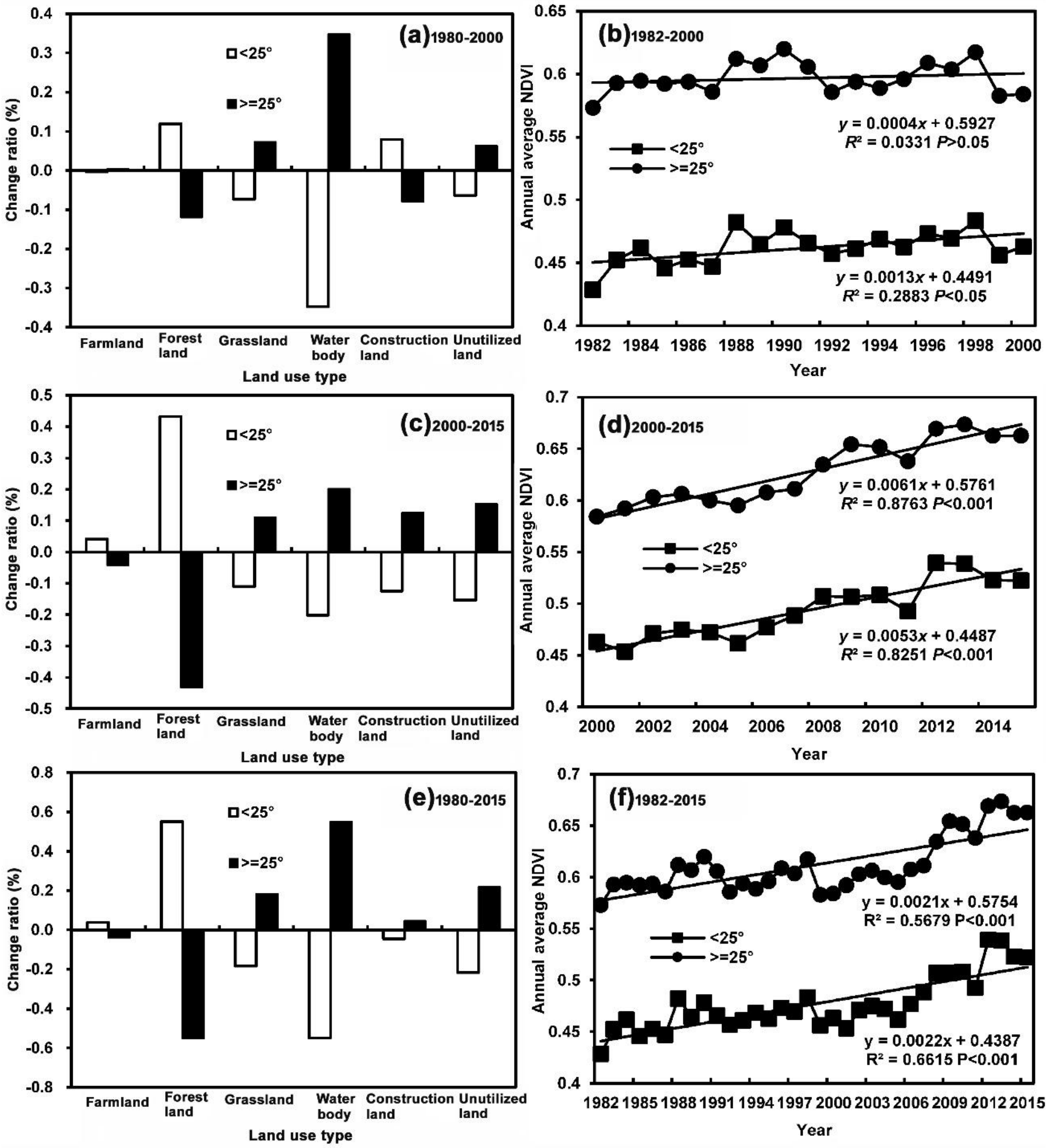

***Figure 5.** The change process of land use and NDVI in different slop area. (a) land use change between 1980 and 2000; (b) NDVI change from 1982 to 2000; (c) land use change between 2000 to 2015; (d) NDVI change from 2000 to 2015; (e) land sue change between 1980 to 2014; (f) NDVI change from 1982 to 2015*

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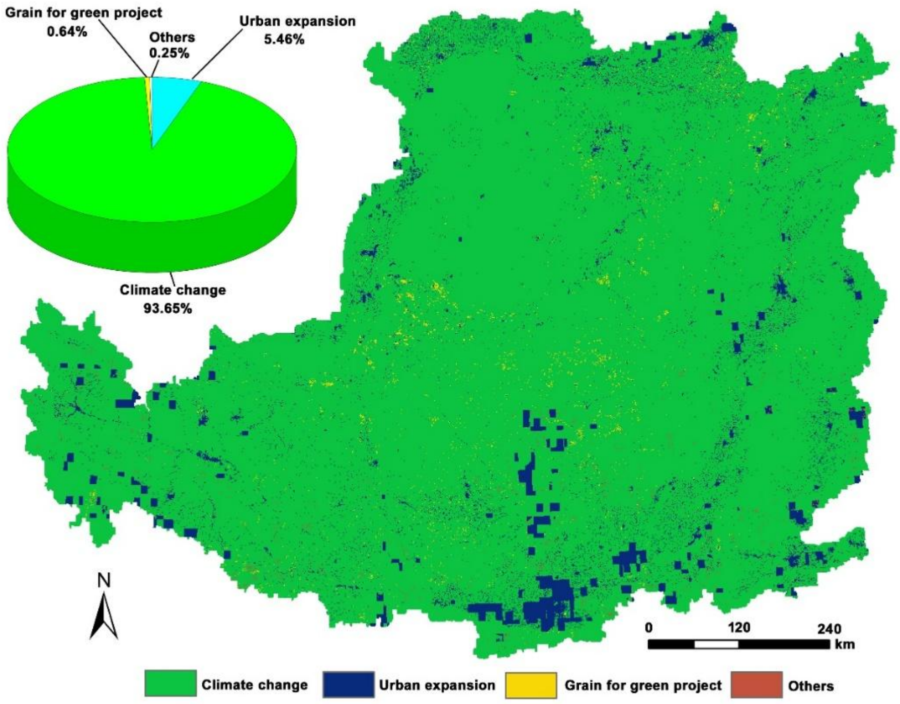

***Figure 6.** The evaluation results for the impacts of climate change, urban expansion, and the grain for green project on LUCC in the Loess Plateau*

Urban expansion was the main driving force for human activities in the LUCC in the Loess Plateau between 1980 and 2015. It reflected the rapid social and economic development and the continued influx of population, and the rapid urbanization process in the Loess Plateau, especially in the southern region where large amounts of farmland, forest land, and grassland, were converted into construction land. The impact of urban expansion on LUCC had point-like characteristics. The impacts of GGP on LUCC were relatively scattered, and related to project implementation and the distribution characteristics in areas with slope >=25°, and were generally patchy in distribution.

Discussion

The LUCC process in other countries or regions is mainly affected by urban expansion, deforestation induced by the behavior of farmers, and industrial activities (such as forest development), while the Loess Plateau region of China is unique. Research on the impacts of climate change, urban expansion, and the implementation of GGP on the LUCC process in the Loess Plateau has attracted the attention of many scholars (Theobald, 2005; Chen et al., 2014; Song et al., 2014), but the focus has been on one or two elements, and less on the LUCC process under the combined action of 3 elements. Although quantitative analysis of influencing factors has been carried out, spatial expression was not achieved and maintained half-quantitative methods, which were also the main approach in the current study on the impact of climate change and human activities on the process of natural geographical elements. The research methods and conclusions presented here provide a reference for similar research in other countries or regions.

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The basic framework for quantitative assessment and spatial expression of the impacts of climate change, urban expansion and GGP on LUCC has already been initiated, but some problems remain. The first is data issues, including NDVI and DEM data. The NDVI data used in this paper were GIMMS NDVI from 1982 to 2015, with a spatial resolution of 0.05° and 1 km by resampling. However, it is clear that resampling did not solve the problem of low spatial resolution (*Fig. 6*). The current time-series NDVI data were mainly GIMMS NDVI and SPOT/MODIS NDVI, but the latter time is short. SPOT NDVI and MODIS NDVI started in 1998 and 2000, respectively, and for the study of climate change, the timeframe is slightly shorter. DEM data was at 90 m resolution and resampled to a 30 m resolution to extract the slope for the analysis of GGP impacts. However, because the NDVI and land use data were 1 km, it was difficult using the extracted slope for analysis of NDVI and land use. As a whole, quantitative assessment results on the role of GGP implementation were lower, while climate change and urban expansion results were higher.

The second problem was the division of quantitative assessment results. In the article, the analysis method for comprehensive factor was subjective, and the classification of results was too simple, only distinguishing the different functions and spatial distribution, but not distinguishing the spatial distribution of their strengths and weaknesses within the same classification. The third problem was the application of the quantitative assessment results. The Loess Plateau is a typical fragile area in China with serious soil erosion problems, and the vegetation cover is reduced from southeast to northwest, while rapid social and economic development drives large significant urban expansion. Research needs to be conducted on the natural spatial and temporal geographical processes, based on spatial mapping of climate change and human activities (such as urban expansion and GGP).

Conclusion

Based on a quantitative assessment of the impacts of climate change, urban expansion, and the implementation of GGP on LUCC in the Loess Plateau, the following conclusions were obtained:

(1) The land use structure of the Loess Plateau was stable, and the area of cultivated land and grassland was reduced, while the area of construction land and forestland increased. The area of cultivated land increased prior to 2000 and decreased afterwards, with a decrease that was 2.89 times the increase. Construction land continued to increase. NDVI had an increasing linear trend in general. After 2000, the NDVI growth rate was five times that of the rate before 2000. The significant increasing trend was concentrated in the middle part of the region, and the decrease was concentrated in the south of the region.

(2) The NDVI changes in most parts of the Loess Plateau were positively correlated with changes in air temperature and precipitation, and significant positive correlations were all greater than 20%, which indicated that climate change had a dominant effect on vegetation changes in the Loess Plateau, but changes in vegetation in some areas were completely affected by human activities.

(3) The boundaries of provincial capitals, cities and counties in the Loess Plateau were 18 km, 14 km and 9 km respectively. The rapid reduction of cultivated land and the rapid increase of construction land were shown within those boundaries. The

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reduction in cultivated land and the increase in construction land outside the boundaries were far below the threshold values.

(4) In the area of >=25°, the proportion of cultivated land decreased, the proportion of grassland increased, and the linear growth rate of NDVI was clearly higher than those areas <25°, which directly showed that the driving effect was GGP implementation.

(5) Among the influencing factors of LUCC in the Loess Plateau, climate change was dominant, accounting for 93.65% of the total, with a surface-like distribution. It was followed by urban expansion, accounting for 5.46% of the total, with a point-like distribution. The impact of GGP accounted for 0.64% and had a patchy distribution.

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IMPACTS OF THE GRAIN FOR GREEN PROJECT ON SOIL EROSION: A CASE STUDY IN THE WUDING RIVER AND LUOHE RIVER BASINS IN THE SHAANXI PROVINCE OF CHINA

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Abstract. The Loess Plateau in the northern Shaanxi Province is one of the areas with serious soil erosion in China. Affected by the implementation of the Grain for Green project, land use types and vegetation status have changed considerably. To assess the impacts of these changes on soil erosion has important theoretical and practical significance. Taking the Wuding River Basin and Luohe River Basin in northern Shaanxi as examples, land use data, normalized difference vegetation index, and daily rainfall data, as well as digital elevation model, soil type data, combined with the revised universal soil loss equation, the change of the soil erosion modulus were analyzed. The results showed that: (1) the implementation of the Grain for Green project has promoted the conversion of farmland to grassland and forest land (concentrated in 2000-2005). In 2000-2014, the change rate of land use and NDVI in the areas with a slope ≥ 25° were higher than those areas with a slope < 25° and across the entire basin. Affected by this, the change rate of the soil erosion modulus difference value at areas with a slope ≥ 25° was higher than that at areas with a slope < 25°. (2) The soil erosion modulus of both basins increased from 2000 to 2014. Without considering the impact of changed precipitation, the soil erosion modulus of both basins showed a decreasing. This proves that the implementation of the GGP, converting farmland into forest land and grassland, has improved the vegetation coverage and reduced soil erosion.
Keywords: *RUSLE, rainfall, vegetation, slope, northern Shaanxi Province*

Introduction

Soil erosion is a worldwide eco-environmental problem and has a close relationship with agricultural production (Montgomery, 2007). As one of the main soil erosion regions in China and the world, the Loess Plateau is affected by the terrain, soil, precipitation, surface vegetation cover, and human activities. To reduce soil erosion, the Chinese government has formulated and implemented the Grain for Green project (GGP), which aims to reduce the soil erosion via improving the vegetation cover. Combined with the check-dam project built in the 1970s, soil erosion on the Loess Plateau will be comprehensively managed.

The GGP is an important environmental construction and restoration project in China and involves 1,897 administrative units at the national level, in 25 provinces and autonomous regions across the country. The Loess Plateau is a key area for implementing the GGP and has drawn considerable attention from scholars (Sun et al., 2013; Jiao et al., 2014). Soil erosion has been studied from the micro-slope scale to the whole Loess Plateau, and slope soil erosion research discussed the effects of land use change on soil surface roughness, soil organic matter, and slope micro-topography

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evolution on soil erosion (Hu et al., 2014; Liu et al., 2014; Li et al., 2015, 2017). Studies on channel soil erosion focus on the role of the check-dam construction in the ecological environment as well as socio-economic, water, and sediment reduction effects in the basin (Wang et al., 2014a; Feng et al., 2016; Zhao et al., 2017). Soil erosion in small watersheds has been studied, as well as the effects of land cover change on soil erosion and ecological and economic sustainability (Zhao et al., 2014; Zhang et al., 2016; Dong et al., 2017). The change of soil erosion in the basins before and after the implementation of the GGP also has been analyzed (Wang et al., 2014b; Liu et al., 2015; Li et al., 2016). Overall, studies on the Loess Plateau focus on climate change, vegetation change, and soil erosion change scenarios, with potential governance prospects (Gao et al., 2016; Xie et al., 2016; Gao et al., 2017; Jiang et al., 2018).

At the core area of the Loess Plateau in northern Shaanxi, a hilly-gully area, the implementation of the GGP has played an important role in the improvement of the regional ecological environment. The impact of GGP implementation on soil erosion is the key evaluation content, with a large number of reports. However, comparative analyses of multiple basins are scarce. The Wuding River and the Luohe River are the major rivers on the Loess Plateau in northern Shaanxi. Solid knowledge on the impacts of the GGP on different basins in terms of soil erosion would be used for assess the effectiveness and direction of future implementations.

Materials and methods

Study sites

The Wuding River Basin (WDRB) and Luohe River Basin (LHRB) were selected as study sites (*Fig. 1*). The two basins are located in the areas Yulin and Yan'an, on the Loess Plateau, in the northern part of the province Shaanxi, China (LPNSC). The area is the core area of the Loess Plateau, with a complex topography and severe soil erosion. The dominant vegetation types are temperate forest, temperate grassland, and temperate desert, from south to north. Due to the long history of human activities, the surface in other regions is dominated by artificial vegetation, except for the forest vegetation in the south. The climate is characterized as temperate continental monsoon climate, with hot and rainy summers and cold and dry winters. The inherent characteristics of loess and the undulating surface conditions, as well as the concentrated precipitation in summer and autumn, lead to serious soil erosion in the region.

The Wuding River is the first tributary of the Yellow River. It is located in the northern part of the LPNSC and is mainly distributed in the territory of Yulin. The northwestern part of the basin is flat and sandy and belongs to the southern margin of the Mu Us Desert, while the southwestern part contains the source of the Wuding River and has a higher elevation; similar to the southeastern part, it is a hilly-gully region. The WDRB is the key basin for soil erosion control on the Loess Plateau. Since the 1970s, more than 10,000 check dams have been built in the basin, with the aim to treat channel soil erosion. Slope vegetation restoration and construction approaches have been adopted since 1999. Large areas of sloping farmland have been turned into forest land and grassland via the GGP implementation, with major land use changes, resulting in improved slope vegetation coverage and increased ecological value; in addition, annual runoff and sediment erosion also decreased.

The Luohe River is mainly located in the area around Yan'an, in the southern part of the LPNSC. It flows into the Weihe River and is a secondary tributary of the Yellow

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River. The vegetation cover in the LHRB is high, and temperate steppe and forest areas are distributed in the northwestern and southern regions of the basin, respectively. As a consequence of GGP implementation, land use in the basin has undergone considerable changes. Compared with the Yanhe River Basin, which is also located in the LPNSC, studies on the Wuding River and the Luohe River are scarce. This paper, therefore, focused on the WDRB and LHRB, analyzing temporal and spatial changes in soil erosion with the implementation of the GGP.

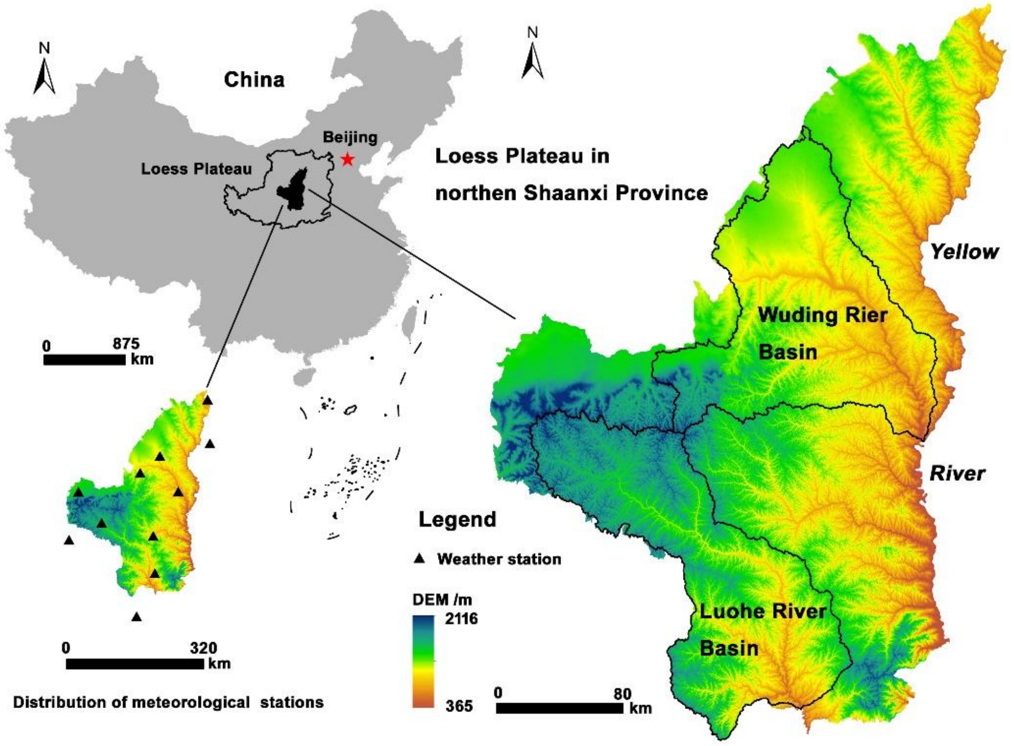

Figure 1. *Study sites and the distribution of meteorological stations*

Data

The research data included the following: (1) daily rainfall data, obtained from 11 meteorological stations in and around the basin from 2000 to 2014 (*Fig. 1*), were provided by the China Meteorological Data Network (http://data.cma.cn); the stations were Yulin, Dingbian, Wuqi, Hengshan, Suide, Yan'an, Hequ, Xingxian, Luochuan, Huanxian, and Yaozhou; (2) ASTER DEM (Digital Elevation Model) data of 30 m resolution, collected from the National Science Data Mirroring Website of the Computer Network Information Center, Chinese Academy of Science (http://www.gscloud.cn); (3) soil type distribution, using a 1:50,000 map; (4) 250 m resolution and 16d synthesized Normalized Difference Vegetation Index (NDVI) from the Moderate Resolution Imaging Spectroradiometer (MODIS) from 2000 to 2014, downloaded from http://lpdaac.usgs.gov; (5) land use data for the province Shaanxi for the years 2000, 2005, and 2010, at a scale of 1:100,000, collected from the "Remote Sensing Survey and Assessment of the Ten Years of National Ecological Environment Change (2000-2010)". All data were resampled to 30-m resolution for calculation and statistical analysis.

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Methods

The Revised Universal Soil Loss Equation (RUSLE) is a mature and widely used calculation method of the soil erosion modulus, using the following formula (*Eq. 1;* Renard et al., 1991):

$$A = R \times K \times L \times S \times C \times P \tag{Eq.1}$$

where
A is the soil erosion modulus, t/(km^2·a);
R is the rainfall erosivity factor, (MJ·mm)/(hm^2·h·a);
K is the soil erodibility factor;
L is the slope length factor;
S is the slope factor;
C is the vegetation cover factor; and
P is the soil and water conservation factor.

Rainfall erosivity factor (R)

The R factor is the indicator for evaluating the impact of rainfall on soil separation and transportation. Annual rainfall erosivity is calculated (*Eqs. 2, 3* and *4*) using daily rainfall data as proposed in Zhang et al. (2002, 2003) and Xie et al. (2000):

$$R_i = a\sum_{j=1}^{n} D_j^b \tag{Eq.2}$$

$$a = 21.586b^{-7.1891} \tag{Eq.3}$$

$$b = 0.8363 + 18.144D_{d12}^{-1} + 24.455D_{y12}^{-1} \tag{Eq.4}$$

where
R_i is the rainfall erosivity in the i year (MJ·mm)/(hm^2·h·a);
D_j is the erosive rainfall on the jth day, mm;
n is the total time of erosive rainfall in a year, d;
D_{d12} is the multi-year average daily rainfall of daily rainfall ≥ 12 mm/d, mm;
D_{y12} is the multi-year average annual rainfall of daily rainfall ≥ 12 mm/d, mm; and
a and b are the model parameters.

Soil erodibility factor (K)

The K factor is the indicator to evaluate soil erosion probability when rainfall erosivity has occurred. The calculated method has been proposed by Sharpley and Williams (1990), using *Equation 5:*

$$\begin{aligned} K = &\{0.2 + 0.3\exp[0.0256M(1 - F/100)]\} \times (F/(F+M))^{0.3}[1.0 - 0.25T(T + \\ &\exp(3.72 - 2.95T)] \times \{1.0 - 0.7\delta/[\delta + \exp(-5.51 + 22.9\delta)]\} \end{aligned} \tag{Eq.5}$$

where
M is the mass fraction of sand, %;

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F is the mass fraction of silt, %;
N is the mass fraction of clay, %;
T is the mass fraction of soil organic carbon, %; and
$\Delta = 1\text{-}M/100$;
The K value is needed to use the international system units through multiplication by 0.1317.

Topographical factor (LS)

Slope length and slope (*LS*) are the topographical factors mainly reflecting the effects of topography on soil erosion. The formulae are expressed as follows (*Eqs. 6, 7, 8, 9* and *10;* Moore and Wilson, 1992; Liu et al., 1994; Renard et al., 1997):

$$L = (\lambda / 22.13)^{\alpha} \quad \text{(Eq.6)}$$

$$\alpha = \frac{\beta}{\beta + 1} \quad \text{(Eq.7)}$$

$$\beta = (\sin\theta / 0.0896) / [3.0 \times (\sin\theta)^{0.8} + 0.56] \quad \text{(Eq.8)}$$

$$S = \begin{cases} 10.8\sin\theta + 0.03, \theta < 9\% \\ 16.8\sin\theta - 0.50, 9\% \le \theta < 14\% \\ 21.9\sin\theta - 0.96, \theta \ge 14\% \end{cases} \quad \text{(Eq.9)}$$

$$LS = L \times S \quad \text{(Eq.10)}$$

where
λ is the slope length, calculated using the hydrological analysis module in the ArcMap on the basis of 30 m resolution DEM;
α is the slope length index;
θ is the slope in %, obtained by the surface analysis tool in ArcMap and based on 30 m DEM;
The *LS* value is calculated by multiplying L by the S value, according to the *Equations 6, 7, 8* and *9*.

Vegetation cover factor (C)

The C factor is the vegetation index, which is calculated based on MODIS NDVI data; the formula is expressed as follows (*Eqs. 11* and *12;* Li and Zheng, 2012):

$$NDVI_A = 0.18 \times NDVI_M + 0.131 \quad \text{(Eq.11)}$$

$$C = \exp[-c \times \frac{NDVI_A}{d - NDVI_A}] \quad \text{(Eq.12)}$$

where
The c value is 2, and the d value is 1;

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$NDVI_A$ and $NDVI_M$ represent the AVHRR (Advanced Very High Resolution Radiometer) NDVI and MODIS NDVI data, respectively.

Soil and water conservation factor (P)

Use the calculation method (*Eq. 13*) proposed by Lufafa et al. (2002):

$$P = 0.2 + 0.03\theta \tag{Eq.13}$$

The results of the soil erosion modulus and various factors in the WDRB and LHRB in LPNSC were calculated using RUSLE (*Fig. 2*).

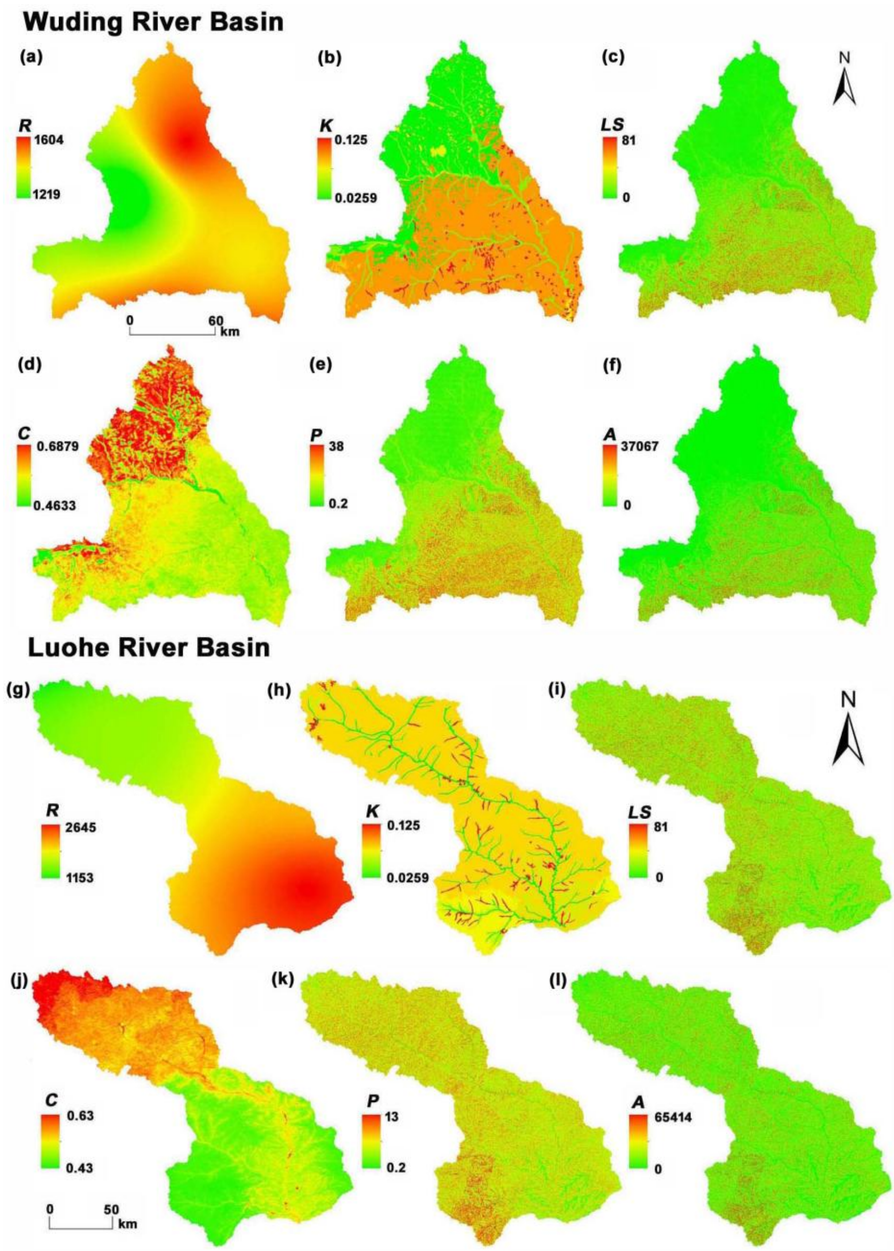

Figure 2. *Results of soil erosion modulation and its factors in the Wuding River Basin and Luohe River Basin. The maps present average values (2000 to 2014) for rainfall erosivity (R; (MJ·mm)/(hm·h·a)), vegetation cover factor (C), and soil erosion modulus (A; t/(km^2·a))*

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Results

Impacts of GGP implementation on land use and NDVI change

Land use and NDVI change

The main land use types in the WDRB were grassland, farmland, and idle land. Affected by the implementation of the GGP, land use in the basin underwent major changes between 2000 and 2010. In general, the areas of farmland and idle land decreased, while other land use types increased. The large reduction in farmland area and the large increase in grassland area indicate that a large amount of farmland was turned into grassland. In addition, the farmland area decreased, while grassland areas increased between 2000 and 2005; the changes were higher than those between 2005 and 2010, indicating that in the WDRB, the impact of GGP implementation on land use change was concentrated in the years between 2000 and 2005 (*Fig. 3a*). Despite the largest reduction and increase in farmland and grassland areas, respectively, between 2000 and 2010, construction land also increased, namely by 162.18%. This reflects the ongoing and considerable growth of the urban area as well as of industrial and mining land (*Fig. 3b*).

The main land use types in the LHRB were forest land, grassland, and farmland. As a consequence of the implementation of the GGP between 2000 and 2010, farmland areas declined mostly, while grassland areas considerably increased, followed by forested land. Such changes were concentrated in the period 2000-2005, while between 2005 and 2010, only slight changes occurred (*Fig. 3c*). Adhering to the main objectives of the GGP, farmland areas were transformed into forests and grasslands. Compared with the year 2000, 19.52% of the farmland was reduced in 2010, while construction land increased more than forest and grassland, reaching 39.58% (*Fig. 3d*).

The impact of GGP implementation on land use changes was concentrated between 2000 and 2005, resulting in a large reduction in farmland and a large increase in grassland. However, there was a lower forest land area in the WDRB, and the farmland was converted into grassland, while in the LHRB, farmland was mainly converted into the forest. Furthermore, in the WDRB, the construction land increased more than in the LHRB, reflecting the economic and social development.

The NDVI is an important indicator of the level of surface vegetation. The vegetation in the WDRB was dominated by temperature grassland, and that in the LHRB was dominated by temperature forest. The NDVI value was higher in the LHRB compared to the WDRB. However, affected by the implementation of the GGP, the average annual NDVI of the WDRB increased from 0.3103 to 0.5038 (61.38%) from 2000 to 2014, while the NDVI in the LHRB increased from 0.6052 to 0.7233 (19.52%) in the same period; both trends were linear. The growth rates were 0.0128/a and 0.0083/a, respectively, above the significance level of 0.001, which indicates that the NDVI in the two basins will continue to increase (*Fig.3e*).

Land use and NDVI change at different slopes

Areas with a slope $\geq 25°$ were the main objectives in the GGP implementation. Changes in land use and NDVI in regions $< 25°$ and $\geq 25°$ were calculated, and the effects of the implementation of the GGP were compared. The results are shown in *Figure 4*.

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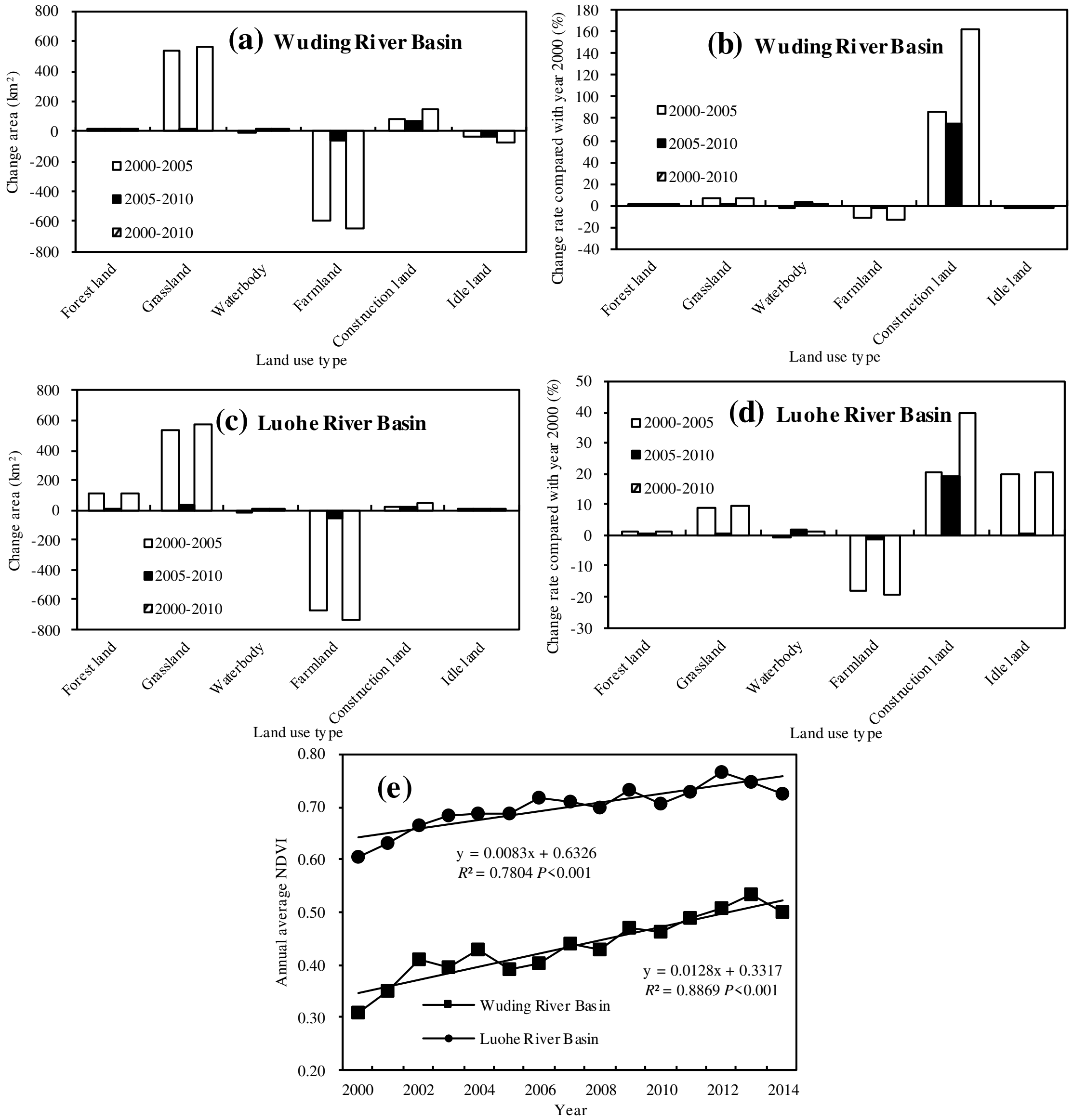

Figure 3. *The area of land use change, change rate compared between year 2000 and 2010, and change trend of annual average NDVI from 2000 to 2014 in the Wuding River Basin and Luohe River Basin. Figure (a) and (b) show the areas of land use change in different periods for Wuding River Basin during 2000-2010 and the percentage of change area compared with 2000, respectively. Figure (c) and (d) show the areas of land use change in different periods for Luohe River Basin during 2000-2010 and the percentage of change area compared with 2000, respectively. Figure (e) shows the annual average NDVI change trend for Wuding River Basin and Luohe River Basin from 2000 to 2014*

Land use change at different slopes can also cause changes in NDVI. In the WDRB, the NDVI value of the area with slopes ≥ 25° was higher than that of the area with slopes < 25°. The NDVI of both regions showed a linear increase from 2000 to 2014, with linear growth rates of 0.0157/a and 0.0127/a, respectively. These results indicate that the growth rate of NDVI in areas ≥ 25° was higher than that in areas < 25°, and the NDVI in the two regions is expected to continue to increase in the future (*Fig. 4e*). The situation in the LHRB was similar to that in the WDRB. The NDVI value in the ≥ 25°

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region was higher than that in the < 25° region, and all showed a linear increase; linear growth rates were 0.0078/a and 0.0084/a, respectively. Based on these results, we assume that the NDVI growth rate in areas < 25° was higher than that in areas ≥ 25°, which may be related to the better vegetation coverage in the LHRB and the effects of GGP implementation (*Fig. 4f*).

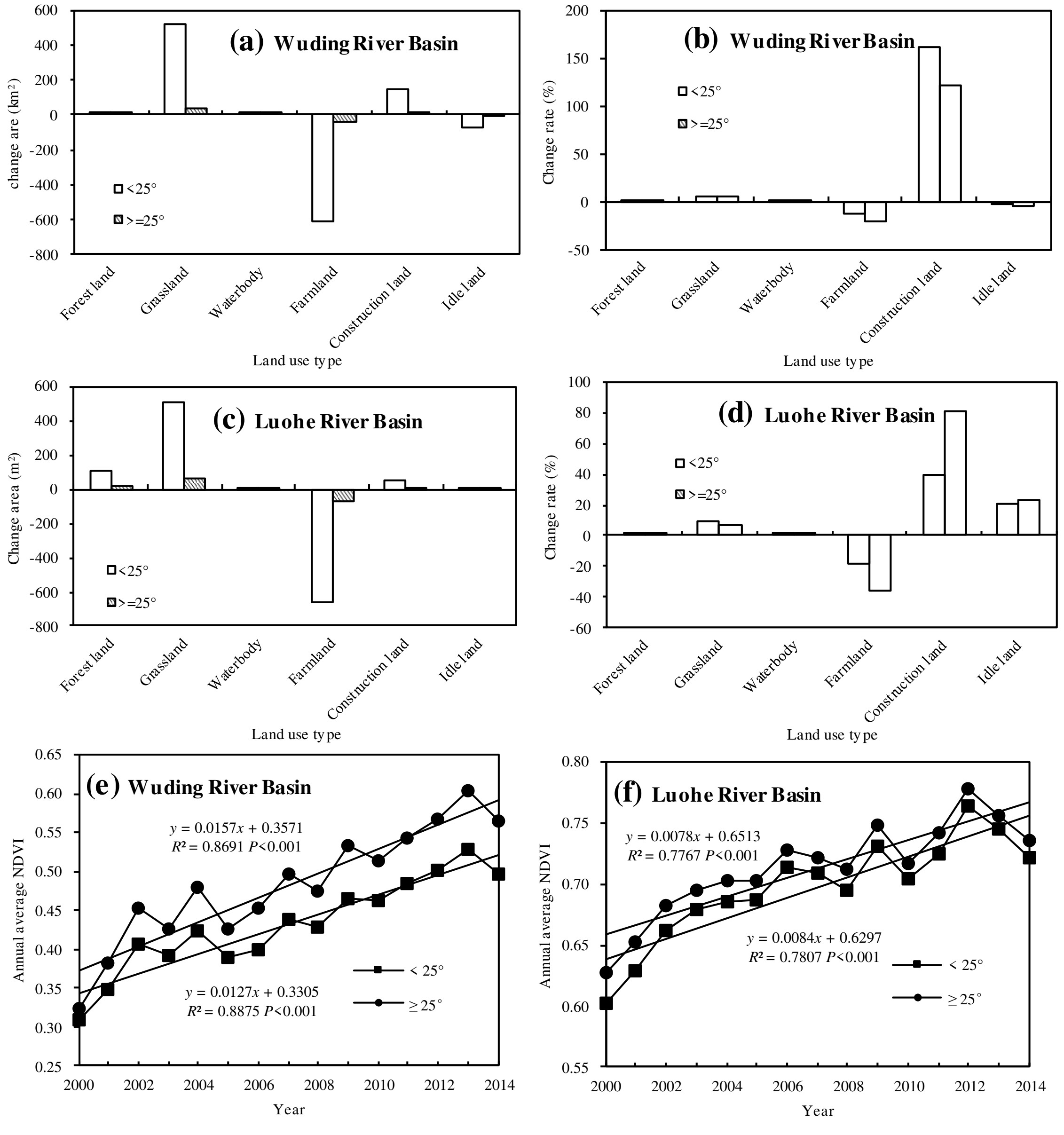

Figure 4. *Area, rate of land use change, and change trend of NDVI at different slopes between 2000 and 2014 in the Wuding River Basin and Luohe River Basin. Figure (a) and (b) show the areas of land use change in different periods for Wuding River Basin during 2000-2010 and the percentage of change area compared with 2000, respectively. Figure (c) and (d) show the areas of land use change in different periods for Luohe River Basin during 2000-2010 and the percentage of change area compared with 2000, respectively. Figure e and (f) show the annual average NDVI change trend for Wuding River Basin and Luohe River Basin from 2000 to 2014, respectively*

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Soil erosion modulus change at different slopes

The soil erosion modulus at different slopes as well as the anomalies for each year were calculated (*Fig. 5*). The annual average soil erosion modulus for both sites from 2000 to 2014 at a slope < 25° was lower than that of the slope ≥ 25°. Annual average soil erosion modulus in the area of < 25° in the WDRB was only 277.26 t/(km^2·a), while that in the ≥ 25° region was 4,362.99 t/(km^2·a). In the LHRB, the annual average soil erosion modulus in the < 25° region was 1,335.96 t/(km^2·a), while in the region ≥ 25°, this value was 5,109.25 t/(km^2·a). Based on the results, the slope of an area had a impact on land use change. However, for any area, the slope was fixed and relatively small. The vegetation attached to the slope surface was affected by climatic changes and human activities, such as GGP implementation.

The decrease in farmland and the increase in grassland and forest land were concentrated in 2000-2005. Given the changes in land use and soil erosion modulus at different slopes (*Figs. 4* and *5*), the soil erosion modulus of the WDRB from 2000 to 2007 (except for 2001) was lower than the average value from 2000 to 2014, while after 2008 (except for 2010), the soil erosion modulus increased and was higher than the multi-year average value (*Fig. 5a*). This can be explained by the relatively sparse precipitation in the WDRB in 2000-2005 and affected by the implementation of the GGP. The surface vegetation coverage increased and the soil erosion modulus decreased; but under extreme precipitation in the following period, the soil erosion modulus increased (Wang et al., 2014c, d).

Vegetation coverage in the LHRB was relatively high, especially in the southeast of the basin. Therefore, GGP implementation further increased the level of vegetation cover in the basin and caused the decline of the soil erosion modulus. The soil erosion modulus anomalies for most years were negative from 2000 to 2014, which indicated that the inter-annual precipitation variability increased, resulting in an extremely high soil modulus. Therefore, the mean value from 2000 to 2014 also increased, and most of the year's anomalies showed a negative value (*Fig. 5b*).

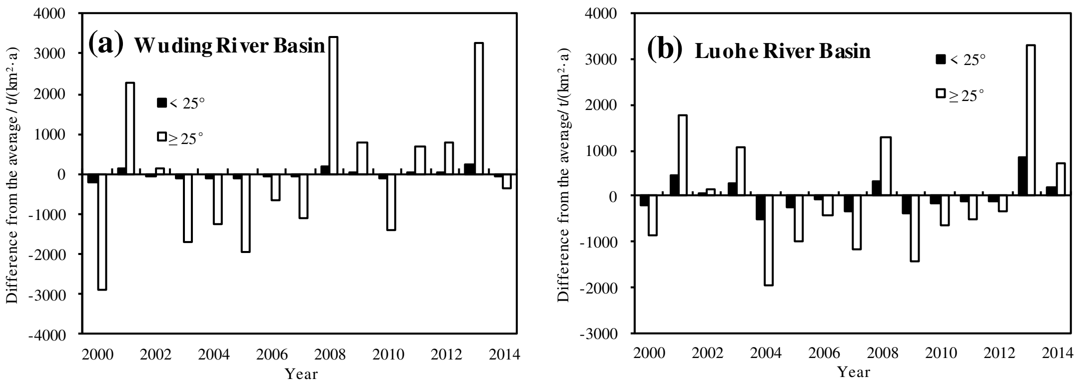

Figure 5. *Differences between annual soil erosion modulus and the average value between 2000 and 2014 at different slopes in the Wuding River Basin and Luohe River Basin*

Temporal and spatial changes of soil erosion modulus

Annual sediment transport data of the Baijiachuan hydrological station in the WDRB and the Zhuangtou hydrological station in the LHRB were obtained by the Yellow River Water Resource and Sediment Bulletin. The multi-year average soil erosion modulus of

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the WDRB in LPNSC, from 2000 to 2014, was 457.90 t/(km^2·a), slightly higher than the average soil erosion modulus of 419.30 t/(km^2·a) measured by the Baijiachuan hydrological station, while the soil erosion modulus in the LHRB was 1,843 t/(km^2·a), slightly lower than the 2,150 t/(km^2·a) measured by the Zhuangtou hydrological station. The estimated soil erosion modulus was higher in the WDRB in LPNSC, while in the LHRB, the estimated values were lower; however, the overall difference was relatively low.

Temporal changes

Soil erosion in LPNSC is serious, but there are obvious differences in soil erosion levels between the different watersheds. *Figure 6* showed that, in general, the soil erosion modulus in the LHRB was higher than that in the WDRB, and the multi-year average soil erosion modulus was different. This mainly reflects the dominant role of precipitation in the soil erosion process. The LHRB is located in the southern part of the northern Shaanxi Province, and annual precipitation is higher than that in the WDRB in the northern part of the northern Shaanxi Province. Moreover, the climatic conditions and the vegetation coverage in the LHRB are relatively better than those in the WDRB. Therefore, the soil erosion modulus in the LHRB was higher than that in the WDRB, which can be attributed to the difference in precipitation. From 2000 to 2014, the soil modulus in the LHRB and the WDRB showed a fluctuating increase, and the linear increase rate of the soil erosion modulus in the LHRB was higher than that in the WDRB, although the linear fitting results were insignificant ($P > 0.05$), reflecting the inconsistency of the soil erosion change in both sites.

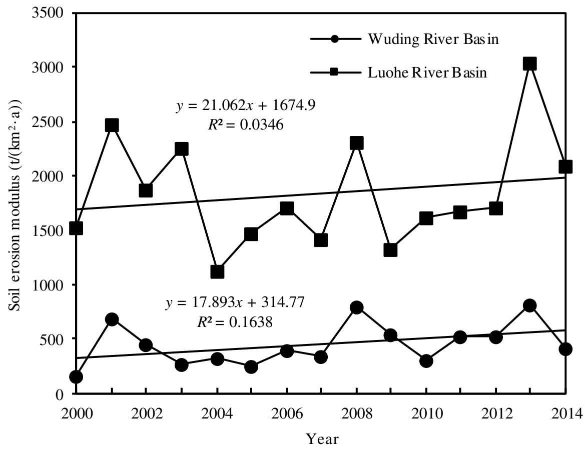

Figure 6. *Changes in annual average soil erosion modulation in the Wuding River Basin and Luohe River Basin*

Spatial change process

The linear change rate, the linear change trend, and the *F* test results for LHRB and WDRB were calculated by the linear trend method, using data from 2000 to 2014. The results are shown in *Figure 7*.

The linear change rate of the soil erosion modulus in the WDRB from 2000 to 2014 ranged between -230 and 1756 t/km^2 per year; higher values were mainly distributed in the southeast of the basin and in the southern part of the hilly-gully area, while the

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northwestern and western parts belong to the southern margin of the Mu Us Sandy Land, with low-lying land, less rainfall, less soil erosion, and a relatively small change rate in soil modulus (*Fig. 7a*). The linear change rate was higher than 0 and lower than 0, which means that the soil erosion modulus in WDRB increased and decreased from 2000 to 2014. The changing trend of the soil erosion modulus mainly increased, accounting for 99.6% of the total basin area, while decreases accounted for 0.45%, concentrated in the northern part of the basin (*Fig. 7b*). The *F* test results showed that the linear change of the soil erosion modulus in the WDRB was mainly insignificant, accounting for 79.3% of the total basin area, while the area with a trend of significant change accounted for 20.7%, concentrated in the south-east of the basin. The soil erosion modulus of the hilly-gully region of the Loess Plateau, located in the southeast of the WDRB, continues to increase, with a critical soil erosion situation (*Fig. 7c*).

The linear change rate of the soil erosion modulus in the LHRB ranged between -584 and 992 t/km^2 per year, which was smaller than that in the WDRB; however, both rates are still relatively high. High values were mainly concentrated in the middle area and the central southwest of the watershed (*Fig. 7d*). The northern part of the LHRB is a loess hilly-gully region, which is dominated by grassland and showed serious soil erosion. The central and southern parts were covered by temperate forest, with a relatively high vegetation coverage and low soil erosion low (*Fig. 2I*). Overall, there was an increasing change, accounting for 73.3% of the total area of the basin, while the decreasing accounting for 26.7%, mainly in the southeastern and northern parts of the basin (*Fig. 7e*). The results of the *F* test showed that the linear change of the soil erosion modulus in the LHRB was mainly insignificant, accounting for 99.3%, while a significant trend was sparsely distributed along the northern margins in the basin (*Fig. 7f*).

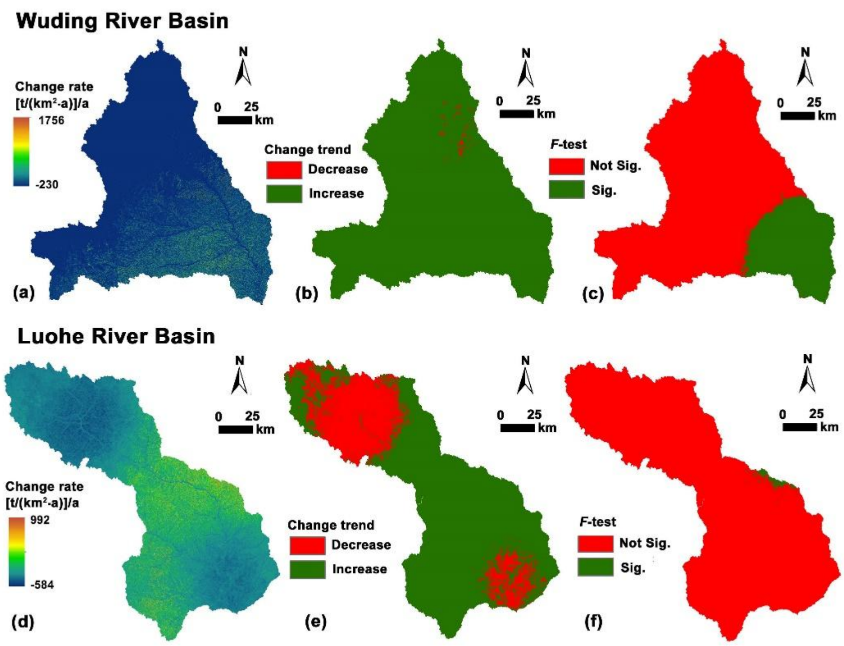

***Figure 7.** The change rate, the change trend, and the F test results of soil erosion modulation for Wuding River Basin and Luohe River Basin. Figure (a), (b), and (c) represent the change rate, the change trend, and the F test result for Wuding River Basin, respectively; Figure (d), (e), and (f) represent the change rate, the change trend, and the F test result for Luohe River Basin, respectively*

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Impacts of GGP implementation on soil erosion

To assess the impacts of GGP implementation on soil erosion, it is assumed that the soil erodibility factor (*K*), the topography factor (*LS*), and the soil and water conservation factor (*P*) are fixed, while the rainfall erosivity factor (*R*) and the vegetation cover factor (*C*) varied over the years. In terms of the *R* and *C* factor, the former was not controllable, while the latter could be affected by GGP implementation. When *R* is fixed, the erosive forces in 2000 would remain unchanged from 2000 to 2014, only the impact of the *C* factor (NDVI) on soil erosion would be considered. The results are shown in *Figure 8*.

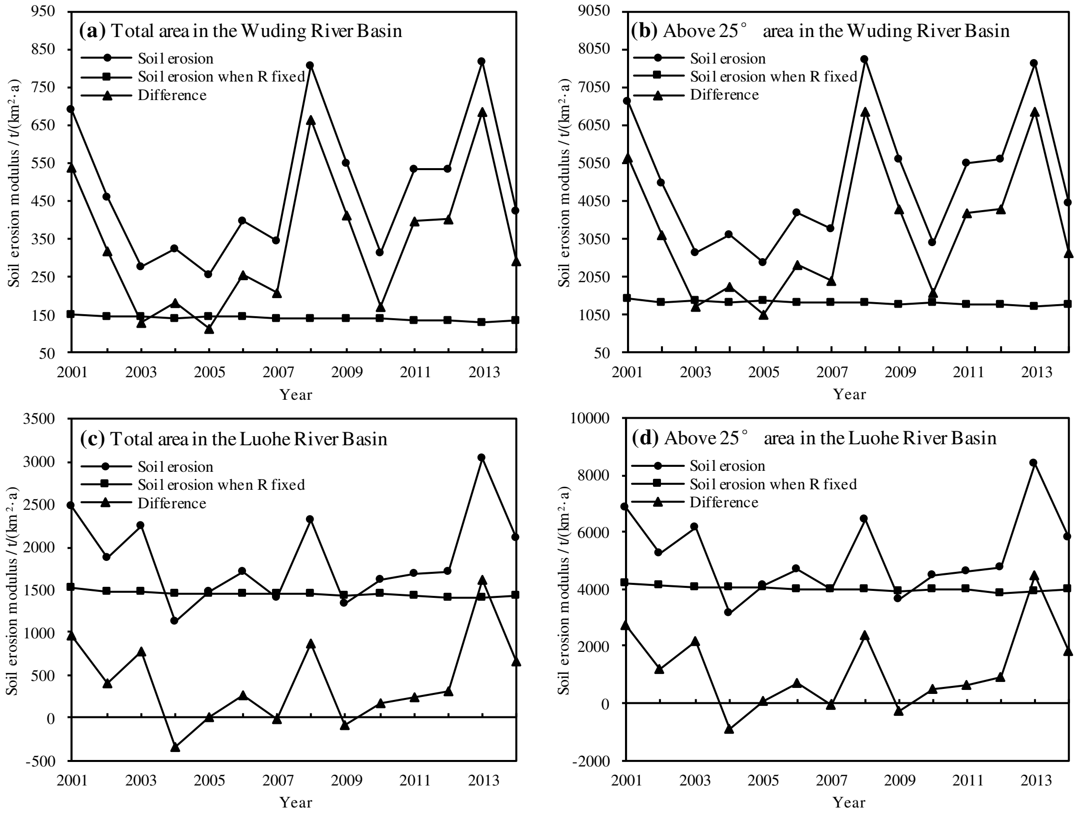

Figure 8. *Soil erosion modulus change in the entire region and areas with a slope ≥ 25 °, with fixed R values, and the difference between soil erosion modulus in actual and when R fixed in 2000, from 2001 to 2014 in the Wuding River Basin and Luohe River Basin*

The soil erosion modulus in both WDRB and LHRB during 2000-2014 increased. When the change of rainfall erosivity was not taken into account, the soil erosion in the two basins was only affected by the surface vegetation changes. The soil erosion modulus in both basins showed decline (*Fig. 8a* and *c*). The ≥ 25° regions were the key areas for GGP implementation, and the soil erosion modulus at these regions also showed reduction (*Fig. 8b* and *c*). At areas with a slope ≥ 25°, the soil erosion modulus in the two basins was 9.5 times and 2.7 times higher than the total soil erosion modulus, respectively, which indicated that the implementation of GGP had reduced the soil erosion modulus by changing the surface vegetation coverage. However, it should also be noted that in the natural state, the soil erosion modulus increased from 2000 to 2014,

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which means that the increase in the soil erosion modulus caused by precipitation changes far exceeded the reduction caused by the implementation of the GGP.

Discussion

Using the WDRB and LHRB as examples, the implementation of the GGP changed the land use types and affected the vegetation status (reflected by changes in NDVI), thereby reducing soil erosion. However, there are a large number of factors influencing soil erosion, which is a quite complex process.

In the RUSLE model, the soil erodibility factor is mainly affected by the organic matter content in the soil and the mechanical composition of soil particles. The GGP implementation in the first stage (2000-2008) has caused obvious changes in soil organic matter content (Zhu et al., 2014; Xie et al., 2016). The soil erodibility factor changes over time, and under objective conditions, the use of a year-by-year soil erodibility factor to calculate the soil erosion modulus will be closer to the actual situation.

Positive soil erosion modulus values in the WDRB and LHRB from 2000 to 2014 were six and five years, respectively. However, the overall increase in soil erosion in the two basins indicates that severe soil erosion will occur within a few years, caused by heavy precipitation (*Fig.9*). The role of precipitation in soil erosion is obvious. The construction of check dams for channel treatment and the implementation of the GGP for slope vegetation treatment have already been performed. However, with the increase of the frequency of extreme precipitation caused by climate change, the changes in soil erosion in the basins require further research.

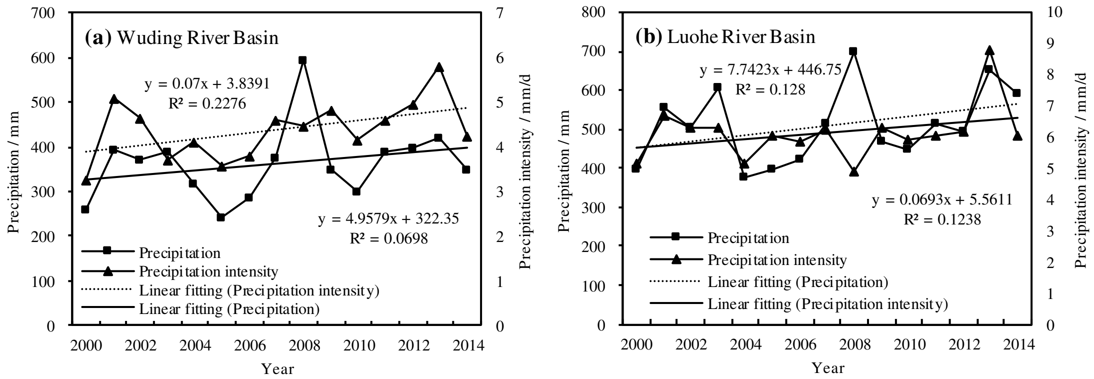

Figure 9. *Precipitation and precipitation intensity change from 2000 to 2014 in the Wuding River Basin and Luohe River Basin*

Conclusions

(1) The implementation of the GGP (2000-2008) promoted the conversion of farmland to grassland and forest land (concentrated in 2000-2005) in the WDRB and LHRB. In 2000-2014, the NDVI values increased by 61.38 and 19.52% in the WDRB and LHRB, respectively, and showed a linear increase; the change rates of land use and NDVI in the area with a slope ≥ 25° were higher than those in < 25° areas and throughout the basin. The change range of soil erosion modulus difference value at a

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slope ≥ 25° was higher than that at a slope < 25°, and the negative value reflected the role of the GGP, while the positive value reflected the rainfall impact.

(2) The soil erosion modulus values of both basins increased from 2000 to 2014, and the average soil erosion modulus and its growth rate in the LHRB were higher than those in the WDRB, which is mainly due to the difference in rainfall. The soil erosion modulus in both basins mainly showed an increase and areas with increase were mainly distributed in the southeastern region of the WDRB (20.7%), and the northern of the LHRB (only 0.7%).

(3) Without considering the impact of changed precipitation, the soil erosion modulus of both basins from 2000 to 2014 showed a decrease; the linear decline rate of the soil erosion modulus in the region with a slope ≥ 25° was 9.7 and 2.5 times than that of the whole basin in the WDRB and LHRB, respectively. This proves that the implementation of the GGP, converting farmland into forest land and grassland, has improved the vegetation coverage (especially in areas with a slope ≥ 25°) and consequently reduced soil erosion.

This paper analyzed the process by which soil erosion in the Wuding River Basin and Luohe River Basin in northern Shaanxi Province of China has changed based on the RUSLE. However, the research lacks support from measured data. Future studies should obtain hydrological monitoring data and compare this with the results calculated by RUSLE.

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EVALUATION OF VISUAL LANDSCAPE QUALITY IN THE WETLANDS NORTH OF SIVAS (TURKEY)

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Abstract. Wetlands are points of interest for social scientists as much as they are for naturalists. Due to their ecological importance, they have been in contact with human communities throughout the history and as a result of this, they also became ecosystems that shape cultural periods. The main reason for choosing wetlands in this study was that Turkey is rich in wetlands as a result of its geographical location. On the other hand, in order to have a sustainable environment, significant steps should be taken in preserving and developing the wetlands. In the scope of this study; the significant wetlands in the immediate vicinity of Sivas were used as material with a holistic view. These wetlands are located to the north of Sivas are Hafik Lake (Koçhisar Lake), Lota Lakes and Tödürge Lake (Demiryurt Lake). The images representing the condition of the study area together with the necessary information and the score card, were submitted to the experts for their evaluation. The aim of this study was to find out wetlands of landscape photographs in terms of potential differences (water property size, plant existence, topographic diversity, neighbors views, natural elements, cultural existence) and was to develop sustainable recreational areas in terms of visual preferences. The results were evaluated with a Q-sort analysis. Suggestions are offered about the visual landscaping quality and the development and preservation of recreational application potential within the scope of sustainable landscaping.
Keywords: *wetlands, landscape quality, q-sort analysis, expert opinion, sustainable landscape*

Introduction

In modern societies, environmental consciousness started with the “Urban ecology” in 1970s, as a result of the worldwide environmental crisis. It continued developing in 1980s (Özgüner, 2003) and in the Rio Conference of Environment and Development held in 1992, the notion of “Sustainability” came up for the first time. Within the scope of this notion, with the aim of having sustainable cities and ecological balance, developing and preserving urban ecosystems gradually became significant. This tendency named as ecological approach in landscape designing and administration, brought a different point of view to the applications of our day (Erdoğan Onur, 2012; Ankaya and Gülgün, 2009; Türkyılmaz et al., 2007; Gülgün et al., 2009).

Wetlands, as one of the most fertile ecosystems of the world, have been the focus of human settlement choices for thousands of years (Tırıl, 2006). Throughout the history, it has been seen that the first settlement areas of humans were concentrated on places that can be defined as wetlands like deltas, floodplains, lakesides or riversides. The water elements that can be listed within the extent of wetlands are swamps, peat bogs, floodplains, rivers, lakes, salt marshes, mangroves, sea grass beds, corals, seaside areas with a depth of no more than 6 m during tides, shore wetlands and man-made areas like water treatment pools and dams. Wetlands have direct and indirect usage values. Direct usage value examples can be; salt production, aquaculture production, reed harvesting, agriculture and stock breeding,

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being used as grazing or timber supply, being used as drinking, municipal or irrigation water, being used for transportation or tourism activities. It is crucial to establish a planned recreational usage in order to develop tourism opportunities and create a sustainable environmental value.

Many methods can be used to evaluate and improve wetlands recreationally. In this study, Q sort analysis was used to analyze the visual quality and to determine the important criteria of each wetland. Q- sort analysis or Q factor analysis as it is named in the foreign literature, is a relatively new tool not only as approach but particularly following the quite recent rediscovery of its usefulness in those fields where psychometric knowledge of individuals have thorough implications (Pitt and Sube, 1979; Kramer et al., 2003).

Review of literature

It is an undisputable fact that the increase in water existence and water surface in terms of recreational activities are important factors in raising the preferability and attractiveness of an area. In many studies (Schroeder, 1982; Arriaza et al., 2004; Özhancı and Yılmaz, 2011; Bolca et al., 2007) it was mentioned that the existence of water positively affects the aesthetic landscaping quality (Aşur, 2017). Despite the various initiatives in Turkey on the preservation and sustainability of the wetland views, especially in the Eastern Anatolia and Southeastern Anatolia regions, these landscapes are under the threat of existing financial activities as well as regional and local development plans prioritizing financial improvement (Baylan et al., 2013).

There were studies showing that areas with architectural aspects compatible with the nature in terms of vegetation, fabric, color and form together with characterizing the aesthetic and visual aspects of water in landscaping; increase the visual quality and are preferable to the users in recreational objectives (Özhancı and Yılmaz, 2011; Özgeriş and Karahan, 2015). According to Ak (2010) visual quality studies should be used as an important leading tool in planning and designing rural and urban areas in terms of the visual data created by the changes in physical environment. They should also be used in forming some of the administrative policies. Criteria for monitoring and defining the landscaping in survey forms used in reviewing the visual landscaping evaluation as published in the Scotland Natural Heritage Environmental Evaluation guidebook (SNH, 2013): Visible physical components: land form, land cover, utilities on the land, water existence, forest land, coppices, woodlands, agricultural areas, animals, settlements, other usages of the land (like area or city parks), linear features (highways or coastal line) or point features (like castles of monuments). In order to reveal the landscaping quality of an area, a visual landscaping evaluation should be conducted. To determine the visual landscaping evaluation parameters, these parameters were grouped under cognitive/emotional/biophysical titles according to studies conducted on the visual perception, preference and evaluation of the landscaping. according to Clay and Daniel (2000), Wu et al. (2006), Arriaza et al. (2004), Sevenant and Antrop (2009), Uzun and Müderrisoğlu (2011), Jahany et al. (2012), SNH (2013), Huang (2014). Biophysical parameters were evaluated as visual field width, form of clarity limits, silhouettes, land silhouette, width, slope, exposure (to sun), land form, relief, land cover, adjacent view, cultural variables, vegetation, richness in species and water existence.

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The categories were obtained using the Q-sort method like described by Pitt and Zube (1979). In particular, participants were asked to sort the 74 landscape photographs into 5 classes of urbanization. As each person's interpretation of urbanization may vary, we used a more objective criterion to perform the sorting: the presence of built area in the image. For the sorting task, the participants were presented with all photographs on a desk and were asked to first remove the 12 scenes which they thought were least characterized by built area. These landscapes were classified as 'Rural'. The second task consisted of picking out the 12 scenes in which they thought the most built area was present (Urban'). For the remaining 50 photographs these two steps were repeated but at each time selecting 16 photographs instead of 12 (respectively 'Semirural' and 'Semi-urban').

Fairweather and Swaffield (2001): This paper reported on an interpretative study of visitor experiences of landscape in Kaikoura, New Zealand and focuses on how these experiences vary among different groups. Photographs representing different landscape experiences were Q sorted by a non-random sample of both overseas and New Zealand visitors. The data were factor analyzed to yield five groups each describing a distinct visitor experience, and the results were interpreted on the basis of the photographs most and least liked, and the comments made about them by the people interviewed. The eco-tourist experience is characterized by being close to marine mammals in a spectacular setting.

Naspetti et al. (2016): Visual Q methodology is particularly suited for the assessment of such perceived impact of photovoltaic systems. A selection (concourse) of landscape images with photovoltaic elements was collected and used during this Q-sort analysis. The final Q sample included 54 images of various photovoltaic plants in urban and rural settings. The P set was composed of 34 participants, including landscape and photovoltaic professionals. This analysis identified three distinctive factors that are representative of the different viewpoints on the integration of photovoltaic systems within the urban and rural landscapes. We conclude with a discussion of the wider land-use policy implications of this analysis.

According to Dupont et al. (2017), the remaining 18 landscape scenes formed the last 'Mixed' class and used Q-sort analysis. Scores of for each photograph were summed across participants and an average urbanization score was calculated. These means determined to which class of urbanization each photograph was assigned. However, a number of photographs seemed to balance between two categories (scores close to e.g., 1.5, 2.5 etc.) and could therefore not be unequivocally assigned to one class.

Q-Sort analysis method was used for this study and the mentioned wetlands which were considered to be important in the close vicinity of Sivas were; Hafik Lake (Koçhisar Lake), Ulaş Lake, Gürün Gökpınar Lake and Tödürge Lake (Demiryurt Lake). The aim of this study was to evaluate the visual landscaping quality and recreational usage potential of the wetlands with different sizes and located to the north of Sivas-Hafik highway. Therefore some parameters were examined (water property size, plant existence, topographic diversity, neighbors views, natural elements, cultural existence). A second aim of our study, these wetlands was to bring suggestions to protect in sustainable landscape.

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Material and method

Material

The study material consisted of the wetlands in the immediate surroundings of Sivas (*Fig. 1*). They are wetlands with different sizes and located to the north of Sivas – Hafik highway: Hafik Lake (Koçhisar Lake), Gürün Gökpınar Lake, Ulaş Lake and Tödürge Lake (Demiryurt Lake). Photographs of these wetlands were taken with a digital camera between 11.00 and 17.00 h. When images were taken, active uses are taken into consideration and areas that people observe individually were taken as basis. In order to allow for the assessment of the areas from all angles, 48 photographs were taken and a total of 16 images (4 photographs for each wetland) were selected for selection.

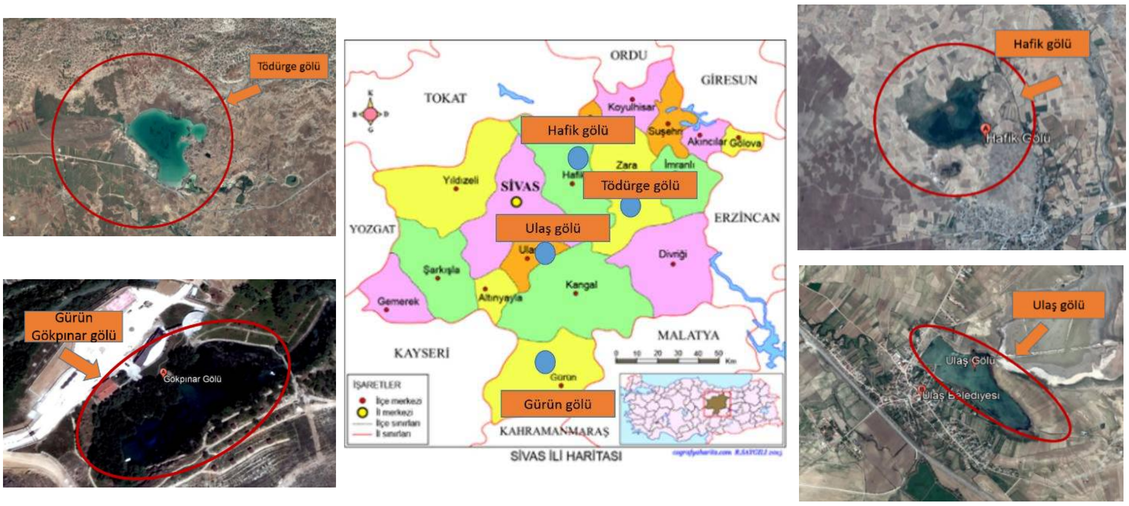

Figure 1. *Satellite images of wetlands in the study and Sivas map (Turkey)*

Gökpınar Lake is located in Gürün County. It is used as a recreational area. Gökpınar (Gürün) Lake has a surface area of 3.000 m^2 and the trout facilities established on the brook flowing from the lake is a source of livelihood for the people living in the area (*Table 1*). Gökpınar has its name because of its structure resembling an aquarium, its water being soft and cold, its color being turquois and azure blue. On the other hand; Hafik Lake is 36 km far from Sivas city center and it is a karst lake. It has a surface area of 7.5 km and its volume is 2.250.000 m^3. It has eutrophic characteristics and has an average depth of 2 m (Çepken, 2008). Ulaş Lake is located in Ulaş County. It has a surface area of 0, 79943 km. It has been recently put into service as a recreational area by the local administration.

Tödürge is a mildly salty lake located on the upper Kızılırmak basin. It is on the Sivas – Erzurum highway, 50 km far from Sivas, between the counties Hafik and Zara. There is a village with the same name in about 1-km distance. The word “Tödürge” is a changed version of the name belonging to one of the old Turkish tribes; Dodurga (*Table 2*).

The surface area of Tödürge Lake is 5 km^2 and it is fed by the waters emerging from the bottom, springs in the surrounding area and Acısu brook. Tödürge Lake is not very deep in the sides and its average depth is 20 m. In its deepest spot, it is

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around 45 m deep. It flows into Kızılırmak with an outgoing channel from the west side. The average depth of the lake is 4 m and there are small reeds on its shores. Keşan Island located to the east of the lake is a favorite spot for cranes. To the west, there are large meadows used as grazing for the cattle.

Table 1. *Expert assessment of images (Hafik and Gürün Lake)*

<table>
<tr><th>Wetlands</th><th>Image 1- Sivas Hafik Lake</th><th>Image 2</th></tr>
<tr><td rowspan="4">Hafik Lake (Code: HL)</td><td></td><td></td></tr>
<tr><td>Image 3- SHL3</td><td>Image 4- SHL4</td></tr>
<tr><td></td><td></td></tr>
<tr><td></td><td></td></tr>
<tr><td rowspan="4">Gürün Lake (Code: GL)</td><td>Image 1- Sivas Gürün Lake</td><td>Image 2</td></tr>
<tr><td></td><td></td></tr>
<tr><td>Image 3</td><td>Image 4</td></tr>
<tr><td></td><td></td></tr>
</table>

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Table 2. *Evaluated images by expert (Tödürge and Ulaş Lake)*

Wetlands	Image 1- Sivas Tödürge Lake	Image 2
Tödürge Lake (Code: TL)		
	Image 3	Image 4
		
Ulaş Lake (Code: UL)	Image 1. Sivas Ulaş Lake	Image 2
		
	Image 3	Image 4
		

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Method

The data obtained from the questionnaires of every 30 experts for each of the 16 images were recorded and separated for 4 wetlands. Then the biophysical parameters of the visual landscape were studied, evaluated (*Table 3*) and scored numerically (very beautiful +2, beautiful +1, ordinary 0, ugly -1, and very ugly -2). The results of this study were set in a separate table. The number of experts are obtained in light of the data of landscape architects living in Sivas according to UCTEA Chamber of Landscape Architects. The score of each landscape characteristic got based on experts' opinions, was calculated. High score of each wetland suggests photo's desirability and higher priorities of public preferences. In order to calculate the score of each photograph, the following method was used: Scoring was done for each building according to five point Likert scale as +2, +1, 0, -1, -2 and the recreationally significant areas are designated by a Q-Sort analysis formula was used and the resulted findings are listed in *Tables 4, 5, 6, 7, 8, 9* and *10.*

$$N = \sum_{i=1}^{5} n_i (3 - i)$$

Total points of every photo = N
The number of selectors with the quality of very beautiful = n1
The number of selectors with the quality of beautiful = n2
The number of selectors with the quality of ordinary= n3
The number of selectors with the quality of ugly = n4
The number of selectors with the quality of very ugly = n5 (Golchin et al., 2012).

Table 3. *Evaluation chart according to visual landscape biophysical parameters Çakcı (2007), Uzun and Müderrisoğlu (2011), SNH (2013)*

Points Criteria	+2	+1	0	-1	-2
Water property size	Very dominant and clear water surface	Water surface is very visible but non-clear water	Water surface obvious	Water surface is obvious and dirty	The physical quality and appearance of the water is very bad, dirty
Plant existence	Very various	Various	Little variety	Not various	No vegetation
Topographic diversity	Very clear	Clear	Little clear	Not clear	Not at all clear
Neighbors views	Very clear	Clear	In the middle	Little clear	Not at all clear
Natural elements	Natural elements are very dominant	Natural elements dominant	Natural and structural elements are balanced	Structural elements dominant	Structural elements are very dominant
Cultural existence	Near, varied and clear	Near, less varieties	Far away, less clear	Far away, not clear	Not available

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Results and discussion

Following the study conducted, 6 parameters on the visual landscaping character and general expert opinion were evaluated with a Q-Sort analysis. In light of the data gathered, it is seen that Gürün Lake had the first place in “attractiveness”. Tödürge Lake also had an important point. As seen in *Table 4,* 2 out of 4 wetlands in different locations had high points. While it is significant that recreational area works of Ulaş Lake continues, the area also must be protected against destruction. The *Table 4* indicated Gürün Lake had 48 points as the general view of expert. Also Tödürge Lake had 39 point second rank in terms of general view.

Table 4. *The general view of visual landscape value assessment by experts in working area of lakes*

The number of the photo selectors of different qualities (from 30 experts) - the general view						
Photo name	**Very Beautiful n1**	**Beautiful n2**	**Ordinary n3**	**Ugly n4**	**Very ugly n5**	**Photo points N**
Tödürge Lake-(TL)	12	16	1	1	0	39
Hafik Lake (HL)	2	12	9	6	1	8
Gürün Gökpınar Lake GL)	19	10	1	0	0	48
Ulaş Lake (UL)	0	11	15	4	0	7

Gökpınar Lake which is one of the important picnic areas of Sivas, has a depth of 15 m. Its water is quite clean and clear. The lake is famous for its trout and it is fed by the waters emerging from the bottom. There are motels and restaurants on the lakeshore and the opportunity of boating on the lake affected its scoring positively (*Table 4; Figs. 2* and *3*). Ulaş Lake is located in Ulaş County. It used to be idle but after the work conducted by Ulaş Municipality, the lake became active again and there was a picnic area constructed on its shore. There are gazebos installed around the lake for the visitors, however the wetland is not under adequate protective measures.

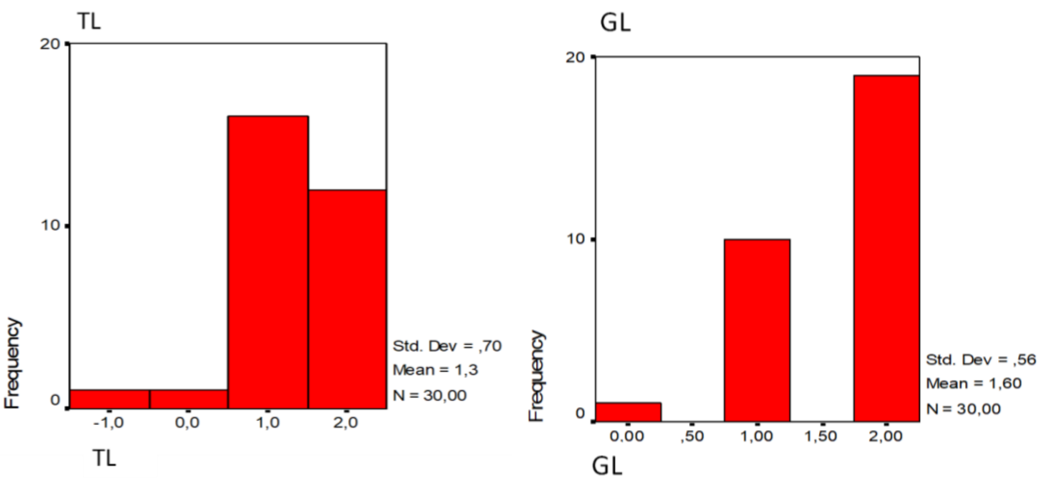

Figure 2. *Standard deviation values of general opinion evaluation by experts of visual landscape value of Tödürge, Gürün Lakes*

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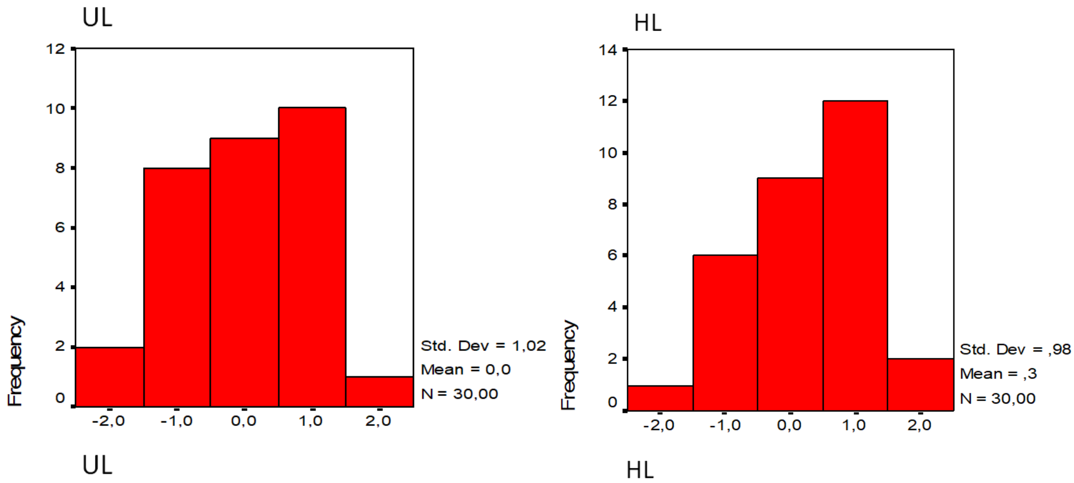

Figure 3. *Standard deviation values of general opinion evaluation by experts of visual landscape value of Ulaş and Hafik Lakes*

When the parameter of water existence was reviewed; Tödürge Lake being on the migration route of the birds and coming in the first place according to area size (4.340 ha) support the result of Q-Sort analysis. As seen on *Table 5,* Tödürge Lake had the highest point in terms of water existence. When we look at Gürün Lake on the other hand, it was seen that running water being used as drinking water supply by the locals was a significant advantage. It was remarkable that the water was clean, healthy and cold at the same time. The effects of this situation could also be seen on the scoring. Hafik and Ulaş lakes had close points. None of the 4 wetlands had negative point in terms of water quality and wetland distinctiveness (*Table 5*).

Table 5. *The visual landscaping items of the lakes assessment according to the "Parameter of water presence"*

The number of the photo selectors of different qualities (from 30 experts) - water presence							
Photo name	**Very beautiful n1**	**Beautiful n2**	**Ordinary n3**	**Ugly n4**	**Very ugly n5**	**Photo score N**	**Standard dev.**
Tödürge Lake-(TL)	12	18	0	0	0	42	,49827
Hafik Lake (HL)	11	14	3	2	0	34	,86037
Gürün Gökpınar Lake (GL)	12	15	3	0	0	39	,65126
Ulaş Lake (UL)	9	15	4	2	0	31	,85029

When the plant existence parameter was reviewed; it was seen that the points of Gürün, Ulaş and Tödürge lakes were close to one another. Ulaş Lakes' Wetland was registered as "Wetland of National Importance" on 10 July 2016. The area is 79.943 decares big. Ulaş Lakes are small and shallow lakes located to the southeast of Sivas. They are 37 km far from Sivas. There are 48 families, 188 types, 338 species and 345 taxa around Ulaş Lakes and their surroundings. 57 out of 338 detected species are endemic. Gurun Lake also had a decent point (45) (*Table 6*).

Table 6. *The visual landscaping items of the lakes assessment according to the "Parameter of plant existence"*

The number of the photo selectors of different qualities (from 30 experts) - plant							
Photo name	**Very Beautiful n1**	**Beautiful n2**	**Ordinary n3**	**Ugly n4**	**Very ugly n5**	**Photo score N**	**Standard dev.**
Tödürge Lake-(TL)	12	17	1	0	0	41	,55605
Hafik Lake (HL)	2	13	11	4	0	13	,81720
Gürün Gökpınar Lake (GL)	17	11	2	0	0	45	,62972
Ulaş Lake (UL)	18	7	4	1	0	42	,85501

When the topographical variety parameter was evaluated; it was seen that the area is rich in topographical variety. It was especially important that there were mountains with different heights that shape up the land view. According to the results of the analysis, Gürün Lake was in the first place in terms of variety. Hafik and Ulaş lakes also had decent points (*Table 7*).

Table 7. *The study area consists of visual landscaping items of the lakes assessment according to the "Parameter of topographic diversity"*

The number of the photo selectors of different qualities (from 30 experts) - topographic diversity							
Photo name	**Very beautiful n1**	**Beautiful n2**	**Ordinary n3**	**Ugly n4**	**Very ugly n5**	**Photo score N**	**Standard dev.**
Tödürge Lake-(TL)	2	19	7	2	0	21	,70221
Hafik Lake (HL)	13	14	3	0	0	40	,66089
Gürün Gökpınar Lake (GL)	18	11	1	0	0	47	,56832
Ulaş Lake (UL)	6	19	5	0	0	31	,61495

When the neighboring view parameter was reviewed; it was seen that plant diversity and topographical diversity, positively affect the view. Existence of settlements close to the wetland was limiting the view and is also a threat to the area. According to the analysis results; Gürün Lake was in the first place as it had clean and unique water as well as natural beauties in its surroundings. Hafik, Tödürge and Ulaş Lakes also had decent points (*Table 8*).

Table 8. *The study area consists of visual landscaping items of the lakes assessment according to the "Parameter of neighbors views"*

The number of the photo selectors of different qualities (from 30 experts) - neighbors views							
Photo name	**Very beautiful n1**	**Beautiful n2**	**Ordinary n3**	**Ugly n4**	**Very ugly n5**	**Photo score N**	**Standard dev.**
Tödürge Lake-(TL)	6	20	4	0	0	32	,58329
Hafik Lake (HL)	10	16	4	0	0	36	,66436
Gürün Gökpınar Lake (GL)	20	10	0	0	0	50	,47946
Ulaş Lake (UL)	3	17	8	2	0	21	,74971

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When the natural elements parameter was reviewed; it was seen that for all three wetlands, natural elements were apparent. The existence of a settlement in close vicinity of Ulaş Lake showed that there was a balance between natural element and structures. In case the city started to become a concrete jungle without the notion of sustainable environmentalism, destruction and pollution in wetlands were thought to happen (*Table 9*).

Table 9. *The visual landscaping items of the lakes assessment according to the "Parameter of natural elements"*

The number of the photo selectors of different qualities (from 30 experts) - natural elements							
Photo name	**Very beautiful n1**	**Beautiful n2**	**Ordinary n3**	**Ugly n4**	**Very ugly n5**	**Photo score N**	**Standard dev.**
Tödürge Lake-(TL)	12	16	2	0	0	40	,60648
Hafik Lake (HL)	13	15	2	0	0	41	,61495
Gürün Gökpınar Lake (GL)	20	10	0	0	0	50	,47946
Ulaş Lake (UL)	1	18	8	3	0	17	,72793

When the cultural existence parameters are reviewed; it was seen that the presence of a historical past surrounding the wetlands had significant effects. There are social facilities belonging to Sivas Cumhuriyet University on the east coast of Tödürge Lake, on a hill overviewing the it. Also, to the north of Tödürge Lake, there is a historical settlement named Tepecik Mound.

When Hafik Lake was evaluated; it was seen that during the drilling excavation on Pılır Mound, the existence of lake houses built on wooden poles nailed to the bottom of the lake was detected and it was concluded that this settlement dated back to Neolithic, Catholic and First Bronze Age. Pılır Mound was the only example of settlement style formed by the lake houses called Palafit in our country. Other examples of these houses were discovered in the Zurich Lake and the Lakes of the Alps in Switzerland (Sivas Provincial Directorate of Culture and Tourism). These historical past effects also affected the result of the analysis. Hafik Lake and Tödürge Lake had the highest points (*Table 10*).

Table 10. *The visual landscaping items of the lakes assessment according to the "Parameter of cultural elements"*

The number of the photo selectors of different qualities (from 30 experts)- cultural existence							
Photo name	**Very beautiful n1**	**Beautiful n2**	**Ordinary n3**	**Ugly n4**	**Very ugly n5**	**Photo score N**	**Standard dev.**
Tödürge Lake-(TL)	18	11	1	0	0	47	,56832
Hafik Lake (HL)	21	8	1	0	0	50	,54667
Gürün Gökpınar Lake (GL)	16	13	1	0	0	45	,57235
Ulaş Lake (UL)	5	10	13	2		18	,85501

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Results and suggestions

It is a known fact that building a city causes pressure and destruction on the natural environment. Yet, in order to minimize the destruction, it is important to make guiding plans for benefiting from natural areas within the limits of preservation – utilization balance and determining the limits for structuring (Aşur, 2017; Aşur and Alphan, 2017; Meriç and Çağırankaya, 2013). In wetlands, many physical factors come into play dominantly and it is easier for users to see the interactions between physical and biological environments.

In the studies conducted on wetlands; asides from using wetlands as recreational areas (Osborn and Spofford, 1972; Troost and Altman, 1972), the advantages of using them for educational purposes and their suitability for local, national and global environmental problems were researched. It was also aimed to teach basic ecological concepts and develop motivations that would help stir the feelings of curiosity and attention about environment by making first-hand observations.

City of Sivas which was the subject of this study, had wetlands with various sizes. These could be listed as the Hafik Lake (Koçhisar Lake) located to the north of Sivas – Hafik highway, Gürün Gökpınar Lake, Ulaş Lake and Tödürge Lake (Demiryurt Lake). Among these wetlands, reed field properties of Sivas – Ulaş wetland and Hafik wetland are under threat because of their being in a close distance to the city center. All four of the wetlands, particularly Tödürge Lake and Gürün Lake, receive many visitors coming with recreational purposes. Even though they were relatively far from the city center, their natural structures have started being subject to depredation. It was suggested that the restoration works on the areas of Sivas Hafik Lake and Ulaş Lake were increased and the location should be open to visitors with arrangements compatible to the nature of the area.

Each lake discussed within the scope of this study were reviewed under 7 different parameters regarding the wetlands and the lakes with the highest point in each parameter are designated (*Table 11*).

Table 11. *The highest values of examined the parameters in the wetlands*

Parameters	Wetland name	Point
General view of experts	Gürün Lake	48
Water property size	Tödürge Lake	42
Plant existence	Gürün Lake	45
Topographic diversity	Gürün Lake	47
Neighbors views	Gürün Lake	50
Natural elements	Gürün Lake	50
Cultural existence	Hafik Lake	50

Table 11 can be summarized as follows: In neighboring view, natural elements, topographical diversity and plant existence parameters, Gürün Lake had the highest point by a narrow margin. In cultural existence parameter, Hafik Lake had the highest point. In water existence parameter, Tödürge Lake had the highest point. The result of the evaluation conducted according to the general view of the experts, the overall highest point belongs to Gürün Lake.

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In order to preserve these beautiful and natural structures existing in Sivas and many other cities alike; shore protection plans should be constituted in order to prevent the destruction caused by the structuring done against building codes and pollution of solid wastes and waste waters. It is suggested that by staying within the limits of Shore Protection Act No. 3621; public-minded facilities on recreation, culture, sports, entertainment, relaxation, health and social life should be established and the natural habitat areas should be protected. Each of the wetlands located in the city of Sivas and its immediate surroundings of shore areas, have high ornithological importance thanks to the significant potential as wetlands and being on the important bird migration routes. Among these wetlands, Kızılırmak Delta has international preservation status. Ulaş and Tödürge Lakes are designated as RAMSAR areas. Sivas has visual landscaping resources and to preserve the landscaping quality of wetlands in Sivas and maintain ecological sustainability, local authorities of Directorate General for Nature Conservation and National Parks and Turkish Republic Ministry of Forestry and Water Affairs should have precedence in the development of human resources and corporate capacity. Studies conducted should be supported with awareness raising activities done by local decision makers and local community, together with preparing integrated wetland administration plans. One of the most important conditions of preserving the wetland sceneries of Sivas was considered to be corporate collaborations aimed at the protection of wetland areas' visual landscaping quality.

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EFFECTS OF OZONE AND DENSITY INTERACTION ON THE GROWTH, DEVELOPMENT AND YIELD FORMATION OF RICE

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Abstract. To further study the effect of ozone stress on growth and yield formation of rice (*Oryza sativa*), we conducted an experiment with rice by using Free Air gas Concentration Enrichment (FACE) facility stored in paddy field in China. A conventional indica cultivar Yangdao 6 and a super hybrid indica cultivar II you 084 were grown at current or elevated ozone concentration (target at 50% above current, E-O_3) under low (LD, 16 hills m^{-2}), medium (MD, 24 hills m^{-2}) or high planting density (HD, 32 hills m^{-2}) from tillering until maturity. The increase of O_3 had no significant effect on phenology and plant height of Yangdao 6, but greatly accelerated phenology development and reduced plant height of II you 084. Ozone stress significantly reduced dry matter production of rice in mid and late stage, and finally, decreased the biomass of Yangdao 6 and II you 084 by 17.6% and 25.4% respectively. Ozone stress had no significant effect on harvest index of Yangdao 6 but reduced it of II you 084 by 9.0%. The increase of O_3 had no significant effect on the panicle number per area of rice. Ozone stress had no significant effect on the spikelet number per panicle of Yangdao 6, but significantly reduced that of II you 084 by 22.0%. Ozone stress decreased spikelets per area, filled grain percentage, and filled grain weight of rice, and eventually reduced grain yield of Yangdao 6 and II you 084 by 15.9% and 27.3% respectively. Variance analysis showed that the impacts of ozone stress on II you 084 were bigger than Yangdao 6, which were proved by the significant ozone by cultivar interactions for biomass, harvest index, spikelet number per panicle, spikelet number per area, filled grain percentage, filled grain weight. Week interactions of ozone by cultivar by planting density were detected: The effects of ozone stress on biomass and grain yield of Yangdao 6 were decreased with the increase of planting density. The above results showed that the increase of O_3 depressed the dry matter production of rice, and then decreased the yield, and the response of super hybrid rice varieties to ozone is greater than that of conventional varieties. The selection of high-yielding conventional varieties combined with relatively high planting density can minimize the yield loss in the future high ozone conditions.
Keywords: *rice, ozone, plant density, yield formation*

Introduction

In recent decades, due to the aggravation of human activities, the use of a large number of fossil fuels and nitrogenous fertilizers and the emission of automobile tail gas, the concentrations of NOx and VOCs in the atmosphere have increased dramatically, resulting in the increasing concentration of ozone in the atmosphere near the ground. According to observations, the concentration of O_3 in the troposphere of the Northern Hemisphere is increasing at an annual rate of 1% (WMO, 1991), even more in some industrial areas (Logan, 1985). It is reported that the average ozone concentration in the troposphere of the earth has risen rapidly from less than 10 nl/L before the industrial revolution (Volz and Kley, 1988) to about 50 ml/L in 2000 (Fiscus et al., 2005). According to Fuhrer et al.'s (1997) study, the ozone concentration has exceeded 25% of sensitive crops' injury concentration (40 nl/L). It is estimated that nearly a quarter of the countries and regions in the world now face the threat of O_3 concentration

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of more than 60 nl/L in summer (Fowler et al., 1999). It is estimated that by 2050, the concentration of O_3 in the troposphere will increase by 20% to 25% (IPCC, 2007), and by 2100 by 40% to 60% (Morgan et al., 2006; Sitch et al., 2007; Meehl et al., 2007). The pessimistic estimate is that the concentration of O_3 in the troposphere will rise to 80 nl/ L by 2100 (Fiscus et al., 2005).

The response of rice growth and yield formation under ozone stress has been widely reported (Yang et al., 2008; Ainsworth, 2008). The results showed that the response of yield to ozone stress varies greatly due to the control equipment of the experiment (Maggs et al., 1995), materials (Kobayashi et al., 1995; Maggs and Ashmore, 1998; Zheng et al., 1998; Jin et al., 2000; Li et al., 2017), the concentration of O_3 treatment materials (Bai et al., 2002; Jin et al., 2001; Feng et al., 2003) and the treatment period (Asakaw et al., 1981; Heagle et al., 1991). But does the response of rice growth and yield formation to the increase of ozone concentration vary with other environment or cultivation factors? Due to the limitation of the air chamber's narrow test space, there are few reports in this aspect. Because of the large experimental space and long duration, FACE platform provides an opportunity to study the interaction between ozone and other factors (Long and Ainsworth, 2005; Lie et al., 2014; Peng et al., 2016). In recent years, the influence of ozone and other factors, especially N, on rice (Luo et al., 2012, 2013) or wheat (Chen et al., 2011a, b) has been studied by FACE platform. As a common cultivation measure, could planting density regulate rice growth and yield formation under high ozone concentration? And does this regulation vary from variety to variety? There have been no previous reports, which is more realistic for the future development of strategies for rice production under higher surface ozone concentrations. According to the experimental materials, most of the previous studies focused on conventional rice varieties, but less on hybrid rice and even less on super hybrid rice. As has been reported in the past, the yield response of rice varieties and hybrid rice varieties to ozone stress was more significant (Shi et al., 2009; Wang et al., 2012; Luo et al., 2013). But what is the difference between the growth and yield response of super rice under ozone stress and other types of varieties? Is the yield loss similar or even greater to hybrid rice? In order to solve these problems, the effect of ozone stress on growth, development and yield formation under different densities were analyzed by using China O_3-FACE platform and conventional indica rice of Yangdao 6 and Super hybrid rice of II you 084 in order to provide some guidance for rice production strategies and high-yield cultivation measures under the condition of high ozone concentration in the future.

Materials and methods

Ozone-FACE platform

The experiment was conducted on the China O_3-FACE platform, which was located at Xiaoji town, Jiangdu County, Jiangsu province, China (119°45' E, 32°35' N). The site, at 5 m above the sea level in elevation, sits in the subtropical marine climatic zone. Throughout the period of 113 days from June 21 to October 17 for the experiment in 2012, mean daily temperature was 24.5 °C, mean daily integral solar radiation (or PAR) was 13.9 MJ m^{-2}, and mean daytime (6:00–18:00) vapor pressure deficit was 0.67 kPa (Tang et al., 2011). This site has been in continuous cultivation for more than 1000 years with rice–wheat or rice–rapeseed rotation. Detailed descriptions of the soil properties for the site can be found in our previous publication (Shi et al., 2009).

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Details of the design and performance of this O_3-FACE system are provided by Tang et al. (2011). In brief, The FACE system has eight 240 m^2 plots, of which four replicate plots were exposed to elevated ozone concentration (hereinafter called E-O_3 plots) and four equal size plots were in current ozone concentration (current ambient ozone concentration, C-O_3 plots). Any one of the E-O_3 plots was separated from other plots by at least 70 m to avoid cross contamination. The quantity and direction of the ozone release for each E-O_3 plot was controlled by a proportional integral derivative algorithm for computer feedback that compares achieved ozone concentration to the target ozone concentration of 1.5 times current ozone concentration (simulating the predicted ozone concentration at the end of the century) with an ozone monitor (model 49i, Thermo Environmental Instruments, MA, USA), a data logger-controller, an anemometer and a wind vane. Current ozone concentration at canopy height was monitored with separate O_3 analyzers in two of the C- O_3 plots at a 2-min interval. Every 10 min, a control computer accessed all the data logger-controllers in C- O_3 and E- O_3 plots to collect the latest ozone concentration. A mixed gas consisting of about 5% ozone and 95% O_2 was produced by an ozone generator (KCF-BT0.2, Jiangsu Koner Ozone Co., Ltd, Yangzhou, China). The mixed gas was released in a stream of compressed air into the plots through the ABS pipes positioned at about 50 cm above the canopy. In the ambient plots, plants were grown under ambient ozone conditions without the ring structures. The ozone fumigation began on 1st July and continued throughout the rice season until harvest, from 9:00 am to 16:00 pm every day, only suspended in these conditions, such as ozone concentration in the control cycle lower than 20 nL/L, leafs moistened by rain and dew, ozone analysis device correction, and equipment overhaul, so the actual mean ozone concentration in FACE cycle during the entire growing season of rice was higher approximately 27% than that in the control cycle. *Figure 1* shows the seasonal change in daily 7-h mean ozone concentration for C-O_3 and E-O_3 plots.

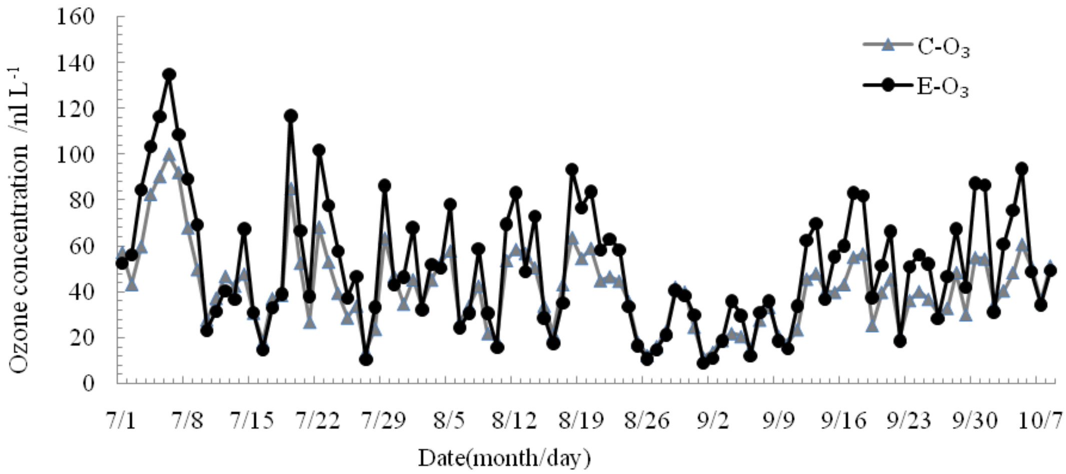

Figure 1. *The change of day time 7-h average ozone concentration during ozone fumigation. C-O_3: Current O_3 concentration; E-O_3: Elevated O_3 concentration; The same below*

Plant material and cultivation

The rice varieties are conventional indica Yangdao 6 (the variety features can not be separated from their offspring) and super hybrid indica II you 084 (the variety that was

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produced by cross breeding, and had heterosis in yield). Rice seeds were sown on 21 May; the seedlings were grown under current air and were manually transplanted into the C-O_3 and E-O_3 plots with one seedling per hill on 21 June 2012. Nitrogen (N) was supplied as urea (N = 46%) and compound chemical fertilizer ($N:P_2O_5:K_2O$ = 15:15:15) at a rate of 15 g N/m^2. Of the total N, 50% was applied as the basal dressing on 20 June, 10% was top-dressed on 7 July and 40% top-dressed on 7 August. Both phosphorus (P) and potassium (K) were applied as compound chemical fertilizer at equal rates of 7 g/m^2 as the basal dressing at 1 d before transplanting. Standard cultivation practices as commonly performed in the area were followed in all experimental plots. In brief, the paddy fields were submerged with water of about 3 cm in depth from 21 June to 4 July, and then the fields were subjected to wet–dry cycles through natural drainage and intermittent irrigation. At 10 d before harvest, irrigation was terminated to allow paddy fields to dry for final harvesting. Pesticides and fungicides were applied when necessary throughout the experiment.

Experimental treatment

This trial was a split plot experiment, in which ozone concentration was the main plot, and the treatment of varieties and transplanting density was the split plot, repeated each treatment four times, so there were 12 treatments and 48 plots in total. The transplanting density was determined as three densities including low density (16 hills/m^2, planting spacing 25 cm), medium density (24 hills/m^2, planting spacing 16.7 cm × 5 cm), and high density (32 hills/m^2, planting spacing 12.5 cm × 25 cm).

Plant phenology, plant height and tillering dynamics

The date for heading (50% of plant headed) and grain maturity was recorded in each subplot. Plant height and tiller numbers were measured for fixed 10 hills in each treatment at 5–10 days intervals until heading. The productive tiller ratio (%) was expressed as the panicle number per square meter/ the maximum tiller number per square meter × 100.

Dry matter production

Eight plants (hills) were randomly selected at tillering, jointing, heading and maturity stage. The samples were separated into green and senescent leaf, stem (including leaf sheath), and panicle (when applicable). Each plant organ was dried in an oven at 105 °C for 30 min, then at 80 °C until dry weight was constant. The dry matter accumulation and distribution among different organs during each growth period were calculated. The dry weight per stem at heading stage (%) was expressed as the dry weight per plant at heading stage / the stem number per plant. The ratio of spikelet number to stem dry weight (number/g^1) was expressed as the spikelet number per panicle/the dry weight per plant at heading stage.

Grain yield and yield components

Actual grain yield was determined of all the plants (60 hills) from a 4, 3, and 2 m^2 patch in each LD (low density), MD (medium density) and HD (high density) subplot, respectively. The grains were air dried and weighted to obtain actual grain yield. Grain yield components, i.e. the number of panicles per square meter, the number of spikelets

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per panicle, filled grain percentage, and individual grain mass, were determined at crop maturity with six hills of plants in each subplot. Grains were soaked in tap water (specific gravity = 1.0) and the number of sunken and floated grains was counted to determine the filled grain percentage. The sunken (filled) grains were oven-dried at 80 °C until constant weight achieved. low, medium and high density.

Statistical analysis

A split-plot design was employed with [O_3] as main-plot treatment, varieties and planting density as the split-plot treatment. Analysis of variance (ANOVA) was performed using the software Statistical Product and Service Solutions (SPSS Inc., Chicago, IL, USA) to determine the main effects of [O_3], variety, planting density and the effects of their interaction. Treatments were compared by Tukey's test and differences were declared statistically significant if $p < 0.05$.

Results

Plant phenology and plant height

O_3 treatment had no significant effects on phenology development and plant height of Yangdao 6. However, high concentration of O_3 advanced the heading stage of II you 084 by 3 days and the mature stage by 7 days (*Table 1; Fig. 2*). High concentration O_3 decreased the plant height of Yangdao 6, but did not reach the significant level, and decreased the height of II you 084 by 0.5%, 1.3% and 6.7% ($p < 0.05$) and 7.8% ($p < 0.05$) after transplanting at 23, 45, 66 and 113 d (*Fig. 3*). The results of variance analysis showed that the interaction between O_3 and varieties had a weak interaction effects on plant heights at the mature stage of rice ($p = 0.16$).

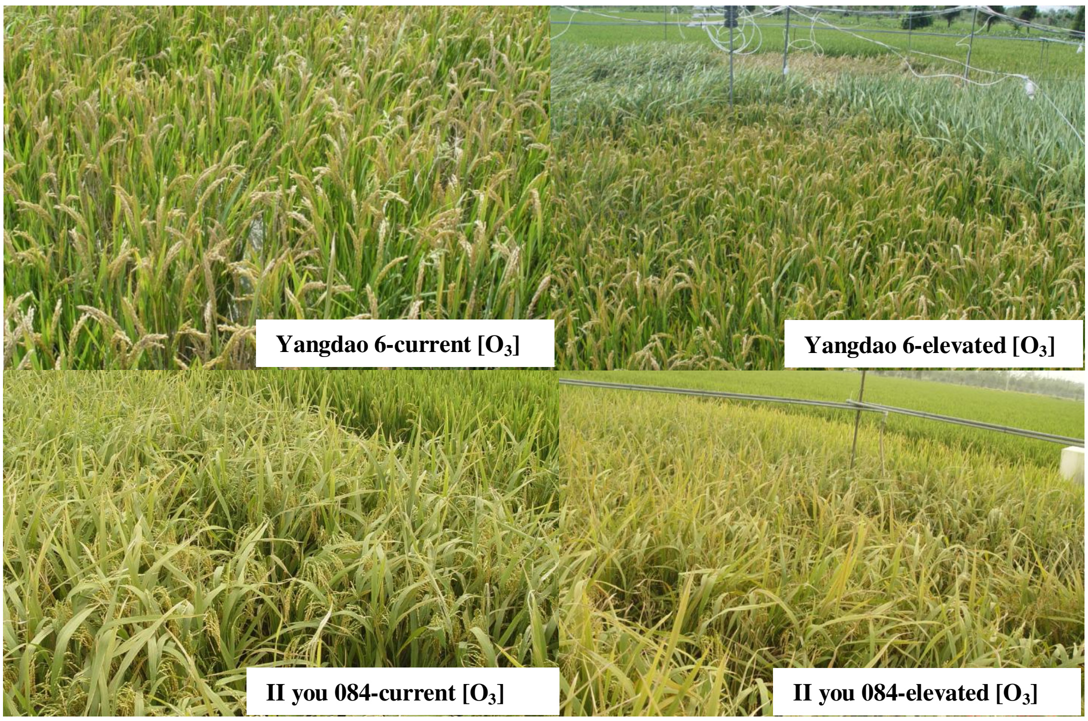

Figure 2. *Ozone stress had no impact on growth stage of Yangdao 6, but obviously accelerated phonological development of II you-084*

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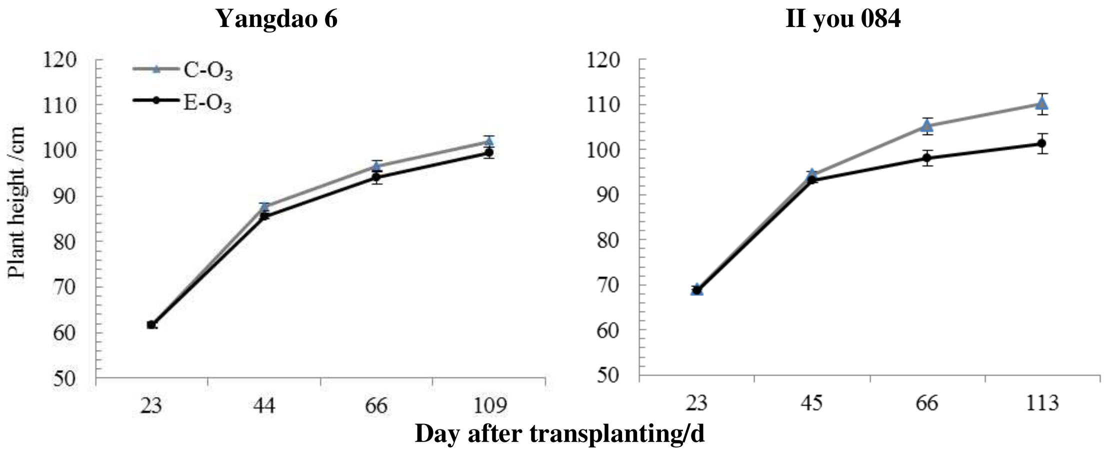

Figure 3. *Effects of ozone concentration on plant height at different growth stage of Yangdao 6 and II you 084*

Table 1. *Effects of surface ozone concentration and plant density on growth stage of rice varieties*

Cultivar	O_3 treatment	Growth stage/mouth/day		Growth period/days		
		Heading stage	Maturity	Transplanting to heading	Heading to maturity	Transplanting to maturity
Yangdao 6	C-O_3	8/28	10/16	68	49	117
	E-O_3	8/28	10/16	68	49	117
II you 084	C-O_3	8/29	10/19	69	51	120
	E-O_3	8/26	10/12	66	47	113

Dry matter production and biomass

High concentration of O_3 reduced the biomass of Yangdao 6 by 17.6% (*$p < 0.01$*), among which 23.5%, 20.1% and 9.3% were decreased at low, medium and high density respectively, with all reaching a significant level. The elevated ozone concentration decreased the biomass of II you 084 by 25.4% (*$p < 0.01$*), among which 29.2%, 25.6% and 22.0% were significantly decreased respectively (*Fig. 4*). The effects of ozone multiplied by varieties and ozone multiplied by density on biomass of rice reached the significant level of 0.05 and 0.1 respectively.

The biomass of rice was closely related to the dry matter production in each growth stage. High concentration of O_3 reduced the dry matter production of Yangdao 6 from tillering to jointing, jointing to heading, heading to maturity stage by 1.8%, 29.2% (*$p < 0.01$*) and 19.0% (*$p < 0.05$*) respectively, and decreased II you 084 with the same stage by 8.6% (*$p < 0.05$*), 13.6% ($p < 0.1$) and 46.5% (*$p < 0.01$*) respectively (*Table 2*). The analysis of variance showed that the interaction of ozone and varieties had a significant effect on the biomass from heading to maturity of rice. The high concentration of O_3 had no significant effect on the harvest index of Yangdao 6 but decreased it of II you 084 by 9% (*$p < 0.05$*) (*Fig. 5*). Variance analysis showed that the effect of ozone multiplied by varieties on harvest index of rice reached 0.1 significant level.

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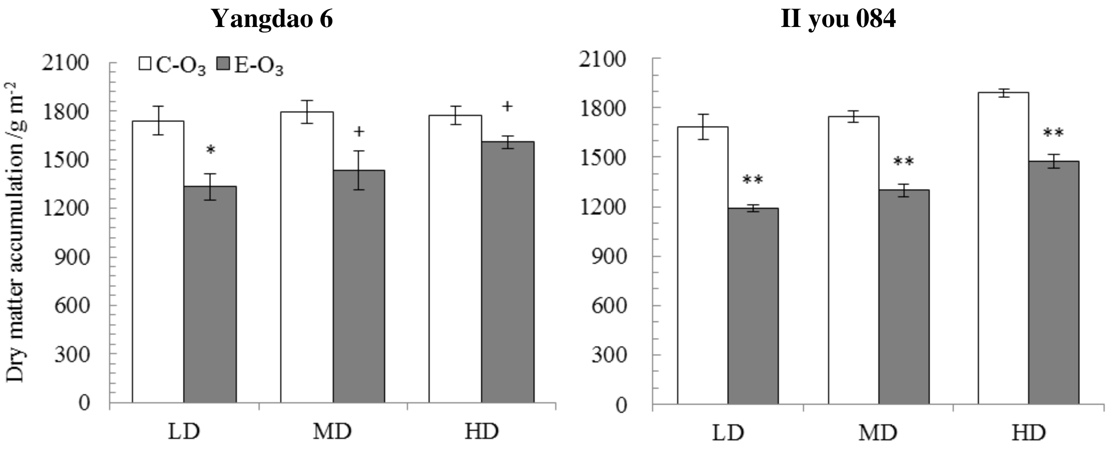

Figure 4. *Effects of surface ozone concentration and plant density on biomass yield of Yangdao 6 and II you 084. ANOVA: Cultivar (P < 0.05); Density (P < 0.01); Ozone (P < 0.01); O_3 × Cultivar (P < 0.05); O_3 × Density (P < 0.1); Cultivar × Density (p > 0.1); O_3 × Cultivar × Density (p > 0.1)*

Table 2. *Effects of surface ozone concentration and plant density on dry matter production of rice varieties*

Cultivar	Density	O_3 treatment	Dry matter production/g/m^2		
			Tillering to jointing	**Jointing to heading**	**Heading to maturity**
Yangdao 6	LD	C-O_3	336±21	389±52	892±75
		E-O_3	339±15	245±41	629±90
	MD	C-O_3	318±5	541±29	776±96
		E-O_3	313±17	393±58	573±92
	HD	C-O_3	351±20	449±26	793±47
		E-O_3	321±34	338±24	792±28
II you 084	LD	C-O_3	341±8	528±55	701±105
		E-O_3	293±13	427±19	371±35
	MD	C-O_3	365±32	567±24	662±58
		E-O_3	342±22	441±48	383±64
	HD	C-O_3	416±28	454±46	852±59
		E-O_3	390±25	470±50	432±71
ANOVA (*p* value)		O_3	**0.068**	**0.001**	**0.000**
		Cultivar	**0.074**	**0.004**	**0.000**
		Density	**0.089**	**0.030**	0.200
		O_3×D	0.859	0.423	0.553
		O_3×C	0.400	0.298	**0.031**
		C×D	0.365	0.641	0.598
		O_3×D×C	0.784	0.662	0.391

The values in the table represent average values ± standard errors

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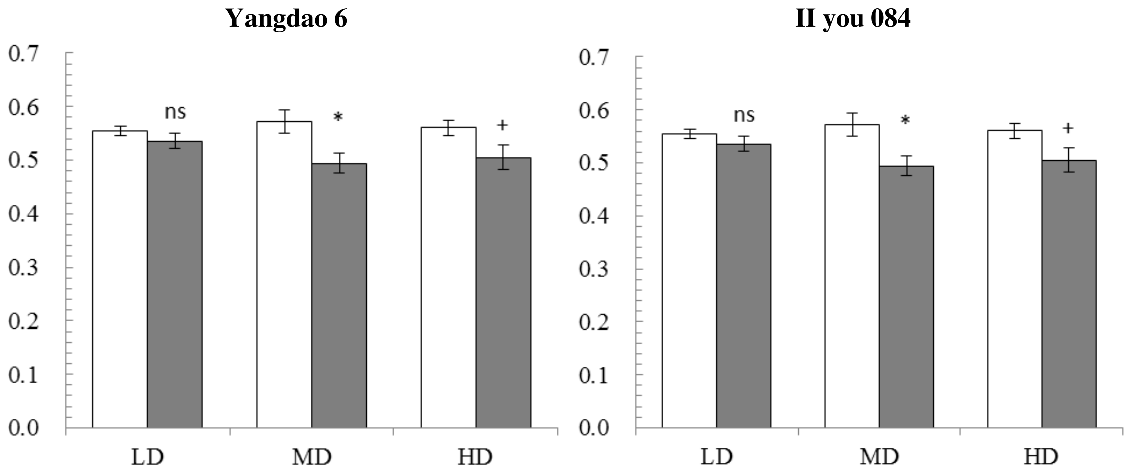

***Figure 5.** Effects of surface ozone concentration and plant density on harvest index of Yangdao 6 and Ⅱ-you 084. ANOVA: Cultivar (p > 0.1); Density (p > 0.1); Ozone (P < 0.1); O_3 × Cultivar (P < 0.1); O_3 × Density (p > 0.1); Cultivar × Density (p > 0.1); O_3 × Cultivar × Density (p > 0.1)*

Grain yield and yield components

O_3 treatment decreased the grain yield of Yangdao 6 by 142.0 g/m^2, which was 15.9% (*p < 0.01*) reduction compared to the control, with the decrease of 23.5% (*p < 0.01*), 14% (*p < 0.1*) and 9.9% (*p < 0.1*) under low, medium and high density. The O_3 stress reduced the yield of II you 084 by 278.5 g/m^2, which was 27.3% (*p < 0.01*) reduction compared to the control, with the significant decrease of 26.9%, 25.0% and 30.0% respectively under low, medium and high density (*Fig. 6*). Variance analysis showed that the interaction of O_3 and varieties had a significant effect on rice yield, and the interaction of three factors of O_3, varieties and density had a weak interaction effect on the rice yield (*p = 0.20*), which show that the effect of O_3 on the grain yield of Yangdao 6 decreased with the increase of density.

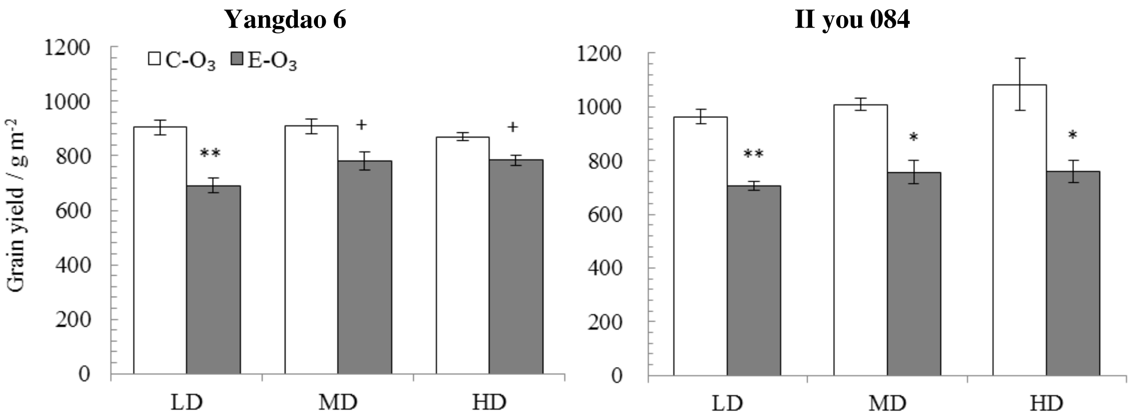

***Figure 6.** Effects of surface ozone concentration and plant density on grain yield of Yangdao 6 and Ⅱ you 084. ANOVA: Cultivar (P < 0.05); Density (p > 0.1); Ozone (P < 0.01); O_3 × Cultivar (P < 0.01); O_3 × Density (p > 0.1); Cultivar × Density (p > 0.1); O_3 × Cultivar × Density (p > 0.1)*

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The high concentration of O_3 had no significant effects on the spike number per unit area of Yangdao 6 and II you 084. But from the composition of the spike number, the elevated ozone concentration significantly decreased the maximum tiller number of rice by 8.5%, increased productive tiller ratio of rice by 6.6% (*$p < 0.05$*) (*Fig. 7*). O_3 stress had no significant effect on the spikelet number per panicle of Yangdao 6 but decreased it of II you 084 by 22. 0% (*$p < 0.01$*), with the significant decrease of 15.7%, 27.6% and 21.9% under low, medium and high density (*Table 3*). Analyzing from the composition of the spikelet number per panicle, the increase of O_3 concentration decreased the dry weight per stem at heading stage of Yangdao 6 by 11.1% (*$p < 0.05$*) but increased the ratio of spikelet number to stem dry weight of Yangdao 6 by 13.2 g (*$p < 0.05$*). High concentration of O_3 reduced the dry weight per stem at heading stage and the ratio of spikelet number to stem dry weight of II you 084 by 12.7% (*$p < 0.05$*) and 10.7% (*$p < 0.05$*) respectively (*Fig. 8*). Ozone stress reduced the spikelet number per unit area of Yangdao 6 by 4.8% (*$p > 0.05$*), and decreased it of II you 084 by 21.7% (*$p < 0.01$*) on average, among which 20.3%, 26.0% and 18.8% were significantly decreased respectively under low, medium and high density (*Table 3*). High concentration of O_3 reduced the filled grain percentage of Yangdao 6 and II you 084 by 6.4% (*$p < 0.05$*) and 11.9% (*$p < 0.01$*) respectively. The filled grain weight of Yangdao 6 and II you 084 were decreased by 2.3% (*$p < 0.05$*) and 4.7% (*$p < 0.01$*) respectively to ozone stress (*Table 3*).

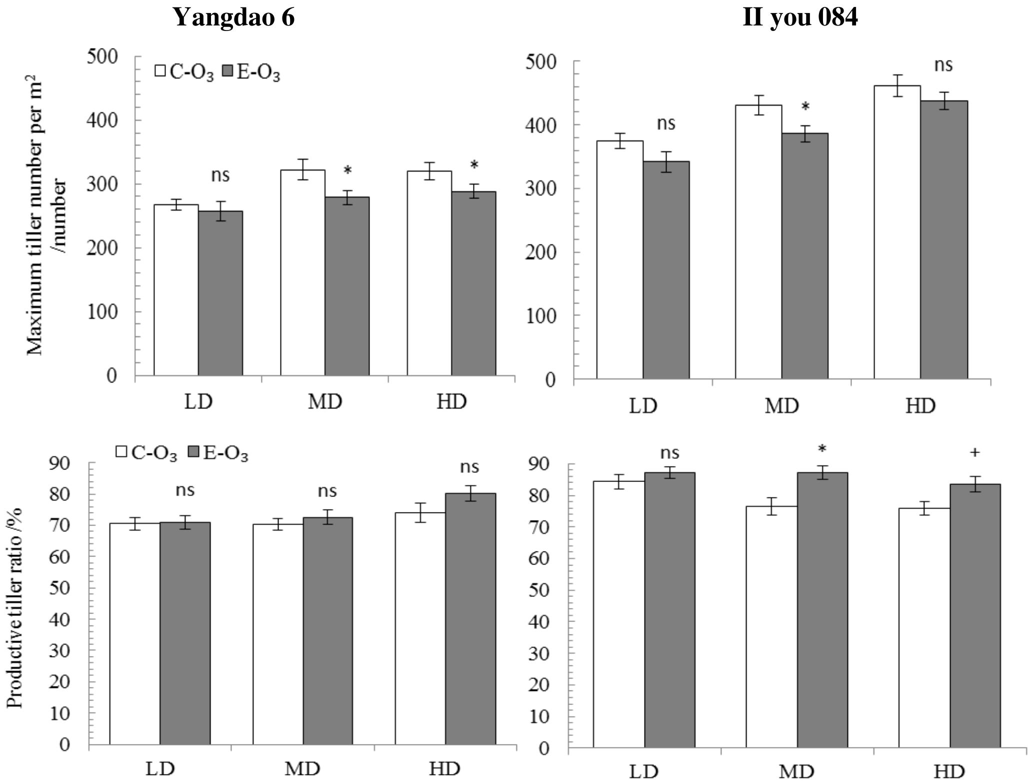

***Figure 7.** Effects of surface ozone concentration and plant density on maximum tiller number per square meter and productive tiller ratio of Yangdao 6 and Ⅱ-you 084. ANOVA (M means maximum tiller number per square meter, P means productive tiller ratio): M: Cultivar ($P < 0.01$); Density ($P < 0.01$); Ozone ($P < 0.01$); $O_3 \times C$ ($p > 0.1$); $O_3 \times D$ ($p > 0.1$); $C \times D$ ($p > 0.1$); $O_3 \times C \times D$ ($p > 0.1$). P: Cultivar ($P < 0.01$); Density ($p > 0.1$); Ozone ($P < 0.05$); $O_3 \times C$ ($p > 0.1$); $O_3 \times D$ ($p > 0.1$); $C \times D$ ($p > 0.1$); $O_3 \times C \times D$ ($p > 0.1$)*

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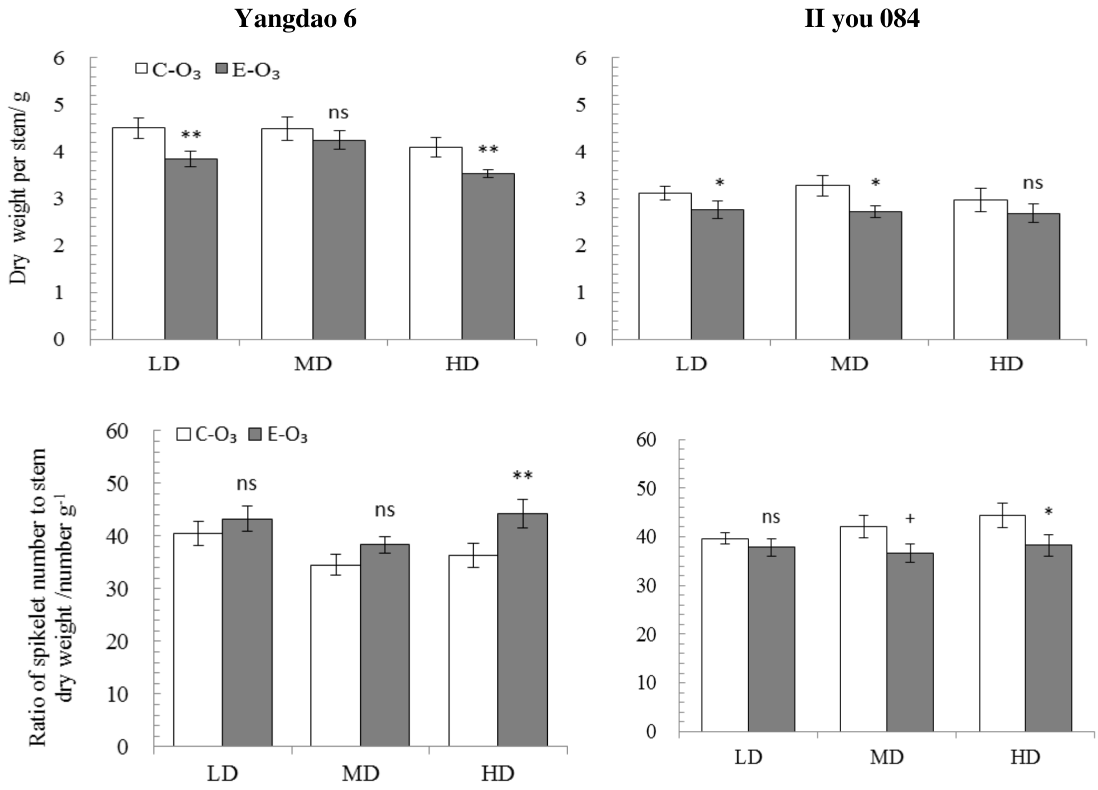

Figure 8. *Effects of surface ozone concentration and plant density on dry weight per stem at heading stage and ratio of spikelet number to stem dry weight of Yangdao 6 and Ⅱyou 084 under FACE condition. ANOVA (D means dry weight per stem at heading stage, R means ratio of spikelet number to stem dry weight): D: Cultivar ($P < 0.01$); Density ($P < 0.05$); Ozone ($P < 0.01$); $O_3 \times C$ ($p > 0.1$); $O_3 \times D$ ($p > 0.1$); $C \times D$ ($p > 0.1$); $O_3 \times C \times D$ ($p > 0.1$). R: Cultivar ($p > 0.1$); Density ($p > 0.1$); Ozone ($p > 0.1$); $O_3 \times C$ ($P < 0.05$); $O_3 \times D$ ($p > 0.1$); $C \times D$ ($p > 0.1$); $O_3 \times C \times D$ ($p > 0.1$)*

Discussion

Phenology and plant height

Previous studies in gas chambers showed that the phenology of rice was unchanged or shortened under ozone stress (Yang et al., 2008). Our study found that O_3 treatment, which is 25% higher than the ambient concentration, had no significant effects on phenology development of Yangdao 6, conventional rice. However, the heading date and maturity stage of super hybrid rice II you 084 were 3 days and 7 days earlier respectively under O_3 treatment (*Table 1; Fig. 2*). The effect on the super hybrid rice was similar to that of hybrid rice “Shanyou 63” under FACE condition (Shi et al., 2009; Wang et al., 2012). It was inferred that the effect of high O_3 concentration on phenology of hybrid rice or super hybrid rice in the future may be more significant than that of conventional rice. Many studies on plant height showed that the increase of ozone concentration generally reduced the plant height of rice, and the decline varied with varieties (Abhijit and Agrawal, 2012; Hiroko et al., 2012; Shi et al., 2009). This research showed that O_3 treatment had no significant effect on the plant height of Yangdao 6 but decreased the plant height of II you 084 by7.8%. From the dynamic response of high sensitive variety II you 084, the effect of ozone stress on the plant

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height gradually increased with the plant growth process (*Fig. 3*), showing a certain cumulative effect.

Table 3. *Effects of surface ozone concentration and plant density on yield components of rice varieties*

Cultivar	Density	O_3 treatment	Panicle number per m^2	Spikelet number per panicle	Spikelets per $m^2/\times10^4$	Filled grain percentage/%	Filled grain weight/mg
Yangdao 6	LD	C-O_3	188±7	182±3	3.4±0.1	84±2	34.5±0.1
		E-O_3	183±11	167±3	3.0±0.2	74±2	33.3±0.5
	MD	C-O_3	227±8	155±6	3.5±0.1	80±2	34.5±0.1
		E-O_3	203±5	163±6	3.3±0.1	80±2	33.7±0.3
	HD	C-O_3	236±6	149±4	3.5±0.1	81±1	34.6±0.1
		E-O_3	231±15	157±3	3.6±0.2	74±2	34.3±0.4
II you 084	LD	C-O_3	315±4	124±3	3.9±0.1	91±1	28.3±0.4
		E-O_3	298±5	104±2	3.1±0.1	81±3	26.9±0.1
	MD	C-O_3	330±5	138±3	4.5±0.1	93±1	28.0±0.3
		E-O_3	336±13	100±4	3.4±0.2	82±4	27.0±0.3
	HD	C-O_3	350±7	132±9	4.6±0.4	90±1	27.5±0.2
		E-O_3	366±7	103±5	3.8±0.1	78±5	26.1±0.4
ANOVA (*p* value)		O_3	0.385	**0.000**	**0.000**	**0.000**	**0.000**
		Cultivar	**0.000**	**0.000**	**0.000**	**0.001**	**0.000**
		Density	**0.000**	**0.028**	**0.005**	0.387	0.784
		$O_3\times D$	0.401	0.438	0.477	0.513	0.664
		$O_3\times C$	0.244	**0.000**	**0.005**	0.112	0.271
		$O_3\times D\times C$	0.282	**0.024**	0.567	0.437	0.599

The values in the table represent average values ± standard errors

Dry matter production and biomass

Previous studies showed that high concentration of ozone significantly inhibited the dry matter production of rice, but the degree of influence varied greatly with different treatment concentration, variety and environment (Kobayashi et al., 1995; Jin et al., 2001; Pang et al., 2009; Hiroko et al., 2012). This result showed that the increase of ozone concentration decreased the biomass of rice by 22%, in which Yangdao 6 and II you 084 were decreased by 17% and 25% respectively (*Fig. 4*). It showed that the loss of biomass of super hybrid rice may be higher than that of conventional rice under high ozone concentration in the future. According to different densities, the response of biomass of the two varieties to O_3 stress decreased with the increase of density, especially Yangdao 6. Our study also showed that high concentration of ozone had little effect on the dry matter production of rice in the early stage of growth, but significantly decreased it in the middle and late stages of rice (*Table 2*). This indicated that the decline of the final productivity of rice under ozone stress was mainly related to the inhibition of growth in the middle and later growth period, especially in the later stage.

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Under ozone stress, the decrease of dry matter production from heading to maturity stage of II you 084 (-47%) was significantly larger than that of Yangdao 6 (-19%) (*Table 2*), which obviously resulted in the decrease of the final biomass of II you 084 was greater than that of Yangdao 6. According to the difference of different densities, there was significant interaction between ozone and density on the dry matter production during the reproductive growth stage (jointing to maturity) of Yangdao 6. The response of dry matter production of Yangdao 6 to ozone stress decreased with the increase of density. The meta-analysis of Ainsworth (2008) on the gas chamber showed that the ozone concentration of 61 nl/L decreased the harvest index of rice by 5%, compared with the filtered air (n = 19). Our results showed that high O_3 concentration had no significant effect on the harvest index of Yangdao 6, significantly reduced it of II you 084 by 9% (*Fig. 5*), and the difference of response between two varieties reached a significant level.

Yield components

Previous studies on yield components of rice show that O_3 stress had no significant effect on panicle number per unit area (Ainsworth, 2008). There were also studies showing that the panicle number per unit area of rice decreased under ozone stress, which was due to the decrease of tiller capability (Walid et al., 1995) or the decrease of productive tiller percentage (Maggs and Ashmore, 1998). This study showed that high concentration ozone had no significant effect on panicle number per unit area of conventional indica rice Yangdao 6 and super hybrid rice II you 084. Analyzing the constituent factors of panicle number per unit area, the increase of O_3 concentration resulted in an average decrease of the maximum tiller number 9% and 7% increase in productive tiller percentage (*Fig. 7*). It indicated that the tiller capability was decreased, and the productive tiller percentage was increased under the ozone stress, which may be the main reason for the no significant change of panicle number per unit area. The majority of previous studies showed that the spikelet number per panicle of rice was decreased with ozone stress, and the decline was due to different ozone treatment (Kats et al., 1985; Jin et al., 2001; Feng et al., 2003; Bhatia et al., 2011) or different varieties (Shi et al., 2009; Wang et al., 2012; Hiroko et al., 2012). This study showed that high concentration ozone had no significant effect on the spikelet number per panicle of Yangdao 6, but significantly reduced it of II you 084 by 22% (*Table 3*). Ozone stress both decreased the dry weight per stem at heading stage and the ratio of spikelet number to stem dry weight of Yangdao 6, which resulted in a significant decrease in the spikelet number per panicle of Yangdao 6. Ozone stress significantly reduced the dry weight per stem at heading stage of II you 084, meanwhile, increased the ratio of spikelet number to stem dry weight. As a result, the spikelet number per panicle of II you 084 was not significantly changed. According to the differences among different densities, ozone multiplied by varieties and density also had significant interaction effects on the spikelet number per panicle of rice ($p = 0.02$). It showed that the effect of O_3 on the spikelet number per panicle of II you 084 had no significant differences between different densities. For Yangdao 6, the high concentration of O_3 decreased the spikelet number per panicle by 9% at low density, but increased slightly (+5%, $p > 0.05$).

The spikelet number per unit area of rice represents the yield capacity. The meta-analysis of Ainsworth (2008) on the gas chamber showed that the ozone concentration of 62 nl/L decreased the spikelet number per unit area of rice by 20%, compared with the filtered air (n = 15). This study showed that high concentration of O_3 significantly

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reduced the spikelet number per unit area of rice by 14%, among which 5% and 9.3% were decreased of Yangdao 6 and II you 084 respectively with significant differences among varieties. Combined with the change of the spikelet number per panicle, the effect of ozone stress on the spikelet number per unit area was mainly due to the influence of the spikelet number per panicle.

The filled grain percentage and filled grain weight reflected the grain filling ability of rice. Previous studies showed that high concentration of ozone reduced the filled grain percentage of rice. This study also showed that ozone stress decreased the filled grain percentage of Yangdao 6 and II you 084 by 6% and 12% respectively, and the effect on the II you 084 was significantly greater than that of Yang Rice No. 6. The meta-analysis of Ainsworth (2008) on the gas chamber showed that the ozone concentration of 83 nl/L decreased filled grain weight of rice by 5%, compared with the filtered air (n = 22). The meta-analysis of Feng (2009) showed that current ozone concentration (31-50 nl/L) decreased filled grain weight of rice by 3.1%, compared with the filtered air. Recent FACE experiments in China showed that the filled grain weight of rice decreased with the increase of ozone stress, and the decline was due to different varieties or years (Shi et al., 2009; Wang et al., 2012). The results of this study showed that O_3 stress reduced the filled grain weight of Yangdao 6 and II you 084 respectively 2% and 5%, and the effect of O_3 stress on the filled grain weight of Yangdao 6 decreased with the increasing density.

Grain yields

Previous studies had mostly shown that the increase of atmospheric ozone concentration significantly decreased the rice yield, and the decline was related to the control equipment of the experiment (Maggs et al., 1995), materials (Kobayashi et al., 1995; Maggs and Ashmore, 1998; Zheng et al., 1998; Jin et al., 2000), the concentration of O_3 treatment (Asakawa et al., 1981; Heagle et al., 1991; Bai et al., 2002) and the treatment period (Morikawa et al., 1980; Asakawa et al., 1981; Chen et al., 2007). The meta-analysis of Ainsworth (2008) on the gas chamber showed that the ozone concentration of 61 nl/L significantly decreased yield of rice by 14%, compared with the filtered air. Our results showed that the grain yield of rice was decreased 235 g/m^2 at high O_3 concentration, with the significant decrease of 25%, in which Yangdao 6 and II you 084 were decreased 16% and 27% respectively (*Fig. 6*), and the interaction between ozone and varieties reached a significant level. Combined with previous research results (Shi et al., 2009; Pang et al., 2009; Wang et al., 2012), it was inferred that compared with conventional rice, although hybrid rice and super hybrid rice have some yield advantages at present, the yield loss of them may be more significant in the future under the condition of high O_3 concentration. From the perspective of yield components, the effect of ozone stress on yield of Yangdao 6 was mainly related to the decrease of filled grain percentage, followed by the decrease of the filled grain weight. That was, ozone stress mainly affects the fertilization process and filling process of Yangdao 6. The main reason for the effect of ozone stress on the yield of II you 084 was the decrease of the spikelet number per area and filled grain percentage, then the filled grain weight. Ozone stress not only affected the spikelet formation of II you 084, but also affected the fertilization process and grain filling process, and the effect of the latter was significantly greater than that of Yangdao 6, so the loss of yield was more obvious. In terms of dry matter production and distribution, high concentration of ozone reduced the photosynthetic production of Yangdao 6 but had no significant effects on the proportion

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of photosynthesis to grain. Analyzing the effect on II you 084, on the one hand, ozone stress significantly decreased the biomass (the decrease was significantly higher than that of Yangdao 6), but also significantly reduced the distribution of dry matter in the grain, thus making the decline of grain yield of II you 084 more significant.

The results showed that there was a weak interaction effect among ozone, varieties and density on yield. There were no significant differences in the effect of O_3 stress on the yield of II you 084 among different densities. However, the yield-loss of Yangdao 6 to high ozone concentration decreased significantly with the increase of density (at low, medium and high density, the yield was decreased 24%, 14% and 10% respectively). According to the factors of yield composition, the effect of ozone on the spikelet number per panicle, yield capacity and filled grain weight of Yangdao 6 decreased with the increase of density. However, the effects of ozone stress on the yield components of II you 084 had no significant differences among different densities. Analyzing from dry matter production, effects of ozone on dry matter production at reproductive stage of Yangdao 6 decreased with the increase of density, and the effects on biomass and yield showed the same trend among different densities. Effects of ozone stress on II you 084 had no significant difference among different densities.

Conclusion

The results showed that the loss of rice yield under the condition of high O_3 concentration was caused by the decline of grain filling ability (conventional rice) or both the decrease of yield storage capacities and grain filling ability (super hybrid rice). The ozone stress effect of hybrid rice varieties was significantly higher than that of conventional rice varieties. Secondly, proper increase of planting density can alleviate the ozone stress effect of conventional rice varieties to some extent. Therefore, under the background of high ozone concentration in the future, we can first select high-yielding conventional rice varieties with strong ozone resistance by properly increasing planting density, thus slowing down the production loss to a certain extent. Limited to the conditions, only two varieties and three densities were studied in our research. Further researches on the interaction of ozone with more varieties and more cultivation measures should be further expanded in the future.

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EMISSIONS OF SOME GREENHOUSE GASES FROM THE MANURE OF EWES FED ON POMEGRANATE PEEL, YUCCA EXTRACT, AND THYME OIL

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Abstract. Sixteen dairy ewes 1 to 2 months pregnant and weighing 50.4 ± 1.02 kg on average were randomly assigned to one of the four treatments, four animals to each treatment: 1) control, 2) yucca saponin (YE, 0.1%), 3) pomegranate peel (PP, 15%), or 4) thyme oil (TO, 0.1%). Total gas production from the fresh manure was measured and the contents of CH_4, N_2O, and CO_2 were determined. Digestibility in vivo and nitrogen balance were also determined. Feed intake and body weight were not affected whereas some properties of the manure (dry matter, total N, ADF, and NDF but neither ash nor pH) were affected by the composition of the feed. Total manure production and the emissions of CH_4 and CO_2 expressed as parts per million per millilitre of manure were similar in all the four treatments whereas the content of N_2O was higher in the control. Supplementing the rations with tannin, saponin, or thyme oil reduced N_2O emissions from the manure.
Keywords: *methane, carbon dioxide, nitrous oxide, livestock wastes, food by-products*

Introduction

Livestock waste is an important source of greenhouse gases (GHG). Manure contributes directly to emissions of nitrous oxide (N_2O) – which has a global warming potential 298 times that of CO_2 (Mosier et al., 1998) – by stimulating nitrification and denitrification (Tang et al., 2016). Methane (CH_4) is also emitted during anaerobic fermentation of organic matter, especially during the storage of manure. The amount of GHG released from manure depends on the nitrogen (N) and carbon (C) content of the manure, the duration over which it is stored, the animal, and the diet. Increasing concern about mitigating the adverse effects of climate change has led to greater interest in reducing the emissions of GHG from all sources including manure from ruminants.

Pomegranate is grown on a large scale (about a million trees) in south-eastern Turkey, especially in Şanlıurfa province, and its productivity has remained high over several decades (TUIK, 2014). Local production and consumption of pomegranate have greatly increased in recent years (8000 tonnes a year) which, in turn, have led to greater quantities of pomegranate peel (PP) and seeds. The peel is a waste product of processing the fruit for juice for human consumption. Pomegranate is reported to contain up to 48% peel (Zarei et al., 2011), which is rich in hydrolysable tannins and saponin. Ullah et al. (2012) estimated the crude fibre content of PP at 21% ± 0.6%, and Feizi et al. (2005) estimated its crude protein content at 11.4%. Pomegranate peel containing tannins and saponin may improve N utilization efficiency and thereby

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decrease the N content of manure, which, in turn, may affect N_2O emissions because less N is available to the denitrifying bacteria that use the manure as substrate. The addition of saponins from PP can thus modify the C and N contents of sheep manure.

Another recent trend has been to use the oil from aromatic plants directly instead of extracting essential oils from such plants, because extraction is difficult and not particularly efficient. Many plant essential oils stimulate rumen fermentation at high concentrations. Thyme (*Thymus vulgaris*) oil is an antioxidant (Seung-Joo et al., 2005) and contains about 15% essential oil (soluble in alcohol or steam). However, in vivo investigation is necessary to determine whether these oils can be used successfully to inhibit rumen methanogens with lasting effects on emissions from manure without any adverse effects on digestion.

Another possible candidate to control the production of GHG from manure is the extract of yucca, which contains two active components: a glyco component that binds ammonia and a fraction of steroidal saponins, which has the properties of a surfactant. Saponins also seem to have shown some potential in reducing enteric CH_4 production (Pal et al., 2015). However, the effects of tannins from PP, thyme oil (TO), and yucca extract (YE) on the production of GHG (CH_4, CO_2, and N_2O) from manure and the overall nutritional effects of PP, TO, and YE are unknown—which is why the present study sought to observe the effects of PP, YE, and TO on the emissions of the above three GHG from sheep manure under routine farming practices such as open-air stool administration.

Material and methods

Animals and experimental groups

Research on animals was conducted according to institutional committee on animal use (ethical document number: KSU-2013/08-3). The experiments took place between 2 and 10 April 2014 at the Sanliurfa province in the Southeast Anatolian Region of Turkey, which lies on 37°9’32.9364 latitude and 38°47’48.8724 longitude. The mean air temperature was 17 °C and the maximum was 27 °C. As a source of tannins, sun-dried PP was collected from pomegranate-processing plants in Şanliurfa City and ground fine enough to pass through a 3 to 5 cm sieve. The other source of forage was lucerne hay cut into 3 to 5 cm long pieces.

A total of 16 adult fat-tailed Awassi ewes 1 to 2 months pregnant, 2 to 3 years old, and weighing 50.4 kg ± 1.02 kg on average were randomly assigned to one of the four treatments (4 ewes in each treatment serving as 4 replicates). Each ewe was kept in a 1.5 m × 1.5 m pen. The sheep were housed in individual cages and had free access to fresh water throughout the study. They were fed separately *ad libitum* with a total mixed ration (TMR) containing a mixture of a concentrate and lucerne hay. The four treatments were as follows: 1-) control, (only the basal diet); 2-) YE, (the basal diet supplemented with YE at the rate of 1.5 kg of YE per tonne of the ratio; 3-) PP, or 15% of ratio (that is, 150 kg of the mix per tonne of the ratio; or a mix of 38% PP and 62% lucerne hay as forage sources, w/w); and 4-) TO, (the basal diet supplemented with TO at the rate of 1 liter per ton of the ratio). While determining the amounts to be included in the ration, PP was used as feed material and TO and YE were considered as extract. The amount of PP was designed to not exceed 20% in the direction of other studies (Shabtay et al., 2008). The amounts of TO and YE were determined based on the manufacturer's recommendation.

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The diets were so designed that all the four treatments were equal in terms of the number of calories and the amount of N over the duration of the experiment, and were also comparable in terms of the proportions of macronutrients and energy:proteins balance (*Table 1*). All rations were set to be a standard milk ration as recommended by the US National Research Council (NRC, 2007).

The experiment, including a 10 day adaptation period, lasted for 17 days. Each ewe was weighed at the beginning and at the end of the adaptation period. Feed intake was monitored over this period. Feed intake and feed refused (amount of feed left untouched) were recorded before the morning feed and weighed after one week during the adaptation period, the total feed intake being the difference between the total amounts of feed offered and refused.

Table 1. *The composition of the experimental diets (g/kg of feed)*

	Basal diet*	**YE**	**PP**	**TO**
Barley	10.0	10.0	10.0	10.0
Corn	493.3	493.3	428.4	493.3
Wheat bran	10.0	9.3	10.0	10.0
Cotton seed meal	117.0	116.2	149.2	117.0
Lucerne hay	357.9	357.9	**237.6**	357.7
Plant oil	10.8	10.8	13.8	10.0
Vitamins-minerals supplement**	1.0	1.0	1.0	1.0
Pomegranate peel	0.0	0.0	**150**	0.0
Thyme oil***	0.0	0.0	0.0	**1.0**
Yucca extract	0.0	**1.5**	0.0	0.0
DM, [%]	89.0	88.8	89.0	88.9
CP, [%]	15.6	15.6	15.5	15.6
ME, [Mcal/kg]	2.7	2.7	2.7	2.7
Ca, [%]	0.5	0.5	0.4	0.5
P, [%]	0.4	0.4	0.4	0.4
ADF, [%]	16.6	16.6	18.1	16.6
NDF, [%]	24.9	24.9	25.2	24.9
Ash, [%]	4.1	4.3	4.4	4.3
Condense Tannin-(CT), [mg//kg]	36.9	36.8	2130.5	36.9
Saponin, [mg/kg]	546.3	558.3	1656.1	546.0

*Calculation based on NRC (2007). **Each kilogram of vitamin–mineral premix provides vitamin A, 800 000 IU; vitamin D_3, 100 000 IU; vitamin E, 3000 mg; Mn, 5000 mg; Fe, 5000 mg; Cu, 1000 mg; Co, 150 mg; I, 800 mg; and Se, 150 mg. ***just oil, not extract, DM: dry matter, CP: crude protein, Me: metabolic energy, P: phosphorus, ADF: acid detergent fibre, NDF: neutral detergent fibre

Sampling of manure and gases

No bedding was used in the experiment; therefore, the manure contained nothing other than feces and urine. Samples of manure for analyzing the gases were collected after leaving the sheep one day on sheets of canvas. During the collection period, urine was not separated from feces, and total manure produced by individual animals was weighed after collecting it from the canvas sheets on which the ewe stood. The manure from each ewe was sampled three times in a day through the 3-day collection periods at

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the beginning, middle and end of the data collection period and fresh weights (kilograms per day) and pH were recorded immediately after collection.

To measure total gas production, specially designed glass bottles (250 ml each) were connected to the sampling device in such a way that its neck was joined to the side of the bottle. The joints were sealed with parafilm. Representative samples of manure (100 g or 150 g) from each animal were placed in these bottles, which were stored in a dryer maintained at 35 to 40 °C for 1 h. Total gas emitted from the feces was tried to be measured with by gas pressure. For this reason, we had left a certain volume empty (50 ml) in bottle for measuring pressure volume dependent. The gas from the 150 grams of fecal matter placed in the bottles was determined and recalculated in proportion to the daily total feces. Total gas from the manure produced in 1 h was measured in grams and estimated from the barometric pressure using *Equation 1* (Petrucci et al., 2010):

$$\left[m = \frac{PV}{RT} \right] \tag{Eq.1}$$

where m is the total gas produced (g), P is the atmospheric pressure (Pa), V is the volume (50 ml or cm^3), R is the national gas constant value, and T is the temperature (°K). However, after sampling the gases, oxygen was allowed to enter the bottles by opening them and the sampling process was repeated several times so as to represent normal conditions (open-air stool administration). The manure samples were kept in sealed bottles and the gases were sampled three times a day by drawing the gas sample through a sealed syringe: the amounts of CH_4, N_2O, and CO_2 in these samples were measured later with gas chromatography. (SRI Instruments-European Greenhouse Gas Chromatograph (GC) System ®). Samples were introduced to the injection port via plastic syringes. 3 m Hayesep D packed column was used for CO_2 and CH_4 analysis and 3m Hayesep D column was used for N_2O analysis. Operating conditions for the GC were as follows: injector temperature 95 °C, column temperature 85 °C, and detector temperature 320 to 350 °C for GC.

Feces and feed analysis

After drying, the manure samples were analyzed following the standard AOAC procedures (AOAC, 1998), and NDF and ADF were analyzed using an ANKOM fibre analyzer (ANKOM Technologies, Macedon, NY, USA) using the methods of Van Soest et al. (1991). The samples were dried in an oven for dry matter analysis and then ashed.

The same methods were used for feed analysis. Additionally, ground pomegranate peels was treated with a condensed tannin (CT) solution (95 ml n-butanol, 5 ml HCl (35%), 0.05 g Fe_2SO_4) and the amount of CT was estimated by taking the absorbance values in the spectrophotometer (Makkar et al., 1995).

Apparent digestibility in vivo

Total manure produced by each animal was collected every day at 08:30 and urine was separated from the samples of manure collected for gas measurements by filtration. As the studies were done in female sheep, it was difficult to separate stools and urine. Because the feces of the sheep were in a pelletized structure, the urine was very small and the effect of urine was very insignificant.

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The digestibility was calculated as follows: digestibility (%) = (A−B) / A, where A is the organic matter or N content of the feed (the input) and B is the organic matter or N content of the faeces (the output). The data were analysed following the completely randomized design with one-way analysis of variance using the generalized linear model (GLM) (SPSS, 2013). The only factor was the difference in the rations. The treatments means were compared using the Tukey's-b multiple range test (TMRT).

Results

Compared to the control, none of the other three treatment groups showed any significant differences ($p > 0.05$) in feed intake, change in live weight, or feed conversion ratio at the end of the trial (*Table 2*).

Table 2. *Effect of diet on some performance parameters of Awassi ewes*

Parameter	Control	YE	PP	TO	SEM	P≤
Starting weight, [kg]	51.50	49.60	50.90	49.50	2.55	0.92
Feed intake, [kg/day]*	2.28	2.34	2.37	2.41	0.08	0.81
LWC, [kg]	−1.00	−0.87	−1.75	0.50	1.68	0.83
DLWC, [kg/day]	−0.14	−0.12	−0.25	0.07	0.24	0.83
FCR	3.36	2.94	1.95	8.70	5.00	0.82

*Fresh weight. PP: pomegranate peel, SEM: standard error of mean, LWC: overall live weight change, DLWC: daily live weight change, FCR: feed conversion ratio

The manure, however, showed significant differences between the treatments in terms of dry matter, nitrogen, ADF, and NDF (*Table 3*). Especially in terms of N content, the three treatment groups were significantly different from the control, the PP group recording the lowest values of N, followed by the yucca group.

Table 3. *Properties of manure as affected by diet*

Property	Control	YE	PP	TO	SEM	P≤
DM, [%]	51.1^{a}	58.2^{a}	52.8^{a}	70.9^{b}	2.56	0.01
N, [%]	1.9^{c}	0.8^{b}	0.4^{a}	2.2^{d}	0.04	0.01
Ash, [%]	13.8	13.1	13.1	14.5	1.30	0.84
pH	8.83	9.2	8.1	8.9	0.29	0.28
ADF, [%]	46.2^{a}	49.9^{ab}	51.8^{ab}	54.1^{b}	1.28	0.01
NDF, [%]	61.2^{ab}	58.9^{ab}	55.7^{a}	64.1^{b}	1.35	0.01
Nutrient intake and output						
E. Intake [Mcal/day]*	5.2	5.5	5.4	5.2	0.07	0.37
N intake [g/day]	48.0	51.3	49.5	46.8	0.79	0.20
N output g/day/manure	23.9^{c}	15.8^{b}	5.8^{a}	13.9^{b}	1.9	0.01
Digestibility in vivo						
N, [%]	0.49^{a}	0.75^{b}	0.83^{b}	0.72^{b}	0.04	0.01
OM, [%]	0.80^{b}	0.63^{a}	0.77^{b}	0.81^{b}	0.03	0.02

*Metabolic energy intake (Mcal/day). OM: organic matter, ADF: acid detergent fibre, NDF: neutral detergent fibre, PP: pomegranate peel, SEM: standard error of mean. Different letters in the same row show significant differences ($P \leq 0.05$) between treatments

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Adding YE, TO, or PP to the diets reduced the N content of the manure compared to that from the control, pointing to the higher in vivo N digestibility (*Table 3*). In other words, N excretion in the manure was significantly ($p < 0.05$) higher in the control. The daily average N excretion in the total manure was 15.8 g with YE, 5.8 g with PP, and 13.9 g with TO (*Table 3*). All the three additives improved in vivo digestibility of N significantly, which is consistent with the above figures. However, YE decreased the in vivo digestibility of organic matter (OM) significantly ($p < 0.05$; *Table 3*). The digestibility of N was maximum in TO, although the manure of ewes fed with TO was rich in N.

Finally, neither daily gas production nor CH_4 and CO_2 content per milliliter varied between the four groups. Overall, YE in the diet led to greater quantities of manure and lower emissions of N_2O than those in the control (*Table 4*).

Table 4. *Gaseous emissions from manure*

Parameter	Control	YE	PP	TO	SEM	P≤
Daily manure production [g]*	644.6^{ab}	1125.0^{b}	765.0^{ab}	448.8^{a}	91.7	**0.01**
Total gas, [g/100 g of manure per hour]	0.7	0.9	0.5	0.9	0.2	0.82
Daily total gas, [g]	106.9	113.0	115.0	91.8	23.6	0.89
CH_4, [ppm/ml]**	1.8	1.8	2.0	2.8	0.4	0.45
CO_2, [ppm/ml]	1737.3	1572.9	3183.3	2342.1	762.7	0.46
N_2O, [ppm/ml]	20.85^{b}	5.04^{a}	10.76^{ab}	8.86^{ab}	3.69	**0.04**
CH_4, [mg/h/manure]***	0.004	0.008	0.0007	0.0005	0.00008	0.52
CO_2, [mg/h/manure]	1.04	0.98	1.76	0.93	0.34	0.84
N_2O, [mg/h/manure]	0.009^{b}	0.003^{ab}	0.001^{a}	0.002^{ab}	0.001	**0.06**

*Fresh weight, **from milliliter of gas sampled through a syringe, ***daily total manure (kg). PP: pomegranate peel; SEM: standard error of mean. Different letters in the same row show significant differences ($P \leq 0.05$) between treatments

Discussion

Manure production and feed intake are often related. Manure output usually increases as the concentration of dietary fiber increases. However, the content of dietary fiber in all the diets was broadly similar although slightly greater in YE and PP (*Table 2*), and the higher manure production can be generally attributed to the high ADF content of the manure (*Table 3*) and low digestibility of ADF in YE and PP. The increased fiber content of the manure in YE and PP was probably due to the higher fiber content of these two treatments (*Table 3*). Weiss and St. Pierre (2010) suggested that diets with a high concentration of by-products are usually less digestible than typical forage, and that cows often consume more dry matter when their diets are rich in the by-products, which means that the manure output is also greater. In PP, ADF content was 51.8% and NDF content was 55.7%; as a result of such high content, feed supplemented with PP increased the total manure production compared to that in the control group in the current study. High manure production by ewes fed with YE was probably due to the unknown response of sheep to YE or, more specifically, to the steroidal saponin in YE, but Li and Power (2015) found no correlation between yucca saponin and manure output. Although feed intake was not affected by the treatments ($p > 0.05$), it was higher in PP and YE than that in the control and TO (*Table 2*).

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In the present study, greater production of manure may also have been associated with a very low rate of digestion of dry matter as a result of the increased tannin or saponin content in PP and YE (*Table 3*)—it is well known that ADF concentration is negatively correlated to digestibility (Niyigena et al., 2016).

Ewes in the control, YE, and PP groups lost weight towards the end of the experiment but no valid inference can be drawn in this case because the differences between the all groups were not statistically significant: perhaps sheep in all groups lost weight simply because they were older (2 to 3 years old). Transient diarrheal events in PP groups and in some other groups may have also caused this.

Our hypothesis was that GHG production can be lowered by additives to the feed, and our results point to greater loss of N from the manure of ewes in the control and TO (*Table 3*) and significantly decreased N_2O emissions from the manure in all the three additives. We speculate that the lower emissions were due to the lower N content of the manure as a result of greater N binding and inhibition of N transformation.

Yucca extract decreased the digestibility of OM in vivo significantly ($p < 0.05$; *Table 3*). These differences between the groups seem to be related to the diet or to the effect of saponin, depending on the source (YE or PP). For example, Lu and Jorgensen (1987) reported increased digestibility of OM and cellulose in sheep fed with lucerne saponins. However, in the present experiment, PP, YE, and TO as dietary supplements increased the digestibility of N (*Table 3*) and may have been increased its availability. This resulted in lower production of N_2O and lower overall emissions (*Table 4*). Earlier research has shown that saponin-rich feed supplements reduce the population of ciliate protozoa in vitro and in vivo (Makkar et al., 1998; Valdez et al., 1986; Cieslak et al., 2013).

A decrease in the ruminal population of protozoa may increase the flow of microbial N from rumen and improve N utilization efficiency, which was reflected in the greater digestibility of N and lower production of N_2O (*Table 3*). According to Makkar et al. (1998), this may also be due to lower protein degradation with tannins or from ammonia binding of *Yucca schidigera*. The digestibility of N was also higher in TO, although the manure too was richer in N, probably because of decreased total manure production in this group.

None of the additives (PP, YE, or TO) had any impact on total gas production or on the production of CH_4 or CO_2, but all the three had an impact on manure production, DM, ADF, NDF, and pH when compared with the control group (*Tables 3* and *4*). After defecation, the emissions are dominated by N_2O and CO_2, probably because of the oxygenated environment. Wulf et al. (2002) suggest that after field application of organic manure, GHG emissions are dominated by N_2O and NH_3, and CH_4 is only of minor importance. On the other hand, when manure is stored, CH_4 emissions may be considerably greater because OM is degraded to CH_4 during anaerobic fermentation. The present experiment offered no anaerobic environment, and the oxygen-rich environment under which the manure was stored in the present study showed that emissions of CH_4 and of CO_2 from manure may be independent of the rumen environment and that the methane-producing bacteria may be inhibited by the oxygen-rich environment. Johnson et al. (2000) suggested that CH_4 emissions from the excreta of grazing cattle amount to only 1% of the CH_4 emissions from anaerobic conditions. Therefore, in ruminant production systems, CH_4 emissions from excreta are often ignored. Emissions of CO_2 from manure depend on storage conditions and were the highest from the mouldy manure towards the later stages of the present experiment.

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Although only a few studies have been conducted so far on CH_4 emissions from manure outdoors, our results reinforce the view that such manure is not a major source of methane and poses no risk to the environment.

Emissions of nitrogenous gases have also been linked to the pH of the manure. For example, Zaman et al. (2009) suggested that an increase in pH and NH_4 concentration could trigger NH_3 emissions during storage and after field application. Our results show a strong influence of PP on N_2O emissions: the lowest emissions of N_2O were seen in that treatment. The lower emissions from PP and YE were probably due to the lower contents of mineral N in the manure (*Table 4*). The concentration of steroidal saponins was 4.4% in YE (Wina et al., 2005). Generally, saponins have been found to have no effect on dry matter consumption (Valdez et al., 1986). However, saponins in YE effectively suppressed ruminal protozoa by interacting with cholesterol in the cell membrane (Wina et al., 2005). A decrease in protozoa may have increased the rate of fermentation of high-forage diets by increasing the populations of bacteria and fungi in the rumen. In ruminants, YE is believed to decrease ammonia in the rumen (Sheng Sun et al., 2017), and a reduction in rumen protozoa may have a positive effect, including N conservation, by altering the rumen flora in favour of bacteria and fungi. Makkar et al. (1999) also demonstrated such conservation of N in the rumen. These mechanisms may have contributed to the difference in the N content of the manure between YE and the control and the lower N_2O emissions in YE. The higher N_2O emissions from the control were probably due to the higher output of mineral N (*Table 3*). Conversely, the high N content but low emissions from TO may be due to the higher concentration of oil in that ration, leading to the N being fixed and thus less amenable to digestion. The lower moisture content of the manure in the TO group may also be responsible for the lower emissions of N_2O from that group (*Table 3*).

Although tannin levels had no effect on the emissions of CH_4 from the manure, PP, which is rich in tannins, decreased the amount of N lost through emissions compared to that in the control. Nauman et al. (2017) suggest that moderate levels of condensed tannins in the rumen protect dietary proteins from degradation by microorganisms, thereby increasing protein flow through the intestines.

The contents of crude protein and the amount of ammonia released from manure are interrelated. However, tannins have been shown to make plant proteins less degradable by binding with them (Nauman et al., 2017). It is well known that PP contains large amounts of tannin; these tannins, by binding to the proteins, slow down proteolysis and thus the degradation of proteins (Garipoğlu and Uçar, 2015). Fayed et al. (2012) reported similar observations for PP and found that rabbit feed that contained PP was low in digestible nutrients (crude protein and ether extract) than that without PP. The present study shows that additives such as PP and YE are an effective component of the strategy for reducing N_2O emissions from ruminants given protein-rich diets (as starter diets and diets aimed at faster growth).

Under experimental conditions, cases of diarrhea were considerably fewer in ewes fed with PP than those in the other groups. In rats suffering from diarrhea, feeding with extracts of pomegranate seeds reduced fluid accumulation in the intestines by decreasing the quantity of faeces and gastrointestinal motility (Aghsaghali et al., 2011), both of which may have contributed to lowering N_2O emissions. However, on the basis of daily manure production, N_2O emissions per animal were lower in all the three groups compared to those in the control group. In the current study, gas production was expressed as parts per million (in milligrams) of total faecal matter (manure) per hour.

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The production was then apportioned into that of specific gases (CH_4, CO_2, and N_2O) and expressed in the same units but per animal. Both daily manure output and total gas production increased, especially in YE, but the production of nitrogenous gases also decreased markedly in YE ($p < 0.05$). The other two additives (PP and TO) also had significant effects on N_2O emissions but the control group did not (*Fig. 1*), and neither YE nor TO had any effect on daily emissions of CO_2 and CH_4 (*Table 4*).

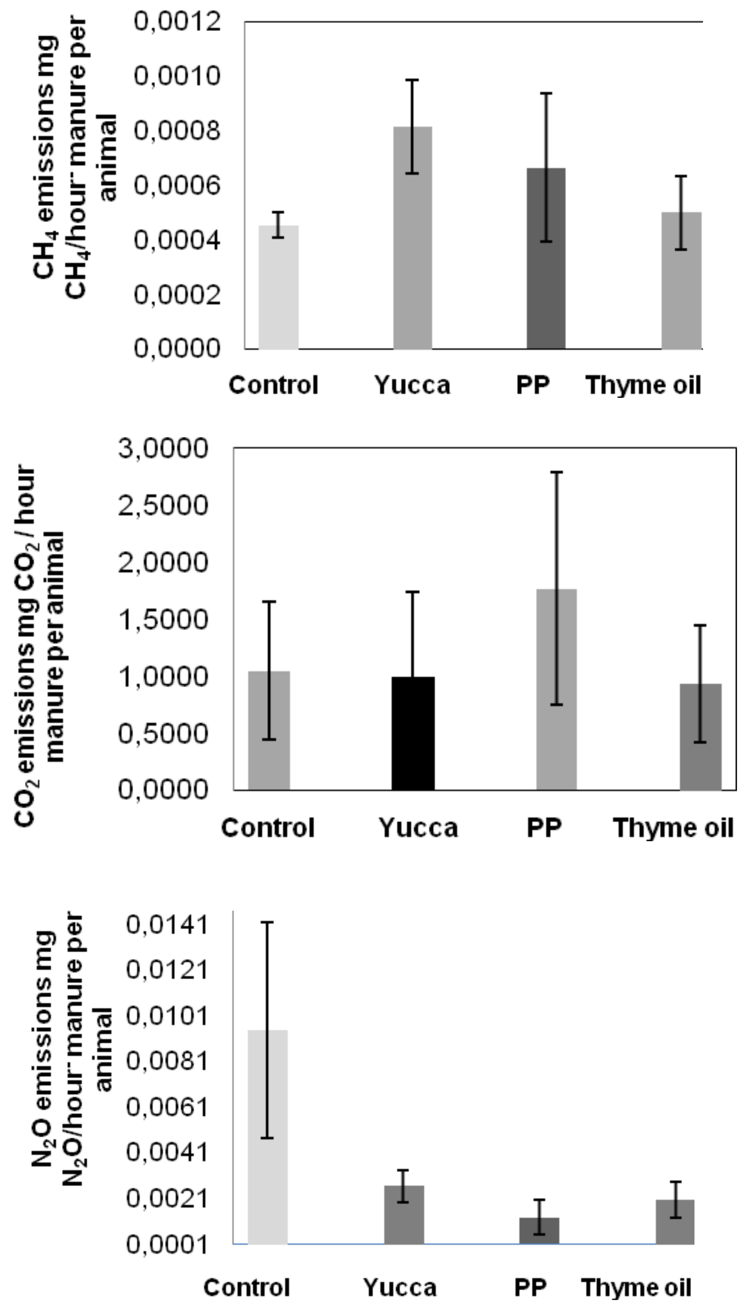

Figure 1. *Emissions of greenhouse gases from manure per hour as affected by diet. The four groups did not differ significantly in terms of CH_4 and CO_2 emissions but differed relatively in terms of N_2O emissions*

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Thus, some by-products of the food industry have a potential as animal feedstuff that can reduce N_2O emissions. Reducing the emissions of GHG from ruminants can put such by-products to better use and help in mitigating the adverse impacts of climate change at the same time. On the other hand, PP is widely used in folk medicine for the treatment of a variety of diseases including diarrhoea, and no metabolic disorders were observed in ewes when hay was mixed with 15% PP (*Table 1*). Therefore, lucerne hay can be safely mixed with 15% dried PP; the effect of increasing this proportion should be assessed through further experiments. We advise that the feces produced in farm should be urgently evaluated as biogas or fertilizer. Otherwise it would be an important source of N_2O and CO_2 gases particularly but not CH_4 due to the oxygenated conditions.

Conclusion

We found that the use of CT-containing PP as 38% of the Lucerne hay as roughage feed was a good choice for sheep production. Supplementing the rations with tannin, saponin, or thyme oil reduced N_2O emissions from the manure. PP, YE or TO were good choices to reduce fecal emission of nitrogen-based gases. But they did not effectively reduce the production of enteric CH_4 in the rumen and energy loss through CH_4 for the Lucerne hay-based.

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PATTERNS OF FIREWOOD USE AMONG ETHNIC MINORITY COMMUNITIES AND LOCAL FOREST MANAGEMENT: A CASE STUDY IN PU HU NATURE RESERVE, VIETNAM

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Abstract. In Vietnam as in other developing countries, firewood, including biomass is very important in rural areas as the main energy source and a part of the subsistence livelihood for ethnic minority communities. Rangers still carry out patrol activity for reducing the firewood collection inside nature reserve areas. However, before this study, little research had been conducted in the buffer zone of Pu Hu nature reserve (NR). The study was conducted to obtain an understanding of household firewood used, and the factors which influence firewood consumption. The methodology included questionnaires, observing household practices, and in-depth interviews with village leaders and local rangers with patrol activity currency. A survey of 248 households was conducted in five villages including households from all three ethnic minorities living in the area, who were found to require firewood as a primary resource. Firewood consumption was greatest among the Thai minority families (187.62 kg/household/month), followed by the Muong and Mong minorities (112.61 and 101.96 kg/household/month, respectively). Economic development was the most important factor in the demand for firewood because of consumption for household businesses. An important finding from this research was that the respondents indicated that rangers did not patrol frequently nor did they strictly enforce the regulations prohibiting the collection of firewood. A well-managed firewood resource could support minority households' requirements, improve fire-efficiency, and contribute to natural reserve objectives through ranger's efforts.
Keywords: *firewood consumption, ethnic minority, household, patrol, rangers, reserve*

Introduction

Since the time a human lit the very first fire, biomass resources have contributed to providing energy for millions of people (Kimemia and Annegarn, 2011). Particularly, in developing countries, forest resources are intensively used by remote communities, even inside nature reserves, and play important roles in their livelihoods (Kumar and Shahabuddin, 2005; Kim et al., 2016). Forest extraction, including timber, hunting, firewood, and grazing, either for direct use or business, has been regarded as a critical issue confronted by management in protecting forest resources (Hegde and Enters, 2000). Sources of firewood are one of society's oldest requirements (Lindroos, 2011), and a vital source of household energy in rural areas essential in the preparation of many staple foods (Biran et al., 2004; Mon et al., 2012). The reality of demands for

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traditional biomass in the form of firewood from forest resources is and will continue to be crucial for energy management and for ethnic people (Ektvedt, 2011; Maes and Verbist, 2012) and pressure on forests due to the continuous loss of biomass will also continue (Chettri et al., 2002).

Forests are one of the most important sources of firewood (Sassen et al., 2015) and are the main and the most frequently used energy source in remote areas in developing and poor countries (Kim et al., 2016; Reyes et al., 2018). Nearly 3 billion people continue to use fuelwood in their daily lives (He et al., 2009) as the first choice of energy for cooking and heating rooms (Felix, 2015) and it is also used for heating water or conducting traditional businesses within households (Nyambane et al., 2014; Wang et al., 2015; Schueftan et al., 2016). However, the distribution of forest resources for traditional use as fuelwood is increasingly affecting biodiversity and decreasing forest cover (Maes and Verbist, 2012). Sassen et al. (2015) found that the amount of dead wood or resident natural forest was affected by fuelwood collection for up to 1000 m inside the boundary of parks in upland Uganda and local villagers who live near to protected tropical forests particularly depend on those forests as their primary source of firewood.

Similarly, in Vietnam, most households depend on firewood to satisfy their demand for energy. Firewood is collected from farms, community and private forests, and private plantations as either live or dead wood (Nyambane et al., 2014) where tree by-products such as pruned branches from bamboo plantations, agricultural waste, and dry plantation wood are collected (Nyambane et al., 2014). Firewood, in Vietnam and other countries is considered as being a free commodity, which may be collected without cost other than that associated with the gatherer's own personal efforts (Kim et al., 2016). In the buffer zone of Pu Hu nature reserve (NR), the collection of firewood is strictly regulated through restrictions on wood-cutting (Nyambane et al., 2014). The scale of the households' requirement for and use of firewood is a critical issue that confronts forest management (Wang et al., 2015). This is particularly critical in mountainous regions, where limited energy sources lead to high pressure on scarce natural resources (Rantala et al., 2004; Mislimshoeva et al., 2014).

People's requirement for firewood as a basic source of energy is causing serious deforestation problems in various developing countries (Fox, 1984). The increased demand for firewood threatens the habitat of many wildlife species (Carey and Gill, 1980) in protected areas, and there is a need for an improved understanding of the use of the resource to enable conservation strategies to be better adapted to local livelihoods (Abbot and Mace, 1999). Ormsby and Kaplin (2005) found that even though residents were mostly aware of a NR's existence they were unfamiliar with its goals resulting in a problematic relationship between NRs and human communities {Formatting Citation}. This means that even with increased time devoted to patrolling or more severe penalties, law enforcement practice alone is unlikely to safeguard protected areas because there are no alternative supplies of firewood which can be used by local people (Abbot and Mace, 1999).

There have been few studies of firewood consumption conducted in Vietnam. The FAO (2017) reported that the firewood and energy sectors were estimated to have extracted around 5 million tons of fuelwood in 2016 consisting of charcoal, chips, residues, and pellets. The daily firewood demand for traditional pig production in Hue province has also been estimated to lie somewhere between 6.1 and 21.3 kg (Lan et al., 2002; Kien and Harwood, 2017).

Wasted agricultural residues are a very abundant source of biomass (Tung, 2009) and are traditionally burned as firewood as a source of energy in Vietnam (Khoa et al., 1999). Nonetheless, available data on firewood consumption in different areas in Vietnam is lacking (Kim et al., 2016), and there has been relatively little research attention devoted to the assessment of firewood use for daily needs in the buffer zone of Pu Hu NR. In fact, there is little information available regarding firewood extraction and use by minority n the buffer zone of the protected area around the NR, or in Vietnam generally. Firewood extraction by ethnic minorities may be having an impact on conservation on the area studied in the research described in this paper. Moreover, the effects of firewood collection and law enforcement practices related to natural resources are frequently not fully investigated nor understood (Brown et al., 2009)

To gain a better understanding relating to these issues, research was conducted on firewood extraction in the buffer zone of Pu Hu NR, Vietnam. The aims of this study were to identify (1) different forms of firewood consumption based on location and ethnic minority; and (2) to foster cooperation between ethnic minority households and NR rangers. The final objective was to support forest management's efforts to adjust their law enforcement practices to take into account ethnic minority households' demands for forest resources.

Materials and methods

Research site

Pu Hu NR, located in Quan Hoa and Muong Lat districts in Thanh Hoa province in northeast Vietnam, was established in 1999 as a NR by the government of Vietnam and is one of the largest in this province covering around 28.000 ha. It is managed by the Pu Hu management board in Quan Hoa district and is located in a mountainous area (latitude 20°30' to 20°40'N and longitude 104°40' to 105°05'E) (*Fig. 1*). It is home to a number of endangered animals, and rare plant species. Following the establishment of the NR, all extraction activity by people living in the area of the NR was made illegal. However, even after the NR was established, the extraction of folder and fuelwood has been informally allowed although people are not allowed to enter the NR without official permission. Communities living around Pu Hu NR practice subsistence farming based on the cultivation of crops and supplement those crops by the exploitation of non-timber forest production (NTFPs), raising livestock and by the cultivation of bamboo (Thapa and Chapman, 2010). The biggest management issues include the relatively unrestricted extraction of forest resources by local residents from the surrounding area (Allendorf and Allendorf, 2012). The major forest resource use focused on in this case study is the requirement for firewood as a source of energy for the ethnic minority households in the area..

This study was conducted during the cold season, extending from November 2017 to March 2018 in the buffer zone of the Pu Hu NR, where there are 24,569 residents in 5,694 households living in 61 villages in 11 communes in two districts (Muong Lat and Quan Hoa). Most local people practice farming, cultivating crops such as paddy, maize, manioc, and vegetables. There has also some forestry activities including the extraction of both timber and non-timber products. Other occupations include harvesting bamboo, retailing and working as hired labor.

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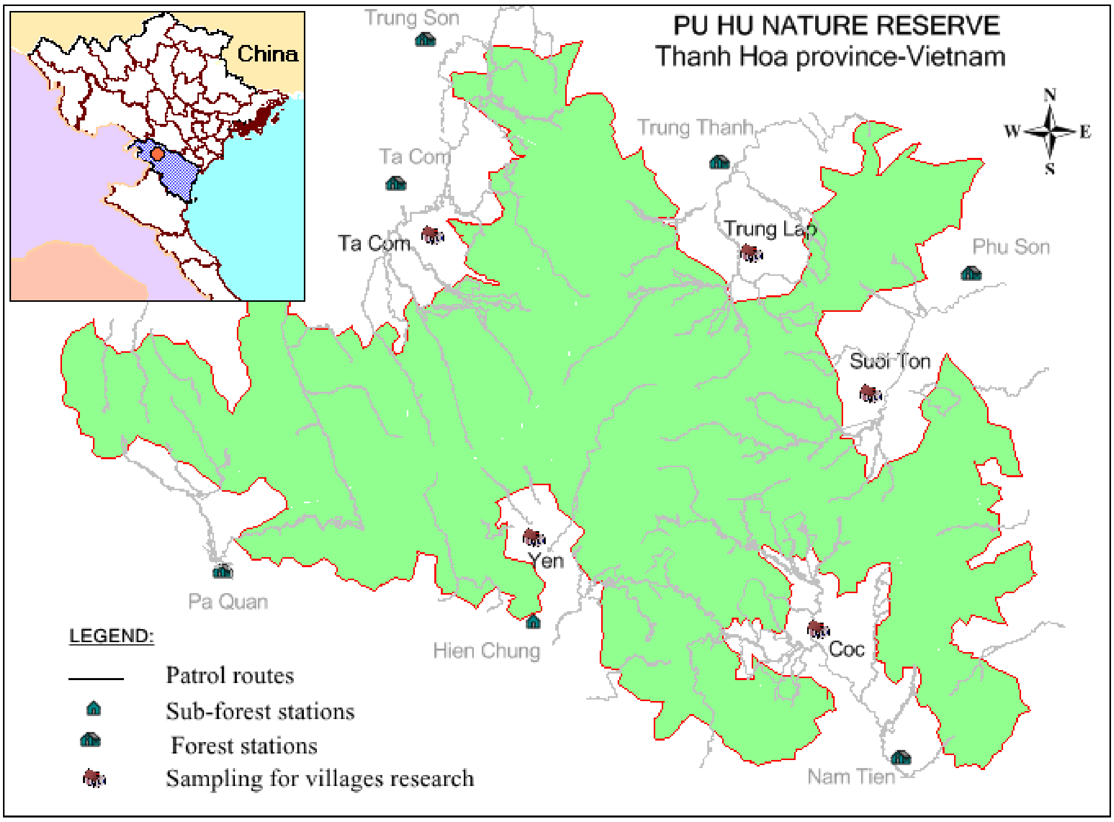

Figure 1. *The location of case study of Pu Hu NR*

Data collection

Data on firewood use was collected in fieldwork using a questionnaire, observation and face-to-face interviews between November 2017 and February 2018. For the buffer zone of Pu Hu, over 90% of the households from different minorities in 5 villages in 5 communes were sampled and interviewed as well as being observed in their daily firewood collecting activity. Household surveys and Interviews were carried out in between 40 and 80 households per village and were conducted either by one of the researchers or by the village leader, based on visiting each household. By preference the head of each family was interviewed, who either completed the questionnaire or answered the questions asked by the interviewer, the answers to which were then filled in on the questionnaire by the interviewer. In most cases the head of household was male and it was a more difficult to interview women among the ethnic minorities because they were mostly illiterate or felt ashamed at being asked questions. If the head of the household was not available a willing member of the household was selected (Thapa and Chapman, 2010). Before the interviews took place there was a meeting in the village with a head of the village who was a key-informant, to gain an understanding of the background of changes. It had taken place in the area in the past as well as obtaining background information on socio-economic conditions, village size, and the distribution and number of households and the size of their families, and a list of households was obtained.

The researcher also interviewed a number of Pu Hu NR rangers in order to gain an understanding of the situation regarding the collecting of firewood, and to gain an

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understanding the law enforcement regime relating to firewood collection in the buffer zone, as well as to discuss the government's firewood programme.

The questionnaire was formulated in English then translated into Vietnamese (Sousa-Silva et al., 2016) and consisted of a mixture of open-ended and closed-ended questions in three parts: the first relating to the respondent's socio-economic situation (*Table 1*), the second to establish the household's requirement for firewood, and the third to gain an understanding of the household's perspective relating to the NR rangers' activities.

Table 1. *Description of socio-economic aspects from survey*

Social-economic parameter	Classification	Description
Gender		
Age (years)		
House size		
Number of families		
Household income (per month)	Wealthy	(≥ 76 USD)
	Average	(51–75 USD)
	Close to poor	(31–50 USD)
	Poor	(≤ 30 USD)
Education level of the leader of household	Tertiary education	Upper 12 years of education
	High school	10–12 years of education in high school
	Secondary school	6–9 years of education in secondary school
	Primary school	1–5 years of education in primary school
	No formal education	Not educated in school
Occupation of person interviewed in household	Full-time farming	Working in the field all the time
	Part-time farming	Doing some other job in addition

Data analysis

Data relating to the households' income levels, firewood use, and the purposes for which firewood was used by the households in the selected villages were collected from interviews. Data on the firewood consumption situation by the different ethnic minority households were summed and averages and percentages for each minority were computed (Kim et al., 2016). Data analysis was performed using the SPSS version 20 software package and Microsoft Excel. Both descriptive and statistical data analyses were performed. The calculation of sample means, standard errors, medians, minimum and maximum values, and frequency distributions was conducted.

Inferential statistical analysis was used to discover the relationships among the relevant variables. Because the data was not normally distributed, the Mann–Whitney U test for two variables and the Kruskal–Wallis test for more than two variables were used in SPSS, to analyze the data with any significant differences expressed at the 0.05 level (or higher where indicated in the text). The estimated volume of firewood consumed by the three ethnic minorities in the five villages was computed based on the data from the questionnaires.

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Results

Background information established from the survey of households

Three-quarters of the 248 interviewees (75%) were male and the age of the interviewees ranged from 20 to 75 years with the median age, 36 years (*Table 2*). the largest age group, represented was 20–35 years with 48% of the respondents. The average house size was 144.6 m^2, varying between 23 and 450 m^2 and more than half the houses (54%) were below 100 m^2. The mean number of persons in each household was five, ranging from 3 to 12 members and more than half of the households (56.5%) had between five and eight persons. Regarding the income situation of the households, around 64% fell within the poor level, whilst those close to poor or with an average living standard accounted for 19.4 and 7.7% of the households surveyed, respectively. More than half of the interviewees in the households (57.3%) had had secondary school level education (five years and four years of education in the primary school and secondary school, respectively). Based on occupation, 87.9% of the interviewees in the households were full-time farmers.

Table 2. *Summary of socio-economic respondents based on the survey*

Social-economic situation	Categories	Frequency (n)	Proportion (%)
Gender	Male	186	75.0
	Female	62	25.0
Age (years)	20–35	118	47.6
	36–50	95	38.6
	51–65	29	11.7
	66–75	6	2.4
House size	≤ 100	135	54.4
	101–200	65	26.2
	201–300	29	11.7
	≥ 301	19	7.7
Number of persons	≤ 4	108	43.5
	5–7	120	48.4
	≥ 8	20	8.1
Household income (per month)	Wealthy	22	8.9
	Average	19	7.7
	Close to poor	48	19.4
	Poor	159	64.1
Education level of the leader of household	Tertiary education	1	0.4
	High school	22	8.9
	Secondary school	142	57.3
	Primary school	69	27.8
	No formal education	14	5.6
Occupation of person interviewed in household	Full-time farmer	218	87.9
	Part-time farmer	30	12.1

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There were significant differences in socio-economic conditions among the household in the Pu Hu buffer zone with income earned by the three ethnic minorities different, at the $p < 0.001$ level. The Thai ethnic minority earned the highest average net annual income of 64.67 ± 51.80 USD/person/month, while the lowest income was 21.98 ± 12.33 USD/person/month for the Mong minority. Further, there were moderately low but significant correlations between the socio-economic data and firewood consumption. The total amount of firewood used was correlated with house-size, number of persons, and income based on Spearman rank order correlations (rs) of 0.24, ($p < 0.01$), 0.24 ($p < 0.01$) and 0.30 ($p < 0.01$) respectively for the communities.

Location of firewood collection areas for daily use

All the ethnic minority households used firewood baskets carried as headloads as illustrated in *Figure 2,* and they frequently carried large loads supported by both their backs and heads as reported by Sassen et al. (2015) in research in protected areas in Uganda. Additionally, 65% of the respondents transported firewood-using motorbikes, and some households also used bicycles.

a b c

***Figure 2**. Photographs of typical headloads in the field (a) Muong; (b) Thai; (c) Mong. (Source: Pu Hu NR and author)*

When asked about the main natural forest resources used by their household, all the respondents stated that their family as a source of energy used firewood daily. Of the respondent households, 74 and 163 were involved in harvesting timber and NTFP respectively. There were three sources of firewood collected as a daily activity: Pu Hu NR, private forest, and agricultural land. The three minorities mainly collected firewood in forestland (Mong: 28%, Muong: 12% and Thai 18%; see *Fig. 3*). In the Pu Hu NR, the Mong minority was the main group who collected firewood accounting for about 10% of the firewood they extracted (*Fig. 3*), while the percentage of firewood collected by other minorities was less than 2%.

Furthermore, for daily cooking and boiling water as shown in *Table 3*, the Thai ethnic minority households required the greatest amount of firewood (36.02 ± 10.08 kg) while the Mong and Muong minorities used less for these purposes (31.51 ± 17.25 and 22.57 ± 11.59 kg, respectively). Similarly, the amount of firewood used for heating was significantly different among the ethnic minorities (*Table 3; Fig. 4*). The amount of firewood used by the Thai minority (21.00 ± 11.54 kg) was nearly double that used by the Mong minority (12.88 ± 4.34 kg) with the Muong minority being the second highest (15.01 ± 7.80 kg). The average firewood used for this purpose was 15.17 ± 7.97 kg/month/household.

There was a significant difference in the monthly amount of firewood used for livestock, with the lowest mean firewood consumed for this purpose of

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26.48 ± 18.27 kg/month/household being found in the Mong minority. It was only around a third and a fifth that used by the Muong and Thai minorities, respectively, even though only 75% of the Muong households used firewood for livestock whereas all the Mong households used firewood for that purpose. The mean amount of firewood used by all three ethnic minorities for their livestock was 63.17 ± 48.26 kg/day/household.

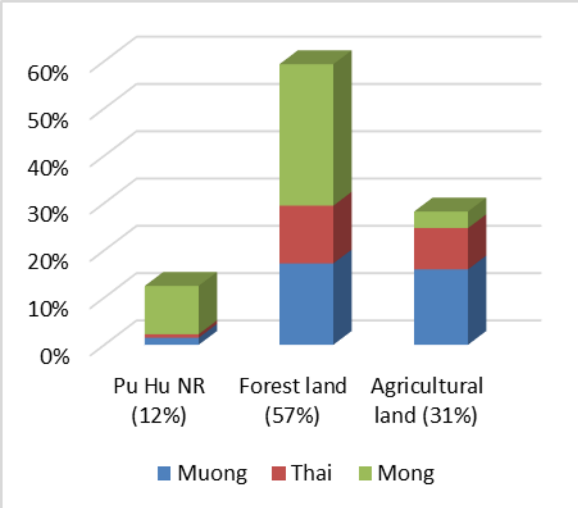

***Figure 3.** Location of firewood collection by three-ethnic minority households (%)*

***Table 3.** Percentage of households using firewood for different purposes and mean monthly volume consumed*

Category of firewood use by ethnic minority	Percentage of households interviewed using firewood for this purpose	Mean monthly volume (kg/household)
Cooking and boiling water		
Muong	100%	22.57 ± 11.59
Thai	100%	36.02 ± 10.08
Mong	100%	31.51 ± 17.25
Heating		
Muong	91%	15.01 ± 7.80
Thai	94%	21.00 ± 11.54
Mong	100%	12.88 ± 4.34
Livestock		
Muong	75%	87.07 ± 31.00
Thai	94%	125.22 ± 31.49
Mong	100%	26.48 ± 18.27
Making wine		
Muong	36%	18.31 ± 10.49
Thai	29%	34.53 ± 26.91
Mong	99%	31.37 ± 8.57
Bamboo drying		
Muong	18%	24.64 ± 18.24
Thai	10%	40.00 ± 12.25
Mong	Not used	

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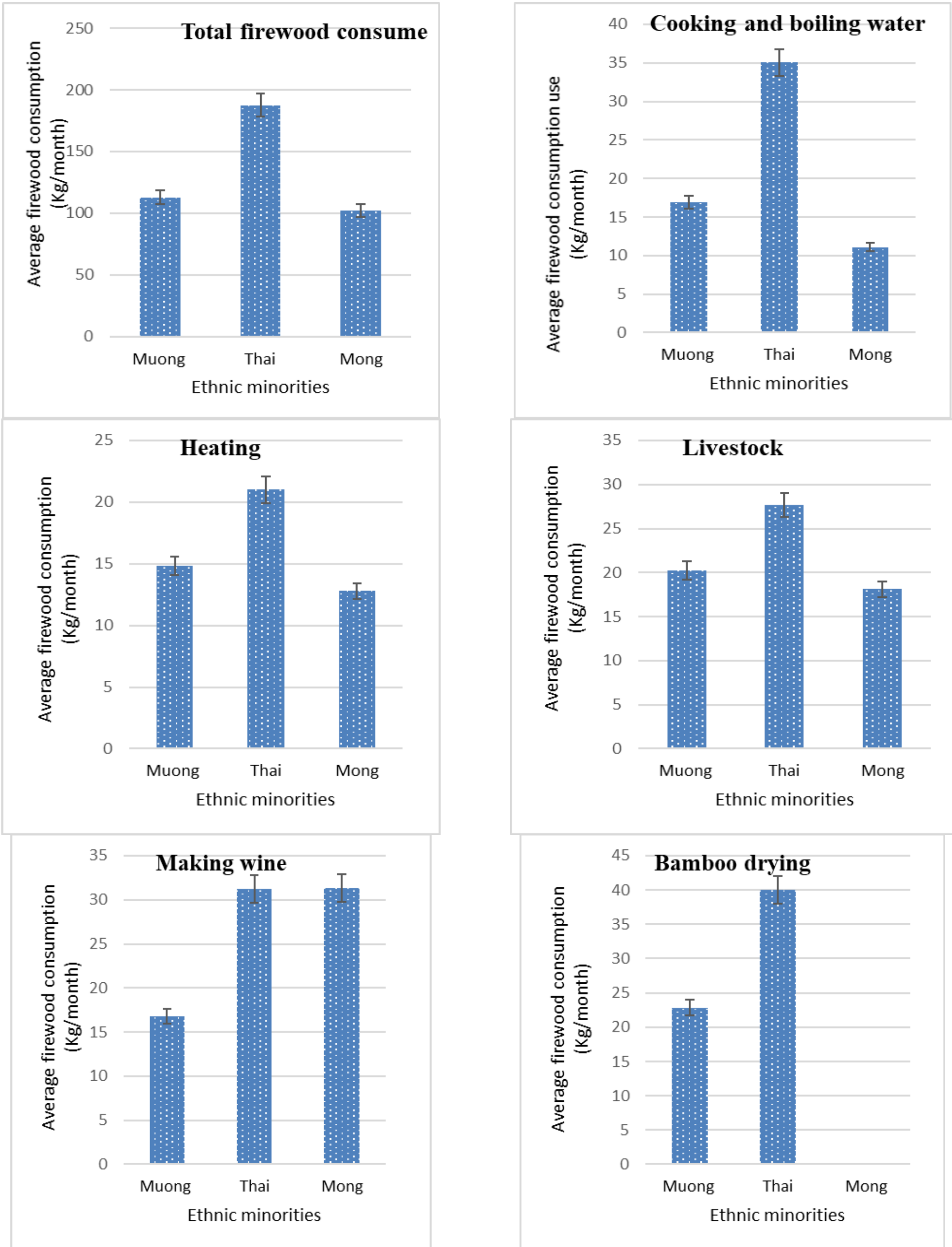

Figure 4. *The estimated monthly weight of firewood used in total and for different purposes*

Further, the Thai minority households used a significantly higher monthly amount of firewood for making wine (34.53 ± 26.91 kg) than the Mong minority (31.37 ± 8.57), whereas the Muong households used only around half the amount used by Thai households for that purpose. Moreover, only around 30% of the Muong Thai households used firewood for this purpose and the number was more than three times that among the Mong households. The average firewood used for this purpose was 29.48 ± 12.71 kg/month/household.

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It was found that none of the Mong households used firewood for drying bamboo but almost twice as many Muong households used firewood for this purpose than Thai households. Nevertheless, the amount of firewood used in the Thai households (40.00 ± 12.25 kg) was higher than that used in the Muong households (24.64 ± 18.24 kg) with the average firewood used for drying bamboo being 28.68 ± 17.94 kg/month/day.

The average firewood used by the three ethnic minorities combined was 123.19 ± 60.49 kg/month/household. However, the Thai households consumed the highest amount of firewood per month (187.62 ± 67.23 kg), while the Muong and Mong households consumed broadly similar amounts of firewood (112.61 ± 58.03 and 101.97 ± 34.57 kg, respectively - see *Fig. 4*). Furthermore, the use of firewood by the Thai households for all purposes of daily living was the highest among the three ethnic minority households. However, the amount of firewood used for heating, livestock and bamboo drying in the Muong households was higher than that of the Mong households and overall, the monthly firewood used by each household among the three minorities was significantly different ($H = 67.06$; $p < 0.01$) as was the personal firewood consumption ($H = 20.82$; $p < 0.01$).

Interestingly, averaging the observed November to February consumption rates and extrapolating from that across the total number of individuals (i.e., 30 kg/person × 12 months). The approximately 360 kg per person of firewood is consumed by the Muong minority during a year while the firewood used by the Thai and Mong minorities was lower at 324 kg/year and 240 kg/year.

Ranger activity in the villages

When interviewed, nearly half the Muong and 72% of the Mong said that they occasionally saw rangers patrolling whereas about 65% of the Thai minority interviewed said that they sometimes met rangers patrolling (*Fig. 5*). Interestingly, a very low percentage of Muong people (1%) generally met rangers while collecting firewood while nobody in the other ethnic minorities generally saw rangers patrolling. When asked what happened when they met a ranger, the main proportion experienced being talked to by the ranger (Muong 55%, Thai 49%, and Mong 85%). The rangers rarely, arrested people in the three ethnic minorities with only 2% of the Thai people reporting having been arrested.

Figure 6 details the communication between the minority households and local rangers with the main percentage (58%) of Muong and Mong households having had communication with rangers, but 67% of the respondents in Thai households having no communication with rangers (*Fig. 6*). Interestingly, the quality of advice from the rangers reported by almost all the Thais (94%) was normal while the percentage of 'normal' advice to the Mong (48%) and Muong (45%) was lower.

There were similar percentages of the three minorities who considered that the rangers provide technical support for forest development. But the percentages of Mong and Thai who considered forest policy to be appropriate (70% and 50%, respectively) was higher than that among the Muong households, many of whom (62%) suggested that the rangers should come to their villages regularly (*Fig. 7*). With regard to the improvement of forest development, the largest number of Muong and Thai households wanted support for forest activities (58% and 62%, respectively, while most Mong households felt that the best way to improve forest development was by the selection of good village leaders.

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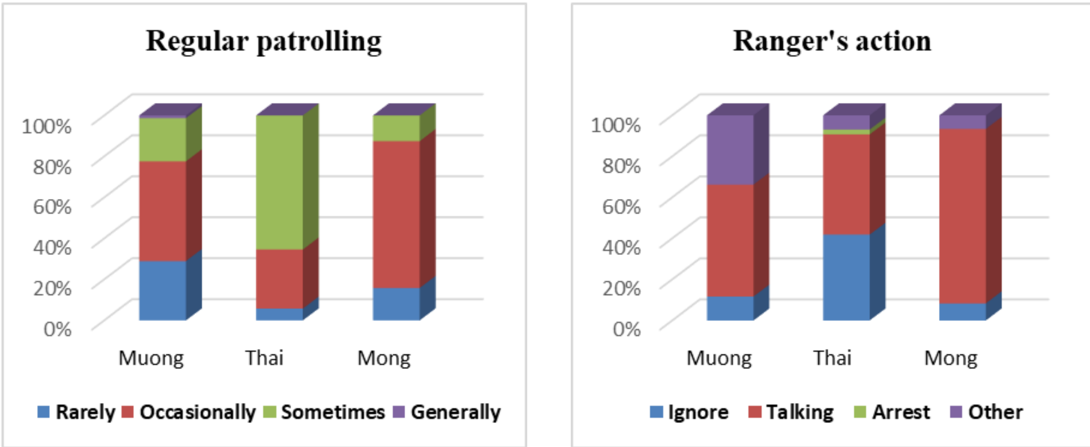

Figure 5. *Ranger's patrolling and action when meeting firewood collectors*

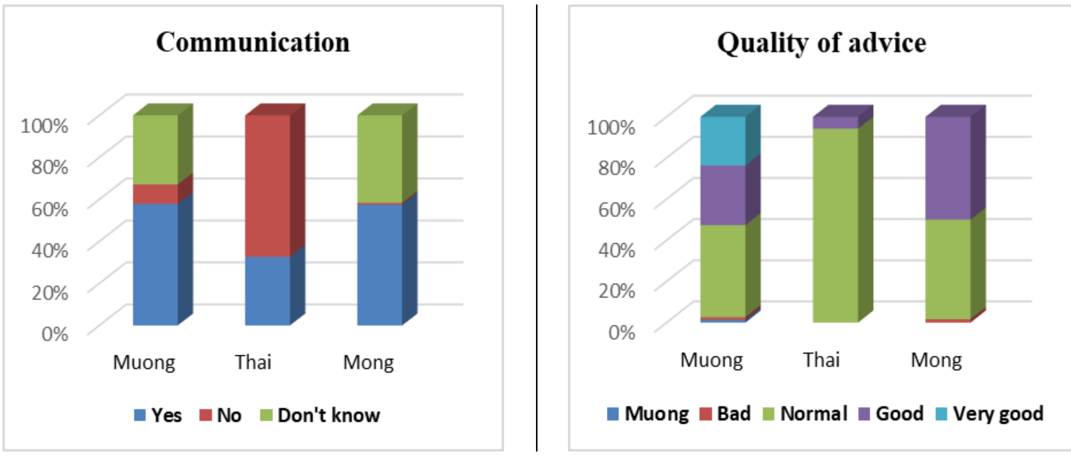

Figure 6. *Communication with and quality of advice from rangers*

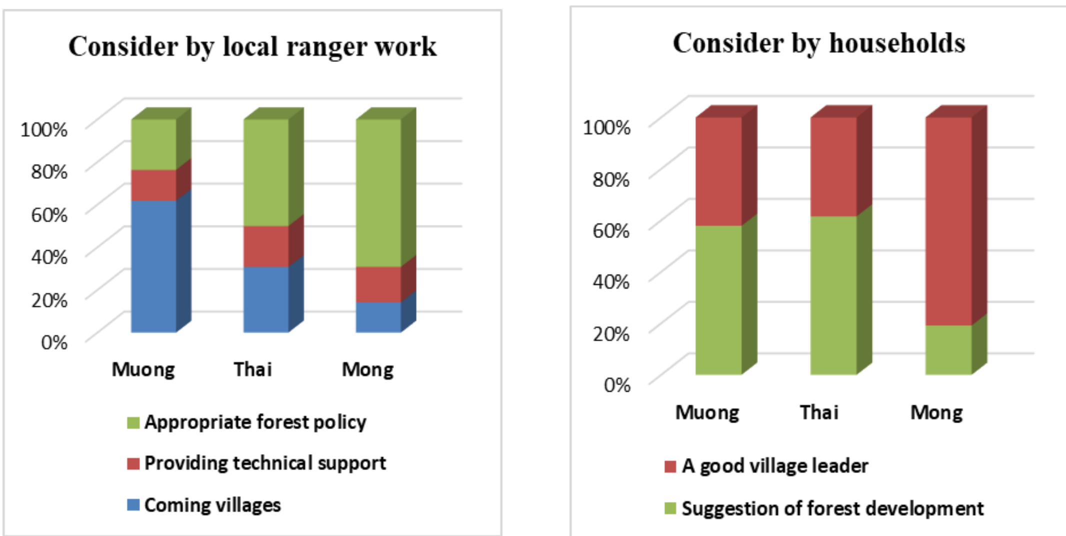

Figure 7. *Households' opinions about improving ranger's roles and forest development*

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Discussion

Firewood use relative to social aspects

Firewood was found to be the main energy source for residents in the Pu Hu buffer zone and is likely to remain an important natural resource because it is essential for human activity (Wangchuk et al., 2014). Overall, the firewood consumed by the three ethnic minorities was significantly correlated with households' economic indicators, house-size, and the number of persons in the family (Démurger and Fournier, 2011; Wang et al., 2015). It was found that all the households had forest land and agricultural land, and almost all the households conducted farming as their daily activity. The forest land which makes up the core zone of the NR is far from the area occupied by the households and thus the collection of firewood is difficult and time-consuming and is mainly carried out by the women in the family (Abbot and Mace, 1999; Maes and Verbist, 2012; Tabuti et al., 2003). This study showed that the household size based on the number members of the family had a relationship with the firewood used and this finding was similar to the results of previous studies (Marquez-Reynoso et al., 2017; Win et al., 2018).

Firewood collection and requirement

In the present work, it was found that firewood is a vital resource in the villages adjacent to the Pu Hu buffer zone (Cardoso et al., 2013). The requirement for firewood is more common, compared to timber and NTFP because firewood is used as the major fuel (Kim et al., 2016). Firewood collection on forest land appears to be more intense than in agricultural land in the region as previously found by Sassen et al. (2015) in the forest of Mt Elgon National Park (Uganda) and the implementing of a buffer zone is a way to reduce some of the pressure on NRs (Naughton-Treves et al., 2007). However, local people (nearly 70%) mainly collected firewood from the forest areas including in forestland both inside and adjacent to the NR. This activity puts pressure on forest resources and is likely to lead to serious deforestation (Fox, 1984; Agurto Adrianzén, 2013). In Pu Hu NR, the percentage of household firewood collected in the NR used by the Mong (about 10%) was the highest among the three minorities. Even though Pu Hu NR is a protected area it makes a highly important contribution to the livelihood of households in the area as similarly found by Kim et al. (2016) in considering Ke Go NR in Vietnam. Moreover, as found by Thapa and Chapman (2010) who conducted research in Bardia National Park, Nepal when farming areas and the collection of resources is very important, local people may not be deterred by measures to deter them from using a protected area. However, the study conducted in Indonesia by Pattanayak et al. (2004) reported that households extracted firewood from a local NR forest and their own fields. To collect firewood in the NR, individuals interviewed in this study said people walked over 2 km taking from 3 h to half of the day to cut and collect a full head-load of wood (Win et al., 2018). The distance between households and the protected areas is a challenge and discouraged most local people from entering the NR to harvest firewood.

The estimated yearly quantities of firewood consumed were around 300 kg per household among the three ethnic minorities living in the Pu Hu buffer zone which was very similar to the household requirement in the Budongo Forest Reserve, Uganda of 316 kg firewood (Tweheyo et al., 2005). Mislimshoeva et al. (2014) found a per household consumption of 355 kg year in the Western Pamirs and Bhatt and Sachan

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(2004) suggested that 174 kg of firewood was used per year by each household in communities in northeast India. Further, Kim et al. (2016) reported a consumption of about 165 kg per household in the neighbouring province of Nghe An.

Firewood use was highest among the Muong minority (1.03 kg/capita/day), followed by the Thais (0.89 kg/capita/day) and Mong (0.67 kg/capita/day), irrespective of their socio-economic status. Comparatively more firewood is used by tribal minorities in Arunachal Pradesh and Garos in northeast India with 3.1 and 5.0 kg/capita/day, respectively (Maikhuri, 1991). Sassen et al. (2015) found that people consumed an average of between 2.14 and 3.88 kg of fuel-wood per capita per year in Mount Elgon national park in Uganda.

In common with the present study, Victor and Victor (2002) and Kim et al. (2016) reported that the average firewood consumption was highest for business activities such as raising livestock. The Thai and Muong minorities are more focused on developing their economic status, and therefore their requirement for firewood was found to be higher for this purpose than for others in similar research conducted in Vietnam by Kim et al. (2016). The livestock business plays a crucial role in household income structure and pig and cattle raising is important to the livelihoods of various minorities living in rural areas (Kim et al., 2016). Based on economic development, the Mong minority generally had lower household incomes, and therefore their priority use of firewood was for cooking and boiling water. Moreover, the Mong people also live in more inaccessible and higher areas than the Thai and Muong minorities.

Connection between households and forest rangers

It is undeniable that firewood is important for a sustainable livelihood among rural people without access to other sources of energy (Kimemia and Annegarn, 2011). Households among the ethnic minorities in the Pu Hu buffer zone mainly used firewood as their source of energy and the main areas for collecting firewood are the forest and agricultural land which are accessible to everybody in the adjacent villages (Kim et al., 2016). Thus, It seems that firewood will remain an important source of energy for the ethnic minority households (Nansaior et al., 2011). Law enforcement is inefficient because the number of effective patrol days is low compared with the potential number of scout working days as reported by Bell and Mcshane-Calzi (1986). A different situation was found by Sassen et al. (2015) in Uganda, where the official allowance per household under forest protection agreements was one headload, twice per week. However, in this study it was observed that although officially excluded, local people have been permitted to collect firewood from the NR area. Rangers at Pu Hu NR said that firewood was collected from both inside the NR and from adjacent forestland, but that they do not consider this a major issue because the volume of firewood taken is not great. This finding is in agreement with those of Kimemia and Annegarn (2011) and Abbot and Mace (1999) in studies in South Africa and Lake Malawi National Park, respectively. Based on the reports of the minority Muong and Mong they patrolling efforts are sporadic and when they encountered the rangers while collecting firewood they would talk to the farmers rather than arresting them.

Most of the respondents considered that the quality of the rangers' advice was normal and there was communication between rangers and some of the people in all three ethnic minorities. Effective communication is necessary because this enables a flow of helpful information about the forest and its management, which is necessary to formulate the authority's management policy (Wang et al., 2015). Insufficient

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communication between rangers and local people might cause negative behavior related to illegal activity inside and outside the NR (Obua et al., 1998). It is notable that more than half of the Muong minority community interviewed expressed the view that the rangers should conduct regularly patrols in their villages, while most of the Mong minority considered it more important for the rangers to implement appropriate forest policy. Due to improvement of forest management, around 80% of Mong respondents focused on having a good village leader, whereas the other ethnic minorities both indicated that the forest development policy was more important

Conclusion

Forests are still a major source of firewood for ethnic minority households in Pu Hu buffer zone and provide them with substantial economic benefits (Wangchuk et al., 2014). In this study, the patterns of household firewood use were analyzed for the three minorities in the Pu Hu buffer zone, namely the Muong, the Thai and the Mong, using primary data obtained during research in villages located adjacent to the Pu Hu NR. The paper also analyzed the relevant factors that influence household firewood consumption, including the number of household members, and house-size. A comparison by way of a descriptive analysis of households of the three minorities was presented and their firewood requirements were established as being the major demand which they make of local natural resources (Behera et al., 2015). In the study location, the Thai minority households tended to consume more firewood than other minorities. However, the percentage of firewood collected by the Mong households in the nature reserve was the greatest among the three minorities and whilst the Thai minority constitute the largest ethnic group, it is the Mong minority who have the traditional culture of collecting firewood in Pu Hu NR. Thus, NR rangers had to talk to them and demand that they reduce their collection of firewood in the NR when they met them while patrolling. In view of this situation, the management of the NR should focus on the firewood needs of the Mong households, for instance by improving their knowledge of forest protection, enhancing reforestation, reducing the risk of forest fires, and contributing to conservation (Wangchuk et al., 2014).

In the long-term, the best way of dealing with this problem would be to provide greater subsidies for the use of electric power for personal consumers, as a way of reducing pressure on the forests in buffer zone (Naughton-Treves et al., 2007). In the short-term, it is suggested that the widespread introduction of improved-design cooking stoves (both in terms of firing efficiency and heating conductibility) to replace the traditional three-stone cooking rings used by local people, is pursued as a matter of urgency (Kim et al., 2016). On the other hand, rangers should regularly patrol and carry out their responsibility of forest protection, even though in practice, there is little extraction of firewood from the NR. To enhance the sustainable management of resources, firewood harvesting by minorities could be controlled by rangers in this location as part of their duty to ensure that it is carried out in a manner that does not create ecological impacts. Moreover, the park management should convince villagers to find ways of working without resorting to illegal resource extraction, which in the long term would be the best way of preventing ecological damage in the Pu Hu NR (Thapa and Chapman, 2010). Further, upcoming research should consider on patrolling rangers efforts in each location that could be influent local’s behavior and firewood collection.

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Conflict of interest. The authors confirm that this article content has no conflict of interest.

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APPENDIX

Household survey questionnaire

I. General information

Date:	Village: ……………	
Name: …………………; Age ………....	Ethnic: …………..; Education: …………	
Gender: ……………;	Number of family: …… / Children: ………………..	
Household size: ………… m^2,	Estimated income: …………………..VND/month	
Living–years in buffer zone: ..... year		
Cultivated working time	☐ Full-time	☐ Part-time
Land use of family	☐ Agriculture	☐ Forest
	☐ Plantation	☐ Other

II. Firewood aspect

1. What is your requirement from the forest?

a. [ ] Firewood b. [ ] Timber c. [ ] None-timber d. [ ] Other, ….

Why?…………………………………………………………………………………..

2. Do your family use firewood? Yes ☐ No ☐
3. How long has your family used firewood? ……… Years

What reason for?……………………………………………………………………….

- Where do you collect firewood?

a. [ ] Pu Hu forest b. [ ] Household forest c. [ ] Agriculture land d. [ ] Buy d. [ ] Other

For the purpose of…

a. [ ] Cooking b. [ ] Heating c. [ ] Boiling water d. [ ] Drying bamboo

e. [ ] Wine f. [ ] Livestock g. [ ] Other……………..

4. What do you usually do when you collect firewood? ……………………………
5. How do you transport firewood from field to house?

a. [ ] Head b. [ ] Bicycle c. [ ] Motorbike d. [ ] Other, …..

6. How important is firewood as energy consuming in your family?

a. [ ] Unimportant b. [ ] Little important c. [ ] Medium d. [ ] Important e. [ ] Very important

7. Why is firewood your main energy consumption?

a. [ ] Cheap b. [ ] Nature c. [ ] Efficiency d. [ ] Other, …..

8. Do you sell firewood? Yes ☐; No ☐

Why? ………………………………………………………………………………

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9. Can you estimate firewood consume per day?

Cooking and heating (…….person/day)	……………………kg/baskets
Heating	……………………kg/baskets
Livestock	……………………kg/baskets
Wine	……………………kg/baskets
Bamboo drying	……………………kg/baskets
Other	……………………kg/baskets

10. There is a different quantity of firewood if compared to 10 years before?

a. [ ] Yes b. [ ] No

Why? ………….……………………………………………………………………

11. Do you select plant species for firewood? Yes ☐ ; No ☐

12. What kind of species for firewood do you prefer? ……………………………..

13. Do you think about improving firewood?

a. [ ] Yes b. [ ] No

If yes? What?

…………………..…………………………………………………………………

14. Do you think what government should support for energy consumption?

…………………..…………………………………………………………………

15. Do you see ranger's patrolling when collected firewood?

a. [ ] Rarely b. [ ] Occasionally c. [ ] Sometimes d. [ ] Generally e. [ ] Always

What happens?

a. [ ] Ignore b. [ ] Talking c. [ ] Arrest d. [ ] Other, ……

16. Do you know forester communicated households?

a. [ ] Yes b. [ ] No c. [ ] Don't know

17. If yes, how good is the communication of forester and household?

a. [ ] Very bad b. [ ] Bad c. [ ] Normal d. [ ] Good e. [ ] Very good

18. Can you estimate the quality of firewood advice?

a. [ ] Very bad b. [ ] Bad c. [ ] Normal d. [ ] Good e. [ ] Very good

19. Do you think household agree on firewood advice?

a. [ ] Yes b. [ ] Not much c. [ ] No d. [ ] Don't know

20. Do you receive firewood's advice from forester? ☐ Yes ☐ No

21. How do you know the communication between household and forester?

- Forester:
  a. Usually go to and contact to commune ☐
  b. Support of technical and information on forest development ☐
  c. Propagandized policy ☐
  d. Others ………………………………………..………………………
- Family requirement:
  a. Suggested forest protection and development ☐
  b. Require the excellent leader ☐

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Household Survey Questionnaire

I. Thông tin chung

Ngày:	Tên thôn: ……………	
Họ và tên: …………………; Tuổi ………...	Dân tộc: ……………; trình độ học vấn: …………	
Giới tính: ……………;	Số khẩu: ………………….. / Số con: ………………..	
Diện tích nhà: ………… m^2,	Ước tính thu nhập: …………………..VND/Tháng	
Số năm sống trong vùng đệm KBT: ..... năm		
Thời gian làm nương	☐ Thời gian chính	☐ Bán thời gian
Các loại đất sử dụng gia đình có	☐ Nông nghiệp	☐ Đất rừng
	☐ Đất trồng cây công nghiệp	☐ Đất khác

II. Quá trình củi

1. Theo anh/chị nhu cầu nào trong gia đình đang cần hơn?

a. [ ] Củi | b. [ ] Gỗ | c. [ ] Lâm sản phụ | d. [ ] Khác, ….

Tại sao? …..……………………………………………………………………..

2. Gia đình anh/chị có dùng củi trong gia đình bạn không? Có ☐ Không ☐
3. Gia đình Anh/chị dùng củi đốt trong thời gian (hoặc không)? ……… năm

Nguyên nhân nào anh/chị quyết định như vậy? …………………………………….

- Anh chị lấy thu gom củi ở vùng nào?

a. [ ] Rừng Pu Hu b. [ ] Đất rừng nhà c. [ ] Đất nông ghiệp d. [ ] Mua d. [ ] Khác

- Mục đích sử dụng củi

a. [ ] Nấu ăn b. [ ] Sưởi ấm c. [ ] Nấu nước d. [ ] Sấy măng

e. [ ] Nấu rượn f. [ ] Chăn nuôi g. [ ] Khác……………..

4. Hoạt động nào anh/chị thường kết hợp với việc lấy củi? ....……………………………………
5. Làm thế nào anh/chị vận chuyển củi về nhà? (chọn một lúc nhiều phương án)

a. [ ] Đi bộ/gùi b. [ ] Xe đạp c. [ ] xe máy d. [ ] Khác, …..

6. Gia đình Anh/chị có quan trọng vấn đề nhiên liệu củi đốt trong thời gian qua?

a. [ ] Không quan trọng b. [ ] Ít quan trọng c. [ ] Bình thường d. [ ] Quan trọng e. [ ] Rất quang trọng

7. Tại sao củi đốt lại là nguồn năng lượng cho gia đình?

a. [ ] Rẻ b. [ ] Có sẵn c. [ ] Có hiệu quả d. [ ] Khác, …..

8. Anh/chị có bán củi nấu không? Có ☐; Không ☐

Tại sao? ……………………………………………………………………………

9. Nếu anh/chị ước tính lượng củi dùng trong 1 ngày?

Nấu ăn và nấu nước (……..người/ngày)	…………………….. kg/bế
Sưởi ấm	…………………….. kg/bế
Nấu chăn nuôi (…..….lợn+….. bò + ……..)	…………………….. kg/bế
Nấu rượn (………./ bó/kg; chai/lít ……….)	…………………….. kg/bế
Sấy măng (……….măng/kg)	…………………….. kg/bế
Khác: ………………………………………………	…………………….. kg/bế

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10. Nếu anh/chị so sánh gia đình có so với 10 năm về trước, có sự khác biệt về số lượng củi đun?

a. [ ] Có b. [ ] Không

Tại sao? ……………………………………………………………………………………

11. Anh/chị có lựa chọn các loài cây làm củi đốt không? Có ☐ ; Không ☐
12. Loại cây nào anh/chị yêu thích dùng củi trong gia đình? ………………………………………………………………………...……………..
13. Anh/chị có nghĩ cần phải có cải thiện nguồn cung cấp củi?

a. [ ] Có b. [ ] Không

Nếu có? Điều gì anh/chị nghĩ cần phải nâng cao nguồn cung cấp củi?

………………..…………………………………………………………………………

14. Hoạt động nhà nước nên hỗ trợ anh/chị thay đổi việc sử dụng năng lượng?

Liệt kê………..……………………………………………………………………….

15. Anh/chị có nhìn thấy cán bộ kiểm lâm tuần tra rừng trong khi đi vào khu bảo tồn thu gom củi đốt?

a. [ ] Hiếm khi b. [ ] Thỉnh thoảng c. [ ] Thường xuyên d. [ ] Liên tục e. [ ] Rất nhiều

Điều gì sẽ xảy ra ?

a. [ ] Bỏ qua b. [ ] Nói chuyện c. [ ] Bị bắt lại d. [ ] Khác, ……

16. Anh/chị có nghĩ đã có liên lạc giữa các hộ và cán bộ Lâm nghiệp trong thời gian qua?

a. [ ] Có b. [ ] Không c. [ ] Không biết

17. Nếu có, anh/chị cho biết mức độ liên lạc giữa các chủ hộ gia đình với KBT?

a. [ ] Rất xấu b. [ ] Xấu c. [ ] Bình thường d. [ ] Tốt e. [ ] Rất tốt

18. Anh/chị đánh giá chất lượng hỗ trợ trong hoạt động của cán bộ kiểm lâm về củi đốt?

a. [ ] Rất xấu b. [ ] Xấu c. [ ] Bình thường d. [ ] Tốt e. [ ] Rất tốt

19. Hộ gia đình có chấp nhận những tư vấn của cán bộ kiểm lâm về củi đốt?

a. [ ] Có b. [ ] Không nhiều c. [ ] Không d. [ ] Không biết

20. Anh/chị có được hỗ trợ tư vấn trong sử dụng củi từ cán bộ KBT?

☐ Có ☐ Không

21. Theo anh/chị cho biết làm thế nào mức độ kết nối liên lạc hộ gia đình với KBT?

- Cán bộ lâm nghiệp:
  a. Thường xuyên xuống và liên lạc với cộng đồng ☐
  b. Cung cấp hỗ trợ kỹ thuật và thông tin về phát triển rừng ☐
  c. Tuyên truyền chính sách ☐
- Hộ gia đình:
  a. Đề xuất các vấn đề liên quan đến lĩnh vực bảo vệ và phát triển ☐
  b. Cần có ban lãnh đạo/trưởng thôn uy tín ☐

ELECTRONIC APPENDIX

This article has an electronic appendix with basic data.

ANALYSIS OF CHROMOSOMAL REGIONS CONTROLLING DROUGHT TOLERANCE IN BARELY (*HORDEUM VULGARE* L.) SEEDLINGS

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Abstract. To locate the loci controlling physiological traits of barley in seedling stage, an experiment was conducted under drought stress and normal irrigation in greenhouse as a completely randomized design with two replications in Iran in 2016. For this purpose, the populations of Cebad Capa (C. Capa) × Susptrit (S) and Vada (V) × Susptrit (S) with their parents were separately cultivated under drought stress condition and normal irrigation. 112 and 89 Recombinant Inbred Lines respectively resulted from V × S and C. Capa × S were used. Susptrit was used as drought tolerant cultivar and Vada and Cebada Capa were selected as sensitive cultivars. In this experiment Chlorophyll content (CC), relative water content (RWC), shoot dry weight (SDW) and leaf temperature (LT) were measured. For each population and environment, QTL (Quantitative Trait Loci) analysis was done based on composite interval mapping on the lines separately. The results showed the effect of genotype was significant for all traits. 43 locations covering QTLs were identified in both populations and both environments. 15 QTLs were located for C. Capa × S under drought stress. The identified QTLs under drought stress were identified as three QTLs for RWC on chromosomes 4 and 5, six QTLs for chlorophyll content as two QTLs for CC-30%FC and two QTLs for CC-50%FC on chromosomes 3 and 7, two QTLs for CC-20%FC on chromosomes 4 and 7, three QTLs for SDW on chromosomes 1 and 6, and three QTLs for LT on chromosomes 4, 6, and 7. In contrast, two QTLs on chromosomes 3 and 6 were detected under normal irrigation for SDW. In addition, 13 QTLs were located for V×S under drought stress. 10 QTLs were observed for chlorophyll content as three QTLs for CC-50%FC on chromosomes 3 and 7, three QTLs for CC-30%FC on chromosomes 1, 2, and 5, four QTLs for CC-20%FC on chromosomes 2, 3 and 6. For RWC, there were identified two QTLs on chromosomes 4 and 6, while a QTL was recorded for SDW on chromosomes 6. Our study showed stable QTLs within and between populations, which it can be used in selection process by markers.
Keywords: *barley, drought stress, mapping, physiological traits, quantitative trait loci*

Introduction

Barley (*Hordeum vulgare* L.), is a major cereal grain grown in temperate climates globally and the fourth most widely grown small-grain cereal in the world (Ashida et al., 2007). It was one of the first cultivated grains, particularly in Eurasia as early as 13,000 years ago. Barley has been used as animal fodder, as a source of fermentable material for beer and certain distilled beverages, and as a component of various healthy

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foods. It is used in soups and stews, and in barley bread of various cultures. It has high flexibility to various environmental conditions especially for water deficient condition (Ceccarelli, 1987). Assessing the grain yield in areas with intensive stress is the most common method to describe the adaptation of plants under stress conditions (Blum, 1988). Different traits of plants are corresponded to drought stress, but the traits indicating the relative water capacity (RWC) can reveal useful findings for water stress shortage. RWC has been reported as a useful parameter in drought stress by Peltonen-Saino and Mäkelä (1995) and Teulat et al. (2003). Gutierrez-Rodriguez et al. (2004) found that there was a significant relationship between chlorophyll content, photosynthesis rate and yield of wheat genotypes cultivated in normal irrigation and water deficit treatments, so the chlorophyll content could be a representative of photosynthesis capacity. Although the analization of some traits could be time consuming, using DNA markers were efficient method for identify QTLs, and selection programmed by marker assisted selection (MAS). Especially in the case of drought stress by identifying certain loci for tolerating sensitive genotypes based on MAS procedure. Molecular methods make a way to produce the drought tolerant cultivars with high capacity of water uptake (Hammam Mohammed, 2004). Many gene loci controlling traits are associated with drought tolerance and they have been evaluated by relative performance of strains (Mackill, 2003). Chen et al. (2004) showed that in barley, *eibi1* gene was caused strong sensitivity to drought condition. Hammam Mohammed (2004) used a new approach based on advanced backcross in barley to identify QTLs for osmotic adjustment, relative water content, carbon isotope discrimination, dry matter yield and seed yield in dry conditions in the greenhouse. Molecular markers were useful and efficient to identifying the controlling factors associated with drought stress and introducing the improved cultivars. Seedling stage as a critical phase in plant establishment is very important in plant growth. Moreover, assessment of traits in early stages which are linked to the adult stage can facilitate early selection of desirable genotypes. Direct selection might not have adequate proficiency for improving yield under drought conditions due to low heritability, polygenic control, presence of epistasis, QTL × environment interactions (QTL × E), and complex mechanisms of drought tolerance. So, in drought tolerance improvement programs, finding molecular markers associated to chromosomal regions which control drought tolerance traits could accelerate breeding programs and raise the selection efficiency (Cattivelli et al., 2002). Determining the number of QTLs controlling quantitative traits is a crucial attempt in plant molecular breeding (Cooper et al., 2009). QTL analysis makes association between continuous variation of the phenotype and genotypic variation of any concerned loci (Collard et al., 2005). Identifying QTLs make possibilities to accomplish MAS programs (Emberini et al., 2009). Climate changes and global warming is a main challenge in present-day agriculture, which are considered as a risk factor for crop plants. Hence, further researches are necessary to obtain the appropriate varieties using molecular breeding. The present study was conducted to determining QTLs associated with some physiological traits involved in drought stress as well as estimating the effects of the QTLs in barley.

Material and methods

A total of 112 recombinant inbred lines (RILs) of Vada × Susptrit and 89 RILs of Cebad Capa × Susptrit were selected. The Susptrit was drought tolerant cultivar,

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whereas Cebada Capa and Vada were sensitive cultivars (Nguyen, 2012). Population of V × S derived from Vada × Susptrit included 112 lines, where its genetic map with 450 markers consisted of 420 amplified fragment length polymorphism (AFLP) and 24 simple sequence repeats (SSR) markers (Jafary et al., 2006) and also population of C. Capa ×S from Cebad Capa × Susptrit consisted of 89 lines with 459 markers included 481 AFLP and 14 SSR markers (Jafary et al., 2008) (*Table 1*).

Table 1. *Characteristics of two barley populations used in this study. "Gene" is related to two morphologic markers include naked vs cowered seed, and two row vs six row ear, also "other markers" indicate restricted fragment length polymorphysm (RFLP) markers*

Populations			**Markers**				
Population	**Type**	**Number of lines**	**AFLP**	**SSR**	**Gene**	**Other markers**	**Total**
V × S	RIL	112	420	24	2	4	450
CC × S	RIL	89	481	14	0	0	459

This study was performed under a completely randomized design in a greenhouse, controled by average temperature 25 °C and moisture 70% in Iran in 2016. The lines of both populations were separately cultivated in 15 × 25-cm plastic pots. Because of uniformity of the greenhouse environment and soil bedding, each experiment was conducted under a completely randomized design with two replications was. In both populations and parent cultivars, 5 sound seeds were selected and after disinfected with 70% Ethilic Alcohol, they were cultivated in plastic pots filled with field soil and sand as 1:1 v/v. The thinning led to reduce of three seedlings per pot. All pots were irrigated uniformly until 4-leaf stage. Standard Hoagland nutrient solution (*Table 2*) was used in two stages (2-leaf and 4-leaf stages) 200 ml per pot in both stages. The drought stress was imposed at the 4-leaf stage by stopping the irrigation for stress experiments, while irrigated experiments was maintained well-watered. Drought stress was applied up to 20% of field capacity (FC). In this stage all traits were measured in both experiments.

Table 2. *Chemical compounds and those concentrations consist of standard Hoagland nutrient solution, used in this study*

Chemical compound	**Chemical formula**	**Concentration (g l^{-1})**
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	3
Potassium nitrate	$K(NO_3)_2$	1.25
Magnesium sulphate	$MgSO_4$	1.1
Ammonium mono phosphate	$(NH4)_3PO_4$	0.3

Measured traits

Chlorophyll content (CC): Chlorophyll content of barley leaves was tested in drought stress during the stress period in 3 stages at 50% FC, 30% FC and 20% FC using SPAD (MINOLTA). In normal experiments, one step was measured. To measure the chlorophyll content of the leaves, the last fully developed leaves and the middle part of the leaves were used. This trait was measured in each of the 3 plants per pot, and average amount was used for further analysis.

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Relative water content (RWC): Several pieces of the last developing barley leaves were used based on Barr and Weatherley (1962). Sampling was done after 11 AM. Fresh weight of leaf samples was measured immediately. All samples were transported to the laboratory in a tank containing ice bricks. Then, the leaf samples immediately immersed in separate containers containing a sufficient amount of distilled water and were incubated at 25 °C. Turgid weight (TW) was measured after 48 h. Then, the leaf samples envelopes for 24 h in an oven at 75 °C, the dry weight (DW) of leaf samples were immediately recorded. Finally, RWC was calculated according to *Equation 1:*

$$RWC\% = \left[\frac{FW - DW}{TW - DW}\right] \times 100 \qquad \text{(Eq.1)}$$

Shoot dry weight (SDW): The shoots of three plants in per pot were harvested and dried at 75 °C for 24 h and then were weighted and average of three plants were recorded.

Leaf temperature (LT): LT was measured in populations of C. Capa × S. The infrared thermometer was used and measurements were taken on the last well developed leaves on every 3 plants in each pot and their averages were recorded.

Statistical analysis

Analysis of variance and calculation of descriptive statistics were performed using software SPSS 22.

QTL analysis

QTL analysis software was used for MapQTL 5. This analysis was performed using the automatic cofactor selection (ACS) and the method of multiple-QTL mapping (MQM). To determine LOD threshold, permutation test was used. In order to map the chromosomal software MapChart 2.2 was applied.

Results

Analysis of variance (ANOVA)

Analysis of variance showed that there was significant difference (P ≤ 0.01) between different genotypes (*Tables 3* and *4*).

Table 3. *Results of combined ANOVA of the traits in population V × S*

		MS		
Source of variations	**df**	**Leaf relative water content**	**Chlorophyll content**	**Shoot dry weight**
Environment	1	22652.96**	431.84**	1.34**
Rep/Environment	2	60.53	38.56	0.046
Genotype	111	169.79**	44.68**	0.011**
Genotype × Environment	111	119.63**	21.46**	0.005**
Error	222	17.92	6.52	0.004

Ns, *, and ** indicate no significant, significant at 5% level, and significant at 1% level, respectively.
MS: mean of squares, df: degree of freedom, Rep: replication

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Table 4. *Results of combined ANOVA of the traits in population C. Capa × S*

Source of variations	df	MS: Leaf relative water content	MS: Chlorophyll content	MS: Leaf temperature	MS: Shoot dry weight
Environment	1	26610.14**	95.01**	310.17**	1.103**
Rep/Environment	2	208.91	16.98	11.59	0.019
Genotype	88	89.41**	45.41**	3.89**	0.013**
Genotype × Environment	88	75.66**	17.53**	3.30**	0.006**
Error	176	31.95	4.32	1.50	0.003

Ns, *, and ** indicate no significant, significant at 5% level, and significant at 1% level, respectively. MS: mean of squares, df: degree of freedom, Rep: replication

The effects of the environment and genotype × environment on all traits in both crosses were significant. In addition, a significant genetic diversity between the traits was observed. Discriptive statistics of traits in parents and RILs of two populations in drought stress and irrigation experiments, were showed in *Tables 5* and *6,* respectively.

Table 5. *Descriptive statistics for traits in populations of C. Capa × S and V × S under drought stress experiments*

	Traits	Means of parents		Means of population			
		S	C. Capa	Mean	SD	Minimum	Maximum
Drought stress condition (C. Capa × S)	RWC	90.4	63.88	70.30	8.55	51.76	86.68
	CC50%FC	41	33.2	34.12	3.93	22.60	42.85
	CC30%FC	40	33.65	33.2	3.27	26.80	41.70
	CC20%FC	37	29.3	33.64	3.72	23.25	40.40
	LT	34.8	37.5	34.83	1.33	32.20	38
	SDW	0.130	0.16	0.16	0.049	0.039	0.272
	Traits	Means of parents		Means of population			
		S	V	Mean	SD	Minimum	Maximum
Drought stress condition (V × S)	RWC	90.4	82	73.28	10.8	52.7	92.9
	CC50%FC	41	37.1	39.04	3.29	30.7	46.2
	CC30%FC	40	37.6	37.65	3.29	29.40	45.5
	CC20%FC	37	35.1	38.8	4.23	25.60	47.6
	SDW	0.130	0.122	0.14	0.039	0.065	0.24

SD: standard deviation

Table 6. *Descriptive statistics for traits in populations of C. Capa × S and V × S under normal irrigation (control)*

	Traits	Means of parents		Means of population			
		S	C. Capa	Mean	SD	Minimum	Maximum
Normal irrigation condition (C. Capa × S)	RWC	96.7	88.37	87.93	3.25	81.38	96.7
	CC	37.4	34.8	34.53	4.17	22.3	44.5
	LT	34.1	34.5	32.93	1.34	29.7	37.4
	SDW	0.322	0.262	0.27	0.085	0.098	0.475
	Traits	Means of parents		Means of population			
		S	V	Mean	SD	Minimum	Maximum
Normal irrigation condition (V × S)	RWC	96.7	84.7	87.61	4.8	70.74	97.21
	CC	37.4	35.6	36.81	3.81	25	46.15
	SDW	0.322	0.186	0.25	0.08	0.057	0.43

SD: standard deviation

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QTL analysis

Analysis of QTL for physiological traits of C. Capa × S has been shown in *Table 7*.

Table 7. *Identified QTLs in C. Capa × S population under drought stress in 4-leaf stage*

Trait	Chromosome number	Position (cM)	LOD	% Exp.	Left marker	Right marker	Additive effect
RWC	4	37-44	2.88	10.7	E35M55-302#4-457	Bmag0384#4-452	3.35
RWC	5	20-53	4.01	11.4	E33M61-110-309	E33M54-313#5-374	3.52
RWC	5	126-132	3.93	14.8	E41M40-304#5-411	E42M51-68-410	3.84
CC-50%FC	3	41-53	4.07	13.5	E33M54-338-152	E42M55-329#3-126	1.52
CC-50%FC	7	43-57	3.31	11.9	E37M50-189#7-194	E35M55-181#7-203	1.39
CC-30%FC	3	41-62	3.15	14.4	E33M54-338-152	E42M55-329#3-126	1.26
CC-30%FC	7	43-57	2.5	10.3	E37M50-189#7-194	E35M55-181#7-203	1.08
CC-20%FC	4	11-26	3.54	9.9	E39M48-219-446	E42M50-245-448	1.23
CC-20%FC	7	43-57	2.89	8	E37M50-189#7-194	E35M55-181#7-203	1.14
SDW	1	0-30	2.81	7.6	E39M48-310-364	E38M61-128#1-363	0.020
SDW	6	38-57	3.65	12.7	E33M55-63-76	E35M61-269#6-67	0.022
SDW	6	145-156	3.61	10.5	E42M48-380#6-20	E38M61-197-18	0.018
LT	4	33-44	4.58	14.8	E32M61-166#4-462	Bmag0384#4-452	0.547
LT	6	75-83	2.5	9.3	E33M54-350#6-41	E45M49-212-38	0.441
LT	7	0-16	2.95	11.9	E38M55-114#7-167	E42M58-388-169	0.504
Identified QTLs in C. Capa × S population under normal irrigation (control)							
SDW	3	53-62	3.56	11.9	E42M55-329#3-126	E39M54-305-113	-0.036
SDW	6	126-141	3.7	13.3	E37M50-532-24	E32M55-102-15	-0.031

There were found a total of 15 QTLs for this population under drought stress. The percentage of phenotypic variance explained by QTLs under drought stress was observed as 7.6, 14.8 and 14.4 for SDW, RWC, LT, respectively. Phenotypic variance ranged from 8 for CC-20%FC to 14.4 for CC-30%FC. There were located 15 QTLs for C. Capa × S under drought stress on chromosomes 1, 3, 4, 5, 6 and 7. RWC had three QTLs on chromosomes 4 and 5, controlling of only 36.90% of its phenotypic variance. Also, six QTLs, as two for CC-30%FC and CC-50%FC on chromosomes 3 and 7 controlling of 25.4% and 24.7%, respectively, and two for CC-20%FC on chromosomes 4 and 7 with 17.9%. SDW covered three QTLs on chromosomes 1 and 6, which controls 31.8% of its phenotypic variance. There were identified three QTLs on chromosomes 4, 6 and 7 with 36% of phenotypic variance. For C. Capa × S under normal irrigation, two QTLs were found just for SDW on chromosomes 3 and 6 with controlling of 25.2%, where QTLs were in 53-62 and 126-141 closed to E42M55-329#3-126 and E37M50-532-24 on the left, and E39M54-305-113 and E32M55-102-15 on the right. The highest LOD in C. Capa × S was observed in CC-30%FC as 2.5, whereas the lowest LOD was recorded in LT as 4.58 in drought stress. In normal irrigating, LOD ranged from 3.56 (for SDW) to 3.7 for (SDW). Increased effect for C. Capa × S ranged from - 0.018 (for SWD) up to 3.84 (for RWC). Three QTLs on

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chromosomes 1 and 6 for SDW was observed. It has the highest control and impact by closing to E33M55-63-76 and E35M61-269#6-67. RWC with one QTL and LOD of 4.01 in 20–35 closing to E33M61-110-309 and E33M54-313#5-374 reached a 3.52-increasing effect. 1.52-increasing effect in CC-50%FC with LOD of 4.07 was recorded for chlorophyll content. Decreasing effect of -0.031 with LOD 3.70 closing to E37M50-532-24 and E32M55-102-15 was observed in SDW under normal irrigation. Allelic effect for RWC, CC-50%FC, CC-30%FC, CC-20%FC, SDW, and LT was positive. In contrast, allelic effect was negative for SDW and LT under drought stress and SDW under normal irrigation.

13 QTLs for V × S under a piece of drought stress and normal irrigation were identified (*Tables 8* and *9*).

Table 8. *Identified QTLs in V × S under drought stress in 4-leaf stage of barley*

Trait	Chromosome number	Position (cM)	LOD	% Exp.	Left marker	Right marker	Additive effect
RWC	4	41-57	4.1	12.5	P17M54-205-301	E39M61-370-298#4	4.91
RWC	6	106-124	3.34	9	E33M54-129-386	E38M54-418-203	3.60
CC-50%FC	3	42-54	5.17	15.9	E38M55-322-389#3	E33M54-446-351	1.32
CC-50%FC	3	115-136	3.39	8.9	E45M55-449-327	E39M61-204-323	1.06
CC-50%FC	7	103-132	4.06	13.2	E42M48-283-46#7	E42M55-75-52	1.31
CC-30%FC	1	52-79	2.83	7.8	E38M54-313-412	E33M54-204-82#1	0.99
CC-30%FC	2	33-38	4.8	13.4	E38M54-241-172#2	E35M48-318-186#2	1.34
CC-30%FC	5	45-63	4.68	14.3	E33M54-313-412	E42M48-128-424#5	1.29
CC-20%FC	2	1-28	4.37	9.9	E39M61-184-143	E38M54-170-154#2	1.72
CC-20%FC	3	31-50	2.61	5.6	E41M40-106-390	Bmag0136-382	1.11
CC-20%FC	3	127-136	2.89	5.9	E38M55-264-326#3	E39M61-204-323	1.19
CC-20%FC	6	64-82	4.34	10	E38M54-274-221	E41M40-288-213#6	1.76
SDW	6	85-131	3.23	12.8	E39M48-310-364	E38M61-128#1-363	0.015

Table 9. *Identified QTLs in V × S under normal irrigation in 4-leaf stage of barley*

Trait	Chromosome number	Position (cM)	LOD	% Exp.	Left marker	Right marker	Additive effect
RWC	1	95-110	3.63	10.3	E35M55-455-120	E42M55-325-127	1.89
RWC	3	37-50	4.72	13.9	E38M55-322-389#3	Bmag0136-382	2.92
RWC	3	51-96	5.24	15.4	Bmag0136-382	E39M55-150-332	3.35
RWC	4	0-15	3.79	9.4	E38M54-145-199#4	E40M32-154-318#4	1.63
CC	3	18-47	3.33	5	E45M55-168-394#3	E33M54-129-386	1.56
CC	3	50-54	3.24	5.6	E33M54-129	E32M55-102-15	1.62
CC	4	21-35	3.63	5.4	E40M32-200-317	E41M40-155-302#4	1.51
CC	4	35-50	4.16	6	E41M40-155-302#4	E38M55-82-299#4	1.53
CC	5	8-35	4.93	7.3	E45M55-57-406	P17M54-210-408	1.39
CC	5	35-45	4.99	7.4	P17M54-210-408	E33M54-313-412	1.42
CC	6	78-85	3.61	5.2	E33M61-304-214#6	E39M61-66-216	1.4
SDW	1	0-10	2.71	8.8	E37M50-401-60	E42M51-232-63	0.042
SDW	7	49-60	3.68	11.1	E36M50-316-37	E38M55-128-39#7	0.032

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Phenotypic variance explained by QTLs ranged from 5.6 (for CC-20%FC) to 15.9 (for CC-50%FC). Chlorophyll content had the maximum number of QTLs ranging from 5.6 (for C-20%FC) to 15.9 (for CC-50%FC). There was observed 13 QTLs for V×S under drought stress located on all chromosomes. 10 QTLs were identified for chlorophyll content, three were observed for CC-50%FC on chromosomes 3 and 7 explaining 38% of phenotypic variances, and three belongs to chromosomes 1, 2 and 5 with 33.3%, four on chromosomes 2, 3 and 6 with 30.4%, and two were determined on chromosomes 4 and 6 with 21.5%. SDW had a QTL on chromosome 6 covering of 12.8% of its phenotypic variance. Two QTLs were identified on chromosomes 1 and 7 explaining 19.9% of its phenotypic variance, where they were respectively in 0-10 and 49-60 closing to E37M50-401-60 and E36M50-316-37 on the left, and E42M51-232-63 and E38M55-128-39#7 on the right. The highest and lowest LOD in V × S ranged from 2.61 for CC-20%FC to 5.17 for CC-50%FC under drought stress. In contrast, LOD was in a 3.24-5.24 range under normal irrigation. Increasing effect in normal irrigation in V × S ranged from 2.91 for RWC to 3.35 for RWC. The maximum and minimum increasing effect in V × S was recorded as 4.91 and 1.43 for RWC and CC-50%FC, respectively.

One QTL was observed on chromosome 6 for SDW with LOD 3.23 closing to E39M48-310-364 and E38M61-128#1-363. On the other hand, the highest phenotypic variance for RWC was 15.4% with LOD 5.24 in the vicinity of Bmag0136-38 and E39M55-150-332 (*Table 9*). Negative allelic effect was obtained for CC-50%FC, CC-30%FC, CC-20%FC, and SDW under drought stress and also RWC and CC was under normal irrigation.

In C. Capa × S under drought stress, there were found great-effect QTLs. For example, a QTL in the vicinity of E33M61-110-309, E33M54-313#5-374, E41M40-304#5-41, E42M51-68-410 on chromosomes 5 for RWC, a QTL closing to E33M54-338-152 and E42M55-329#3-126 on chromosomes 3 for CC-50%FC and CC-30%FC, a QTL in the vicinity of E33M55-63-76 and E35M61-269#6-67 for SDW, a QTL in the vicinity of E32M61-166#4-462 and E36M50-155#4-464 on chromosome 4 for LT. Moreover, the greatest QTL was observed in the vicinity of E37M50-532-24 and E32M55-102-15 on chromosome 6. In V×S under drought stress, a QTL in the vicinity of P17M54-205-301 and E39M61-370-298#4 on chromosome 4 for RWC, some QTLs closing to E38M55-322-389#3 and E33M54-446-351 on chromosomes 3 for CC-50%FC, and a QTL in the vicinity of E38M54-241-172#2 and E35M48-318-186#2 on chromosome 2 for CC-30%FC, and a QTL in the vicinity of E39M48-310-364 and E38M61-128#1-363 on chromosome 6 for SDW. In contrast, in V×S under normal irrigation, major QTLs in the vicinity of Bmag0136-382, E39M55-150-332, E38M55-322-389#3 and Bmag0136-382 on chromosome 3 for RWC, and a great QTL neighbouring to E36M50-316-37 and E38M55-128-39#7 on chromosome 7 had the maximum impacts.

Chromosomal map, the number and the type of populations have been presented in *Figure 1*. Highlighted area on QTLs relates to significant LOD. The areas shown by continuous lines belong to start and end of LOD curve. QTLs in the linkage disequilibrium with same allelic effect had a positive correlation. In contrast, a negative correlation was observed for QTLs in the linkage disequilibrium with different allelic effects. QTLs of CC-50%FC and CC-30%FC on chromosome 3, and QTLs CC-30%FC and CC-20%FC on chromosome 7 were mapped as a linkage disequilibrium.

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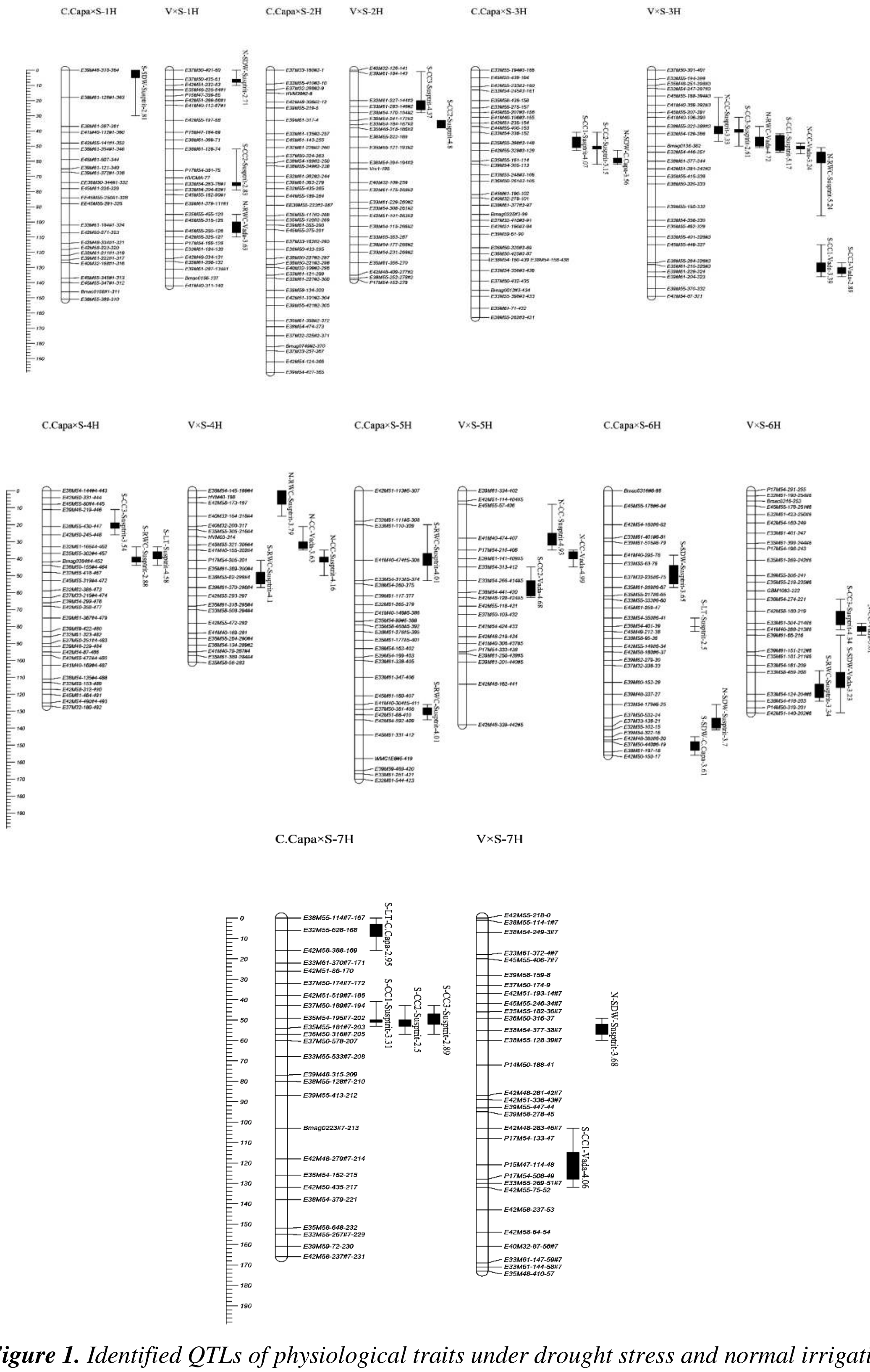

Figure 1. *Identified QTLs of physiological traits under drought stress and normal irrigation, and the number and type of the markers. Note: S and N indicate drought stress and normal irrigation, respectively. Susptrit is representative of positive direction of additive effects while C. Capa and Vada is show negative direction; the numbers relate to maximum LOD. Highlighted area is corresponded to great LOD. CC1, CC2 and CC3 indicate CC-50%FC, CC-30%FC and CC-20%FC, respectively*

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Discussion

Statistical parameters diversity of traits in both crosses and both environmental conditions (normal and stress) showed that the difference between parents for some traits were significant, shows parents in two extremes. The aggressor separation (positive genetic progress) for the best parent and line was observed. Significant genetic progress in a positive direction and negative indicated that there are increasing and decreasing alleles in parental. For assessing the stability of QTLs, the society should be investigated in different genetic and environmental conditions. In our study, most QTLs mapped to control physiological traits were stable in different conditions and crosses, a finding that agrees with previous studies (Liu et al., 2015; Srividhya et al., 2011). Finding the chromosomal areas controlling the traits is a starting point to use the QTLs in order to improve of the agricultural products. In our study, some genes showed different reactions in various environments based on different sizes and numbers of QTLs. Hence, repeating the experiments in different environments is important, because some QTLs are specialized to given environments. QTL × E was indicated as a change in the number of QTLs in different environments or their effects in different environments (Yadav et al., 2003). The QTLs have been reported under drought stress in different studies such as resistance to premature senescence in sorghum (Tuinstra et al., 1997; Crasta et al., 1999), yield in corn (Ribaut and Ragot, 2007), and RWC in barley (Teulat et al., 2001, 2003).

The overlap of traits and identified QTLs on chromosomes may be occurred. In our study, C. Capa × S and V × S were conducted in both drought and normal conditions and parent S was same in both crosses. It was observed for RWC in C. Capa × S under drought stress, and in V×S in both drought stress and normal irrigation on chromosome 4. For CC-50%FC in both V × S and C. Capa × S identified QTLs were observed on chromosomes 3 and 7. It was occurred for SDW on chromosome 6. It can be mentioned that according to mutuality of parent S in two crosses, the corresponding QTLs have overlapping areas. QTLs of linkage disequilibrium in the vicinity of each other may reveal a strong correlation between traits. For example, it was observed for RWC, CC-50%FC, and SDW. The presence of a QTL corresponds to growth in *Q7HB* and *Q7 HC* has been reported (Teulat et al., 1997a). The analysis of linkage disequilibrium QTLs can correspond to phenotypic and genotypic correlation. In addition, six QTLs for chlorophyll content in *Q4HA, Q2H, Q7HC, Q7HB, Q5HC,* and *Q4HC* were mapped in barley (Thisi et al., 2000). This trend was recorded for PHS of barley (Han et al., 2003) and yield of barley (Siahsar and Narouei, 2010). Cluster genes of different traits can make overlap QTLs. Traits controlling the yield should be determined and transfer to high-performance genotypes (Cattivelli et al., 2002). Yang et al. (2007) found a locus under drought stress and five loci under normal irrigation by studying chlorophyll fluorescence of wheat. Baum et al. (2003) identified different QTLs using RAPD and RFLP markers on chromosomes 2, 3, 6 and 7.

Stability of RWC is a significant trait in yield efficiency, which is considered as an eminent factor in tolerance to drought stress (Rezapour Fard et al., 2015). Chen et al. (2010) reported three QTLs for RWC located on chromosomes 1, 2 and 6 in barley under drought stress. 3 QTLs for RWC under early short time drought stress were recorded in barley on chromosomes 2 and 5 (Wójcik‑Jagła et al., 2013).

Castro et al. (2008) in QTL analysis of phenological traits for population deriving from *BCD47* and Baronesses showed that the parental lines of mapped population have a same growth profile. However, their offspring showed transgressive segregation. In

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the present study, there were found different QTLs whether their location was different or their allelic effect was variable. Drought stress influences physiological traits, which results in creating diversity and instability in QTLs. According to overlapping between QTLs places and mutual parent in both two crosses, we indicated that the QTLs have sufficient stability. The allelic effect in C. Capa × S for RWC, CC-50%FC, CC-30%FC, CC-20%FC, SDW and LT was positive under drought stress, whereas it was negative for SDW and LT and SDW under normal irrigation. It shows alleles which decrease SDW and LT, they increase SDW, LT, RWC and CC. A negative allelic effect was observed for CC-50%FC, CC-50%FC, CC-30%FC, CC-20%FC and SDW under drought stress and for RWC and CC under normal irrigation. It indicates the alleles which decrease some traits, they can increase others. To understand the controlling areas with more traits is derived from linkage, pleiotropic effect or cluster genes; the high density map is needed (Golshani and Fakheri, 2015). These traits can also easily stabilize through the selection in primary generations of genotypes (Ehdaei and Waines, 1989). QTLs obtained for C. Capa × S and V × S were stable in most different places. So it can be used in similar findings to select by markers. Due to the controlling of the traits by some loci with high effects, the stability of QTLs is. Stable QTLs relatively stabilize genetic controlling of traits, which cope with QTL × E.

Conclusions

Our study showed, there was a significant diversity in two different populations in terms of physiological traits of barley in normal and drought stress conditions. QTLs were distributed on all 7 chromosomes which most of them observed on chromosome 3. Several overlapping regions among QTLs were observed within and between of two populations, that can be related to common parent S in both crosses. QTLs were observed in both positive and negative direction, indicated that there are increasing and decreasing alleles in parents. Stable QTLs were identified, especially for chlorophyll content in different soil moisture conditions. Some QTLs showed different reactions in various environments based on different sizes and numbers. Hence, repeating the experiments in different environments is important, because some QTLs are specialized to unique environments. In both populations, the most QTLs, were related to chlorophyll content, that it seems the trait is more affected from drought stress in barley.

We recommend that the QTLs identified in this study, can be applied in order to marker assisted selection (MAS) and also gene pyramiding, which finally resulted in appropriate genotypes of barley under drought stress. Also chlorophyll content can be used as an important trait in QTL studies under drought stress in barley.

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THE IMPACTS OF SOCIO-ECONOMIC FACTORS ON THE PERCEPTION OF RESIDENTS ABOUT URBAN VEGETATION: A COMPARATIVE STUDY OF PLANNED VERSUS SEMI-PLANNED CITIES OF ISLAMABAD AND RAWALPINDI, PAKISTAN

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Abstract. The present study deciphers the impacts of urban planning and role of socio-economic determinants on the perception about urban vegetation. The residents inhabiting the planned (Islamabad) and the semi-planned (Rawalpindi) urban centres were the study population. Both urban areas, lying in close proximity, face rapid transformations in LULC due to urbanization. Despite their closeness, such variants as discrepancies in the standards of urban-planning and socio-economic characteristics of inhabitants make them apt study-sites. The inhabitants' perception was tapped regarding the importance of urban vegetation, temporal and spatial changes and their impacts. The majority concurred to its efficacy, a substantial proportion observed transformations in it over time while a reasonable number perceived these changes as negative and unwelcome. Such socio-economic determinants as location, education, gender, ownership status of residence and income of respondents were studied, deploying Statistical analyses (KW). Responses varied, with location and income weighing-in more heavily. Pair-wise comparison (WRST) further vindicated the results. Urbanization is sure to tarnish the environmental sustainability of both cities. Synchronized efforts from all stake-holders are a must.
Keywords: *urbanization, socio-economic, perception, urban ecology, urban ecosystem services, urban planning*

Introduction

Life on the planet Earth is dependent on constant support and productivity of Ecosystems Services (ES) (De Groot et al., 2002). The biophysical processes and ecological systems have profound effects on the natural and social systems (Pickett et al., 2001; Alberti et al., 2003; Rockström et al., 2009; Collins et al., 2011). Since the early stages of social and societal organization, the human perception regarding the role of ecological products in their lives, witnessed many transformations in response to spatial-temporal changes.

The researchers acknowledge that human perception and interactions with the ecological resources are significantly influenced by the contextual settings (Ward Thompson et al., 2005; Nasar, 2008; Jim and Shan, 2013). The nature of these interactions significantly transform with the awareness about the benefits of these resources. In present times, these benefits are acknowledged as ES, the contributions of ecological resources towards human wellbeing (Costanza et al., 1997, 2014; De Groot et

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al., 2002). The ES are grouped into four categories i.e. provisioning, regulating, supportive and cultural (Millennium Ecosystem Assessment, 2005a; Rodríguez et al., 2006; De Groot et al., 2010).

It has been opined that the paradigm of human wellbeing is dependent upon the cumulative contribution of four types of capitals in a given geographical setting. These capitals are recognized as; the natural capital (natural resources), the human capital (human resources), the built capital (physical infrastructure) and the social capital which includes the social norms and institutions (Chiesura and De Groot, 2003; Mulder et al., 2006; Vemuri and Costanza, 2006). The human, built and social capitals have reflective effects on the contributions of natural capital towards human prosperity. The previous studies observed a symbiotic relationship between vibrant ecosystems and the quality of human life (Millennium Ecosystem Assessment, 2005b; Elmqvist et al., 2013; Gómez-Baggethun and Barton, 2013; Luederitz et al., 2015).

Thus, the evaluation of ES, in a given spatial setting, demands inclusion of trans-disciplinary perspectives based upon contextual requirements. These assessments are also indispensable for postulating sustainable measures to enhance the performance of eco-capital i.e. ecological resources e.g. natural and manmade vegetative covers.

The previous studies have stressed on the evaluation of ecosystem services based upon holistic appraisal about the contextual demands (Millennium Ecosystem Assessment, 2003; Heal Geoffrey et al., 2005; Troy and Wilson, 2006). Thus, the identification of socio-economic factors are needed to ensure the sustainability of ecology and environment (Holling, 2001; Ostrom et al., 2001; Anton et al., 2010; Castro et al., 2011; Colding, 2013; Jim and Shan, 2013; Mcphearson et al., 2014; Kaczorowska et al., 2016; Sutton and Anderson, 2016). This realization is a precondition for sustainable development and warrants a coordinated research effort across the disciplinary divides. In response to these demands, the understanding of linkages between man and the natural environment have begun to gain momentum (Costanza and Folke, 1997; Egoh et al., 2007).

The socio-cultural transformations in a society have noteworthy impacts on the ecological resources and their performance. Agricultural activities have magnified the role of ecological resources in the wellbeing of human society (Goldblatt, 2013; Hannigan, 2014). Agricultural revolution also supported the phenomenon of permanent settlements. The urban centers are the culmination of these earlier settlements. These settlements are classified into three major types on the basis of their physical structure i.e planned, unplanned and semi planned urban settlements. Planned cities are built and progress according to a 'Master Plan', thus, displaying a perfect equilibrium of infrastructure for urban social life and ecological sustainability. While, the unplanned cities reflect no formal structure and design to achieve these goals. As compared to these types of urban settlements, the semi-planned urban settlements, grow haphazardly i.e. without any specific design or form but in the subsequent stages, its expansion and development might be regulated with planning and management instruments.

The 21st century is being labeled as the 'urban century' due to the alarming concentration of human population in the urban areas (Benko and Strohmayer, 2014; Nersesian, 2014). The researchers supported the notions that the proportion of global population living in the urban areas is increasing (Elmqvist et al., 2013; Nations, 2014; Luederitz et al., 2015; Green et al., 2016; Larondelle and Lauf, 2016). The uncontrolled urbanization and socio-economic transformations in the urban-based activities are held responsible for unregulated land use/ land cover changes (LULC), loss of urban

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biodiversity, weather and climatic abnormalities and compromises over urban ecological managements (Grimm et al., 2008; Mcdonald et al., 2008; Seto et al., 2012; Wamsler et al., 2013; Luederitz et al., 2015; Green et al., 2016; Kaczorowska et al., 2016; Schetke et al., 2016). Veeman and Politylo (2003) and Corburn (2017) opined that the ecological degradations in the urban areas are also accountable for rising vunerabilities among the economically deprived and socially marginalized segments of society. The pressures on urban ecological resources will intensify in magnitude and complexity (Grimm et al., 2008; United-Nations, 2014; Schetke et al., 2016). Thus, the uncontrolled urbanization and ecological deteriorations in urban areas are the real challenges of the present times (Marten, 2001; Solecki et al., 2013; Sutton and Anderson, 2016) and synchronization of these two realities is incumbent for the social, economic, ecological and environmental sustainability of urban areas (Luederitz et al., 2015). The assessment of human perception about the ecological resources in a given urban milieu is, thus, a precondition for ensuring wellbeing of urban areas (Mcintyre et al., 2008; Jim and Shan, 2013; Rapoport, 2016). The developing nations are less equipped and hence less prepared to address these challenges (Schetke et al., 2016; Jim, 2013). This lack of preparedness in the developing regions is a potent threat for their urban ecological assets and social life (Morinière, 2012; Schetke et al., 2016).

The phenomena of permanent settlements in Pakistan emerged during the phase of Indus Valley Civilization (Kenoyer et al., 2013) and these settlements were urban in structure and character. The inhabitants were acquainted with the benefits of healthy environment. The subsequent socio-economic and structural transformations in this region such as canalization of the Indus Plain (Shiva, 2016) and more economic opportunities in the big cities stimulated the rural population to migrate towards these urban areas as they were already facing a paucity of basic facilities in their native rural areas. Thus, it triggered an uncontrolled urbanization of certain regions at the cost of their ecological environment. The resulting degradation in ecological resources has added stress for the urban social life in these settlements. The occurrences of erratic weather extremities such as urban heat waves and smog in winter have become a common phenomenon of big cities. These undesirable phenomena are thought to be associated with hyperactive urbanization and urban ecological degradation. Grimm et al. (2008), Wu (2008) and Qureshi et al. (2010b) anticipated that in future the process of urbanization will more accelerate in the developing countries.

The reorientation of policy to reverse the ensuing urban environmental/ecological degradation demands scientifically-based research initiatives (Kaplan and Kaplan, 1989; Jim and Shan, 2013). The inclusion of stakeholder’s perception about the urban ecological resources in research and management initiatives is a prerequisite for ensuring ecological integrity and social wellbeing in the urban areas (Elkington, 1997; Sutton and Anderson, 2016).

Pakistan is among those countries where the research regarding urban environment is in its embryonic stages. Therefore, an increased focus on urban studies is required towards the assessments of urban environment and its ecological resources. In response to these demands, the research focusing on urban vegetative resources got impetus in Pakistan during the last decade. Most of the earlier research concerning urban vegetation was carried out in the contextual settings of the coastal city-Karachi. The studies such as Qureshi et al. (2010a, b, 2013) and Schetke et al. (2016) were designed to decipher the impacts and nature of relationship between urban social life and vegetative cover. However, the physical and human geography of Karachi is

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diametrically different from the urban settlements of Pothwar Plateau such as Islamabad and Rawalpindi.

In the similar time period, the researchers also tried to investigate the potentials of ecological resources and impacts of urbanization on the environmental sustainability of Islamabad and Rawalpindi. However, these studies such as Malik and Husain (2006), Jabeen et al. (2009) and Ali and Malik (2010a, b) were either inclined towards plant sciences are the studies such as Adeel (2010), Ali et al. (2011), Butt et al. (2015) and Hassan et al. (2016) were designed to decipher the impacts of urbanization on LULC changes.

Whereas, the evaluation of human perception about ecological resources is a requirement for ensuring integrated management of urban environment (Breuste, 2008; Qureshi and Breuste, 2010; Qureshi et al., 2010b). In this respect, it is apt to note that human interaction and perception about urban environment is significantly determined by societal perception about ecological resources, economic status, technological advancements, standards of urban planning, and management of existing vegetative covers in the urban regions.

The current study was designed to evaluate the impacts of socio-economic factors on the perception of urban residents about urban greenery in the planned (Islamabad) and semi-planned (Rawalpindi) urban settlements. The study hypothesizes that urban planning and socio-economic status of the inhabitants significantly influence the awareness about urban greenery.

Method

Study area

The study context is located between 72°55”E to 73°10”E and from 33°30” N to 33°45” N and comprises urban and peri-urban areas of twin cities, Islamabad and Rawalpindi. Islamabad owes its development to an administrative decision in 1959 (Maria and Imran, 2006) and was designed to serve as the capital city of Pakistan (Doxiadis, 1965). The green landscape of Islamabad was mainly inhabited by government employees besides some rural population in the vicinity. The older city of Rawalpindi, on the other hand, is a sprawling urban settlement with no formal design and infrastructure. It has less developed green areas and which are typically less taken care of.

In the recent times, the structural and social transformations in this region are responsible for the phenomena of rural to urban migration. The educated and resourceful migrants prefer to shift in Islamabad for better opportunities and peaceful urban social life. While, the economic, environmental and social migrants with less financial support find an abode in urban centers such as Rawalpindi. Resultantly, the density of human population in both urban centers is rapidly increasing (*Fig. 1*).

The impacts of population growth in the study area have become more visible over the period of the last ten years in the form of unregulated urban expansion. *Figure 2* depicts the spatial-temporal transformations in the LULC of the study area.

The quantitative and qualitative changes in the LULC of the study area from 2005 to 2016 have been condensed in *Figure 3* for comparison and brevity.

These urban centers are located in close proximity but their contrasting socio-ecological contextual settings and level of urban planning make it a suitable context for conducting this study of human perception. *Figure 4* indicates that the respondents were

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selected from across the study area with the intent of representing the socio-economic heterogeneities of the study population.

Data collection

The data about socio-economic characteristics of respondents and their views about urban vegetation was retrieved through questionnaire method. The questionnaire was designed for deciphering the effects of socio-economic factors on the perception of respondents about urban vegetation in planned and semi planned urban areas. For this purpose, a structured questionnaire based upon literature review and feedbacks of the pilot survey was prepared.

The questionnaire is composed of two sections. The first section was designed to collect information about the economic and demographic characteristics of the respondents. The second part of the questionnaire deals with the views of the respondents about the urban vegetative cover of the study area. The respondents were required to select an option from the given format for depicting their views (*Appendix 1*). The questionnaire with a brief introduction about the scope and significance of the study was translated in the Urdu language for clarity and convenience of the respondents.

The residents, who were living within the metropolitan limits of Islamabad and Rawalpindi for the last ten years, were the target population. In the contextual setting of Pakistan, the head of a household significantly influences the socio-economic status and orientations of the other family members. Thus, the designated head of the family by National Database and Registration Authority (NADRA) was requested to participate in the survey as a respondent.

The sub-division of the study area into neighborhoods is a reliable sampling technique for representing social, economic and ecological heterogeneities of urban areas (Dupont, 2004). The technique was relied upon and deployed. The initial respondent from each selected locality was contacted through convenience sampling method. The rest of the respondents from the same neighborhood were approached with the help of the initial respondent on the principle of the snowballing or chain-referral sampling (Etikan et al., 2016; Marcus et al., 2017).

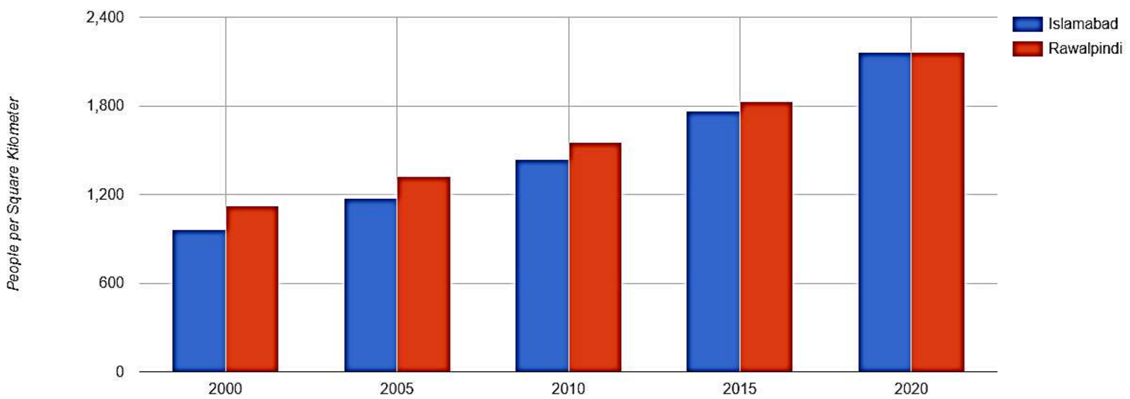

Figure 1. *Estimated and Projected population density of Islamabad/ Rawalpindi. (Source: Gridded Population of the World, Version 4. http://sedac.ciesin.columbia.edu/data/collection/gpw-v4)*

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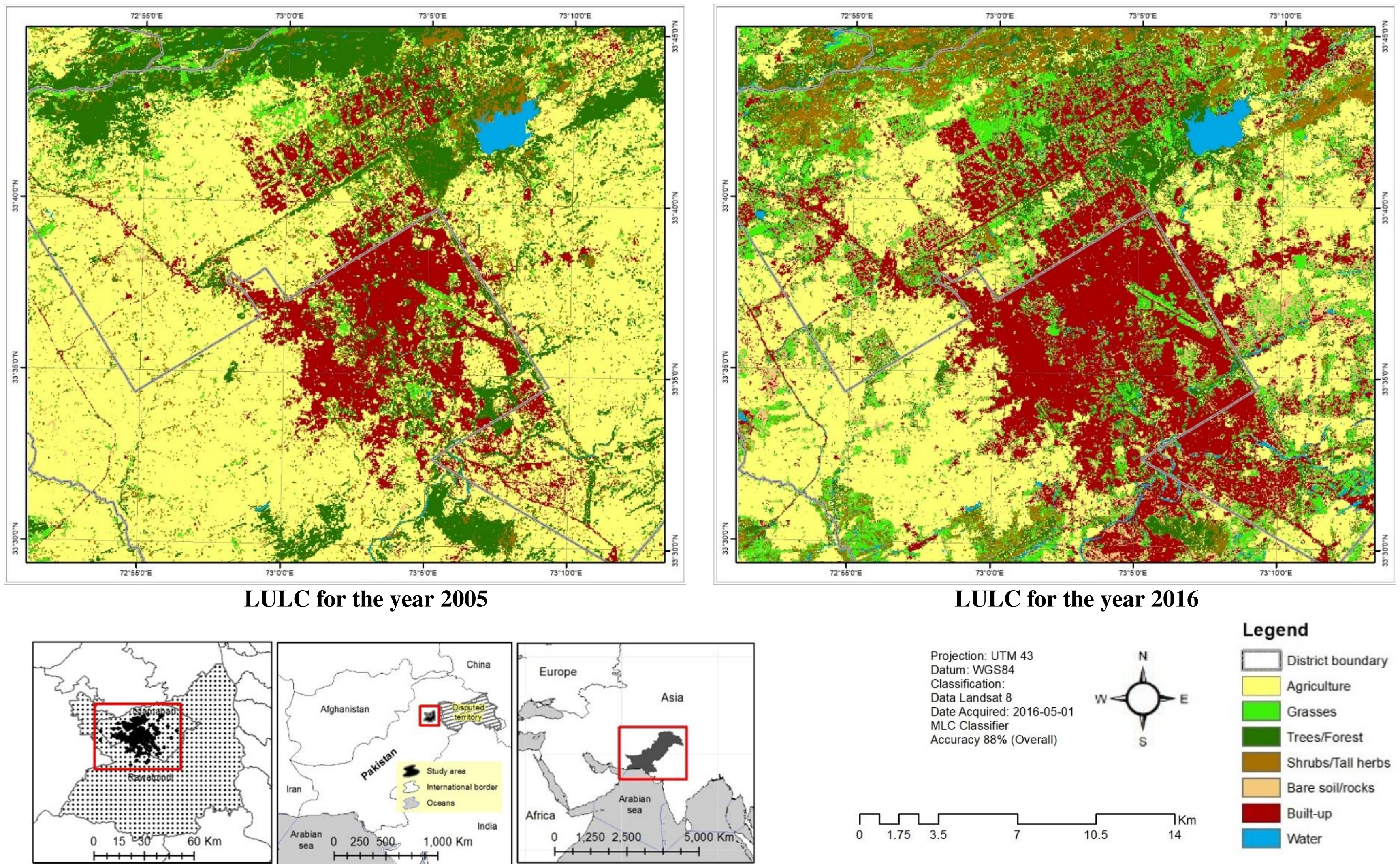

Figure 2. *Portraying the LULC of the study area for the years 2005 and 2016*

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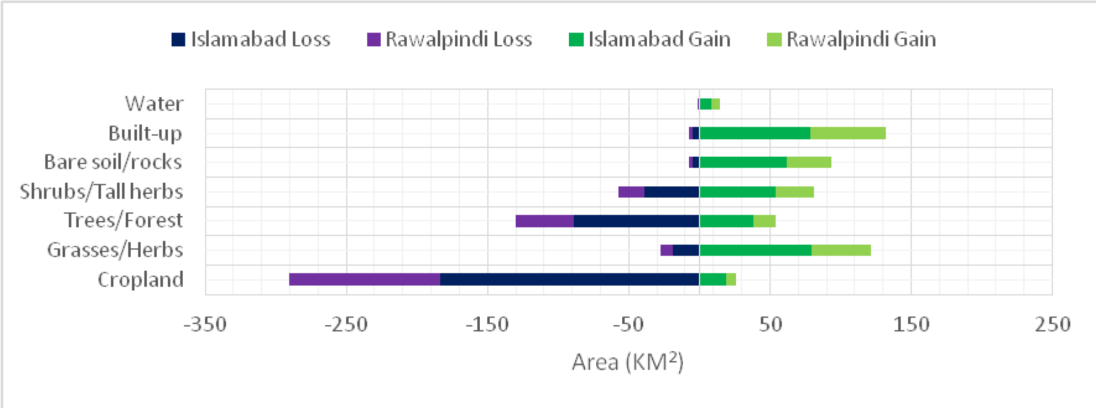

Figure 3. *Comparing the changes in LULC of the study area from 2005 to 2016*

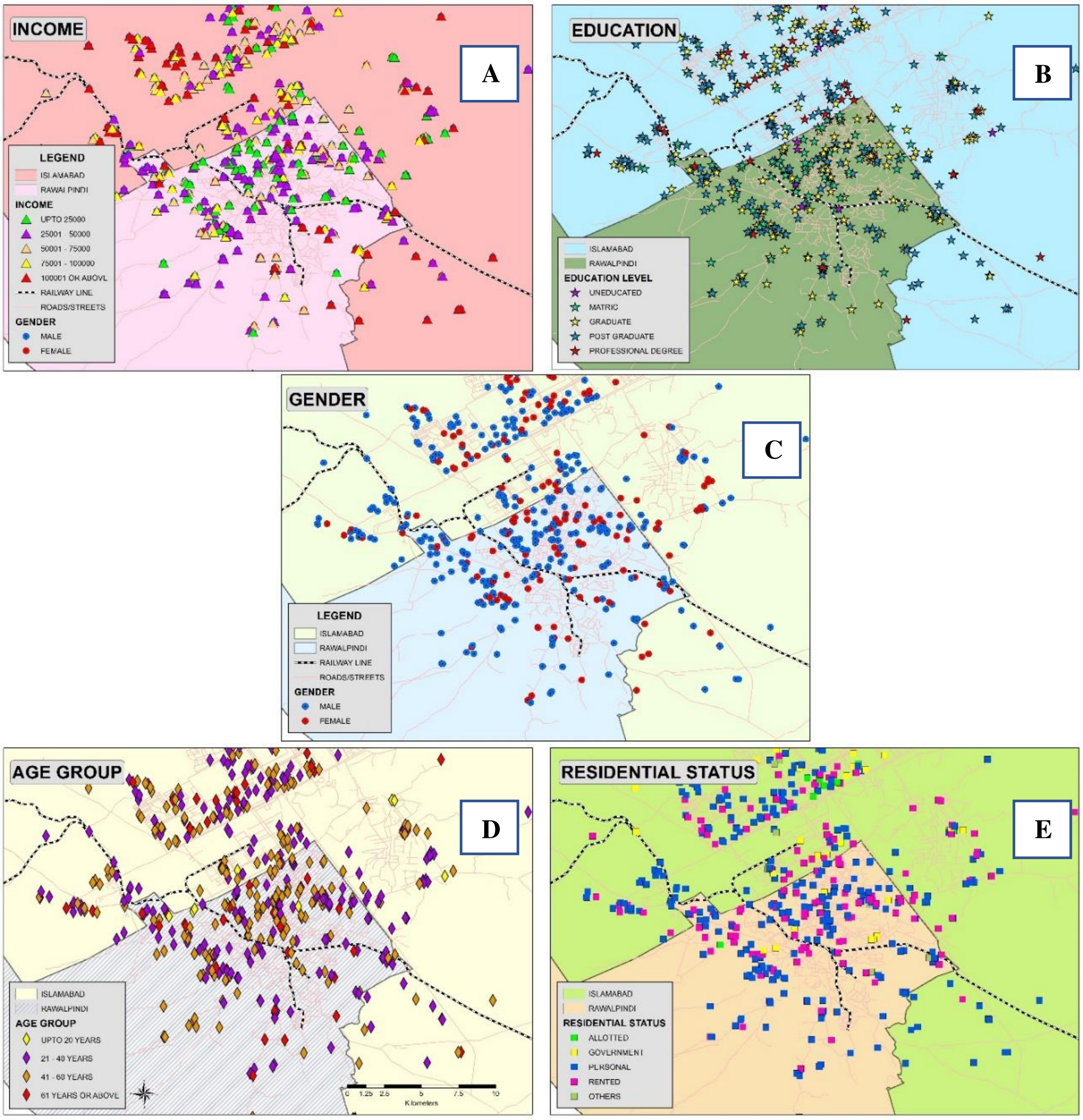

Figure 4. *Maps (A-E) illustrating the spatial distribution of respondents in the study area on the basis of income, education, gender, age and residential status.*

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Vollmer et al. (2015) stressed on the recording of the geo-coordinates for each data point as these information are helpful in portraying the fine scale heterogeneities of a study area. The geographic coordinates were noted down on the questionnaire at the residence of each potential respondent (*Appendix 2*).

The questionnaires were retrieved from the respondents after one week of delivery. On the whole, 531 questionnaires out of the distributed 800 were collected, returning an average of (66.37%). The process was concluded during the months of July & August 2016.

The questionnaires with incomplete records were discarded during the scrutiny. On the whole, 250 questionnaires each, from both urban centers were selected. The initial data entries were made by the researchers in Microsoft Excel (Version 2016) for subsequent processing and analysis in R (R Version 3.4.2) program language and Geographic Information System (GIS). A portion of this data set is being used in this study.

Data analysis

Three questions about urban vegetation were asked from the respondents. The respondents were grouped on the basis of their residential location i.e. Islamabad and Rawalpindi for inter-city comparison. In the next stage, the respondents were classified and their responses were segregated on the basis of gender, education, residential status of dwellings, monthly income, and age for evaluating the role of these predictor variables in opinion building (*Table 1*).

Table 1. *The socio-economic and demographic characteristics of respondents*

Respondents	Islamabad (%)	Rawalpindi (%)
Gender		
Male	178 (71.2%)	174 (69.6%)
Female	72 (28.8%)	76 (30.4%)
Education		
Uneducated	3 (1.2%)	7 (2.8%)
Up to matric	24 (9.6%)	60 (24%)
Graduate	70 (28%)	89 (35.6%)
Postgraduate	33 (13.2%)	9 (3.6%)
Professional	120 (48%)	85 (34%)
Residential/ownership status of dwelling		
Allotted	9 (3.6%)	2 (0.8%)
Government/official	27 (10.8%)	8 (3.2%)
Personal	137 (54.8%)	151 (60.4%)
Rented	73 (29.2%)	85 (34%)
Others	4(1.6%)	4 (1.6%)
Monthly household income (Pak Rupees)*		
Up to 25000	31 (12.4%)	57 (22.8%)
25001 to 50000	57 (22.8%)	113 (45.5%)
50001 to 75000	35 (14%)	36 (14.4%)
75001 to 100000	53 (21.2%)	27 (10.8%)
100001 and above	74 (29.6%)	17 (6.8%)
Age		
Up to 20 years	31 (12.4%)	28 (11.2%)
21 to 40 years	103 (41.2%)	121 (48.4%)
41 to 60 years	110 (44%)	97 (38.8%)
61 and above		4 (1.6%)

*One hundred and thirty six Pak. Rupees are equal to 1€ (EURO) on June 7, 2018

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The responses and attributes of the respondents were subsequently cross-tabulated for subsequent statistical analysis (*Appendix 3*).

Keeping in view the non-parametric nature of data the Kruskal-Wallis (KW) test was performed to discover the significant variations between the responses on the basis of predictor variables. In the next stage, pair -wise comparisons were carried out with the help of Wilcoxon Rank Sum Test (WRST) for those predictive variables in which the significant differences were observed in the initial KW test. The findings of WRST helped in deciphering the intra-group variations in responses. The findings were tabulated for assessments and comparisons.

Results

Perception about the usefulness of urban vegetation

The role and value of ecological contributions in a contextual setting is determined by human perception (Bixler and Floyd, 1997; Jim and Shan, 2013). The urban surroundings and socio-economic factors such as gender, education, residential status of dwellings, monthly income, and age, markedly influence the human perception about urban vegetation. The outcomes of the study (90% respondents agreed or strongly agreed; 4.8% responded "disagree or strongly disagree" while 5.20% stayed neutral) vindicate that the contributions of urban ecological resources stand acknowledged across the study area. However, the statistical findings (KW $\chi^2 = 5.90$; df = 1; $p < 0.02$) pointed out the significant differences between the responses of residents from both cities about the usefulness of urban vegetation.

The findings of KW based upon the predictor variables such as Education (KW $\chi2$ 30; df 4; $p < 0.01$), Income (KW $\chi230$; df 4; $p < 0.01$), Age (KW $\chi220$; df 3; $p < 0.01$) and Residential status (KW χ^2 10; df 4; $p < 0.02$) indicated that these predictor variables also have a significant influence on the perception of respondents regarding the usefulness of urban vegetation (*Table 2*). However, the test statistics (KW χ^2 0.3; df 1; $p > 0.05$) indicate that the Gender of respondent has a less significant role in this connection.

Table 2. *The findings of Kruskal-Wallis (KW) test based upon of socio-economic variables*

Kruskal-Wallis (KW) test and views of respondents									
	Chi square value			**Degree of freedom (df)**			**P value**		
Predictor variables	**UVBR**	**VCC**	**ICUV**	**UVBR**	**VCC**	**ICUV**	**UVBR**	**VCC**	**ICUV**
Gender	0.3	0.5	4	1	1	1	0.6	0.5	0.05
Education	30	10	4	4	4	4	0.0000006	0.05	0.4
Residential status	10	10	7	4	4	4	0.02	0.03	0.2
Income	30	20	20	4	4	4	0.000002	0.001	0.0001
Age	20	20	1	3	3	3	0.0007	0.001	0.8

Urban Vegetation is Beneficial for Residents (UVBR); Vegetation Cover Changes (VCC); Impacts of Changes in Urban Vegetation (ICUV)

The predictor variables identified in KW as responsible for significant differences were further tested for pair-wise comparison by WRST. The findings ($p < 0.05$) of

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WRST based upon educational background revealed significant differences in the responses between the lesser or uneducated and educated respondents (*Appendix 4a*). This clearly implies that the level of education has significant bearings on the human perception about the benefits of natural capital. The ownership status of the dwelling is another important socio-economic indicator and meaningfully influences the opinions of people about the benefits of urban ecology. The marked variations ($p < 0.05$) among the views of respondents residing in different categories of accommodations were also observed in the findings of WRST (*Appendix 4b*).

The age-based comparison of WRST among different age groups (*Appendix 4c*) revealed significant differences ($p < 01$) between the responses of the most senior age group (61 years and above) with all other age groups (up to 20 years; 21-40 years; 41-60 years). The significant variations in views were not found between all the other categories of age groups. The income of respondents was also observed to be an influential factor in shaping the perception of respondents about the usefulness of urban vegetation. The pair wise comparison of income based categories in (*Appendix 4d*) indicated that the two lowest income groups (Up to Rs. 25000, Rs. 25001 to 50000) have a significantly different perception about the importance of urban greenery ($p < 01$) than the respondents from three higher income categories (Rs. 50001 to 75000, Rs. 75001 to 100000, and Rs. 100001 and above).

Perception of respondents about change in urban vegetation

The respondents were enquired about the vegetation cover changes in the study area. The majority of the respondents (69.20%) observed that the vegetative cover of Islamabad and Rawalpindi is changing and 12.80% reported that they do not perceive any visible change in it. Whereas, the remaining 18% of respondents have no considered opinion about the phenomenon.

However, the test statistics (KW χ^2 5.26; df 1; $p < 0.02$) identified the significant variations in the responses of inhabitants from both urban centers regarding the changes in vegetation cover. The significant variations in the responses were also found on the basis of socio-economic factors such as Education (KW χ^2 10; df 4; $p < 0.05$), Residential status (KW χ^2 10; df 4; $p < 0.03$), Income (KW χ^2 20; df 4; $p < 0.001$) and Age (KW χ^2 20; df 3; $p < 0.001$). However, the role of Gender was found negligible in this regard (KW χ^2 0.5; df 1; $p > 0.5$) (*Table 2*).

The subsequent findings of WRST ($p < 0.05$) revealed meaningful variations among the different categories of respondents on the basis of education (*Appendix 5a*)**.** In this connection, significant differences ($p < 0.05$) were also observed in the opinions of respondents living in the rented dwellings with those who are living in government residences or in their personal abodes (*Appendix 5b*). These statistical findings infer that the ownership status of dwelling influences the opinions of people about changes in urban vegetation.

The significant differences in the opinions ($p < 0.01$) about the phenomenon were also observed in the findings of WRST between the responses of age group (61years and above) with all other ages based categories (*Appendix 5c*). While, the Income based pair wise comparison based upon WRST indicated that the lowest income group (up to Rs. 25000) had a significantly different perception ($p < 0.01$) about the changes in vegetation cover of the study area than all the other income based categories of respondents (*Appendix 5d*).

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Impacts of vegetative cover changes and respondents

The changes in the vegetative cover of the study area were negatively perceived by the majority (55.80%) of respondents. The significant differences in views regarding the impacts of these changes were also observed on the basis of residential location i.e. Islamabad or Rawalpindi (KW χ^2 7.37; df 1; $p < 0.01$) and socio-economic factors such as Gender (KW χ^2 4; df 1; $p < 0.05$) and Income (KW χ^2 20; df 4; $p < 0.01$). However, the test statistics based upon KW in (*Table 2*) depicted that the predictor variables such as Education, Residential status and Age of respondents have an ineffective influence on the views of residents in the study context.

The (KW) findings revealed that the gender of respondents had, yet, a different type of influence on perceptions regarding outcomes of change in the urban vegetative cover of the study area (*Table 2*).

The succeeding findings based upon WRST suggested that the economic status of urban residents has significant bearings on their views about the consequences of changes in vegetation cover (*Appendix 6e*). The pair wise findings of WRST based upon categories of education (*Appendix 6b*) pointed towards significant variations in perception about the impacts of changes ($p < 0.05$) between uneducated and higher educated respondents. However, such differences were found to be insignificant between uneducated and moderately educated respondents.

Discussion

The study evaluated socio-economic impacts and role of urban planning in shaping the perceptions of urban residents about ecological resources. The study was carried out in the contextual setting of Islamabad and Rawalpindi in Pakistan. The findings of the study establish that the process of urbanization is gaining momentum. The previous studies (Ali and Malik, 2010b; Ali et al., 2011; Ghafoor Chaudhry et al., 2014) returned similar conclusions. The critical findings of the study also formulate that urbanization through LULC changes is responsible for transformations in the ecology of the study area. These findings give credibility to the assertions of Ali and Malik (2010b) and Faeth et al. (2011) that urbanization causes and stimulates changes in the urban vegetation.

The majority (90%) of the respondents affirmed the positive contributions of urban ecological resources. The finding is in line with the opinions of Kaplan and Kaplan (1989) and Qureshi et al. (2010b) that urban residents acknowledge the importance of ecological contributions.

However, the findings divulge that the residential location and socio-economic characteristics of the study population are accountable for significant variations in views about the various aspects of urban vegetation. The outcomes of subsequent analysis vindicate the assertions that human perspectives about vegetative cover are significantly influenced by the level of education (Tidball and Krasny, 2011; Rupprecht and Byrne, 2014), ownership status of the inhabitant (Van Heezik et al., 2013; Shakeel and Conway, 2014), age (Lee and Maheswaran, 2011), income (Lee and Maheswaran, 2011; Majumdar et al., 2011) and gender (Gidlöf-Gunnarsson and Öhrström, 2010; Lee and Maheswaran, 2011).

The statistical findings based on empirical data validate the differences in the views of respondents from both urban centers about the transformation in vegetative cover. These variations in opinions are attributable to quantitative and qualitative differences in socio-ecological settings of both cities. The urban vegetative cover, city structure, level of urban planning and management of urban ecological resources in Islamabad and Rawalpindi are inherently different. The former urban settlement is comparatively greener, broader in structure, more planned and administered by a well-structured and resourceful organization. As compared to it, Rawalpindi is a semi planned city, a victim of compromised environmental governance and unregulated and disorderly urban expansion.

However, this acknowledgement of change in urban vegetation is not homogenous among the different socio-economic segments of the study population. The summary statistics of data illustrate significant heterogeneities among the opinions of respondents on the basis of their awareness and sensitivity. The socio-economic trajectories of respondents were found influential in shaping their perception regarding changes in urban vegetation. These observations are in line with the previous assertions (Faeth et al., 2011; Kowarik, 2011) that socio-economic factors suggestively influence the human perception about changes in urban vegetative cover.

The variations in views about the impacts of changes on the basis of gender, support the previous findings (Gidlöf-Gunnarsson and Öhrström, 2010; Lee and Maheswaran, 2011) that the gender of respondents influences the human perception about human-environment relationships. In conservative social settings of the developing world, unequal exposures between male and female, is responsible for differences in normative knowledge about urban vegetation. Thus, it offers a plausible explanation for reported dissimilarities in views.

The study also substantiates the notions of reported findings (Jim and Shan, 2013; Mcguirk, 2013) that income, a proxy variable for economic status of individuals, not only determines socio-economic standings of the individuals but also significantly influences their propensities towards ecological resources. It is the considered opinion of the authors of this study that in the present age of consumerism and knowledge based economy the role of income and education is becoming more influential in shaping the perception of respondents regarding urban ecological resources.

Conclusions

The present study evaluated the role of urban planning and socio-economic factors i.e. gender, education, residential status of dwellings, monthly income, and age, in shaping the perception of respondents about ecological resources in the study area. The findings of the study indicate that the level of urban planning, exposure to ecological resources, socio-economic and demographic characteristics of the urban population have significant bearings on their orientations towards green infrastructure of urban areas. The study also points towards the growing urbanization and rapid transformations in LULC of the study area. The resultant impacts of these intrusions may adversely impact the performance and sustainability of the urban environment in both cities. The synchronized efforts of researchers, opinion builders, policy makers, concerned institutions and proactive participation of urban residents are required for integrated management and sustainability of the urban environment. In this connection further research is needed for evaluating the orientation of spatial and temporal changes in the LULC of the study area. The use of Remote Sensing (RS) data with the help of Geographic Information System (GIS) techniques seems to be a pragmatic option for measuring these trends. The findings of this study also signify the role and importance

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of environmental management for the sustainability of urban green resources. Therefore, further investigations are also required for assessing the role and performance of institutions responsible for the environmental management of the study area. The holistic appraisal about such dimensions of environmental management are imperative for a healthy, green urban infrastructure.

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APPENDIX

Appendix 1. *Questionnaire*

Date		GPS Coordinates	
		LAT.	LONG.

1	Location of respondent	(a) Islamabad
		(b) Rawalpindi
2	Year of birth	
3	Gender	(a) Male
		(b) Female
4	Highest education level	
5	Monthly household income	
6	Do you have any knowledge about ecosystem?	Yes
		No
7	What is the ownership status of your Dwelling?	(a) Personal
		(b) Official
		(c) Rented
		(d) Allotted
		(e) any other

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8	Do you think that urban vegetative cover is beneficial for urban residents?	(a) Strongly disagree
		(b) Disagree
		(c) Agree
		(d) Strongly agree
		(e) Don't know
9	In your opinion, have the vegetative cover between your work place and home have changed in the past 10 years?	(a) Strongly disagree
		(b) Disagree
		(c) Agree
		(d) Strongly agree
		(e) Don't know
10	What is your opinion about the impacts of these changes in vegetative cover?	(a) Positive change
		(b) Negative change
		(c) Don't know

Appendix 2. *Latitude/longitude coordinates of the respondents*

Sr#	LOC	LAT	LOG	Sr#	LOC	LAT	LOG	Sr#	LOC	LAT	LOG
1	ISB	**33.536923**	**73.174316**	26	ISB	**33.639852**	**73.15182**	51	ISB	**33.672661**	**73.076228**
2	ISB	**33.536846**	**73.172665**	27	ISB	**33.686116**	**72.99567**	52	ISB	**33.667629**	**73.066207**
3	ISB	**33.63617**	**72.978908**	28	ISB	**33.658383**	**73.156036**	53	ISB	**33.649446**	**73.029046**
4	ISB	**33.607847**	**72.850839**	29	ISB	**33.674268**	**73.064078**	54	ISB	**33.64249**	**73.033104**
5	ISB	**33.530552**	**73.153841**	30	ISB	**33.725245**	**73.043077**	55	ISB	**33.664002**	**73.073039**
6	ISB	**33.674121**	**73.140556**	31	ISB	**33.695646**	**73.059183**	56	ISB	**33.628747**	**72.970096**
7	ISB	**33.672931**	**73.141064**	32	ISB	**33.711004**	**73.047149**	57	ISB	**33.635737**	**72.973216**
8	ISB	**33.67256**	**73.141703**	33	ISB	**33.663343**	**72.997037**	58	ISB	**33.637068**	**72.973638**
9	ISB	**33.671609**	**73.14178**	34	ISB	**33.680973**	**73.034108**	59	ISB	**33.630741**	**72.972535**
10	ISB	**33.671251**	**73.14158**	35	ISB	**33.689797**	**73.025659**	60	ISB	**33.647733**	**73.031731**
11	ISB	**33.727595**	**73.056729**	36	ISB	**33.700229**	**73.05811**	61	ISB	**33.634999**	**73.016013**
12	ISB	**33.702391**	**72.973088**	37	ISB	**33.736223**	**73.18053**	62	ISB	**33.701716**	**72.977988**
13	ISB	**33.726474**	**73.056701**	38	ISB	**33.714305**	**73.022111**	63	ISB	**33.626205**	**72.943854**
14	ISB	**33.696425**	**73.002886**	39	ISB	**33.67777**	**73.006601**	64	ISB	**33.679226**	**73.006626**
15	ISB	**33.718631**	**73.03959**	40	ISB	**33.70351**	**73.052849**	65	ISB	**33.68595**	**73.042524**
16	ISB	**33.714255**	**73.030288**	41	ISB	**33.706771**	**73.057767**	66	ISB	**33.671719**	**73.138991**
17	ISB	**33.714187**	**73.035895**	42	ISB	**33.670639**	**73.011922**	67	ISB	**33.67251**	**72.993287**
18	ISB	**33.709318**	**73.047175**	43	ISB	**33.659214**	**73.046819**	68	ISB	**33.656493**	**73.059137**
19	ISB	**33.712017**	**73.032955**	44	ISB	**33.669774**	**72.989071**	69	ISB	**33.669039**	**72.992042**
20	ISB	**33.657152**	**73.156284**	45	ISB	**33.645494**	**73.112561**	70	ISB	**33.694461**	**73.045402**
21	ISB	**33.654241**	**73.153504**	46	ISB	**33.721724**	**73.035479**	71	ISB	**33.694822**	**73.032598**
22	ISB	**33.641731**	**73.153008**	47	ISB	**33.699268**	**73.069618**	72	ISB	**33.697278**	**72.948379**
23	ISB	**33.641161**	**73.151337**	48	ISB	**33.569066**	**73.146967**	73	ISB	**33.647354**	**73.038753**
24	ISB	**33.640303**	**73.154155**	49	ISB	**33.722568**	**73.039925**	74	ISB	**33.655301**	**72.852578**
25	ISB	**33.738727**	**73.184161**	50	ISB	**33.672295**	**73.032692**	75	ISB	**33.670407**	**73.033975**
76	ISB	**33.680982**	**72.979134**	101	ISB	**33.710882**	**73.045141**	126	ISB	**33.707578**	**73.085324**
77	ISB	**33.69022**	**72.978645**	102	ISB	**33.720249**	**73.061964**	127	ISB	**33.708621**	**73.088182**
78	ISB	**33.620439**	**72.996606**	103	ISB	**33.657195**	**73.157919**	128	ISB	**33.708703**	**73.083634**
79	ISB	**33.691769**	**72.999643**	104	ISB	**33.686878**	**73.004887**	129	ISB	**33.706632**	**73.082528**
80	ISB	**33.670827**	**72.948749**	105	ISB	**33.718432**	**73.080917**	130	ISB	**33.706717**	**73.043412**
81	ISB	**33.695233**	**72.976793**	106	ISB	**33.733158**	**73.174369**	131	ISB	**33.710411**	**73.080945**
82	ISB	**33.539643**	**73.095454**	107	ISB	**33.664736**	**73.002125**	132	ISB	**33.700287**	**73.072771**
83	ISB	**33.570388**	**73.117425**	108	ISB	**33.672513**	**73.015797**	133	ISB	**33.698098**	**73.067043**
84	ISB	**33.618189**	**73.141211**	109	ISB	**33.665239**	**73.001066**	134	ISB	**33.710545**	**73.083518**
85	ISB	**33.618473**	**73.140682**	110	ISB	**33.685229**	**73.027236**	135	ISB	**33.702063**	**73.06734**
86	ISB	**33.668057**	**73.076849**	111	ISB	**33.710437**	**73.071021**	136	ISB	**33.700811**	**73.07123**
87	ISB	**33.646749**	**73.102839**	112	ISB	**33.70951**	**73.048247**	137	ISB	**33.705475**	**73.035411**
88	ISB	**33.658427**	**73.106216**	113	ISB	**33.682738**	**73.215079**	138	ISB	**33.495701**	**73.108808**
89	ISB	**33.64129**	**72.95239**	114	ISB	**33.692377**	**73.056488**	139	ISB	**33.71154**	**73.049321**
90	ISB	**33.70373**	**73.066209**	115	ISB	**33.62327**	**72.943758**	140	ISB	**33.708969**	**73.062294**
91	ISB	**33.670355**	**73.138939**	116	ISB	**33.695217**	**72.986642**	141	ISB	**33.557094**	**73.162729**
92	ISB	**33.671886**	**73.071259**	117	ISB	**33.673274**	**73.009671**	142	ISB	**33.556691**	**73.16306**
93	ISB	**33.671893**	**73.139966**	118	ISB	**33.679621**	**72.980374**	143	ISB	**33.623932**	**73.012788**
94	ISB	**33.679005**	**73.024874**	119	ISB	**33.698752**	**73.062879**	144	ISB	**33.643816**	**73.164535**
95	ISB	**33.724127**	**73.03138**	120	ISB	**33.626269**	**72.938704**	145	ISB	**33.690077**	**73.132412**
96	ISB	**33.631234**	**72.924653**	121	ISB	**33.668952**	**73.064887**	146	ISB	**33.689706**	**73.134008**
97	ISB	**33.611977**	**73.132363**	122	ISB	**33.695896**	**73.049999**	147	ISB	**33.619684**	**73.232303**

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152	ISB	33.624294	72.9349	177	ISB	33.624144	72.994379	202	ISB	33.694964	73.037178
153	ISB	33.622098	72.939162	178	ISB	33.671857	73.14728	203	ISB	33.694515	73.038306
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157	ISB	33.648742	73.030719	182	ISB	33.700907	72.975272	207	ISB	33.690384	73.001077
158	ISB	33.672778	73.010005	183	ISB	33.661296	73.069463	208	ISB	33.699618	73.04318
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161	ISB	33.702552	73.060185	186	ISB	33.639918	72.950984	211	ISB	33.719713	73.03381
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164	ISB	33.731958	73.089311	189	ISB	33.738999	73.176386	214	ISB	33.716911	73.035713
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173	ISB	33.663631	73.080545	198	ISB	33.648195	73.028895	223	ISB	33.741304	73.186029
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229	ISB	33.68239	73.032029	254	RWP	33.618054	73.118467	279	RWP	33.613769	73.045342
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232	ISB	33.630873	72.919074	257	RWP	33.625075	73.072464	282	RWP	33.625576	73.094389
233	ISB	33.631128	72.918588	258	RWP	33.644763	73.059759	283	RWP	33.59639	72.989813
234	ISB	33.634467	72.919605	259	RWP	33.620509	72.981786	284	RWP	33.597883	73.128872
235	ISB	33.644277	72.960679	260	RWP	33.622052	72.987597	285	RWP	33.638069	73.045929
236	ISB	33.648557	73.029628	261	RWP	33.630294	73.108549	286	RWP	33.598722	73.111448
237	ISB	33.673181	72.992671	262	RWP	33.610133	72.999132	287	RWP	33.598205	73.109388
238	ISB	33.644066	72.965343	263	RWP	33.564942	73.157254	288	RWP	33.526769	73.048802
239	ISB	33.647732	72.960076	264	RWP	33.580883	73.032126	289	RWP	33.630557	73.091877
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241	ISB	33.638862	72.955265	266	RWP	33.496187	73.109512	291	RWP	33.568446	73.062295
242	ISB	33.605555	72.965436	267	RWP	33.573501	73.110618	292	RWP	33.631717	73.092102
243	ISB	33.734351	73.076717	268	RWP	33.57349	73.112873	293	RWP	33.561403	73.070909
244	ISB	33.665402	73.066916	269	RWP	33.57244	73.112299	294	RWP	33.626592	73.09027
245	ISB	33.666143	73.069079	270	RWP	33.496669	73.110121	295	RWP	33.497421	73.047907
246	ISB	33.72704	73.048121	271	RWP	33.583832	73.017894	296	RWP	33.62862	73.085249
247	ISB	33.382902	72.583522	272	RWP	33.59176	73.129152	297	RWP	33.632333	73.086797
248	ISB	33.375526	72.59311	273	RWP	33.600465	73.05216	298	RWP	33.623945	73.100375
249	ISB	33.373609	72.585332	274	RWP	33.61797	73.117843	299	RWP	33.621574	73.106565
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301	RWP	33.612588	72.98724	326	RWP	33.616909	73.066494	351	RWP	33.625679	72.970355
302	RWP	33.606585	72.990838	327	RWP	33.618407	73.039625	352	RWP	33.628053	73.091542
303	RWP	33.553054	73.009223	328	RWP	33.618459	73.040014	353	RWP	33.583316	73.016337
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306	RWP	33.60287	73.104445	331	RWP	33.523971	73.046419	356	RWP	33.621758	72.995479
307	RWP	33.587318	73.02142	332	RWP	33.587002	73.032289	357	RWP	33.61356	73.007475
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312	RWP	33.600164	73.033664	337	RWP	33.650872	73.073036	362	RWP	33.556354	73.0123
313	RWP	33.561977	73.061278	338	RWP	33.651427	73.072265	363	RWP	33.557308	73.012867
314	RWP	33.627985	73.125366	339	RWP	33.558827	73.098715	364	RWP	33.621178	73.03776
315	RWP	33.572637	73.038673	340	RWP	33.636482	73.102621	365	RWP	33.621521	73.037754

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317	RWP	**33.544268**	**73.067471**	342	RWP	**33.621457**	**73.061821**	367	RWP	**33.597469**	**73.069398**
318	RWP	**33.486988**	**73.099908**	343	RWP	**33.607402**	**73.00942**	368	RWP	**33.597341**	**73.071352**
319	RWP	**33.58562**	**73.091711**	344	RWP	**33.584748**	**73.027598**	369	RWP	**33.600499**	**73.050567**
320	RWP	**33.608074**	**73.04495**	345	RWP	**33.585021**	**73.03487**	370	RWP	**33.62114**	**72.980886**
321	RWP	**33.591667**	**73.046588**	346	RWP	**33.614597**	**73.00681**	371	RWP	**33.582371**	**73.09762**
322	RWP	**33.616621**	**73.065852**	347	RWP	**33.550436**	**73.115782**	372	RWP	**33.583582**	**73.095847**
323	RWP	**33.616167**	**73.065788**	348	RWP	**33.530077**	**73.112428**	373	RWP	**33.641394**	**73.068788**
324	RWP	**33.596916**	**73.053857**	349	RWP	**33.596473**	**73.022209**	374	RWP	**33.638368**	**73.056437**
325	RWP	**33.616496**	**73.06608**	350	RWP	**33.596454**	**73.019341**	375	RWP	**33.635115**	**73.085288**
376	RWP	**33.544563**	**73.055324**	401	RWP	**33.624667**	**73.054338**	426	RWP	**33.557562**	**73.061322**
377	RWP	**33.625636**	**73.064122**	402	RWP	**33.652706**	**73.07189**	427	RWP	**33.589325**	**73.025251**
378	RWP	**33.642315**	**73.081253**	403	RWP	**33.607978**	**73.066514**	428	RWP	**33.628171**	**73.124221**
379	RWP	**33.551925**	**73.027701**	404	RWP	**33.625575**	**73.075722**	429	RWP	**33.596572**	**73.024144**
380	RWP	**33.552281**	**73.013677**	405	RWP	**33.628529**	**73.109527**	430	RWP	**33.596275**	**73.025553**
381	RWP	**33.634119**	**73.090047**	406	RWP	**33.582073**	**73.019416**	431	RWP	**33.594028**	**73.130073**
382	RWP	**33.594477**	**73.02448**	407	RWP	**33.603771**	**73.008261**	432	RWP	**33.621935**	**73.041589**
383	RWP	**33.5936**	**73.021246**	408	RWP	**33.626628**	**73.017598**	433	RWP	**33.586621**	**73.078158**
384	RWP	**33.651597**	**73.065626**	409	RWP	**33.62701**	**73.032636**	434	RWP	**33.605451**	**73.093072**
385	RWP	**33.627663**	**73.057469**	410	RWP	**33.622498**	**73.011807**	435	RWP	**33.598654**	**73.026315**
386	RWP	**33.652161**	**73.090715**	411	RWP	**33.626204**	**73.064411**	436	RWP	**33.605257**	**73.091759**
387	RWP	**33.65293**	**73.091171**	412	RWP	**33.594171**	**73.126695**	437	RWP	**33.58513**	**73.088427**
388	RWP	**33.496017**	**73.110314**	413	RWP	**33.59433**	**73.126932**	438	RWP	**33.614625**	**73.02527**
389	RWP	**33.49659**	**73.108891**	414	RWP	**33.637067**	**73.077261**	439	RWP	**33.596077**	**73.134311**
390	RWP	**33.63229**	**73.038046**	415	RWP	**33.593028**	**73.130417**	440	RWP	**33.626994**	**73.094579**
391	RWP	**33.633104**	**73.03795**	416	RWP	**33.637044**	**73.069514**	441	RWP	**33.616851**	**73.062575**
392	RWP	**33.633113**	**73.037213**	417	RWP	**33.590299**	**73.132788**	442	RWP	**33.522612**	**73.048165**
393	RWP	**33.568377**	**73.052454**	418	RWP	**33.629281**	**73.092291**	443	RWP	**33.474636**	**73.014316**
394	RWP	**33.567956**	**73.052714**	419	RWP	**33.628574**	**73.060012**	444	RWP	**33.583477**	**73.024615**
395	RWP	**33.633629**	**73.07574**	420	RWP	**33.598285**	**72.994212**	445	RWP	**33.62893**	**73.116726**
396	RWP	**33.616649**	**72.990835**	421	RWP	**33.552195**	**73.119747**	446	RWP	**33.651335**	**73.064304**
397	RWP	**33.617502**	**72.991409**	422	RWP	**33.607463**	**73.09511**	447	RWP	**33.635075**	**73.038575**
398	RWP	**33.618942**	**73.079751**	423	RWP	**33.599097**	**73.015775**	448	RWP	**33.634102**	**73.063326**
399	RWP	**33.58213**	**73.03863**	424	RWP	**33.629861**	**73.090781**	449	RWP	**33.620943**	**73.051603**
400	RWP	**33.581969**	**73.039268**	425	RWP	**33.654378**	**73.071851**	450	RWP	**33.634531**	**73.069006**
451	RWP	**33.622218**	**73.050947**	476	RWP	**33.615132**	**73.046074**				
452	RWP	**33.633956**	**73.069073**	477	RWP	**33.617942**	**73.039156**				
453	RWP	**33.63611**	**73.076866**	478	RWP	**33.652163**	**73.082399**				
454	RWP	**33.627316**	**72.941757**	479	RWP	**33.617326**	**73.030224**				
455	RWP	**33.61338**	**72.991244**	480	RWP	**33.605947**	**73.008537**				
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457	RWP	**33.619449**	**72.997178**	482	RWP	**33.647004**	**73.058734**				
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463	RWP	**33.628651**	**73.060912**	488	RWP	**33.611767**	**73.068567**				
464	RWP	**33.626496**	**73.035736**	489	RWP	**33.630137**	**73.062296**				
465	RWP	**33.604472**	**73.074591**	490	RWP	**33.631233**	**73.061962**				
466	RWP	**33.62491**	**73.03405**	491	RWP	**33.639587**	**73.049848**				
467	RWP	**33.596699**	**73.012665**	492	RWP	**33.621038**	**73.064693**				
468	RWP	**33.62567**	**73.031908**	493	RWP	**33.620015**	**73.077435**				
469	RWP	**33.604092**	**73.072392**	494	RWP	**33.364216**	**73.15347**				
470	RWP	**33.62645**	**73.03099**	495	RWP	**33.37426**	**73.222**				
471	RWP	**33.588147**	**73.025732**	496	RWP	**33.363432**	**73.25764**				
472	RWP	**33.627691**	**73.084004**	497	RWP	**33.3752**	**73.24996**				
473	RWP	**33.536371**	**73.079054**	498	RWP	**33.36576**	**73.4368**				
474	RWP	**33.6254**	**73.050937**	499	RWP	**33.364445**	**73.43563**				
475	RWP	**33.586415**	**73.023293**	500	RWP	**33.374917**	**73.43779**				

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Appendix 3. *The socio-economic and demographic characteristics of respondents and their predilections*

City →	City code ↓	Predictor Categories ↓	Natural capital beneficial for urban residents?					Did vegetation cover change?					Change type?		
			ST.AG	AG	NL	DA	ST.DA	ST.AG	AG	NL	DA	ST.DA	- ve	+ve	NA
City	I	Islamabad	99	131	9	7	4	38	143	40	15	14	126	82	42
	R	Rawalpindi	78	142	17	8	5	20	145	50	24	11	153	53	44
Gender	I	FEMALE	24	40	3	2	2	13	38	12	3	5	27	29	15
		MALE	75	91	6	5	2	25	105	28	12	9	99	53	27
	R	FEMALE	22	45	6	1	3	7	43	18	6	3	40	20	17
		MALE	56	97	11	7	2	13	102	32	18	8	113	33	27
Education	I	1.UE	1	1	1	0	0	1	1	1	0	0	2	1	0
		2.MT	4	16	3	0	1	3	14	4	1	2	11	6	7
		3.GR	24	39	2	3	2	9	34	13	8	6	24	33	13
		4.PG	53	61	3	2	1	22	72	17	5	4	70	33	17
		5.PD	17	14	0	2	0	3	22	5	1	2	19	9	5
	R	1.UE	0	5	1	0	1	0	3	3	1	0	3	1	3
		2.MT	8	40	5	5	2	1	35	14	8	2	35	12	13
		3.GR	32	47	7	1	2	6	56	18	5	4	55	18	16
		4.PG	35	45	4	1	0	13	44	14	9	5	54	21	10
		5.PD	3	5	0	1	0	0	7	1	1	0	6	1	2
Residential Status	I	1.ALT	6	3	0	0	0	2	3	3	1	0	5	2	2
		2.GOV	10	15	0	2	0	3	19	4	1	0	13	11	3
		3.PER	50	73	7	4	2	24	80	15	10	7	67	50	19
		4.REN	31	38	2	1	2	7	40	18	3	6	40	17	17
		5.OTH	2	2	0	0	0	2	1	0	0	1	1	2	1
	R	1.ALT	1	1	0	0	0	1	1	0	0	0	1	1	0
		2.GOV	2	4	2	0	0	0	6	2	0	0	4	1	3
		3.PER	50	91	5	3	2	9	96	27	16	3	95	32	24
		4.REN	21	46	10	5	3	9	39	21	8	8	52	16	17
		5.OTH	4	0	0	0	0	1	3	0	0	0	1	3	0
Age	I	1.A	3	2	0	1	0	1	4	1	0	0	4	2	0
		2.B	37	58	4	2	2	18	57	13	8	7	51	32	20
		3.C	53	62	5	3	2	19	71	22	6	7	67	41	17
		4.D	6	9	0	1	0	0	11	4	1	0	4	7	5
	R	1.A	1	1	3	0	0	1	1	2	0	1	3	0	2
		2.B	40	75	9	2	0	13	63	27	15	8	71	28	27
		3.C	29	53	4	4	5	5	64	16	8	2	63	20	12
		4.D	8	13	1	2	0	1	17	5	1	0	16	5	3
Monthly Incom	I	C.1	6	21	3		1	4	12	11	1	3	13	9	9
		C.2	18	33	2	4		4	40	5	4	4	9	10	38
		C.3	15	17	1	1	1	6	19	6	4		12	5	18
		C.4	28	21	2	1	1	11	30	8	2	2	16	6	31
		C.5	32	39	1	1	1	13	42	10	4	5	32	12	30
	R	C.1	10	33	6	6	2	2	28	13	10	4	14	14	29
		C.2	33	70	8		2	6	70	25	8	4	20	20	73
		C.3	12	22	1		1	6	21	5	3	1	5	5	26
		C.4	15	9	2	1		3	15	6	2	1	7	3	17
		C.5	8	8		1		3	11	1	1	1	7	2	8

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Appendix 4. *Pair-wise findings of Wilcoxon Rank Sum Test (WRST) based upon predictor variables about Urban Vegetation is Beneficial for Residents (UVBR)*

a: Education				
	Matric	**Graduate**	**Postgraduate**	**Professional**
Uneducated	0.449	0.039	0.006	0.014
	Matric	0.0005	0.0000001	0.0001
		Graduate	0.055	0.144
			Postgraduate	0.712
b: Residential Status				
	Rented	**Government**	**Allotted**	**Others**
Personal	0.24	0.78	0.04	0.02
	Rented	0.73	0.03	0.02
		Government	0.07	0.04
			Allotted	0.64

c: Age			
	up to 20 years	**21 to 40 years**	**41 to 60 years**
61years and above	0.001	0.0003	0.0002
	up to 20 years	0.452	0.67
		21 to 40 years	0.065

d: Monthly Income				
	Up to Rs.25000	**Rs.25001 to 50000**	**Rs.50001 to 75000**	**Rs.75001 to 100000**
Rs.100001 and Above	0.00001	0.015	0.378	0.374
	Up to Rs.25000	0.005	0.001	0.000003
		Rs.25001 to 50000	0.221	0.002
			Rs.50001 to 75000	0.106

Appendix 5. *Pair-wise findings of Wilcoxon Rank Sum Test (WRST) based upon predictor variables about Vegetation Cover Changes (VCC)*

a: Education				
	Matric	**Graduate**	**Postgraduate**	**Professional**
Uneducated	0.795	0.573	0.207	0.279
	Matric	0.442	0.009	0.146
		Graduate	0.029	0.381
			Postgraduate	0.554
b: Residential Status				
	Rented	**Government**	**Allotted**	**Others**
Personal	0.01	0.5	0.72	0.11
	Rented	0.05	0.32	0.06
		Government	0.98	0.15
			Allotted	0.48

c: Age			
	up to 20 years	**21 to 40 years**	**41 to 60 years**
61years and above	0.0002	0.00009	0.0002
	up to 20 years	0.9	0.6

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		21 to 40 years	0.5

d: Monthly Income

	Up to Rs.25000	Rs.25001 to 50000	Rs.50001 to 75000	Rs.75001 to 100000
Rs.100001 and Above	0.001	0.06	0.851	0.942
	Up to Rs.25000	0.012	0.003	0.001
		Rs.25001 to 50000	0.14	0.087
			Rs.50001 to 75000	0.891

Appendix 6. *Pair-wise findings of Wilcoxon Rank Sum Test (WRST) based upon predictor variables about Impacts of Changes in Urban Vegetation (ICUV)*

a: Gender

	FEMALE
MALE	0.05

b: Education

	Matric	Graduate	Postgraduate	Professional
Uneducated	0.87	0.039	0.006	0.014
	Matric	0.22	0.73	0.79
		Graduate	0.06	0.24
			Postgraduate	0.97

c: Residential Status

	Rented	Government	Allotted	Others
Personal	0.31	0.4	0.97	0.05
	Rented	0.16	0.72	0.02
		Government	0.69	0.16
			Allotted	0.15

d: Age

	up to 20 years	21 to 40 years	41 to 60 years
61years and above	0.6	1	0.8
	up to 20 years	0.4	0.7
		21 to 40 years	0.5

e: Monthly Income

	Up to Rs.25000	Rs.25001 to 50000	Rs.50001 to 75000	Rs.75001 to 100000
Rs.100001 and Above	0.327	0.00002	0.007	0.021
	Up to Rs.25000	0.0006	0.046	0.119
		Rs.25001 to 50000	0.46	0.208
			Rs.50001 to 75000	0.686

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ASSESSMENT OF PERFORMANCES OF YIELD AND FACTORS AFFECTING THE YIELD IN SOME SOYBEAN VARIETIES/LINES GROWN UNDER SEMI-ARID CLIMATE CONDITIONS

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Abstract. The aim of this study was to determine the performances of yield and factors affecting the yield of 9 soybean lines and 2 standard soybean varieties grown in the soybean production seasons of 2012 and 2013 under semi-arid climatic conditions. The results indicated that performances of yield and other characteristics of soybean varieties/lines obtained in both trial seasons were significantly different from each other. The average temperature of experimental site in the first plant growing season was higher than that of the second year. The difference in temperatures between two growing seasons adversely influenced the yield, parameters affecting the yield and some quality parameters. Negative relationship between soybean yield and the temperature, an environmental factor that cannot be controlled during the growing season of the soybean, was obtained.
Keywords: *soybean, heat stress, plant and environment interactions*

Introduction

Soybean (*Glycine max*) is an important industrial legume as human and animal feed in the world with an average of 18-22% oil, 38-56% vegetable protein in its seeds. Soybean is the most widely grown oilseed in the world as a main or second crop (USDA, 2018). The annual plant of soybean is a vertically branching plant and has pile root system that allows the use of water and nutrition in deeper soil profile (Baydar and Erbaş, 2014). Soybean that has a symbiosis with a special *Rhizobium japonicum* bacteria, is an extremely important plant in terms of improving the physical and chemical quality of soils. Soybean is consumed as soybean oil and soybean flour in human nutrition as well as in preparation of human foods such as dough products, baby foods, confectionery products, non-allergic milk and dairy products, special dietary products, artificial meat products and dry/cold instant (Öner, 2006). Soybean is used in many other sectors as in producing insecticides. Wax, soap, oil lamps, biodiesel, glue, paper raincoats and plastic materials are also produced using soybean. Soybean pulp contains 40-46% protein, 1-6% oil, 30-31% carbohydrates and 5-6% cellulose, thus is utilized as a good source of protein and fiber in livestock feeding (Singh and Shivakumar, 2010). Since soybean is extensively used in human nutrition, numerous researches have been conducted to determine the effects of soybean products on human health. The results revealed that individuals fed by soybean and soy products have a reduced risk of developing different types of cancers and soybean has a protective effect against cancers and also has positive effect in prevention of many other diseases

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(menopause, cholesterol, osteoporosis, cardiovascular and chronic diseases-coronary heart diseases) (Uesugi et al., 2013; Lovati et al., 1987; Messina, 1999; Xiao, 2008; Brouns, 2002).

Soybean is originated from China. Domestication and cultivation of soybean by humans in China dates from about the 11th century BC or a little earlier according to oldest records (Hymowitz and Shurtleff, 2005). Soybean is introduced from the ancient world to the new world in the middle of 17th century and gained its worldwide importance at the beginning of the 20th century. The arrival to Turkey coincides with World War I (Arıoğlu, 2007).

The soybean planted area in the world 2016 was estimated at 121 million hectares and soybean production was 335 million tons (www.fao.org). The consumption of soybean per person is 40.64 kg year^{-1} based on the assumption that the entire soybean production in the world is consumed by humans. But a large part of the produced soybean is used in animal feeding. Soybean is suitable for growing in both temperate and tropical climates with diverse environmental conditions (Hasanuzzaman et al., 2016). However, soybean production in Turkey is only 150 thousand tons in 34 thousand hectares. Despite the appropriate climate, soil and water resources to produce higher amounts in Turkey and the importance of soybean in human and animal nutrition as well as a raw material in industry, Turkey imports significant amount of soybean and processed soy products. Soybean is ranked 4th in Turkey among the imported agricultural products with 1,073,757 tons and ranked 3rd with 1.08674 million tons as processed soybean (www.fao.org). For this reason, studies on the cultivation and adaptation of soybean varieties are of great importance to increase the soybean production in the country.

Abiotic stress or extreme environmental conditions such as soil salinity, long term drought, low and high temperatures, nutrient deficiency or toxicity of heavy metals etc. may cause significant reduction in quality traits and yield of soybean varieties (Jumrani and Bhatia, 2018; Shah and Paulsen, 2003; Hasanuzzaman et al., 2016). Also many researchers have examined the effects of environmental factors on yield and quality. It has been shown that environmental factors significantly affect yield and quality in soybean cultivation (Piper and Boote, 1999; Thomas et al., 2003; Kumar et al., 2003; Pipolo et al., 2004). This study was carried out in semi-arid climatic conditions to determine oil and protein ratios of soybean seeds, yield and factors influencing the yield in different soybean genotypes.

Material and method

The study was conducted during the main soybean crop production season in 2012 and 2013. In the study, 2 standard soybean varieties (Ataem-7 and Türksoy) and 9 advanced soybean lines (6, 11, 17, 24, 27, 834, 1021 and 1022) were used as material. Standard varieties used as materials in the experiment were used because they conformed to the local conditions. The lines used in this study were the crossbreeds of the parents shown in *Table 1* and were obtained in 1999.

The field experiments were carried out in the Gündaş research station of GAP Agricultural Research Institute during soybean growing seasons in 2012 and 2013. The Gündaş station is located at 360 44’ 05.22 N and 380 48’ 49.76 E within the borders of Akçakale town of Sanliurfa province, Turkey (*Fig. 1*). The summers are dry and hot, and the winters are relatively warm. The average temperature in July and August is

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40 °C, while during some nights the temperature goes above 30 °C (https://mevbis.mgm.gov.tr, 2017).

Table 1. *Pedigrees of lines*

Line number	♂	♀
6	Wayne	Keller
11	Spancer	SGI 1308
13	Williams	Keller
17	Spancer	SGI 1308
24	Williams	Keller
27	Williams	Keller
834	Williams	Keller
1021	Spancer	SGI 1308
1022	Spancer	SGI 1308

Figure 1. *The area of trial*

Some physical and chemical characteristics of the soil samples taken from the experimental fields are given in *Table 2*. The experimental fields were similar in terms of soil water saturation (%), soil pH level, soil salinity (EC ds m^{-1}), calcium carbonate (%), plant available phosphorus (P_2O_5, kg ha^{-1}), potassium (K_2O, kg ha^{-1}) and soil organic matter content (*Table 2*).

Table 2. *Soil properties of trial areas*

Years	Water saturation rate (%)	pH	Ec ds/m	Lime RATE (%)	Phosphorus P_2O_5 (kg ha^{-1})	Potassium K_2O (kg ha^{-1})	Organic matter rate (%)
2012	64	7.53	0.51	26.6	56.0	2490	2.32
2013	69	7.47	0.89	24.3	51.9	3000	2.45

The meteorological data indicated that the temperatures in 2012 were generally higher than those of 2013. The average temperatures of June, July and August which are the vegetative and generative development periods of the soybean, was warmer in 2012 (*Table 3*).

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Table 3. *The climate data of 2012, 2013 and the long terms averages of Akçakale Town (21)*

Months	Years	Monthly average min. temp. (°C)	Monthly average max. temp. (°C)	Monthly average temp. (°C)	Monthly average relative humidity (%)	Monthly total precipitation (mm=kg÷m²)
April	2012	10.70	26.90	18.60	48.30	44.80
	2013	10.10	26.00	17.80	53.00	9.20
	LTA*	8.19	23.86	16.34	67.64	25.96
May	2012	14.60	29.80	22.00	49.80	43.20
	2013	15.60	31.60	23.30	47.20	43.40
	LTA	13.00	30.20	22.56	56.42	22.82
June	2012	20.80	38.20	30.10	26.00	4.60
	2013	19.60	36.80	28.70	28.90	0.20
	LTA	17.46	36.01	28.19	47.34	3.12
July	2012	23.10	40.40	32.10	26.30	0.00
	2013	21.90	39.60	31.30	27.20	0.00
	LTA	20.73	39.90	31.41	46.23	0.60
August	2012	21.90	39.80	31.10	32.70	0.00
	2013	20.90	39.40	30.40	30.60	0.00
	LTA	19.87	38.99	30.43	48.37	0.60
September	2012	17.80	36.30	26.80	37.80	3.80
	2013	16.50	33.70	25.10	38.70	0.00
	LTA	15.28	34.44	25.39	52.11	0.55
October	2012	14.10	28.40	20.80	54.80	35.20
	2013	10.00	26.90	18.40	33.50	0.40
	LTA	11.28	27.98	19.74	59.09	21.41

*Long term average (1929-2017)

The experiments were established in a randomized block design with three replicates on May 08 and May 06 in 2012 and 2013, respectively. Each plot was 6 m long and plants were grown in 4 rows with a 70-cm row and a 5-cm inter-plant (intra row) spacing. Irrigation, hoeing, and all other cultural practices have been uniformly applied to all the experimental plots to minimize the experimental error. Plants were harvested between 01 and 11, October in 2012, and 05 and 10, October in 2013. *Rhizobium japonicum* bacterial inoculation was not carried out during planting. Data were recorded for grain yield (kg ha^{-1}), plant height, first pod height, number of pods per plant, 1000-grain weight. Seed oil and protein ratios which are important quality characteristics were also determined.

The data were subjected to analyses of variance using JMP (JMP is a division of SAS that produces interactive statistical discovery software) statistic software package and average values for each of the traits were grouped according to LSD test.

Results

The mean values of properties examined for varieties/lines used as materials in the experiment and the groups obtained for the values of 2012 are presented in *Table 4*.

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Table 4. *Mean values and groups of the investigated characteristics of different soybean varieties/lines at 2012-2013 growing seasons*

Years	**Varieties /lines**	**Yield** (kg ha^{-1})**		**Plant height (cm)**		**First pod height (cm)**		**Number of pod per plant**		**Weight of 1000 grain (g)**		**Oil rate (%)**		**Protein rate (%)**
2012	**11**	2950.00	a	121.33		24.17		50.53		185.33	b	15.89		36.97
	Ataem-7	2900.50	a	125.00		23.77		55.67		165.33	bc	15.83		35.68
	13	2760.50	a	119.77		18.50		53.77		181.33	b	15.44		37.46
	834	2560.50	b	119.77		18.77		44.67		178.67	b	17.52		35.39
	27	2552.90	b	118.77		23.60		46.00		176.00	bc	18.00		34.56
	17	2500.00	b	110.27		26.33		47.87		146.67	c	17.67		36.48
	1022	2433.30	b	125.70		24.27		40.37		222.67	a	18.18		35.78
	24	2413.80	b	115.10		23.37		42.27		173.33	bc	16.18		34.73
	Türksoy	2150.00	c	121.60		28.77		42.03		172.67	bc	17.20		35.28
	1021	2145.70	c	124.77		21.60		43.53		177.33	bc	16.85		37.22
	6	2044.30	c	122.93		18.00		45.20		194.67	ab	17.36		37.00
	Average	2492.00		120.45		22.83		46.54		179.45		16.92		36.05
	CV (%)	4.54		5.81		25.01		14.41		10.10		7.09		5.70
	LSD	192.70		Ö.D.		Ö.D.		Ö.D.		30.88		Ö.D.		Ö.D.
	Varieties /lines	**Yield** (kg ha^{-1})**		**Plant height (cm)****		**First pod height (cm)***		**Number of pod per plant****		**Weight of 1000 grain****		**Oil rate****		**Protein rate (%)**
2013	**11**	4464.30	a	105.20	cd	17.47	bcd	75.47	a	185.33	c	20.80	ab	35.60
	13	4339.30	ab	114.67	ab	19.60	abc	71.27	ab	180.33	cd	18.49	c	37.43
	27	4214.30	bc	116.47	a	16.00	d	54.67	de	187.00	bc	21.21	ab	35.61
	1021	4071.40	cd	104.13	cd	19.87	abc	48.80	ef	196.00	b	22.56	a	34.30
	Ataem-7	4000.00	cd	111.20	abc	18.73	abcd	54.33	de	173.33	de	19.73	bc	37.15
	1022	3982.10	d	107.00	bcd	21.00	a	54.00	e	207.67	a	21.87	a	35.31
	834	3928.60	d	100.93	d	16.33	d	42.73	f	163.33	e	21.13	ab	35.27
	Türksoy	3928.60	d	108.60	bcd	20.00	ab	48.73	ef	185.00	c	21.90	a	35.33
	17	3875.00	de	114.47	ab	21.07	a	62.40	cd	172.67	de	20.80	ab	35.03
	6	3678.60	e	106.40	cd	17.20	bcd	54.13	e	185.67	c	21.Şub	ab	35.66
	24	2892.90	f	102.53	d	17.00	cd	64.60	bc	182.00	cd	19.46	bc	36.29
	Average	3943.20		108.32		18.57		57.38		183.49		20.82		35.73
	CV (%)	3.38		4.22		9.08		8.32		3.25		5.08		4.81
	LSD	22.70		7.79		2.87		8.13		10.15		1.80		Ö.D.

The grain yields (kg ha^{-1}) for different soybean varieties/lines were statistically significant at $p < 0.01$ level and the 1000-grain weight significant at $p < 0.05$ level. However, the values for plant height, the first pod height, the number of pods per plant, the oil and protein ratio among the different soybean varieties/lines were not statistically different. The yield of varieties / lines ranged from 2044.30 to 2950.00 kg ha^{-1}. The highest yield was obtained as 2950.00 kg ha^{-1} with the line 11, while the lowest yield was obtained as 2044.30 kg ha^{-1} with line 6. The plant height of varieties/lines varied between 110.27 and 125.70 cm. The highest plant height was

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125.70 cm with line 1022, while the lowest plant height was 110.27 cm with line 17. The first pod height values ranged from 18.00 (Türksoy variety) to 28.77 cm (line 6). The highest number was obtained from the Türksoy variety (28.77 cm) and the lowest value was obtained from the line 6 (18.00 cm). The highest value number of pods per plant was obtained from Ataem-7 as 55.67 pods/plant, and the lowest value was obtained from line 1022 as 40.37 pods/plant. The highest 1000-grain weight (222.67 g) was occurred with line 1022 and the lowest weight (146.67 g) was obtained with line 17. The oil rate values of varieties/lines varied between 15.44 and 18.18%. The highest oil ratio (18.18%) was found in line 1022, while the lowest oil ratio (15.44%) in line 13. The highest protein ratio (37.46%) was obtained with line 13 and the lowest protein ratio (34.73%) in line 24.

The mean values of properties examined and the groups obtained for varieties/lines 2012 are presented in *Table 4*. The values of grain yield (kg ha^{-1}), plant height (cm), number of pods per plant (pod $plant^{-1}$) and 1000-grain weight (g), and oil ratio (%) were significantly different ($p < 0.01$) among the different soybean varieties/lines. The difference in the first pod height was significant at $p < 0.05$ level. The differences in protein values among varieties/lines were not statistically significant in 2013. The grain yield of varieties/lines was ranged from 2892.90 (line 24) to 4464.30 kg ha^{-1}(line 11). The plant height of varieties/lines was between 100.93 cm (line 834) and 116.47 cm (line 27). The highest first pod height value (21.07 cm) was obtained for line 17, while the lowest value (16.00 cm) was occurred with line 27. Line 11 yielded the highest number of pods per plant (75.47) whereas the lowest number of pods per plant was obtained for line 834 as 42.73 pods $plant^{-1}$. The highest 1000-grain weight (207.67 g) was found with line 1022 and the lowest 1000-grain weight (163.33 g) was obtained with line 834. The oil rate among varieties/lines was ranged from 18.49% (line 13) to 22.56% (line 1021). The highest protein content was obtained with line 13 (37.43%) and the lowest was in line 1021 (34.30%).

Discussion

In the second year of the experiment, grain yields and oil ratios of all varieties/lines were higher, whereas plant lengths were lower compared to the first year of the study. The first pod height values of varieties were lower except for line 13 which was significantly higher than in the first year of the experiment. The number of pod per plant in the second season was lower in Ataem-7 variety and line 834 compared to the first growing season of the study. The 1000-grain weights of lines 13, 834, 1022 and 6 in 2012 were lower compared to the second year, while it was similar for line 11, and higher for Ataem-7 and Türksoy varieties and lines of 27, 17, 24 and 1021. The protein ratios of Türksoy and Ataem-7 varieties and 27 and 24 lines increased in the second year and decreased in other varieties/lines.

Grain yield

The average soybean yield in 2012 (249.20 kg da^{-1}) was lower than that of 2013 (394.32 kg da^{-1}). The average, maximum, minimum and total temperatures during the growing season of 2012 (June, July, August) were higher than those of 2013 (*Table 3*). Higher temperatures during the vegetative and generative periods in 2012 compared to those of 2013 resulted in lower grain yields of varieties/lines in 2012. Increasing temperature causes water stress (Jumrani and Bhatia, 2018; Pipolo et al., 2004; Shah

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and Paulsen, 2003) which negatively affects the fertilization of the flowers, and also causes flowers to fall down. Eventually, significant losses occur in grain yield of crops (Dornbos and Mullen, 1992).

Relationship between grain yield and yield components

A correlation test was conducted to determine the relationship between the grain yield and the factors affecting the yield of soybean varieties/lines (*Tables 5* and *6*). Although not statistically significant in the first year, there was a negative correlation between the grain yield and plant height, the 1000-grain weight, the oil ratio and the protein ratio, and a positive relationship between yield and the first pod height. However, a statistically significant positive correlation was found between yield and number of plant pods. In the second year, a statistically non-significant positive relationship was found between the grain yield and the plant height, the first pod height, the number of pods per plant, the 1000-grain weight, the oil ratio and the protein ratio. The positive correlations between grain yield and the first pod height and the pod number per plant revealed that increases in the first pod height and the number of plant pods resulted in considerable increases in grain yields. The height of first pod and the number of pods per plant are important factors positively affecting the yield and both have been considerably changed with variation in temperature between the experimental years (*Table 7*). Due to the higher temperatures, the first pod height was higher in the first year compared to that in the second year.

Table 5. *Correlation coefficients between the inspected features, 2012*

	Yield	Plant height	First pod height	Number of pod per plant	Weight of 1000 grain	Oil rate
Plant height	-.045					
First pod height	.101	.118				
Number of pod per plant	.499**	-.214	-.203			
Weight of 1000 grain	-.065	.093	-.094	.089		
Oil rate	-.327	-.017	.087	-.365*	-.014	
Protein rate	-.094	-.021	-.158	.227	.253	-.364*

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)

Table 6. *Correlation coefficients between the inspected features, 2013*

	Yield	Plant height	First pod height	Number of pod per plant	Weight of 1000 grain	Oil rate
Plant height	.281					
First pod height	.185	.069				
Number of pod per plant	.155	.252	-.037			
Weight of 1000 GRAİN	.095	-.284	.249	.008		
Oil rate	.076	.021	.095	-.446**	.154	
Protein rate	.051	-.389*	.144	.027	.290	-.552**

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)

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Table 7. *Correlation coefficients between two years temperature differences and the inspected features*

	Year	Yield	Plant height	First pod height	Number of pod per plant	Weight of 1000 grain	Oil rate
Yield	-.896**						
Plant height	.620**	-.511**					
First pod height	.447**	-.356**	.347**				
Number of pod per plant	-.514**	.566**	-.293*	-.324**			
Weight of 1000 grain	-.106	.097	-.092	-.073	.096		
Oil rate	-.816**	.710**	-.506**	-.323**	.216	.115	
Protein rate	.069	-.061	-.129	-.006	.047	.237	-.330**

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is significant at the 0.01 level (2-tailed)

Despite the decreasing number of pods per plant compared to the second year due to the high temperature, the grain size remained unchanged and therefore the 1000-grain weight was not significantly decreased compared to the first year. Higher temperatures in the first year caused decline in the number of grains per pod and this had a negative impact on grain yield. A negative correlation between the 1000-grain weight and the grain yield may actually be attributed to the decline in the number of grains per pod. In summary, a decrease in the number of grains per pod in the first year resulted an increase of the 1000-grain weight, while an increase in the number of the grains per pod caused a lower the 1000-grain weight. Also Ionescu et al. (2016) has reported that the relationship between the number of capsules in the plant and the 1000-grain weight is negative. Therefore, the grain yield depending on the number of the pod per plant is assumed to have a negative relationship with the weight of the 1000 grain.

Oil and protein ratio (%)

Environmental factors as well as genetic characteristics of genotypes are also effective in influencing the chemical content of soybean (Jumrani and Bhatia, 2018; Shah and Paulsen, 2003). The oil and protein ratios of the soybean are as important as the grain yield. The oil and protein ratios of both years are given in *Table 4*. The oil ratio of varieties/lines were between 15.44 and 18.18% in the first year and it was between 18.49 and 22.56% in the second year (*Table 4*). Similar to the oil ratio, the grain yield was also lower in 2012 compared to that in 2013. Differences in oil ratios of genotypes between two growing seasons are related to responses of genotypes to environmental factors. Some researchers have reported similar results (Piper and Boote, 1999 and Wang, et al., 2015). In other words, the high temperatures during the growing season in 2012 caused the oil ratio of grains to significantly decrease in parallel with the grain yield. The difference between the average protein ratios of the two years is not significant, though the protein ratio of the first year is relatively higher than that of the second year. Also, Kumar et al. (2006) has similarly found that the mean air temperature during bean development showed a significant positive correlation with protein and a negative correlation with oil. The results revealed that the protein ratio of the grain does not react to the temperature as in the grain yield and the oil ratio of the grain (*Table 7*). The correlation test showed that the oil and protein ratios of the soybean grains had significant negative relationships ($p < 0.05$ and $p < 0.01$ in the first

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and second years, respectively). Similar to our findings, Johnson et al. (1955) also reported a significant negative relationship between oil and protein rates of soybean grains.

Conclusion

A two-year field experiment (2012-2013) was conducted to determine the performances of two standard soybean varieties and 11 soybean lines under semi-arid climate conditions by evaluating the yield, factors affecting the yield and some quality traits. Performance of yield and other characteristics of soybean varieties/lines differed between two growing seasons. The average temperature of the study area during the growing season in the first year was higher than that of the second year. The higher temperature in the first growing season had a negative impact on the yield, the factors affecting the soybean yield and the oil ratio of the soybean grain. A negative relationship between oil ratio and protein ratio of the soybean grains is one of the most important findings of the study. In conclusion, the yields of soybean varieties/genotypes have been negatively affected by the extreme temperature, one of the most important environmental factors.

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SALT-INDUCED VARIATION OF INORGANIC NUTRIENTS, ANTIOXIDANT ENZYMES, LEAF PROLINE AND MALON-DIALDEHYDE (MDA) CONTENT IN CANOLA (*BRASSICA NAPUS* L.)

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Abstract. Present experiment was performed to determine the effect of salinity stress on canola (*Brassica napus* L.) using the parameters of inorganic nutrients, malondialdehyde (MDA) and leaf proline content and the activity of antioxidant enzymes. Four canola cultivars viz., Oscar, Ac Excel, Cyclone and Dunkled and two levels of salt (0 mmol/L NaCl, 120 mmol/L NaCl) were used. Root fresh and dry weight of four canola cultivars markedly declined due to salinity stress. Salt stress significantly increased leaf proline content in four canola cultivars and higher values were recorded in cultivar Dunkled and Ac Excel than those in Cyclone and Oscar. Salt stress significantly enhanced antioxidant enzyme activities including SOD, POD and CAT in all canola cultivars. SOD showed highest value in Cyclone and Dunkled, POD in Ac Excel, Cyclone and Dunkled while CAT in Ac Excel and Dunkled. Malondialdehyde (MDA) showed variable response in all canola cultivars under saline conditions. Shoot and root (Na^+, Cl^-, K^+, and Ca^{2+}) were significantly affected under salt regimes. Salt stress increased Na^+ content in root and shoot in canola cultivars. High shoot Na^+ was found in Oscar and Cyclone, root Na^+ in Oscar, Ac Excel and Cyclone. Value of K^+ and Ca^{2+} in shoot and root was markedly reduced under saline conditions. It is concluded that high salt tolerance of canola cultivar "Dunkled" could be accredited due to exclusion of Na^+ and accumulation of K^+ ions, enhanced leaf free proline content and also antioxidant enzyme CAT activity.
Keywords: *oilseed crop, salt stress, enzyme assay, biomass production, Na^+ and K^+*

Introduction

Stresses including biotic and abiotic limits life forms all over the world. Abiotic stresses such as drought, salinity, waterlogging, low light intensity, and extreme in temperature inhibit plant growth and development like biomass production. Overall, agriculture productivity markedly reduces by salinity stress (Khodary, 2004; Ashrafuzzaman et al., 2002). Salinity, as a major abiotic stress in crop species, disrupts homeostasis, water potential, ion distribution and induces inhibition of growth and oxidative changes as a secondary stress. Formation and accumulation of reactive oxygen species (ROS) can be induced by salinity stress (Erdal et al., 2011). Reactive oxygen species include singlet oxygen, hydroxyl radicals and hydrogen peroxide which destroy mitochondria and DNA. Enzymatic as well as non-enzymatic antioxidant defence mechanisms reduce the accumulation of reactive oxygen species by detoxifying free radicals (Siddiqi et al., 2011). The activities of antioxidant enzymes, such as catalase, peroxidase, superoxide dismutase and ascorbate have a key role in the removal of reactive oxygen species (Anderson et al., 1995).

Canola (*Brassica napus* L.) is a moderately salt tolerant oilseed crop which is mostly cultivated for edible oil purpose (Francois, 1994). In literature, it is widely reported that requirements of canola oil as oilseed crop has increased since the last four decades due to

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increased awareness about health benefits of canola. Attention increased to cultivate canola in soils where salinity issues already exist (Ashraf and McNeilly, 2004). To improve salt tolerant crop, it is necessary to increase the productivity of saline soils, different approaches have been proposed by various plant scientists (Ashraf et al., 2008).

Major objectives of the present research work were to explore the response of canola cultivars to salt stress through their antioxidant enzymes, leaf proline and nutrients content at vegetative growth stage.

Materials and methods

Objective of the present investigation were to determine the effect of salt stress on canola (*Brassica napus* L.) cultivars. Pot experiment was performed in the Botanical Garden, Department of Botany, University of Gujrat, Pakistan. Seeds of four canola cultivars were acquired from Ayub Agriculture Research Institute (AARI), Faisalabad, Pakistan. Seeds were treated using sodium hypochlorite (5% NaClO) solution for surface sterilization for 5 min. Seeds were rinsed with double distilled water after the process of sterilization. Plastic pots (24 cm diameter) were filled with 10 kg fresh river washed sand. Eight (8) healthy seeds were sown in each plastic pot. Each pot was irrigated with Hoagland nutrition (2 L) solution. Thinning was done to maintain five plants in each pot after ten days. Treatment of the experiment was control (0 mmol/L NaCl) and salt stress (120 mmol/L NaCl). After twenty days of initiation seed germination, salt treatment was started with full strength Hoaglands nutrient solution. Objective of the Hoagland nutrient solution application to plants is to maintain mineral nutrients requirements. Step wise salt treatment was given by adding 40 mmol/L NaCl on a daily basis to maintain final salinity level. Also, distilled water (250 ml) was applied on a daily basis to each pot to avoid evaporation loss. Data of the followings attributes were noted after the start of seven weeks of salt treatment.

Biomass production

One plant from each replicate was removed and separated into their respective shoot and root. Samples of shoot and root were oven dried at 65 °C for five (05) days and their dry weight was recorded by using an analytical balance.

Malondialdehyde (MDA)

Method of Carmak and Horst (1991) was followed for the determination of leaf malondialdehyde (MDA) contents. Fresh leaf sample (0.5 g) was grinded, trichloroacetic acid (10 ml) was added and then that mixture was centrifuged for 10 min at 12,000 × g. Four ml of 0.5% thiobarbituric acid prepared in 20% trichloroacetic acid was added in 1 ml of the supernatant. After that reaction mixture was placed in water bath at 90 °C for three min. These samples were again centrifuged for 10 min at 12,000 × g. Absorbance of the samples was noted at two wavelengths 532 and 600 nm with a spectrophotometer (U2020 IRMECO).

Antioxidant enzyme activities

Fresh leaf sample (0.5 g) was grinded in 0.5 ml phosphate buffer for the measurement of antioxidant enzyme activities. Homogenate was centrifuged at 15,000 × g for 15 min at 4 °C. Values of superoxide dismutase, peroxidase and catalase was determined using the supernatant. Superoxide dismutase activity was determined by enzymatic photoreduction of

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nitroblue tetrazolium (NBT) following the method of Giannopolitis and Ries (1977). Reaction mixture of 3 ml contained methionine (13 mmol/L), riboflavin (1.3 µmol L^{-1}), phosphate buffer and NBT (50 µmol L^{-1}). In test tube 20–50 µL enzyme extract was homogenized. Under white fluorescent light, solution mixture was illuminated. Catalase and peroxidase activities were determined using the method of Chance and Maehly, 1995. Solution mixture was prepared containing guaiacol (20 mmol/L), hydrogen peroxide (40 mmol/L), phosphate buffer (50 mmol/L phosphate) and enzyme extract (0.1 ml). Catalase activity was determined using hydrogen peroxide (5.9 mmol/L), phosphate buffer (50 mmol/L) and 0.1 ml enzyme extract. To determine catalase activity, enzyme extract was mixed with reaction mixture and absorbance was recorded at 240 nm after every 20 sec. Concentration of protein was determined using the method of Bradford (1976).

Determination of free proline

Method of Bates et al. (1973) with some amendments was used for the measurement of leaf proline content. 0.5 g of fresh leaf sample was grinded and extracted using sulfosalicylic acid (3%). Filtrate of the reaction mixture was obtained using filter paper (Whatman No. 2). Two ml of filtrate was added in the test tube with 2 ml of acidic ninhydrin solution and 2 ml of glacial acetic acid and further homogenized. Then it was heated in water bath at 75 °C for 60 min. After that mixture was placed in ice water bath for the termination of reaction. Toluene (4 ml) was added to the reaction mixture to isolate proline as supernatant. Absorbance of the proline supernatant was recorded at 520 nm using a spectrophotometer. Only toluene was used as a blank.

Nutrient analysis

Method of Wolf (1982) was followed for the determination of different nutrient contents. Dried sample (0.1 g) was grinded and digested in H_2SO_4 and H_2O_2 for the determination of nutrients (Na^+, K^+ and Ca^{2+}). Sulphuric acid (2.5 ml) was added in the digestion tube and further tubes were incubated overnight at room temperature. Then hydrogen peroxide (1 ml) was added to each digestion tube. Tubes was set in digestion block and heated to 350 °C until fumes were produced. Heating continued for a further 30 min and after that digestion tubes were removed from the digestion block. Appearance of colorless material in the digestion tube is the indication of completed digestion. Using deionized water, volume of the digestion material was made to 50 ml. Values of Na^+, K^+ and Ca^{2+} were determined with a flame photometer (PFP-7 ELE, Jenway Instrument Co. Ltd, Stone, UK).

Experimental design and statistical analysis

Experiment was set as a completely randomized design (CRD) with four replicates. Two factorial experiments consist of two levels of salt treatment and four canola cultivar. The data obtained were analyzed statistically by using Analysis of Variance (ANOVA) technique using the MSTAT- C computer package (MSTAT Development Team, 1989).

Results

Growth parameter

Salinity in the rooting growth medium markedly reduced root fresh and dry weight in four canola cultivars. A notable variation was recorded in this set. Maximum value of

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root fresh weight was found in cultivar “Oscar and Dunkled” and minimum in “Ac excel and Cyclone” while root dry weight showed highest value in “Dunkled” as compared to all other canola cultivar under saline regimes (*Fig. 1*). Analysis of variance of the data showed that salt stress significantly reduced the root fresh and dry weight of four canola cultivars and a clear variable response was recorded (*Table 1*).

Leaf proline and MDA content

Addition of salinity in the root zone significantly increased leaf proline content in all canola cultivar. A significant variation was found in this regard. Highest value of leaf proline was found in “Ac excel and Dunkled” as compared to other cultivar under salt regimes (*Fig. 1*).

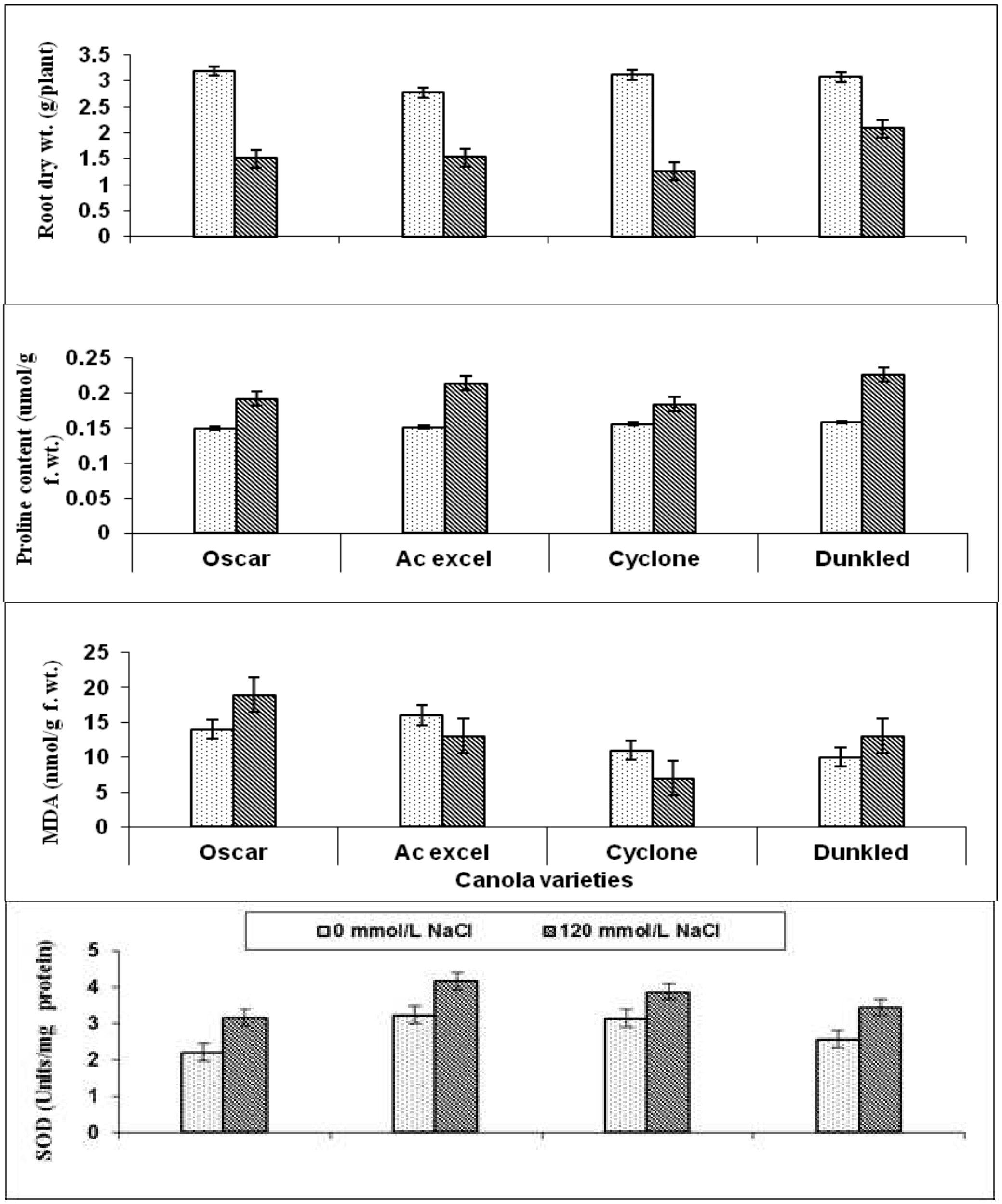

Figure 1. *Fresh and dry weight of root, leaf proline and malondialdehyde (MDA) content of four canola cultivars when treated to salinity stress for 48 days*

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Addition of salt to rooting growth medium showed variable response in malondialdehyde (MDA) values in four canola cultivars. Cultivar "Oscar and Dunkled" showed increased while Ac excel and Cyclone showed decreased values of MDA under saline conditions (*Fig. 1*). Analysis of variance of the data showed that salt level and cultivars showed significant result in the set of leaf proline and MDA content (*Table 1*).

Nutrient analysis

Salinity in the growth medium increased Na^+ content in shoot and root of four canola cultivars. A variable response was noted in this attribute. Cultivar "Oscar and Cyclone" showed higher values in shoot Na^+ content while Ac Excel, Oscar and Cyclone in root Na^+ under saline conditions (*Fig. 2*).

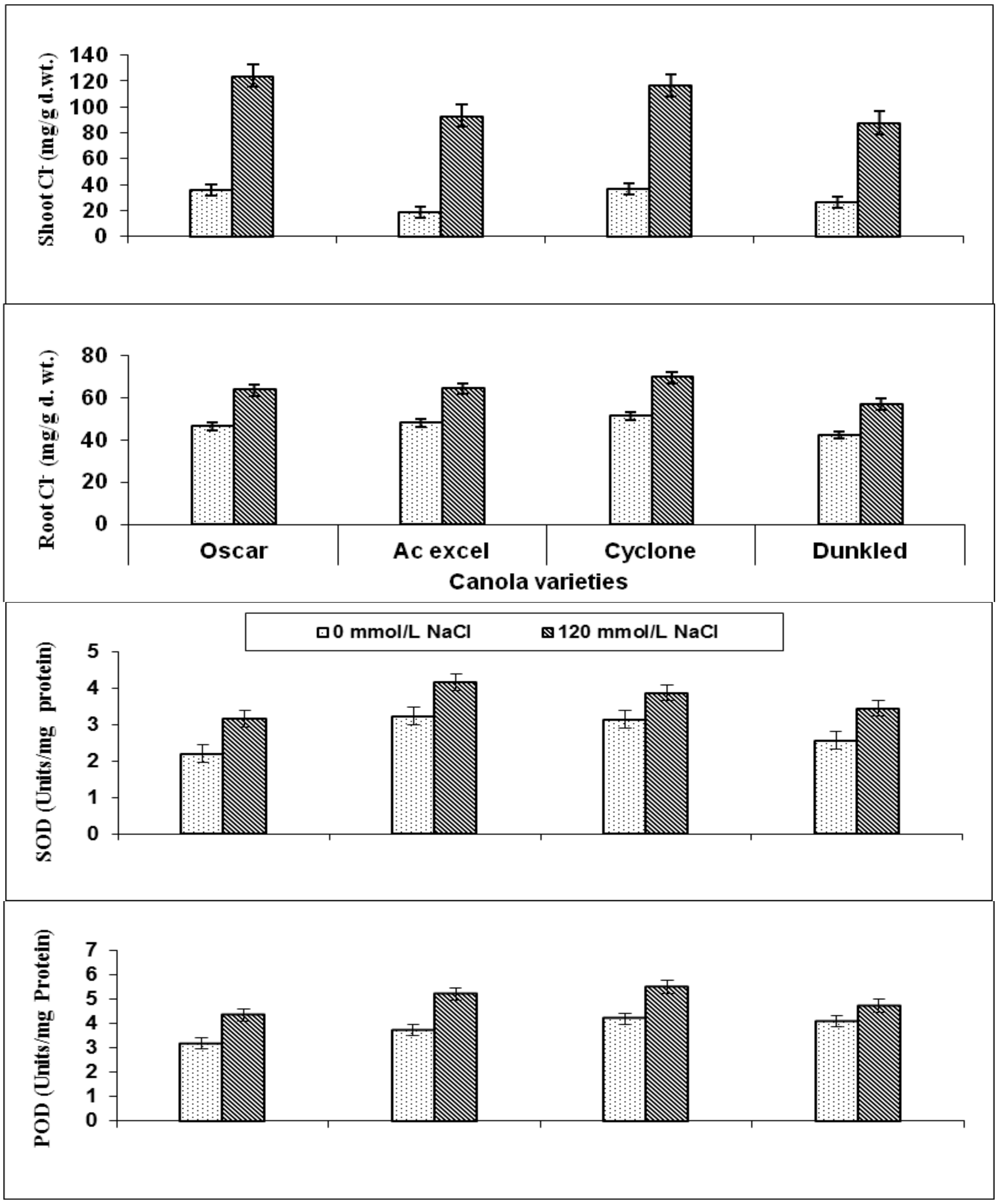

Figure 2. *Shoot and root Na^+ and Cl^- of four canola cultivars when treated to salt stress for 48 days.*

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Addition of salinity in the rooting medium highly increased the values of root and shoot chloride (Cl^-) in four canola cultivars. A clear variation was found in this attribute. Higher conc. of chloride was found in the shoot of Oscar and Cyclone and root of Oscar, Ac Excel and Cyclone than those of other cultivars as compared to control (*Fig. 2*).

Increasing salinity in the rooting medium significantly decreased values of K^+ in four canola cultivars. A variable response was found in this set of parameter. Less reduction of shoot K^+ was found in Ac excel and Cyclone while root K^+ in Oscar, Ac excel and Cylone under saline conditions (*Fig. 3*). Salt stress significantly decreased Ca^{2+} in shoot and root in all canola cultivars. Less variation was observed in shoot Ca^{2+}. Less reduction in shoot Ca^{2+} was noted in Cyclone while in root Ca^{2+} in Oscar and Cyclone under salt regimes (*Fig. 3*). Analysis of variance of the data showed that salt level and cultivars showed significant result in the parameters of shoot and root Na+, Cl^-, K^+ and Ca^{2+} (*Tables 2* and *3*).

Table 1. *Mean squares from ANOVA of the data for root fresh and dry weight, leaf proline and MDA content of four canola (Brassica napus L.) cultivars when subjected to salt-stress and non-stress conditions*

SOV	d.f.	Root fresh weight		Root dry weight		Leaf proline		MDA	
		F	P	F	P	F	P	F	P
Salt level	1	643.793	.000***	235.715	.0000***	95.713	.0000***	29.779	.0000***
Cultivar	3	12.194	.000***	4.355	.0111*	4.266	.012*	.003**	.003**
Salt level × cultivar	3	12.113	.000***	4.363	.0110*	3.248	.0345*	.786 ns	.786 ns
Error	32								

Table 2. *Mean squares from ANOVA of the data for shoot and root Na^+ and Cl^- content of four canola (Brassica napus L.) cultivars when subjected to salt-stress and non-stress conditions*

SOV	d.f.	Shoot Na^+		Root Na^+		Shoot Cl^-		Root Cl^-	
		F	P	F	P	F	P	F	P
Salt level	1	116974.540	.0000***	2961.794	.0000***	116974.540	.0000***	691.538	.0000***
Cultivar	3	4918.852	.0000***	213.738	.0000***	4918.852	.0000***	43.601	.0000***
Salt level × cultivar	3	3992.863	.0000***	7.363	.0007***	3992.863	.0000***	4.373	.0109*
Error	32								

Table 3. *Mean squares from ANOVA of the data for shoot and root K^+ and Ca^{2+} content of four canola (Brassica napus L.) cultivars when subjected to salt-stress and non-stress conditions*

SOV	d.f.	Shoot K^+		Root K^+		Shoot Ca^{2+}		Root Ca^{2+}	
		F	P	F	P	F	P	F	P
Salt level	1	472.804	.0000***	593.249	.0000***	4921.891	.0000***	365.492	.0000***
Cultivar	3	49.435	.0000***	166.376	.0000***	58.012	.0000***	22.676	.0000***
Salt level × cultivar	3	26.375	.0000***	21.658	.0007***	99.384	.0000***	5.406	.0040**
Error	32								

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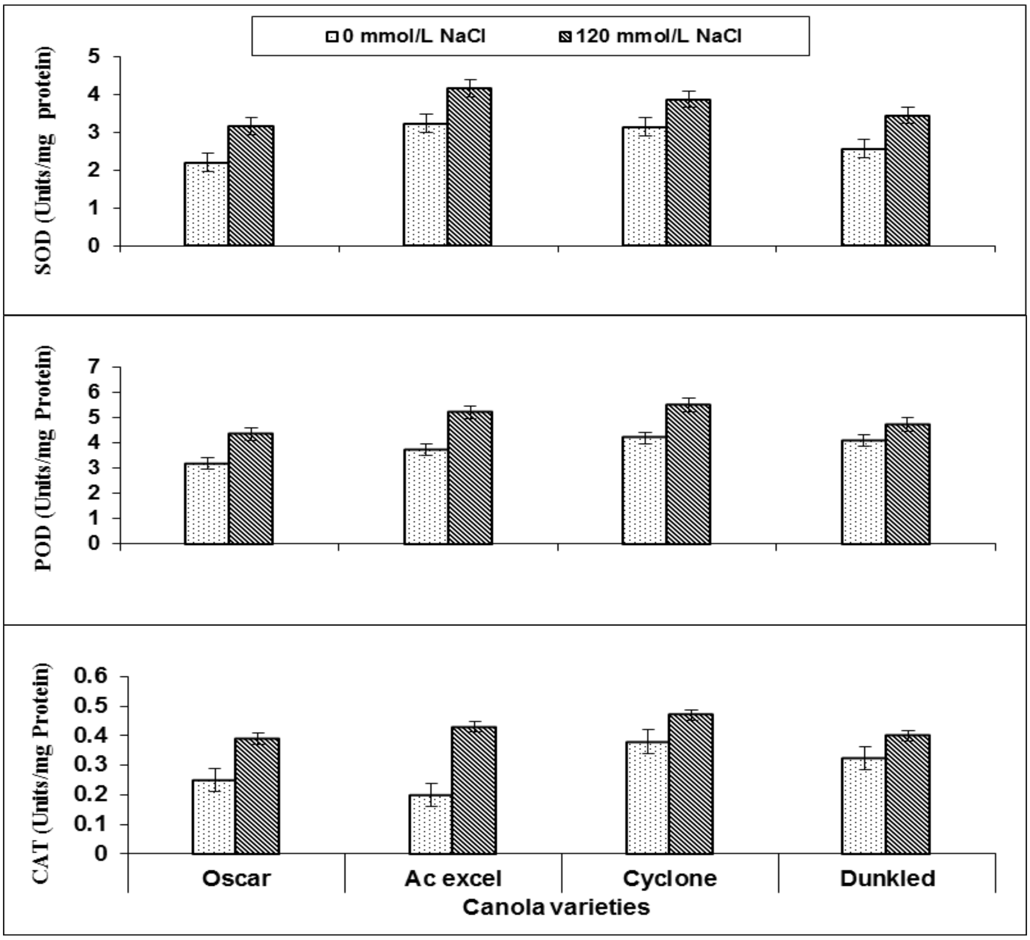

Figure 3. *Shoot and root K^+ and Ca^{2+} of four canola cultivars when treated to salt stress for 48 days*

Antioxidant enzyme activities

Values of antioxidant enzyme activity (SOD, POD. CAT) were increased in four canola cultivars under saline conditions. A notable variation was found in these parameters. Higher value of SOD was found in cultivar “Cyclone and Ac excel” than in “Oscar and Ac excel” under salt stress. POD showed higher value in Ac excels and Cyclone while lower in Ac excel and Oscar under salinity stress. Less reduction in catalase activity was found in Oscar as compared to Ac excel, Cyclone and Dunkled under salt regimes (*Fig. 4*). ANOVA of data showed that salt level, cultivar and salt level × cultivar interaction showed significant results in the value of antioxidant enzyme activities (SOD, POD. CAT) (*Table 4*).

Table 4. *Mean squares from ANOVA of the data for SOD, POD and CAT content of four canola (Brassica napus L.) cultivars when subjected to salt-stress and non-stress conditions*

SOV	d.f.	SOD		POD		CAT	
		F	P	F	P	F	P
Salt level	1	5017.421	.0000***	5017.421	.0000***	5017.421	.0000***
Cultivar	3	379.241	.0000***	379.241	.0000***	379.241	.0000***
Salt level × cultivar	3	71.190	.0000***	71.190	.0007***	71.190	.0000***
Error	32						

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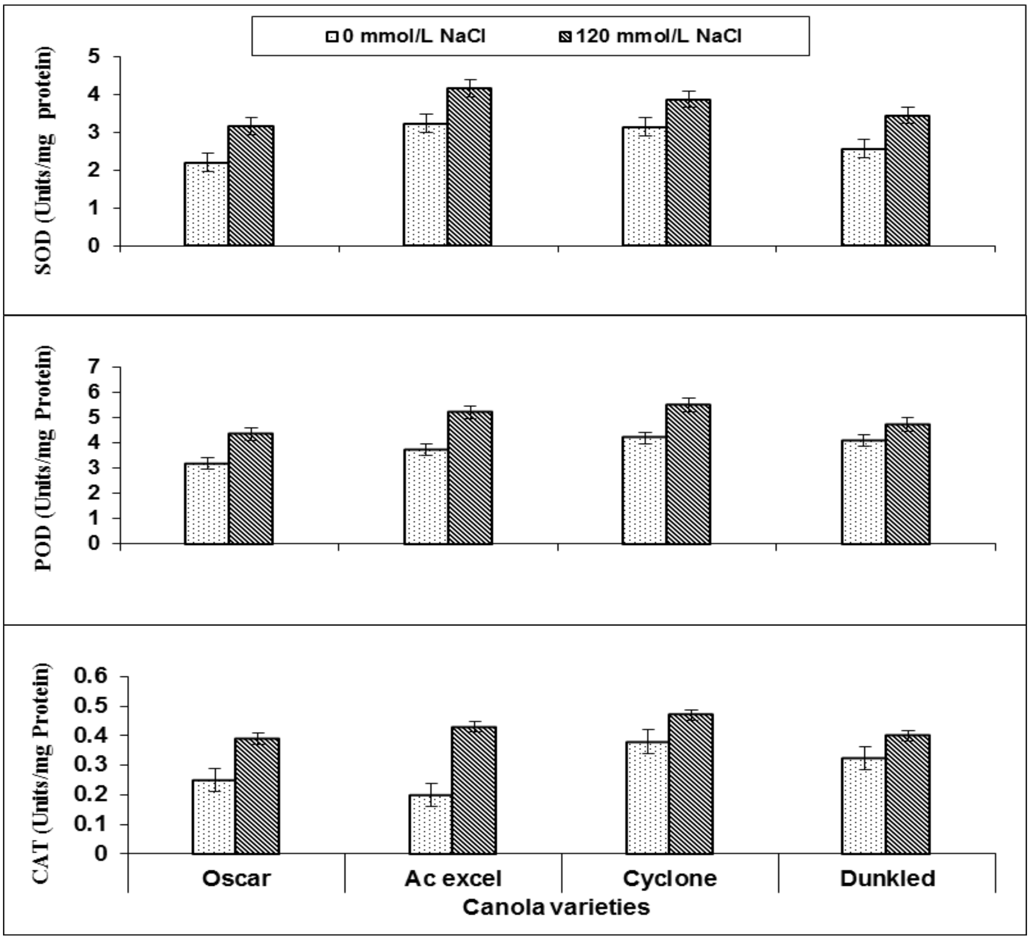

Figure 4. *Antioxidant enzyme (SOD, POD and Catalase) of four canola cultivars when treated to salt stress for 48 days*

Discussion

To explore variation at inter and intra-cultivar level is a prime importance for a breeding program needing crop improvement for different abiotic stresses including salinity (Munns, 2007). Results of the present research work are in accordance with the finding of different researchers where biomass production (root fresh and dry weight) were significantly reduced in different crops e.g., mangrove plant (Parida et al., 2004), tomato (Foolad and Lin, 1997) and safflower (Siddiqi et al., 2011).

Salinity stress has severe damaging effect on plants through ion toxicity or nutrient imbalance which occur due to higher accumulation of sodium and chloride in differentparts of plant in different plants (Munns et al., 2002; Ashraf, 2004). Higher quantity of salinity (NaCl) in soil or water hinders the uptake of different essential nutrients, such as potassium and calcium due to which plants face the problem of nutrient deficiency (Parida et al., 2004; Siddiqi et al., 2011). In the present investigation, accumulation of salt in the rooting zone medium markedly increased the concentration of Na^+ and Cl^- while decreased that of K^+ and Ca^{2+} in four canola cultivars. In literature, it is reported that low accumulation of Na^+ and high K^+ showed salt tolerant cultivars of the different crop species such as barley (Wei et al., 2003) and safflower (Siddiqi et al., 2011). Plant vacuoles maintained the balance of various nutrients due to accumulation

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of active osmolyte through osmotic adjustment. Of different osmolytes, proline is the essential one which amasses in various plants under saline conditions (Ashraf, 1994; Abbas et al., 2010). Present investigation showed that salt-stress increased the concentration of proline in the leaf of all canola cultivars. These results are in accordance with earlier findings where accumulation of proline under salt stress conditions has a key contribution to salt tolerance of various plant species (Maggio et al., 2002; Abbas et al., 2010). Furthermore, it is clearly reported in literature that proline accumulates more in salt-sensitive than in salt-tolerant cultivars (Ozturket al., 2012; Abbas et al., 2010).

Abiotic stresses including salt stress generate reactive oxygen species in plants (Turkyilmaz et al., 2014; Flowers et al., 2010). Plants have ability to counterpoise salinity-induced reactive oxygen species with the help of various antioxidant enzyme activities. In literature, it is reported that activities of antioxidant enzymes have a key role in salt tolerance of plant in different plant species. Findings of the present research work showed that addition of salinity in the growth medium increased the values of superoxide dismutase, peroxidase and catalase in all canola cultivars. Our results are in agreement with the findings on different crops such as wheat, safflower, finger millet and tomato where antioxidant enzymes produced under salinity stress (Raza et al., 2007; Ediga et al., 2013; Shalata et al., 2001).

In the present research work, values of MDA showed variable response in all canola cultivars under saline conditions, which are in accordance with the findings in the case of e.g. wheat (Ashraf et al., 2010), and finger millet (Ediga et al., 2013).

Conclusion

It is concluded that salt stress significantly increased sodium (Na^+), chloride (Cl^-) and leaf proline content of root and shoot, and enhanced the activities of SOD, POD and CAT, while it decreased the values of potassium (K^+) and calcium (Ca^{2+}). Overall, tolerance of canola to salt stress could be accredited to exclusion of Na^+ and Cl^-, higher accretion of K^+ and free leaf proline boosting antioxidant enzyme activities in canola cultivars.

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THE ANALYSIS OF VERTICAL HYDRAULIC CONDUCTIVITY OF THE RIVERBED OVER TIME – A CASE STUDY OF DOWNSTREAM OF ZIARAT RIVER, NORTHERN IRAN

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Abstract. Vertical hydraulic conductivity (K_v) of riverbed is an important variable exerting impact on the exchange of water and salts between riverbeds and the surrounding underground water systems. However, there is little detailed information on the spatial variations of the riverbed K_v, and there is hardly any information on its timing variations. Time changes in the amount of K_v of the riverbed reveal the need for further studies as a potentially significant controlling factor in temporal variations in the amount and spatial patterns of water fluxes and solutes between groundwater and surface waters. With this in view, this study adopted a time series analysis approach to investigate the hydraulic conductivity measured over a year from January 2016 to January 2017 on a weekly basis for a downstream point of the Gorgan Ziarat River, Iran. The researchers first began to verify the hydraulic conductivity data, which was a downward trend during this period, from the particle size distribution of the riverbed for several times over the course of a year. Then, after identifying the existing data trends, autocorrelation and partial autocorrelation coefficients were determined. Finally, an ARMA model (1,1) was identified as the superior model. The mean squared error was used to evaluate the selected model. Moreover, Pert-Manto test was used to test the validity and reliability of the model.
Keywords: *riverbed, downstream, particle size, time series analysis, ARMA models*

Introduction

Hydraulic conductivity (K_v) of streambed is regarded as one of the most important controlling variables in the exchange of water and minerals between the rivers and the surrounding groundwater systems (Saenger et al., 2005; Storey et al., 2003). Variations in the location of K_v of the streambed may lead to increase in the water exchange rate between one stream and the Hyporheic region of the bed, which can lead to changes in the durability of the minerals and further cause intermixture in this area (Cardenas et al., 2004). Little information is available about the way such aspects of the exchange between the streambed and groundwater may be affected by the temporal changes of the K_v of the streambed.

Hydraulic conductivity (K_v) of streambed is an effective variable in the exchange of water and minerals between the rivers and the surrounding groundwater systems. There exists little information about the environmental changes of the K_v of the streambed and almost no information on its temporal changes. The environmental and temporal

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variation of K_v of a river in North Carolina during a one-year period was studied using 487 field measurements (Genereux et al., 2008).

The temporal changes of streambed's K_v determine further studies as a potential controlling factor in the temporal changes of amount and environmental patterns of water and the minerals between the groundwater and surface water. Using the available information, it seems appropriate to consider the streambed's K_v as a dynamic feature which is dynamic both in terms of time and location.

A pilot area (11.42 km in length) in Pajaro River located in the central beach of California was studied using the average reach and special point-based methods in order to determine the rate of leakage in the bed (Hatch et al., 2010). These data were utilized to assess the variation in the hydraulic conductivity of the streambed over time, as a function of the canal and changes are related to sedimentation.

The point-by-point leakage rate, which was determined using the time series analysis of the thermal records of the streambed, shows leakage rates of 1.4 meters per day (moving to the lower parts and into the center). The leakage rates had changes in time and place, such that the leakage mostly is occurred in lower end of the reach and during summer and fall.

These results show that the interaction models of surface and groundwater can benefit from the relationships between the stream, sediment load and the hydraulic features of the streambed (Hatch et al., 2010).

In the study accomplished by Min et al. (2013), the scholars have focused on the spatial changes of the vertical hydraulic conductivity of a stream in a meandering river of China. They observed that the change in the vertical hydraulic conductivity of the stream along the reach of the river was remarkable and high amounts of K_v is related to part of the canals with more deep water.

The evaluation of the 14 methods to determine the permeability was conducted through indirect measurement. The estimation of the value of hydraulic conductivity (K_v) was done based on the distribution curve of particle measurement (Fenza et al., 2017). All the methods were evaluated with regard to certainty, by comparing the measured parameters and the results obtained from the permeation experiments. The findings demonstrated that the amounts of K_v obtained from the distribution curves are smaller than the measured data, which could be due to the different depth of sediment sampling during the experimental analyses.

In this study, we analyzed the hydraulic conductivity during a one-year period. We tried to study the hydraulic conductivity of one area of a river during 52 weeks starting from January 20, 2017 and to see if we can come up with a formula to estimate the hydraulic conductivity during one year. Moreover, we extracted samples from the streambed at five different time intervals and sent them to the laboratory to analyze the changes in the grain size during the one-year time period. In addition, using experimental formulas, we measured the hydraulic conductivity and compared them with the data obtained using the stable permeation method. We reached an overall analysis about the interaction between the surface and groundwater.

Materials and methods

Study area

Ziyarat River, originating from Alborz mountain range, is located in Golestan Province, which is a northeastern area in Iran (*Fig. 1*). It flows to the north and after

36 km, and merges into Gharesoo River. The drainage basin in upstream is about 100 km^2 with dimensions of 19.64 km in length and 5.11 km in width. The mean of water abstraction rate at stream gauge is about 10.5 m^2. The study area is located in a temperate climate. The mean of annual precipitation is equal to 601 mm, and mean temperature is 13.4 °C. The variation in seasonal precipitation is distinctive and not uniform. The temperature of stream water varies from 19.04 to 29.2 °C. Although, the weather is rainier in winter, the majority of floods occur from August to November. The streambed sediments were alluvial coarse, sand-gravel, and clay and silt in downstream. The highest height of Ziyarat stream gauge is equal to 3068 m and the beginning of main river is 2620 m. The length of the river to Naharkhoran is 21 km with the slop average rate of 10.1% and the remainder of river to Ghorbanabad is 15 km with the slop average rate of 4.6%.

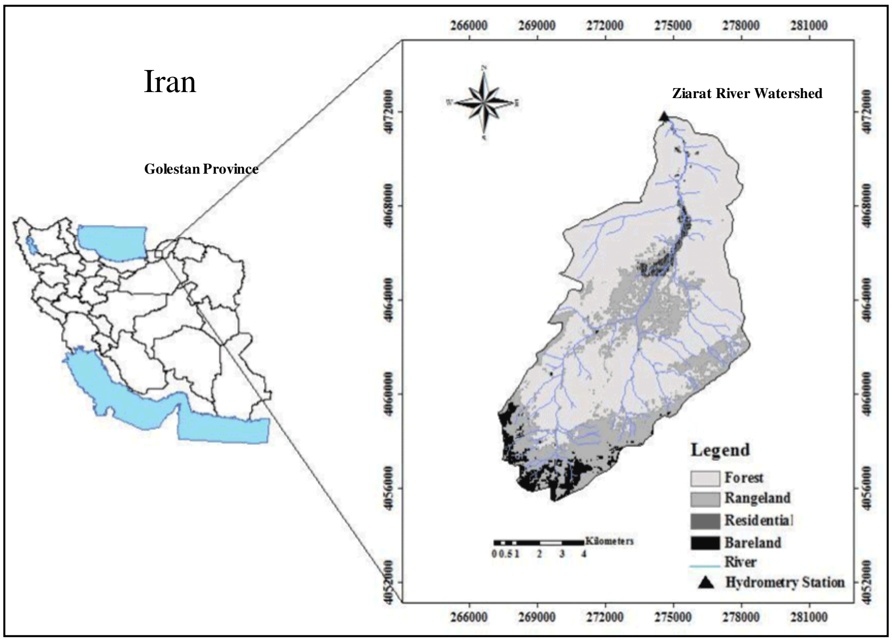

Figure 1. *The location of the study area along the Ziarat River*

The geographical position of the study area is shown based on latitude and longitude and the UTM coordinates system. It is evident that, most rivers stemming from the Alborz Mountain Range, are similar in terms of morphology and slope. Therefore, studying any of them can help us get familiar with other rivers of the same kind. In other words, the results of the study could be generalized to other rivers with alluvial bed. The area under study was located in downstream in Ghorbanabad, with a slope around 2%. The position of the area, as identified by the UTM system, is X:271732,Y:4089803 (*Fig. 2*).

Since the interaction between the surface and groundwater in the bed rivers has been widely investigated and is considered significant, in this study, we examine the potential of groundwater layers in the downstream, as they satisfy the needs of human beings for water through wells. Another reason for selecting this part of the river is the decrease in the hydraulic conductivity of this area due to being located in the downstream. This results in sediments with small grain size consisted of silt which act like an

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impermeable layer. In addition, studying the downstream is an undeniable necessity, as this area is under the danger of flood during summer and this natural disaster overfills the streambed with sediments consisted of mud which thickens the impermeable layer in the bed of the river.

Figure 2. *The location of the test point on the map*

The high frequency of flood occurrence in the summer for this region is due to the fact that this area is located adjacent to the Alborz Mountains, within south zone and it is also close to the Caspian Sea, in the north zone, as a result the high heat leads to evaporation and the formation of rainy clouds due to the high volume of water evaporation during summer season. Hence, the reduction in the temperature renders this volume of water evaporation into the intensive rains. Moreover, due to the high heat of soil and secrecy of humid heat air in the soil holes, the intensive rains during summer reason cannot permeate into the soil holes and the accumulated water will flow which will lead to formation of flood.

Methods

Vertical hydraulic conductivity

Falling-head test method

A falling-head test is conducted by rapidly raising the water level in the control well and subsequently measuring the falling water level. Slug-in test is another term for falling-head test. In the previous studies, a field permeameter method has been adopted for measuring vertical hydraulic conductivity (see e.g. Chen, 2005; Genereux et al., 2008; Song et al., 2010; Dong et al., 2012). The purpose of this method is to determine the streambed hydraulic conductivity (K_v), and, as it is illustrated in *Figure 3,* it operates by vertically inserting a pipe into the streambed, filling the pipe with water, and calculating the amount of decline of the water level inside the pipe. After repeating this process for a few times, the K_v can be measured using this rate. In the present study, steel standpipe with an inner diameter of 4.8 cm and length of 120 cm was used. The pipe was injected into the sediments of the streambed and it was ensured that the length of the sediment column was almost equal to 40 cm. A sensor (Onset HOBO Water

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Level-U20L-Series) with an accuracy of 1 mm was also employed to measure the water levels. Water was added during the test at the top of the pipe to create a hydraulic head. Then, the head was passed to fall in the pipe. In the current research, a water-level declining interval of 1 cm was used to record the hydraulic head and time. The hydraulic head estimations were obtained more than 3 times. To measure the K_v value using *Equation 1* proposed by Hvorslev (1951), all pairs of measured data of hydraulic head and time were used.

$$K_v = \frac{\frac{\pi D}{11m}+L_v}{t_2-t_1} ln(h_1/h_2) \quad \text{(Eq.1)}$$

Where D denotes the inner diameter of the pipe, m denotes the square root of the ratio of the horizontal conductivity K_h represents the vertical conductivity K_v (i.e., $m=\sqrt{\frac{Kh}{Kv}}$), L_v refers to the length of the sediment column, h_1 and h_2 are hydraulic head inside the pipe measured at times t_1 and t_2, respectively. Overall, K_h is greater than K_v. An adopted Hvorslev (1951) solution has been proposed by Chen (2004) and Song et al. (2009) in order to specify the K_v when L_v is greater than D (*Eq. 2*):

$$K_v = \frac{L_v}{t_2-t_1} ln(h_1/h_2) \quad \text{(Eq.2)}$$

However, the error in K_v from the formula provided by the modified formula of Hvorslev (1951). *Equation 2* is related to the ratio (L_v/ D) of the estimated sediment length (L_v) to the inner diameter (D) of the steel pipe. When $1 < m < 5$, if the ratio (L_v/D) is greater than 5, the error of the adopted estimation will be less than 5% (Chen, 2004).

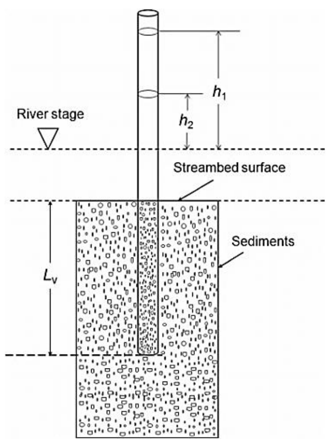

Figure 3. *Schematic diagram showing an in situ permeameter test to determine streambed*

The values of hydraulic conductivity were calculated weakly for this section of the river, in a regular manner and these values were analyzed in such a way that almost 52 values for hydraulic conductive were recorded during 52 weeks.

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Statistical analysis of the data for vertical hydraulic conductivity

The initial time series analysis shows that it has a decreasing trend and its data do not follow a normal distribution. The initial time series plot and the primary results of the data are presented in *Figure 4* and *Tables 1* and *2*.

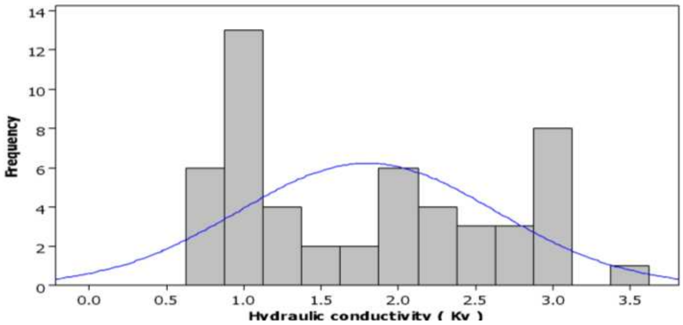

Figure 4. *Histogram and Box diagram (K_v) of Riverbed*

Table 1. *Values of K_v within 52 weeks*

Time (week)	K_v	Time (week)	K_v
1	2.92	27	1.62
2	2.27	28	1.70
3	2.98	29	1.42
4	2.16	30	1.12
5	2.92	31	1.33
6	2.98	32	1.34
7	2.62	33	1.21
8	3.56	34	1.02
9	2.96	35	0.86
10	2.15	36	0.98
11	2.76	37	1.21
12	2.96	38	0.99
13	3.09	39	1.06
14	1.96	40	0.98
15	1.66	41	0.96
16	2.86	42	0.78
17	2.42	43	1.00
18	2.98	44	0.98
19	2.02	45	1.09
20	2.62	46	0.88
21	2.63	47	1.02
22	2.02	48	0.70
23	1.96	49	0.86
24	2.26	50	1.02
25	2.02	51	0.66
26	2.05	52	0.74

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Table 2. *Statistical parameters linked to K_v*

Mean	Minimum	Maximum	Std. Deviation	Kurtosis	Skewness
1.79	.66	3.56	.148	-.947	-.271

Verification of the hydraulic conductivity

Researchers have made a lot of efforts and introduced the grain size analysis of the particles in the sediments of the streambed for the autocorrelation of the parameters that have a direct relationship with the hydraulic conductivity (K_v) (Hazen, 1982; Rosas et al., 2014). Therefore, the hydraulic conductivity analysis of the streambed should be regarded as one of the methods to obtain the vertical hydraulic conductivity. Accordingly, the best method for sampling from the sediments of the bed is the vertical hydraulic conductivity to verify the process of sampling in the horizontal hydraulic conductivity (K_v)

Sampling from the sediments of the streambed

Since the present research aims at conducting a time history analysis on the hydraulic conductivity of a point of riverbed within one year, so the grain size of riverbed soil can be regarded as a proper parameter which has a high correlation with hydraulic conductivity. Therefore, a specific section of the river channel associated with river bed (at the afore-mentioned geographical length and width of X: 271732, Y: 4089803, in accordance with UTM coordination system) was opted and the results obtained for this section were compared with the values recorded for hydraulic conductivity of that section.

Since the sediments were soft in that part of the river, this task was done easily and the only point was ensuring that the device was operating properly.

A steel pipe was inserted 50 cm into the hyporheic zone in the bed of the river. A plastic pipe (40 cm) made of Polyvinyl chloride (PVC) was inserted into this steel pipe where the particles of the sediments pile up. There was leather cover above the open part of the pipe which disconnected the pipe from the surrounding atmosphere. The sediments accumulate in the pipe remain unchanged and will not fall when the pipe is pulled out from the bed (Song et al., 2009).

These samples were sent to the soil mechanics laboratory of Golestan Province. The grain size experiment was conducted carefully and it was noted that the smallest grain was .075 and the largest one was 10 mm. The particles smaller than .075 were identified as silt and clay and those between .075 and 2.0 were classified as sand. The grains larger than 2.0 were identified as gravel (Song et al., 2010).

Results

Grain size analysis

Grain size is the main controlling factor for streambed hydraulic conductivity (Song et al., 2010). Sampling from the sediments was accomplished in five sessions after obtaining the hydraulic conductivity. So, the analysis of grain size was done in weeks 1, 6, 26, 47 and 52.

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Indeed, the primary objective of this research was to extract sediment from intended section, middle section and endpoint section of the riverbed within the first week, 26-th week and 52-end week, respectively. This project was defined for grading analysis. That is, these three extractions within the above-mentioned weeks were pre-defined, while the research team decided to perform random sampling method on the riverbed particles within 6-th and 47-th weeks too. In fact, this mode of week selections meets the requirements of purposive and random sampling method.

The curve of grain size is depicted by *Figure 5*. This study attempted to analyze the changes in the sediments of the streambed during the three time intervals. It is evident that during 1 and 6 weeks, the curves of the grain size are quite similar and this was expected, since the hydraulic conductivity for these two weeks were the same. During the middle stages of the study, the curve of the grain size tended to the left in such a way that the percentage of sand increased and the percentage of gravel decreased. These variation in the grain size occurred with a decrease in the hydraulic conductivity. During weeks of 47 and 52, the percentage of silt and clay increased and the percentage of gravel decreased. As it was expected, the hydraulic conductivity decreased to a great extent. The percentages of silt, clay and sand of the sediments for these five specimens are given in *Table 1*. It is evident that due to the occurrence of floods during summer in this region, which caused great changes in the morphology of the streambed, the hydraulic conductivity of the bed started to decrease noticeably from 26-th week. The increase in the percentage of silt and clay is evident in the samples taken from 26-th week. d_{50} in *Table 3* refers to the average diameter of particles of the sediments in millimeter the weight of which is less than 50% of the overall particles.

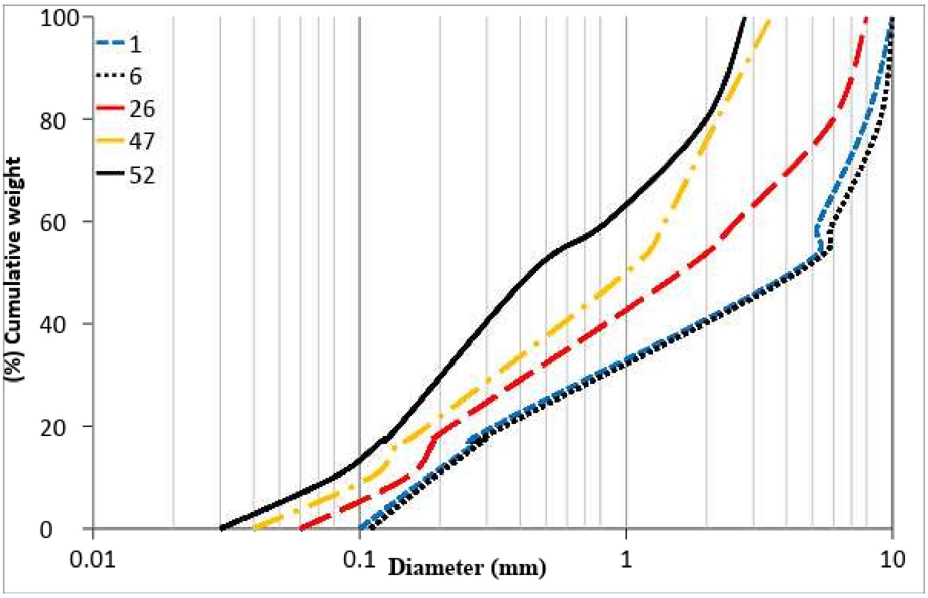

Figure 5. *Curve of grading distribution of the sediment fines of riverbed within selected five weeks*

The first step in the analysis of time series data is drawing the variable values during the period under investigation. This is influential in identifying the trends of the data which need to be extracted from the data before doing the analysis. *Figure 6* shows the values of the K_v index during the 52 weeks period which was analyzed by MINITAB 18

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Software. To determine the trend of the data, which is a descending trend, as the diagram demonstrates, the different visual functions, the second step and the S curve were evaluated. The results indicate that the S curve exhibited the best fitness for the trend of the data. The functional format of this curve is shown in *Equation 3*:

$$y_t = \frac{\alpha}{\beta + \gamma(\delta^t)} \tag{Eq.3}$$

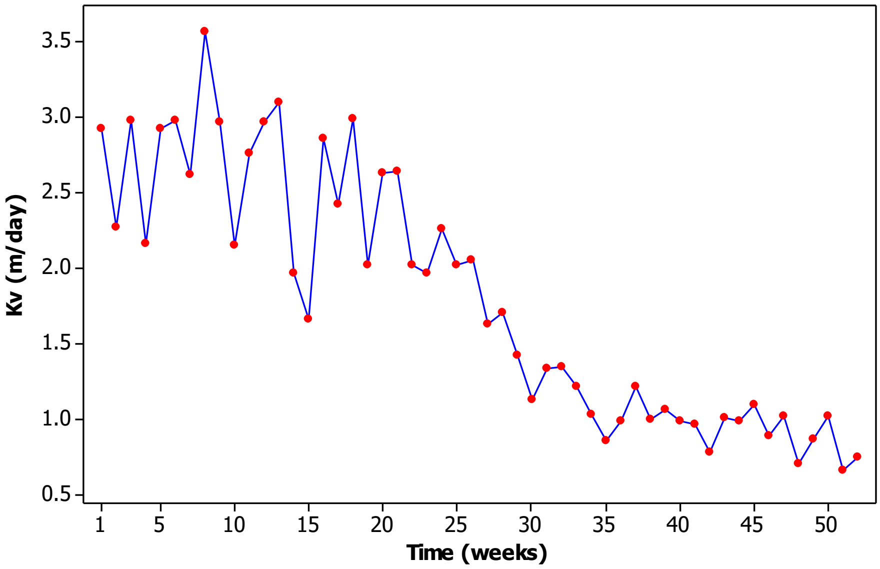

***Figure 6.** Time series diagram of K_v within 52 weeks*

***Table 3.** Grading distribution of 5 samples of particles within riverbed over a year*

Sample number	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Per week	1	6	26	47	52
% < 2 mm	40.4	41.5	52.2	78.6	80.4
% < 0.075 mm (silt and clay %)	0	0	2.1	6.3	8.1
Average median grain size d_{50} (mm)	4.1	4.2	1.4	1	0.42

The α, β, γ, *and* δ parameters were estimated by the data and the values of the y_t trend were subtracted from the main values of the data. The remaining data from this section will be analyzed as the detrended data using the time series models. The values of the trend under this function are shown over the main values of the data. As it can be seen in *Figure 7, Equation 4* obtained from the fitness analysis is as follows:

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$$y_t = \frac{100}{5.43+23.69(1.033^t)} \quad \text{(Eq.4)}$$

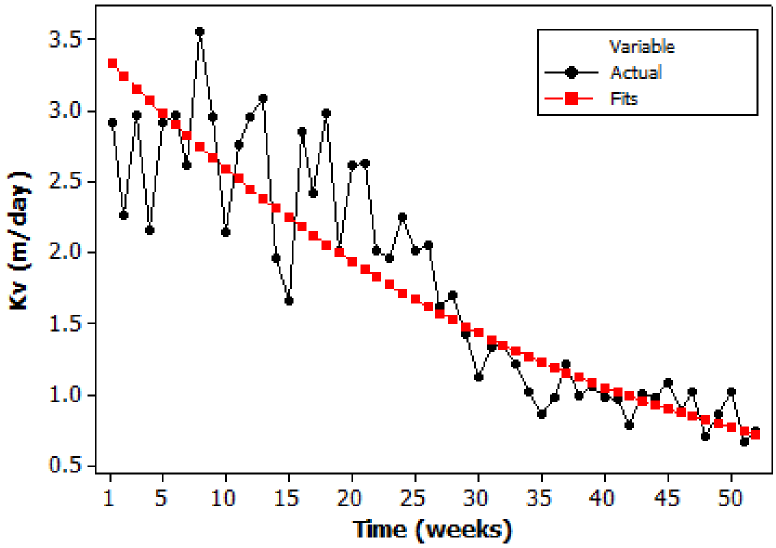

Figure 7. *Identifying the time series trend (K_v) under S curve*

After identifying the trends in the time series data, the first step in recognizing the model is to determine the values of autocorrelation. For this purpose, the values of ACF and PACF were calculated and drawn for the ACF and PACF of detrended data of 13 intervals which were identified automatically with reference to the length of the data. *Figures 8* and *9* show the values of ACF and PACF of detrended data (K_v), respectively.

As can be seen in *Figures 8* and *9,* the values of autocorrelation and partial autocorrelation of the data were insignificant only in the first interval which is an indication the fact that ARMA model with (1,1) parameters is fitted. To analyze the values more accurately, the statistics of their significance level which have the Student's t-distribution are also given in *Table 4*.

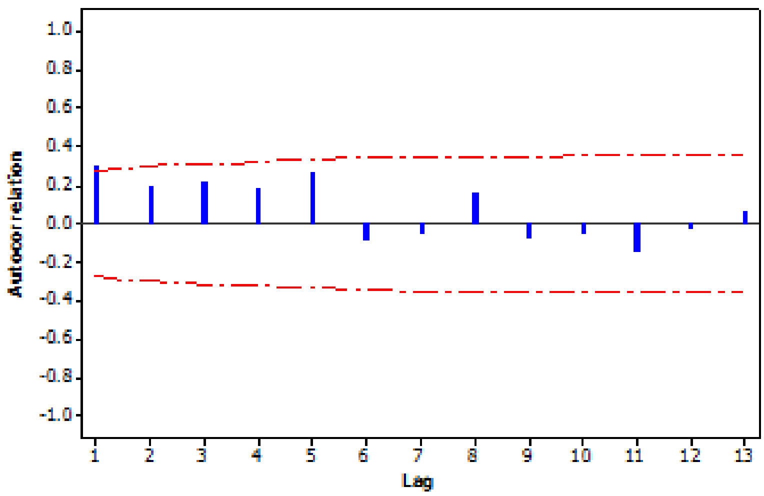

Figure 8. *Autocorrelation values of (ACF) detrended hydraulic conductivity*

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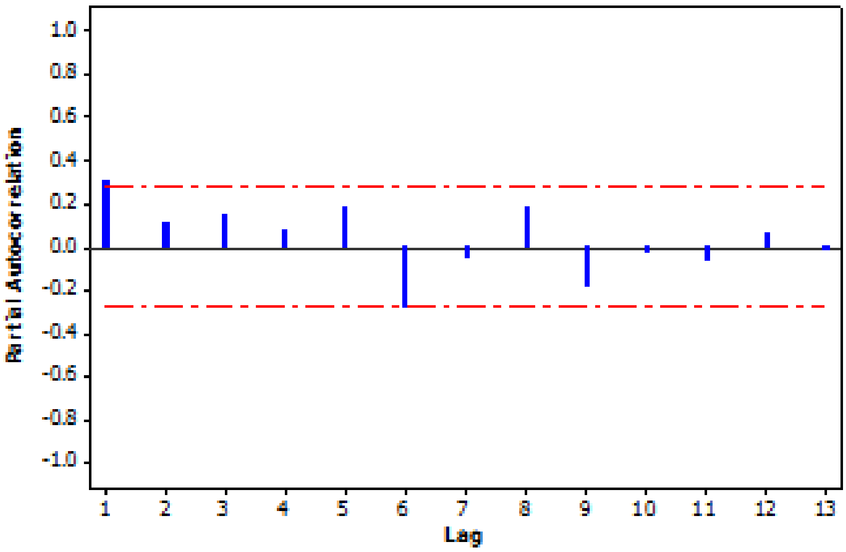

Figure 9. *Partial autocorrelation values (PACF) of detrended hydraulic conductivity*

Table 4. *Values of autocorrelation and partial autocorrelation of detrended data*

Interruption	Autocorrelation	T Static	Partial autocorrelation	T Static	Ljung–Box statistic
1	0.302403	2.18	0.302403	2.18	5.03
2	0.187394	1.24	0.105604	0.76	7.01
3	0.214519	1.38	0.146813	1.06	9.64
4	0.181128	1.13	0.079277	0.57	11.56
5	0.264257	1.6	0.182559	1.32	15.74
6	-0.08194	-0.47	-0.27918	-2.01	16.15
7	-0.0496	-0.29	-0.04806	-0.35	16.3
8	0.159692	0.92	0.179708	1.3	17.93
9	-0.07248	-0.41	-0.17439	-1.26	18.27
10	-0.04918	-0.28	-0.02614	-0.19	18.43
11	-0.14704	-0.83	-0.05485	-0.4	19.91
12	-0.0151	-0.08	0.065661	0.47	19.93
13	0.055656	0.31	-0.00882	-0.06	20.15

According to the results given in *Table 5,* it is evident that in the first interval both ACF and PACF were significant. The value of Ljung-Box, which was found to be equal to 5.03, shows the suitability of the first interval for the self-recursion sections and the average dynamic model.

To determine the optimum model among all the possible models which have parameters equal to the suggested values in the diagrams of ACF and PACF, the average square values of the model errors in data prediction were taken into account. Accordingly, models with different parameters were fitted for the data and the average square values of the model errors were compared and contrasted. In addition, since estimating the time series models results in error values in the model, the primary

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assumptions of the models (normality, equal variance, and independence) need to be checked. Among all the fitted models, most did not have desirable models as far as the primary assumptions were concerned. The statistics of the Ljung-Box test were also evaluated to check the information in the model error and, accordingly the model which significantly caused pure noises was selected. The ARMA model (1,1) consisted of one self-recursion term and one term of dynamic average.

Validation of the model

Taking into account the details of the model fitness, it can be seen that the self-recursion term in the first step of the time series model and the dynamic average component of the model were significant, so the index of K_v is significantly affected by the first interval. After fitting the model, determining the accuracy of the selected pattern is necessary. The root-mean-square error (RMSE) was used in this model. The mean square error for this model was found to be equal to .1357, which, compared to other estimated models, had an acceptable value. The criterion of the root-mean-square error (RMSE) was estimated based on *Equation 5*.

$$RMSE = \left[\frac{\sum_{i=1}^{n}(P_i - O_i)^2}{n}\right]^{1/2} \quad \text{(Eq.5)}$$

In *Equation 5*, n refers to the number of cases given to the model. Oi and Pi refer to the values of measurement and estimation, respectively.

Discussion

Checking the suitability of the model

One of the methods to analyze the residuals is analyzing the diagrams of the residuals. Accordingly, the first step is to check the normality of the residuals. *Figure 10a* shows that the error components are normally distributed. The histogram (*Fig. 10c*) also depicts that the residuals are normally distributed. The distribution of error values versus the fitted values shows that the expanded and contracted trends do not exist in this diagram. Accordingly, the error statements were independent from each other.

In order to determine the equal variance of the error components, the values of error were plotted based on the order of their occurrence (*Fig. 10b*). In this figure, it seems that the primary statements of error have more variance but this variance has not been different over time. Therefore, it is possible to consider the converting models like the MSM model as more suitable for these data than the ARMA or ARIMA models.

ARIMA model is considered as one of the best prediction models used for short-term time series analysis (Chang et al., 2012). In addition, the Holt-Winters time series analysis can be used when there exist seasonal or cyclic variation. Costa et al. (2015) have attempted to estimate the water qualitative parameters using Holt-Winters model whose findings have demonstrated the model efficiency. However, it is should be noted that models like Holt-Winters lead to greater error, compared to ARIMA Model. The diagram of the residuals versus time show that they are around zero with a fixed variance (*Fig. 10d*).

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The Pert-Manto test

A more formal method to examine the suitability of the model is the Pert-Manto test. This test is given at the end of all the outputs of the model fitness. This method is known as the Ljung-Box Test in the statistical software. The significance level of Ljung-Box statistics in this model is greater than the .05 error for the intervals of 12, 24, 36, and 48 (*Table 5*). This shows that there were not significant variations in the error statements for the modelling and the assumption that the residuals were not correlated, is confirmed. The primary assumptions of the error statements in the model are checked in *Figure 10*. *Figure 11* illustrates the fit values of K_v in the model in contrast to their real values. It seems that the obtained time series model can suitably explain the trend and changes existent in the data.

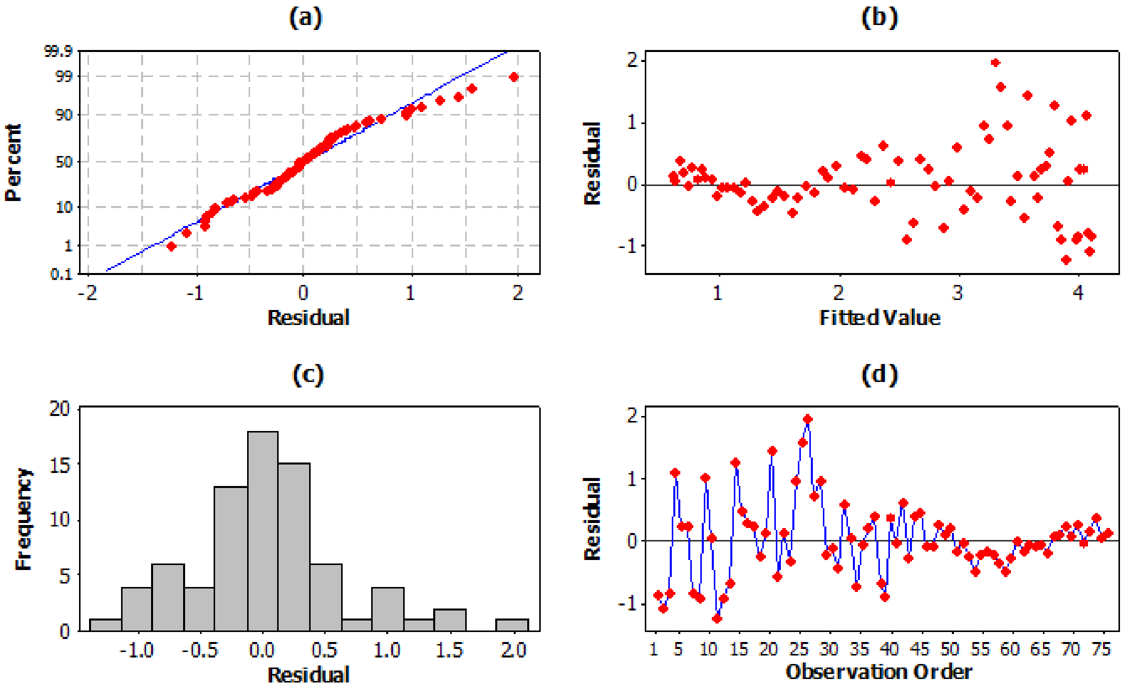

Figure 10. *Primary assumptions of model error terms.* ***a*** *Normality diagram.* ***b*** *Residual versus fitted values.* ***c*** *Histogram diagram.* ***d*** *Residual versus time*

Table 5. *Results of Ljung-Box test*

Delay time	12	24	36	48
Chi Square	14.2	29	32.6	37
Freedom degree	9	21	33	45
P-Value	0.117	0.114	0.486	0.797

To determine the goodness of fit and ensure that error statements of the model do not have any information, the diagrams of ACF and PACF were plotted for the components of the model error (*Figs. 12* and *13*).

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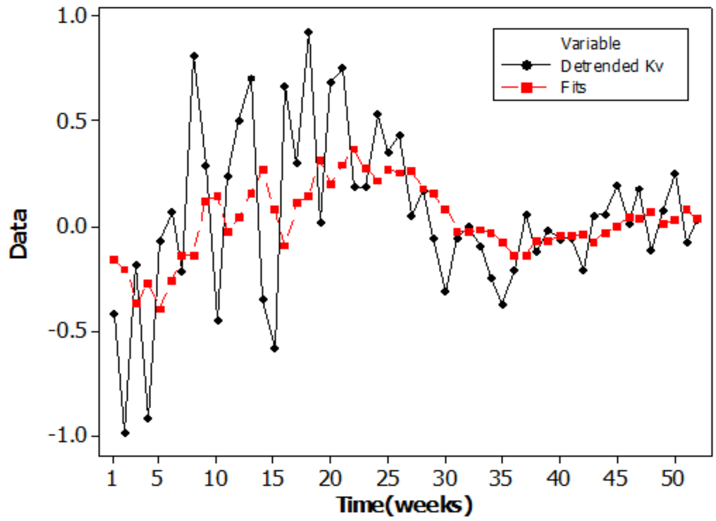

Figure 11. *Fitted values versus detrended real values of K_v*

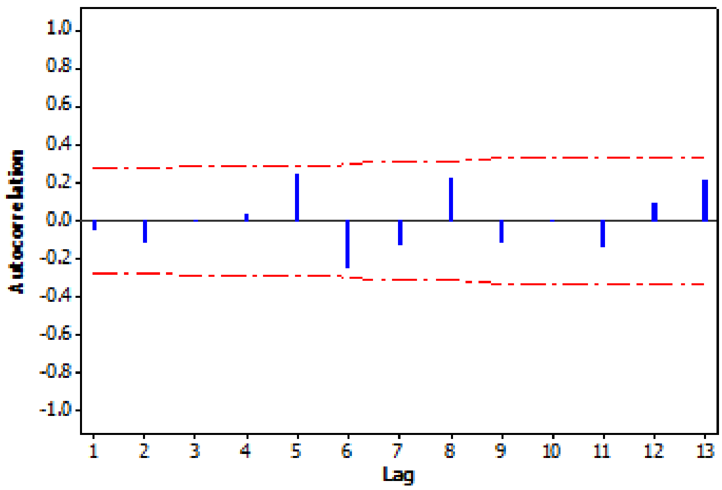

Figure 12. *Values of ACF function for model error elements*

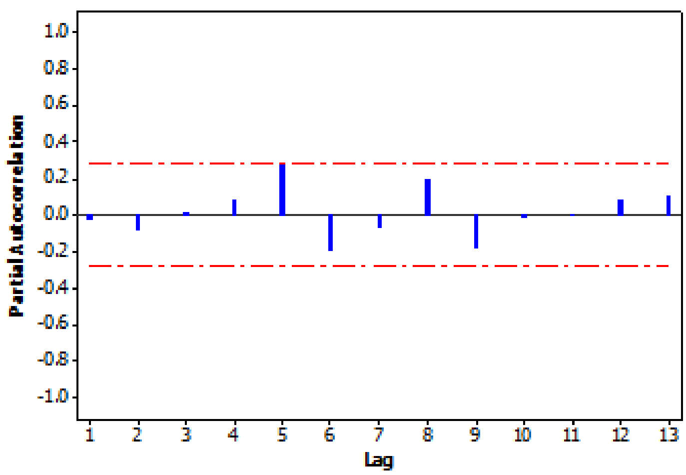

Figure 13. *Values of PACF function for model error elements*

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The results showed that in all the intervals, these two indices were significant and the errors obtained from the model were around zero and no further information was available for modelling. Therefore, the assumption of independence of the residuals was accepted.

With reference to the suitable fitness of the model, the predicted values of K_v which were added to the trend values in the next steps and show the main predicted values for the K_v index during the following 52 weeks. *Figure 14* shows the predicted values by the model for K_v. However, with a closer examination and analysis of the impact and role of major factors on K_v, such a prediction is not very optimistic. Therefore, considering the next flood and its effect on the river bed layers, it is likely to expect quite surprising results on the time series model so that the flood phenomenon can be considered as a direct shocking factor for the time series model.

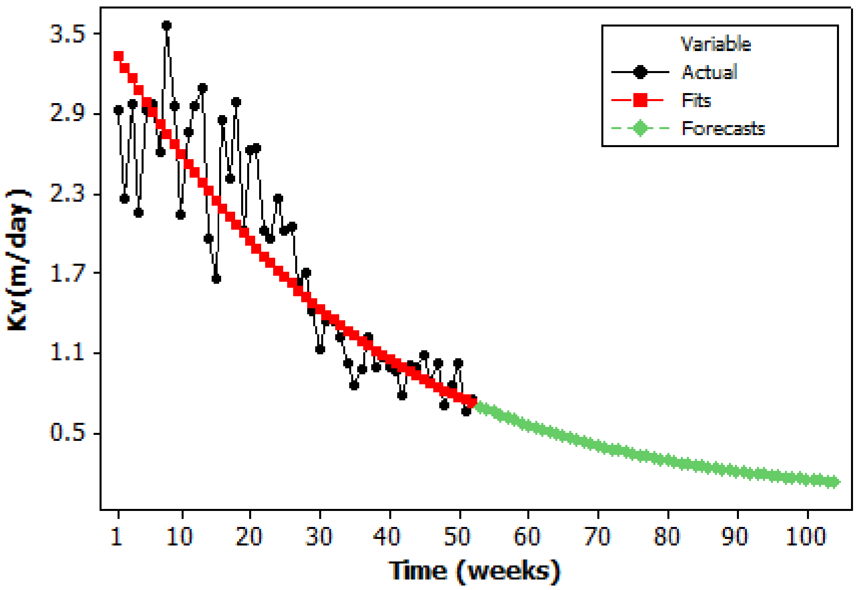

Figure 14. *Identifying the time series trend (K_v) for the next 52 weeks*

Unfortunately, there is little information about time series analysis of the hydraulic conductivity of the riverbed. For instance, Genereux et al. (2008) have focused on West Bear River, located at North Carolina, USA and the significant variation of the river within all 46 zones with 262 interval were measured in bimonthly manner. However, it is worth noting the investigation of hydraulic conductivity per two months cannot yield promising results and the resulted trend cannot be desirable in terms of predicting the hydraulic conductivity, while in our research, the measurements were done every 7 days. Wu et al. (2015) found that, after the flood season, the influence of bed-form on streambed K_v values was small, compared to that of the flood season. In the present study, it was observed that bed form of the river has a great impact on the hydraulic conductivity values prior to flood occurrence, and this finding is in line with that of Wu et al. (2015). It can be deduced that the impermeable layer consisted of silt and clay particles emerging within the river bed after flood, can affect everything, while the impact of other factors on the values of hydraulic conductivity is negligible (e.g. bedform).

It should be pointed out that the estimation of hydraulic conductivity of a specific zone of a river within a time can yield useful information on the exchange rate of surface and subsurface water. Moreover, time series analysis is a cost-effective solution for estimating the hydraulic conductivity. So the behavior of specific zone of a riverbed can provide us with relatively general information about river. For instance in the present paper, the influence of flood on degradation intensity of hydraulic conductivity was revealed and it can be inferred that the flood encompasses the riverbed with impermeable layer comprised of silt and clay particles, as a result the exchange rate of surface and subsurface water will decrease.

Conclusion

The time series analysis of hydraulic conductivity is one of the new methods to predict the trend of estimating the interaction between the surface and groundwater which helps human beings protect the rivers against natural disasters. Consequently, this study conducted a time series analysis to examine the hydraulic conductivity of one part of the downstream in Ziarat River in Gorgan, Iran. First, the data of hydraulic conductivity were verified and after identifying the trends, the values of autocorrelation and partial autocorrelation were determined. Finally, the ARMA model (1,1) was identified as the best model, but some external variables like flood were effective in the variation of hydraulic conductivity which led to evident changes in the morphology of the streambed and noticeably reduced the hydraulic conductivity. A look at the weather forecasts for this region shows the occurrence of flood in late July or August, but due to the inaccurate prediction of the flood, a variable that reduces the hydraulic conductivity is impossible. Considering the growing impact of major factors on K_v such as flood, It can be mentioned that predicting the hydraulic conductivity is difficult so it is suggested that time series analysis be performed in a period of 3 to 5 years. The obtained results can be generalized to other rivers of the same type, which have the same morphological conditions or annually experience one or more flood.

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DETERMINATION OF THE MOST SUITABLE ECOTOURISM ACTIVITIES WITH THE ANALYTIC HIERARCHY PROCESS: A CASE STUDY OF BALAMBA NATURAL PARK, TURKEY

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Abstract. Ecotourism can be defined as tourism conserving the existing natural and socio-cultural structure, considering the balance between conservation and utilization, promoting the sustainability of natural resources, encouraging tourism activities, and developing the economic structure of the local community. Natural parks are areas where local and foreign tourists may join ecotourism activities due to their natural and cultural landscape potentials. Balamba Natural Park (BNP) located in the city of Bartin in Turkey, was chosen as a research area due to its natural landscape values and easy accessibility. The research method is composed of the following steps; data acquirement, field analysis, Analytical Hierarchy Process (AHS), Strengths, Weaknesses, Opportunities, and Threats (SWOT) Analysis, evaluation of findings, and conclusion and recommendations. This study aimed to determine the most suitable ecotourism activities in the existing potentials BNP. As a result, the study concluded that ecotourism activities such as botanical tourism, trekking, photo safaris, bicycle safaris and bird/butterfly watching could be implemented in the BNP.
Keywords: *natural and cultural landscape, nature conservation, land use, collaborative planning, Bartın*

Introduction

According to Hector Ceballos-Lascurain, ecotourism is a sightseeing tour of flora and fauna for touristic purposes and for observance and monitoring of cultural activities in natural areas that have not been interfered with by human beings (Cheia, 2013). This definition of ecotourism has recently become even more comprehensive. Ecotourism can be defined as responsible travel aimed at promoting the expansion of conservation awareness among both tourists and local residents, as well as conserving natural and cultural areas, maintaining sustainability, and raising the economic level of local communities (Gössling, 1999; Ceylan, 2004; Kuvan and Akan, 2005; Jaafar and Maideen, 2012; ESTA, 2016; Wishitemia et al., 2015; Verdugo et al., 2016; TIES, 2017). Briefly, the definition of ecotourism includes fundamental issues/characteristics such as natural and cultural areas, conservation, benefits for local residents, education, sustainability, ethics and awareness (Fennell, 2001).

With flora-fauna richness, aesthetic and natural beauty, water resources, active and passive recreational opportunities, the natural conservation areas and forests, where ecotourism activities are realized, have several potentials (Toskay, 1989). Ecotourism activities have several influences on the natural and cultural environment. However, the negative effect of these activities are at the lowest level, and the positive effects are at the highest level (Nyaupane and Thapa, 2004). In addition, ecotourism activities may contribute to the conservation of natural resources by providing economic support in this manner (Lopez and Monteros, 2002). Ecotourism may also accommodate several nature-based activities. Botanical tourism, mountain tourism, photo safaris, wildlife observation, cave tourism, adventure tourism, boating, and walking through forests or in

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nature could be provided as examples. Furthermore, cultural tourism activities are also included within ecotourism activities (Erdoğan, 2003; Hussin et al., 2015).

There are various activities related to ecotourism in Turkey. For example, with the aim to develop ecotourism, the improvement of protected wildlife reserves, national parks and similar conservation areas and activities related to the protection of natural, cultural and aesthetic values, were included in the Five-Year Development Plans (EFYDP, 2000). In addition, ecotourism sites and the Tourism Strategies of Turkey were identified by the Ministry of Culture and Tourism, and the importance of issues such as sustainable use of natural and cultural resources and increasing the level of social consciousness and awareness were emphasized (TTS2023, 2007; EPD, 2017). In Turkey, as is the case in many countries, the national parks, protected wildlife reserves, etc., have been mostly utilized for ecotourism purposes (ESR, 2012). The definition of a natural park, from a legal dimension, was established with Turkey's National Parks Law No. 2873, and the authorized organizations were determined in this field (NPL, 2016.). According to the 2018 official data provided by the General Directorate of Nature Conservation and National Parks within the Ministry of Forestry and Water Affairs, there are 214 natural parks in our country (PA, 2018). Indeed, one of these parks is Balamba Natural Park. In terms of landscape potentials, BNP has the power to be an important centre of attraction in Bartın Province and the immediate surrounding areas. Therefore, the purpose of this study was to attain answers to the following questions: what are the existing landscape potentials of BNP? And, what are the most suitable ecotourism activities to utilize these potentials? This study also foresaw that any activities held in BNP should be assessed within the scope of ecotourism in terms of not harming nature, ensuring environmental sustainability, and providing economic benefits to the local community.

Material and methods

Study area

The main material in the study was BNP itself. BNP was located at 41°37'39.62" N and 32°21'47.59" E. Based on the 1/5000 scale master plan, there were residential areas in the southern and north-western areas in BNP and agricultural lands in the northern and eastern areas. The park covered an area of 13,105 ha. BNP was located in the eastern side of the central district in Bartın Province, at a distance of 2.2 km from the city centre, and on the Bartın–Karabuk Highway (*Fig. 1*). The park was located at an altitude of 13-30 m. Approximately 31.5% of the park had north-east-facing slopes, and 30% was considered to be within the range of the 10-20% slope group. It is located within the first degree earthquake zone. The First Revision Development Plan for the park was created in 2006. In this context, the Plan was aimed at protecting the natural resources and recreational potential of the area, developing without destroying the existing values, and ensuring sustainable development (Anonymous, 2006). While the Park Balamba was once used as an in-forest-recreational-ground, the land was converted from this status into a natural park, in accordance with the provisions from Article 3 of the National Parks Law No. 2873, dated 11.07.2011 (BNP, 2017).

Since BNP was located in Bartın city centre, it was selected as a research area and had a landscape potential to serve the communities in the city and in the immediate surrounding areas and also presented high local recognition and accessibility. In addition, this park was located in Bartın Province, which was previously selected for its

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potential in terms of biological diversity and ecotourism development in the Black Sea Region within the scope of Turkey's Tourism Strategy, 2023 (TTS2023, 2007).

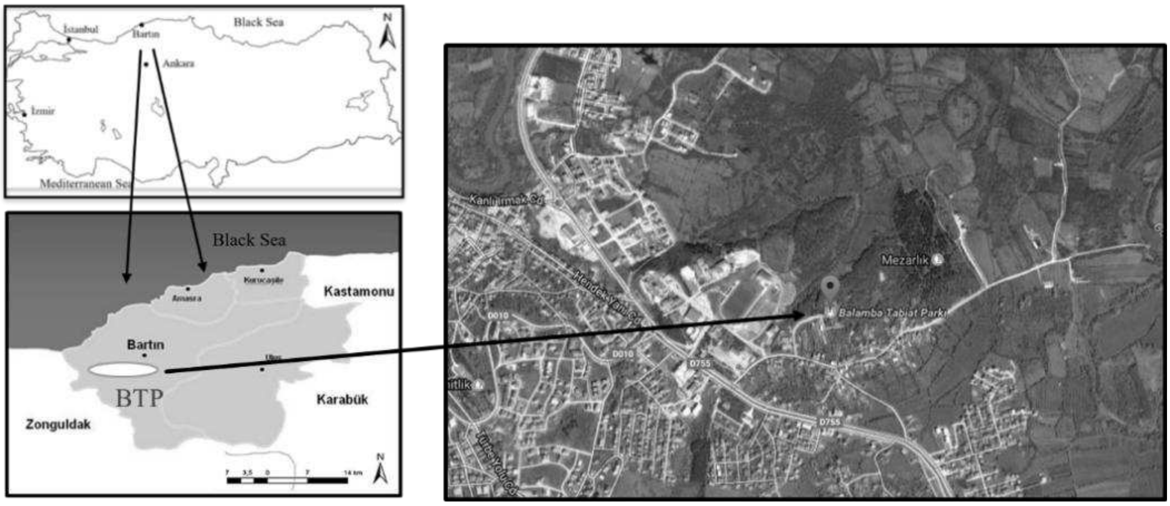

Figure 1. *Maps of the location of Bartın Province and BNP*

Methods

The methods in this research were developed by analysing studies in the literature (Saaty, 1994; Akpınar, 1995; Scholl, 2005; Timor, 2011; Akpınar Külekçi and Bulut, 2013; Yılmaz and Surat, 2015). The research was conducted within a six-stage method. These stages included literature review and data retrieval; land analysis and observation; administration of a questionnaire using AHP with the participation of experts among the relevant stakeholders; a SWOT (Strengths, Weaknesses Opportunities, Threats) analysis; evaluation of findings; and the conclusion and recommendations.

In the first stage, a national and international literature review was conducted on the topics of natural parks, AHP, BNP, ecotourism, etc., and the necessary required data were obtained through interviews at the related institutions and organizations. In the second stage, field analyses and observations were performed, and the current situation was determined by taking photographs when visiting the area of interest during different seasons.

In the third stage, a public survey was conducted by interviewing the stakeholders and experts who were considered relevant, such as representatives from the Bartın Provincial Directorate of Forestry and Water Affairs, Bartın Provincial Directorate of Culture and Tourism, Kure Mountains National Park Directorate, Bartın Municipality, Bartın University, Bartın Provincial Directorate of Environment and Urbanism, Bartın Provincial Representative of the Chamber of Landscape Architects, the Grand Astra Hotel (tourism enterprise) and a specialized tour agency. A total of nine experts participated in the questionnaire, including various professionals such as a landscape architect, forest engineer, art historian, archaeologist, construction engineer, tourism operator, botanist, and wilderness guide. The questionnaire made it possible to determine the most suitable ecotourism activity among the potential activities. Questionnaires were conducted by the author using the AHP method in June 2017. The AHP method was used for the ranking process and selecting the alternatives in accordance with multiple criteria. Questionnaires were administered to more specialized

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persons. In the fields of sensitive areas, education, health, planning, tourism, architecture, etc., the most suitable location, ecotourism activity, and selection of project or education methods, etc., were available for use by different professional disciplines (Saaty, 1977; Akpınar, 1995; Saaty and Vargas, 2006; Akten et al., 2009; Daşdemir and Güngör, 2010; Akpınar Külekçi and Bulut, 2012; Medjoudj et al., 2012; İmren et al., 2017; Chen et al., 2014; Yılmaz and Surat, 2015). In addition, with the use of the AHP method together with GIS (Geographic Information System), selection of the most suitable area can be achieved for planning green spaces in urban areas, selection of the most suitable solid waste area, and determination of the most suitable marina location, etc. (Lin et al., 2008; Gumusay et al., 2016; Şener et al., 2011).

The AHP method, developed by Saaty, allows us to use both qualitative and quantitative attributes. In the AHP, the factors within the scope of the goal and the sub-factors belonging to these key factors are determined first. Thus, the AHP decision hierarchy is established (Scholl, 2005). Subsequently, a binary comparison is made between the factors. The significance level or weights of the factors are determined. The consistency rate (CR) of the questionnaires is checked. If the CR is less than 0.10, the identified values are considered valid and acceptable (Saaty, 1994; Akpinar, 1995; Timor, 2011).

During the fourth stage, a SWOT analysis of the area was conducted based on the results of the field analyses, observation, the questionnaire data and literature review. SWOT analysis is a method used to determine the strengths and weaknesses of the studied environment, organization, technique, city, period, etc., and to identify opportunities and threats originating from internal and external environments. The objective of this technique is to develop strategies to obtain the maximum benefit from the strengths and opportunities available and to reduce the effect of the weaknesses and keep threats to a minimum. While the strengths and opportunities have a positive effect; the weaknesses and threats result in a negative effect. There are strong and weak aspects, which may be mostly under our control, in the internal environment. However, the external environment contains opportunities and threats, which are factors usually outside of our control. The possibilities to convert weaknesses, such as political, economic, sociocultural, and technological factors, into strengths have been carried out via SWOT analysis, and efforts are underway to remove the threats (Gürlek, 2002). SWOT analysis is also used for determining the current state and development of strategies on issues such as tourism, utilization of natural resources, sustainable development, conservation areas, and the energy and economic sectors (Ghinolfi et al., 2014; Scolozzi et al., 2014; Ghorbani et al., 2015; Açıksöz et al., 2016; Njoh, 2017).

In the fifth stage, the findings were analysed and evaluated from the perspective of the discipline of the landscape architecture profession by relating the findings to issues such as ecotourism and collaborative planning. In the sixth stage, however, the obtained results regarding the most suitable ecotourism activities for the existing potential were presented, and recommendations were formed.

Results

The current field usage

The current situation of the BNP was determined by field studies and observations. The park is under the influence of both the Black Sea climate, which is hot in summer and cool in winter seasons, and a forest ecosystem. The BNP is entered by a single

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access point. At this entrance, there are fees for vehicles and visitors. In the entrance area, there are a parking lot, a water-well, a rural-casino, an amphitheater, a information desk, a basketball court, children's playgrounds, and pergolas. There are also 40 picnic tables, 30 barbecues and 6 fountains that are located in suitable places throughout the area. The 2-meters-wide walking trail and 1153-m-long and 4-m-wide existing forest road, of which both were decided in the 1st Revision of the BNP Development Plan, are still in use at the present time. There are tennis courts and areas for fitness equipment on the walking trail. In the park, there are also four natural water channels for drainage, especially for the rainy periods against rain falls. Bridges are constructed for passing over these water channels. Today, the use of park continues as a picnic area and walking area, and as well as photographing area for weddings.

Questionnaire results

Using the AHP method with the expert group, a questionnaire was conducted to determine the ecotourism activity that best suits the current landscape potentials of BNP. The AHP full-hierarchy model generated for BNP is shown in *Figure 2*.

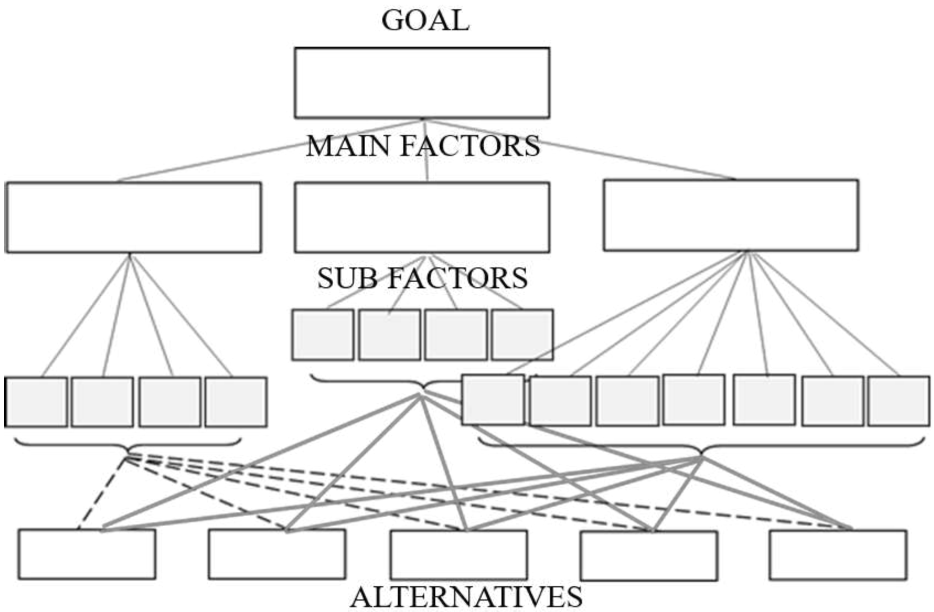

Figure 2. *Full AHP Hierarchy modes for BNP*

The purpose of this questionnaire was to make a classification between the main factors, sub-factors, and alternatives. For this purpose, 19 questions containing the key factors, sub-factors and alternatives were asked to the experts. The natural and cultural landscaping potentials were considered when determining the main factors and sub-factors. These factors were identified based on the literature review (Akpınar, 1995; Akten et al., 2009) performed on BNP-related studies and field analyses. While the main factors were composed of 3 variables, the sub-factors consisted of 15 variables, and the alternatives included 5 variables. The main factors consisted of the following variables: natural factors, socio-cultural and economic factors, and factors related to targets and policies regarding land usage. The main factors, sub-factors and alternatives are presented in *Table 1*.

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The Expert Choice (EC) software program was utilized for calculations and use of the AHP method. When the data obtained from the AHP were analysed, binary comparisons were also performed between the main factors and sub-factors. By using this program, the significance levels and weights of the factors, as well as the weight scores of the alternatives/ecotourism activities for each factor were determined. The questionnaires were determined to be meaningful since their consistency rates were identified as below 0.1. In addition, sensitivity analysis was also performed on the main factors. The survey results via use of the AHP are summarized below.

Table 1. *Main factors, sub-factors and alternatives used in the questionnaire*

Main factors	Sub-factors	Alternatives
Natural Factors (NF)	Climate	• Botanical tourism • Trekking • Bird/butterfly watching • Photo safari • Bicycle safari
	Flora/fauna	
	Topography	
	Geographical location	
Socio-cultural and economic factors (SCEF)	Transportation	
	Economic status of visitors	
	Demographic structure of visitors	
	Existence of nearby attraction centres	
Factors related to land use targets and policies (FRLUTP)	Biodiversity conservation	
	Satisfaction of recreational needs	
	Preservation of visual landscape potentials	
	City branding	
	Diversification of tourism activities	
	Increase in business opportunities	
	Improvement of infrastructure	

For the binary comparison between the factors, experts were asked to generate an assessment in accordance with the basic scale listed in *Table 2*.

Table 2. *The significance levels and their definitions used in the binary comparison among criteria (Saaty and Vargas, 2006)*

Significance level	Definition
1	Equal importance
3	Moderate importance
5	Strong importance
7	Very strong importance
9	Extreme importance
2, 4, 6, 8	Intermediate levels

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Based on the existing potentials, which were obtained from the experts, a binary comparison matrix, in which each main factor was compared with the others for determination of the most suitable ecotourism activities, is presented in *Table 3*.

Table 3. *The binary comparison matrix of the main factors*

	NF	SCEF	FRLUTP
NF	1	4	3
SCEF	1/4	1	1/2
FRLUTP	1/3	2	1

In *Table 3*, factors in rows were compared to factors in columns. These comparisons were presented as questions in the questionnaire form. The natural factors were scored with 4 points when compared to the sociocultural and economic factors and 3 points when compared to the factors related to targets and policies for land usage. In this case, the natural factors had priority for significance at the outset.

According to *Figure 3,* when the main factors, which were assumed to be effective for selecting the ecotourism activities by experts, were classified based on their weight ratios, 'natural factors' was ranked as the factor with the highest weight ratio (0.625).

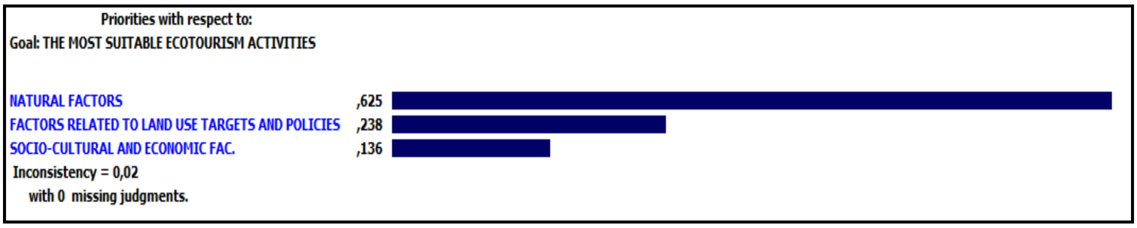

Figure 3. *The weight ratios for the main factors*

The sub-factor of flora/fauna was rated with the highest score by the experts. The binary comparisons among the sub-factors of the natural factors are also shown in *Figure 4.* According to *Figure 4,* the significance levels of the sub-factors of the natural factors were ranked as follows: flora/fauna, climate, topography, and geographical location.

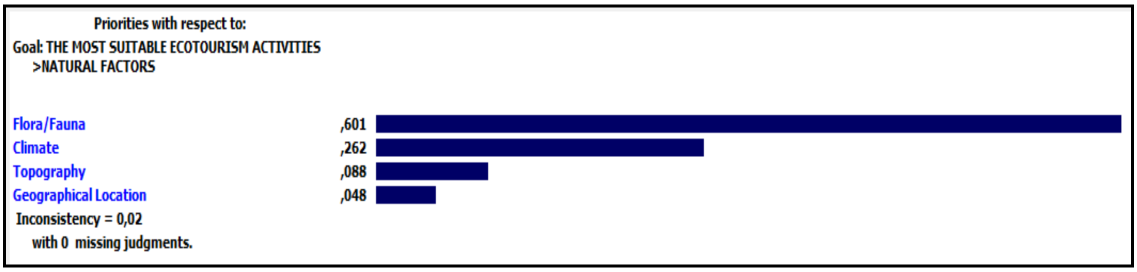

Figure 4. *The weight ratios of the sub-factors related to natural factors*

As a result of the binary comparison of natural factors with alternatives, botanic tourism had the highest weight ratio (0.338) (*Fig. 5*).

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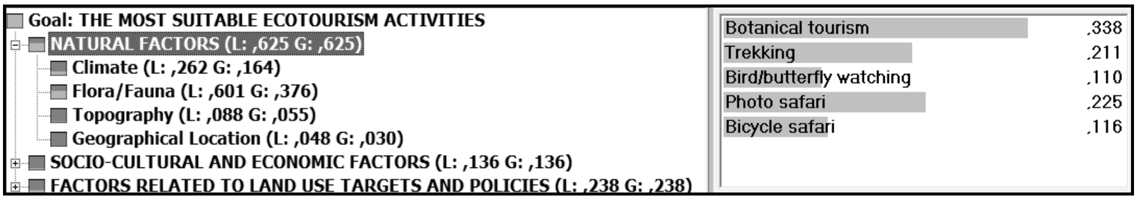

Figure 5. *The weight ratios of the ecotourism activities based on the natural factors*

Trekking had the highest weight ratio based on the binary comparison of climate and topography sub-factors with alternatives. When the flora/fauna and geographical location sub-factors were compared with alternatives, however, botanical tourism received the highest weight ratio.

The sub-factor of transportation was rated with the highest score (weight ratio 0.542) by the experts. According to *Figure 6* the significance levels of the sub-factors belonging to the socio-cultural and economic factors were ranked as follows: transportation, demographic structure of visitors, existence of nearby attraction centres, and economic status of visitors.

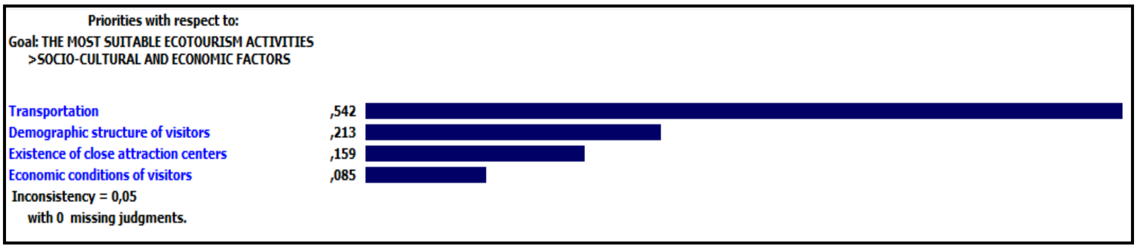

Figure 6. *The weight ratios of the sub-factors related to socio-cultural and economic factors*

As a result of the binary comparison of socio-cultural and economic factors with alternatives, trekking was identified as the highest weight ratio (0.507) (*Fig. 7*).

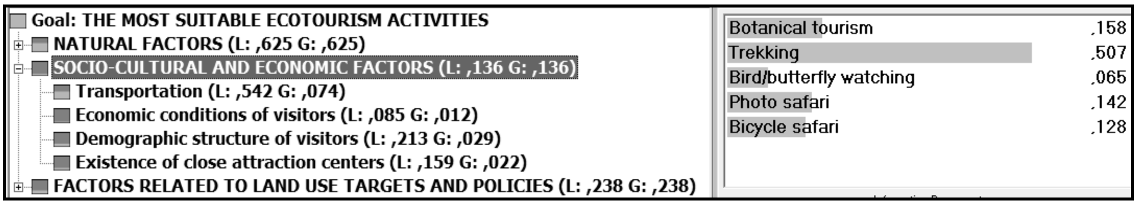

Figure 7. *The weight ratios of the ecotourism activities based on the socio-cultural and economic factors*

The binary comparison of alternatives with sub-factors, such as transportation, economic status of visitors, demographic structure of visitors, and the existence of nearby attraction centres, resulted in the identification of trekking as the activity with the highest weight ratio.

The binary comparison among the sub-factors of the main factor related to land use targets and policies is shown in *Figure 8*. The sub-factor of conservation of biodiversity was rated with the highest score (0.401) by the experts. Others were ranked in the

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following order: diversification of tourism activities, city branding, improvement of infrastructure, preservation of visual landscape potentials, increase in business opportunities, and satisfaction of recreational needs.

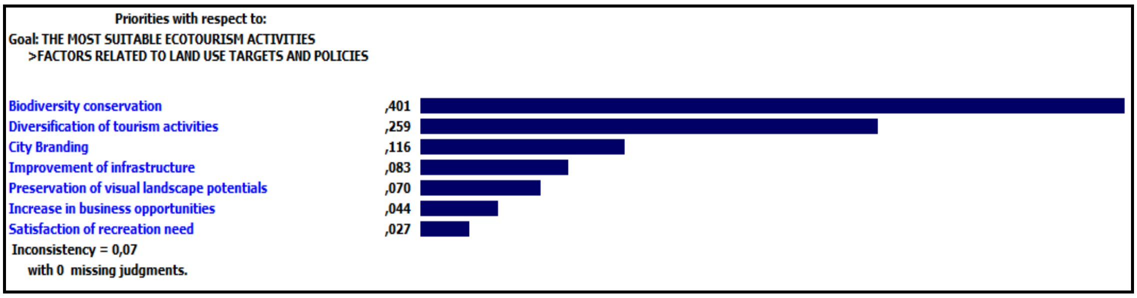

Figure 8. *The weight ratios of the sub-factors of the main factor related to land use targets and policies*

As a result of the binary comparison of alternatives with the factors related to land use targets and policies, botanical tourism received the highest weight ratio (0.330) (*Fig. 9*).

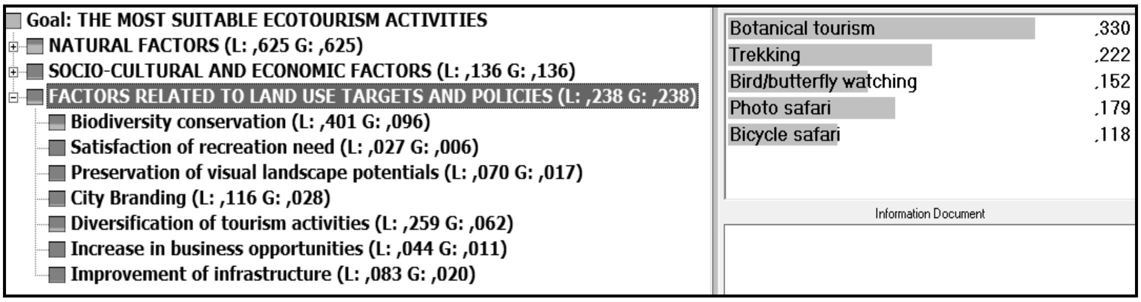

Figure 9. *The weight ratios of the ecotourism activities based on the factors related to land use targets and policies*

When the binary comparison was performed among the alternatives and the sub-factors, such as biodiversity conservation, satisfaction of recreational needs, city branding and increase in business opportunities, botanical tourism obtained the highest weight ratio. In the case of the binary comparison among the alternatives and the sub-factors, such as preservation of visual landscape potentials and diversification of tourism activities, trekking resulted in the highest weight ratio. The bicycle safari, however, received the highest weight ratio as a result of the binary comparison of alternatives with the sub-factor of improvement of infrastructure.

The sum of the weight ratios for all of the criteria was ‘1’. Hence, the comparison was consistent since the resulting rate was CR < 0.1.

The sensitivity analysis performed on the main factors is presented in *Figure 10*. While the natural factors had a 62.5% significance level, botanical tourism was in the top position with a significance level of 31.3%.

When the significance levels of socio-cultural and economic factors were increased from 13.6% to 65.2%, the order of the alternatives did not change. Additionally, trekking still remained in the leading position (*Fig. 11*).

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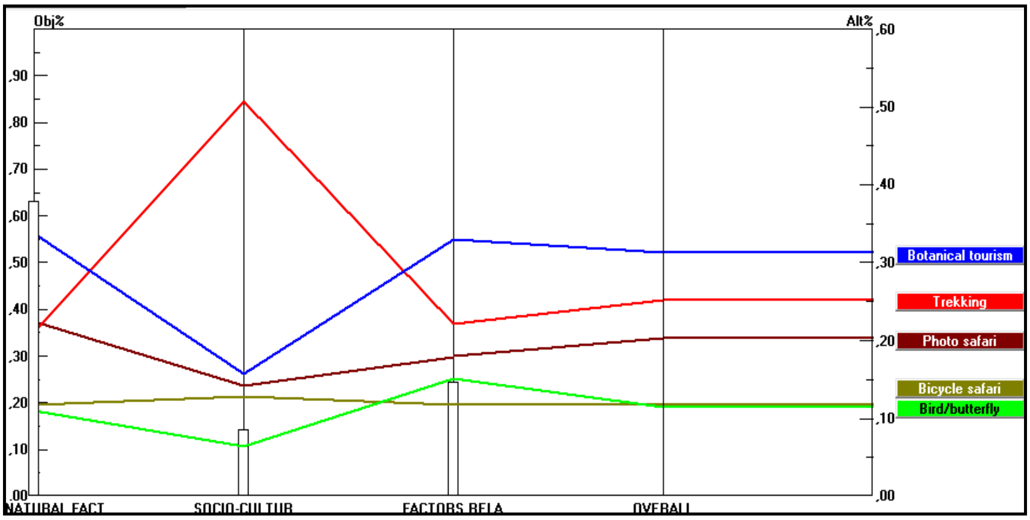

Figure 10. *The sensitivity analysis of the main factors*

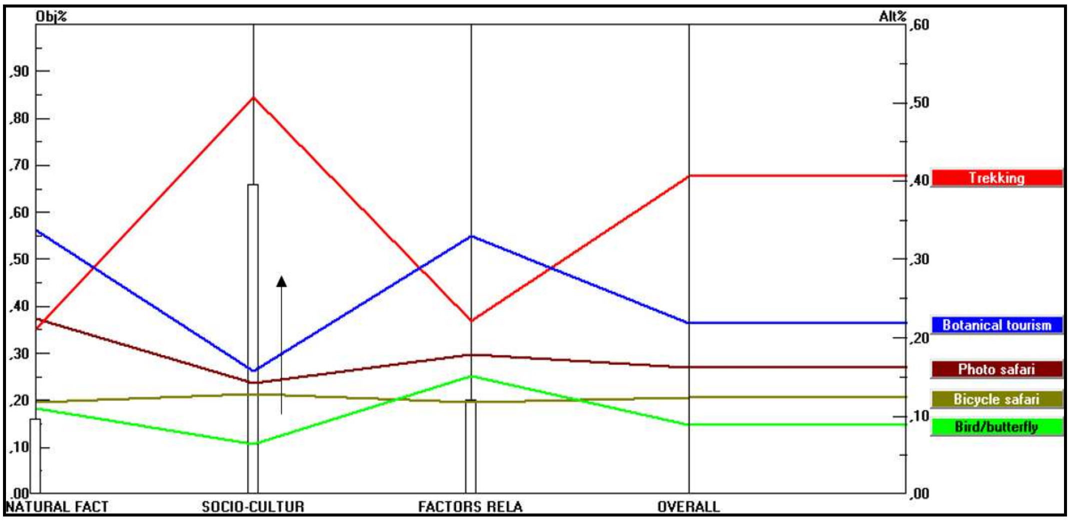

Figure 11. *Significance level of socio-cultural and economic factors*

Botanical tourism was also ranked the highest when the significance level of factors related to land use targets and policies was increased from 23.8% to 61.3% (*Fig. 12*). Hence, botanical tourism had a preferential significance level for BNP.

SWOT analysis

Field analyses and observations were made via visiting the field in different seasons. The current situation was determined by photo shoots. In addition, literature review was performed. In this context, the following studies and reports were analyzed; the 1st Revision of Development Plan and Report for the BNP prepared by the Bartın Provincial Directorate of Environment and Forest (Anonymous, 2006); the ‘Strategic Goals and Provincial Development Plan’ prepared by the Bartın Provincial Directorate of Planning and Coordination (Anonymous, 2008); the ‘Bartın Province Tourism Action Plan’ for 2012–2016 period prepared by the Bartın Provincial Directorate of Culture

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and Tourism (Anonymous, 2011); the ‘Strategic Plan’ for 2015–2019 period prepared by the Bartın Municipality (Anonymous, 2014); the ‘Regional Plan’ for 2010–2013 period prepared by the Western Black Sea Development Agency (Anonymous, 2009); and the article themed on the importance of open green areas in branding of Bartın Province (Çelik, 2017; *Table 4*). With this analysis, the strengths and weaknesses and opportunities and threats of BNP were determined. The study also determined how strengths and opportunities might be assessed and how weaknesses could be converted into strengths as well as what could be undertaken to remove the threats.

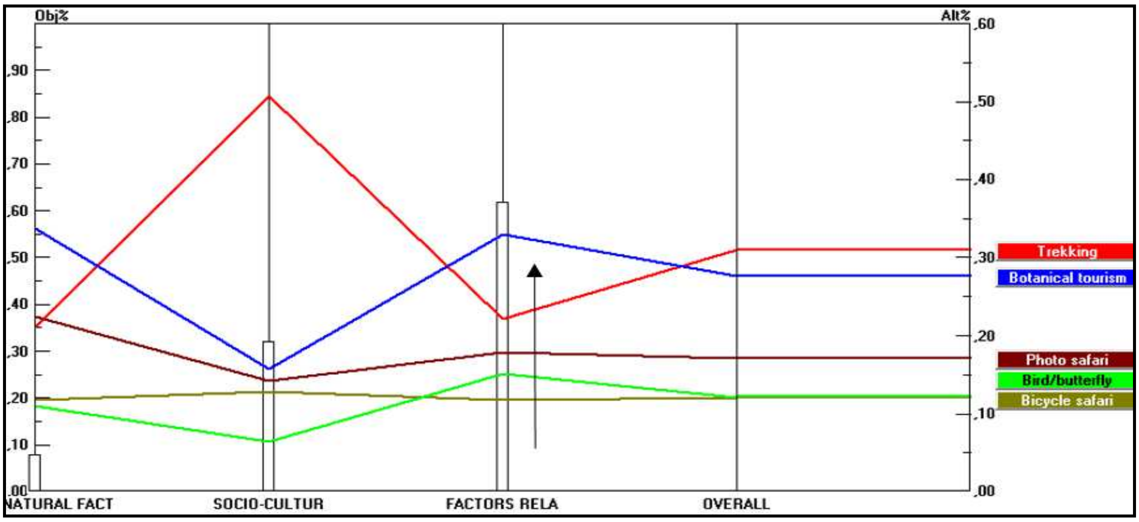

Figure 12. *Significance level of factors related to land use targets and policies*

As a result of the field analysis, the following issues were determined the strengths of BNP: absence of any residential areas and no deterioration of natural structures due to less frequent use, preparation of an in-progress development plan for the Park, presence of potential recreational activities, the existence of a safe environment, close proximity to important routes such as the Bartın-Karabuk Highway, and the famous destinations such as Amasra, Inkumu, Yedigoller, which have favourable climatic conditions and rich floral as well as faunal diversity, and finally, the absence of a property problem. As a result of the scientific research project (No.2016-FEN-A-001-author included) conducted in BNP, researchers determined that the Park contained a total of 50 families, 115 genera and 130 taxa. These aspects might be important forces for implementing ecotourism activities and for contributing positively to city branding as well as local and regional economic development. Again as a result of the surveys and observations, the positive reaction of the local community to development of the tourism sector, enthusiasm for environmental protection by both the local community and tourists, lack of agriculture and livestock activities, and tourist attraction potential of Bartın Province were considered opportunities in BNP. However, insufficient urban equipment and walking/running road information signage, lack of timely landscape maintenance and repair work, and insufficient promotional activity for BNP were considered weaknesses in the area. The absence of an application-oriented landscape project in this field of study, the lack of adequate investment activity in the tourism sector, and the increasing use of ecotourism activities that caused complications for biodiversity conservation were also listed as threats.

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Table 4. *The SWOT analysis related to ecotourism*

INTERNAL ANALYSIS	STRENGTHS	• Suitable climate conditions for ecotourism activities • Rich floral and faunal diversity • Existence of an in-progress Development Plan for BNP • Natural Park status • High potential landscape value, visually • Close proximity to Bartın city centre • Easy intracity and intercity transportation • Presence of a forest ecosystem • Close proximity to important destinations such as Safranbolu, and Yedigoller • Location in a secure area
	WEAKNESSES	• Insufficient promotional activity for BNP • Lack of infrastructure (information desks, buffets, urban equipment, etc.) • Lack of maintenance for tennis courts, volleyball courts and conditioning places • Lack of timely landscape maintenance and repair work • Lack of trekking trails maintained with desired plate stones and grass joints
EXTERNAL ANALYSIS	OPPORTUNITIES	• Local community's positive reaction to development of the tourism sector • Safety of Bartın as a secure city • Areas for souvenirs and shopping • Local community's positive reaction to environmental protection and BNP • Tourism brand potential of Bartın Province • Traditional handicrafts and food, existence of historical buildings, and development of ecotourism activities and business opportunities • Possibility for improvements in infrastructure • Possibility of diversifying and extending tourism activities throughout the year • Possibility of satisfying the recreational needs of the community • Possibility for conservation of visual landscape potentials • Perception of Bartın Province as a natural beauty • Location of Bartın in close proximity to Ankara and Istanbul cities
	CONSTRAINTS/THREATS	• Lack of application-oriented landscape projects • Wedding parties in the area • Possibility of the pressure for extensive use • Placement of newly added informational signboards for flora and fauna in a way that disrupts the existing integrity • Lack of an adequate amount of tour agents to contribute to the advertisement of Bartın Province • Lack of an adequate level of touristic events and activities in Bartın Province • Possibility of encountering difficulties in biodiversity conservation due to increasing demand for ecotourism activities • Lack of quality service and professionalism in the accommodation facilities in Bartın • Lack of infrastructure opportunities in Bartın Province (parks, parking areas, banks, post offices, social facilities, etc.) • Insufficient promotion and marketing activities for Bartın and its surroundings

Discussion

Various studies have been conducted both in national and international literature to identify the natural and cultural potentials of national parks, natural conservation areas, and natural parks and to determine the most suitable recreational or ecotourism activities for these potentials. Similar or different aspects of this research with the other conducted studies were summarized below. For example, in a study based on the socio-

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economic and cultural structure of a local community in Ol Donyo Sapuk National Park in Kenya, the research noted that ecotourism would contribute to economic development at the local and regional scale (Owino et al., 2012). This result was also supported by Yunus et al. (2013). In a survey study conducted for a Malaysian National Park, researchers emphasized that the implementation of ecotourism would be appropriate for increasing the income level of a local community and for achieving land use conservation. Similarly, the conservative use of natural resources, maintenance of sustainability, and engaging in ecotourism activities to accelerate local development were also predicted for BNP. This study also determined that ecotourism activities would be primary contributors to the socio-economic structure of Bartın's local residents.

When studying the Iron Gates Natural Park in Romania, Boengiu (2012) initially examined the available resources and infrastructure in order to determine the development potential for tourism. As a result, the study stated that attractive natural resources and natural monuments had potential values for tourism. In addition, Öztürk (2005) investigated the natural and cultural landscape potential of Kastamonu-Bartın Kure Mountains National Park on the basis of recreation and ecotourism concepts and determined the most suitable activities for ecotourism. These included activities such as wildlife viewing, trekking, photography, etc. Using SWOT analysis, Orhan and Karahan (2010) identified Uzundere's natural and cultural landscape potentials and determined the relevant ecotourism activities. In this context, religious tourism, trips to Tortum Waterfall and Ruins, picnicking, and festivals were listed as promising tourism activities. Similar to these studies, the current potentials of BNP were determined using SWOT analysis. However, the proposed ecotourism activities for BNP were varied. BNP had the potential for activities such as trekking, botanical tourism, photo safaris, bicycle safaris, etc.

In a study conducted by Nahuelhual et al. (2013) on a local level in southern Chile, the researchers noted that the unprecedented natural beauty had recreational potential. Additionally, Aminzadeh and Ghorashi (2007) investigated the relationship between recreational activities and aesthetic and ecological potentials in their research, which was conducted in Siangtan Forest Park in Iran. As a result, they indicated that recreational activities attracted attention in areas of natural beauty instead of in designed spaces. Similarly, this research concluded that BNP also had rich flora. As a result of the floristic studies conducted in BNP, a total of 130 taxa were identified. The park contains recreational potential due to its natural landscape values. With organized events, tourists and local residents will receive benefits from these recreational and ecotourism activities.

Within the context of landscape planning/restoration, alternative land uses following mining activities were determined by Akpınar (1995) using the AHP method. These alternatives were also prioritized. The priority order of ecotourism activities in the Yusufeli District of Artvin Province was also determined using the AHP method from Yılmaz and Surat (2015). According to the AHP analysis results, a nature exploration walk was identified as the ecotourism activity with the highest priority value. Similarly, BNP questionnaire data obtained from the experts were utilized in the AHP method, and the ecotourism activities, which could be implemented in BNP, were determined. The priority order of the ecotourism activities was determined; thus, botanical tourism was the top ranked activity. Other important activities identified were trekking, photo safaris, bicycle safaris, and bird/butterfly watching.

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To select the most suitable ecotourism activity in the Oltu and Olur Districts in Erzurum Province, a questionnaire was conducted using the AHP Method. The study emphasized that the ecotourism activities would contribute to promotion of the region (Akpınar Külekçi and Bulut, 2012). This result also aligns with the conclusion that ecotourism contributes to the promotion and branding of Bartın Province.

In a study conducted by Jalani (2012) in the Puerto Princesa Subterranean River National Park, the research stated that the local communities would receive new employment opportunities and their economic conditions would prosper due to ecotourism activities. In addition, the study concluded that city development would increase through ecotourism activities. These findings paralleled the results of the research performed in BNP. This study also concluded that the ecotourism activities in BNP would generate new employment opportunities for the local community and increase residents' economic incomes as well as the quality of urban infrastructure.

The existing natural and cultural values of the Karapinar District in Konya Province were determined using SWOT analysis. As a result, planning, management and monitoring requirements were emphasized in order to ensure the long-term sustainability of ecotourism (Önder and Polat, 2004). Similarly, the SWOT analysis technique was also used to determine the existing natural and cultural resources of Oltu District in Erzurum Province for the ecotourism potential. The research stated that development of ecotourism policies at regional and national scales was required to avoid harming the natural biota (Akpınar Külekçi and Bulut, 2013). The SWOT analysis performed for BNP also presented similar results. Ecotourism may be used as a tool for sustainable economic development at local, regional and national levels. In the tourism industry, attention should be given to planning, implementation, supervision and monitoring as well as to compliance with necessary laws and regulations.

Güngör and Polat (2017) stated that the local community in Konya was intensively using the urban parks for picnicking. They indicated that the visitor capacity of these parks could be at risk under such high pressure, and environmental pollution might increase to the degree that other visitors might feel uncomfortable. Therefore, the authors emphasized that allowing picnics and recreational activities, etc. in the forest area near the city might reduce this overuse pressure. Similar to that result, BNP has the characteristic of being a potential area for use as an alternative to publicly open green spaces in the city centre. By engaging in recreational events or ecotourism activities in BNP, both local communities and tourists will contribute to reducing pressure on publicly open green spaces in the city centre. There are one hundred and four parks in Bartın city. These parks are different sizes, and including playground areas, sporting areas, hiking trail, etc. These urban open green areas have been extensively used in Bartın (Anonymous, 2014).

Conclusions

In conclusion; with the aim of determining the existing landscape characteristics of the BNP, literature reviews, field analyses and observations were performed. Literature reviews and data obtained for Bartın city were analyzed for the BNP. In this context, data were obtained regarding geographical location, transportation status, topographical and geological structures, climatic characteristics, flora and fauna characteristics, historical development, technical and social infrastructure, tourism and recreation relations.

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Questionnaire were also conducted to an expert group in order to determine the most suitable ecotourism activities in the identified landscape potentials. The surveys were analyzed using the AHP method. Experts recommended ecotourism activities in the following order such as botanical tourism, trekking, photo safari, bike safari, bird/butterfly watching in line with the current potentials of the BNP. Ecotourism may be used as a tool for conservation, and sustainable use of landscape values for the BNP.

The ecotourism activities have the power to contribute to economy and local tourism sector. Thus, it will be possible to diversify tourism activities and spread them throughout the year. Ecotourism activities also have a strong effect on the economy and contribute to positively activating the local tourism sector. Thus, diversification of tourism activities and extending the activities throughout the whole year is possible.

In this context, the following recommendations were developed for ecotourism-oriented use of BNP:

- Initially, the visitor capacity of the park should be identified,
- Structural and botanical landscape design projects should be prepared to include disadvantaged groups,
- These projects should be conducted by a multi-disciplinary team,
- After the implementation of a project, landscape maintenance and repair work should be performed by a certain level of experts,
- Training activities should be planned to elevate the environmental protection awareness level,
- Tourism infrastructure should be developed in Bartın city centre,
- Promotional activities for Bartın and BNP should be increased.

In this process, it is very important to compel stakeholders to work together such as those from the public and private sectors, universities and NGOs (Non-Governmental Organizations) without any conflicting authorities. Planning, implementation, supervision controls should also be implemented, and compliance with laws and regulations should be considered.

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APPENDIX

Questionnaire

Determination of the priority with the analytic hierarchy process

This questionnaire aimed to determine the most suitable ecotourism activities in the existing potentials Balamba Natural Park (BNP).

Table A1. *The significance levels and their definitions used in the binary comparison among criteria (Saaty and Vargas, 2006)*

Significance level	Definition
1	Equal importance
3	Moderate importance
5	Strong importance
7	Very strong importance
9	Extreme importance
2, 4, 6, 8	Intermediate levels

The binary comparison matrix of the main factors

Natural factors	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9	Socio-cultural and economic factors
	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9	Factors related to land use targets and policies

1- *What is the relative significance of the following* ***main factors*** *in relation to the purpose of determining the most suitable ecotourism activity according to the existing potentials?*

Socio-cultural and economic factors	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Factors related to land use targets and policies

2- *What is the relative significance of the following the sub-factors related to natural factors in relation to the purpose of determining the most suitable ecotourism activity according to the existing potentials?*

Climate	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Flora/fauna
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Topography
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Geographical location

Flora/fauna	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Topography
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Geographical location

Topography	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Geographical location

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***3-** What is the relative significance of the following **the sub-factors related to socio-cultural and economic factors** in relation to the purpose of determining the most suitable ecotourism activity according to the existing potentials?*

Transportation	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Economic status of visitors
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Demographic structure of visitors
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Existence of nearby attraction centres

Economic status of visitors	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Demographic structure of visitors
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Existence of nearby attraction centres

Demographic structure of visitors	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Existence of nearby attraction centres

***4-** What is the relative significance of the following **the sub-factors of land use targets and policies** in relation to the purpose of determining the most suitable ecotourism activity according to the existing potentials?*

Biodiversity conservation	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Satisfaction of recreational needs
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Preservation of visual landscape potentials
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	City branding
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Diversification of tourism activities
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Increase in business opportunities
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Improvement of infrastructure

Satisfaction of recreational needs	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Preservation of visual landscape potentials
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	City branding
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Diversification of tourism activities
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Increase in business opportunities
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Improvement of infrastructure

Preservation of visual landscape potentials	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	City branding
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Diversification of tourism activities

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	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Increase in business opportunities
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Improvement of infrastructure

City branding	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Diversification of tourism activities
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Increase in business opportunities
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Improvement of infrastructure

Diversisfication of tourism activities	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Increase in business opportunities
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Improvement of infrastructure

Increase in business opportunities	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Improvement of infrastructure

Relevant importance of ecotourism activities according to sub-criteria of natural factors criterion

1- *What is the relative importance of the following activities related to* ***climate*** *sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

2- *What is the relative importance of the following activities related to* ***flora/fauna*** *sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

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Trekking	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

3- *What is the relative importance of the following activities related to* ***topography*** *sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

4- *What is the relative importance of the following activities related to* ***geographical location*** *sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

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Relative importance of ecotourism activities according to sub-criteria of socio-cultural and economic criteria

1- *What is the relative importance of the following activities related to* ***transportation*** *sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

2- *What is the relative importance of the following activities related to* ***economic status of visitors*** *sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

3- *What is the relative importance of the following activities related to* ***demographic structure of visitors*** *sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

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Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

***4-** What is the relative importance of the following activities related to **existence of nearby attraction centres** sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Relevant importance of ecotourism activities according to the sub-criteria of the field use targets criteria

***1-** What is the relative importance of the following activities related to **biodiversity conservation** sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

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Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

***2-** What is the relative importance of the following activities related to **satisfaction of recreational needs** sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9	Bicycle safari

***3-** What is the relative importance of the following activities related to **preservation of visual landscape potentials** sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

***4-** What is the relative importance of the following activities related to **city branding** sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

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Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

***5-** What is the relative importance of the following activities related to **diversification of tourism activities** sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

***6-** What is the relative importance of the following activities related to **increase in business opportunities** sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

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***7-** What is the relative importance of the following activities related to **improvement of infrastructure** sub-factors in order to determine the most suitable ecotourism activity according to the existing potentials?*

Botanical tourism	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Trekking
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Trekking	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bird/butterfly watching
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Bird/butterfly watching	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Photo safari
	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

Photo safari	9	8	7	6	5	4	3	2	**1**	2	3	4	5	6	7	8	9	Bicycle safari

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LAND USE AT ST. MARTA RANGE, LOS TUXTLAS, VERACRUZ, MEXICO – HOW DOES IT AFFECT THE COLLEMBOLA COMMUNITY?

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Abstract. The abundance of Collembola families were studied in three localities with four different land-use types: forest, agroforestry, grassland and corn crop, all located in the Santa Marta Range, Los Tuxtlas Biosphere Reserve, Veracruz, Mexico. Samples of litter and soil were collected in each land use during the dry season, February and March 2005, and processed by Berlese-Tullgren funnels. In addition, physical and chemical parameters of soil were measured. A nested MANOVA was applied to evaluate land-use and site effect on edaphic parameters, and a nested ANOVA was used to evaluate their effect on the Collembola abundance. In addition to this, a Cluster Analysis (CA) and Canonical Correspondence Analysis (CCA) were used. Besides, Shannon diversity index was calculated. A total of 1,088 collembolans from seven families were gathered, with the most abundant being Isotomidae, Entomobryidae and Hypogastruridae. The nested MANOVA and ANOVA revealed significant effect of the site and land-use on the soil parameters and Collembola abundance, respectively. CA formed two main groups based mainly on sites and biotopes. The CCA, showed that abundance of Onychiuridae and Odontellidae are related with altitude, Na and Cation Exchange Capacity (CEC). Diversity was higher in forest than in corn crop and grassland. The corn crop showed a higher incidence of Isotomidae and Entomobryidae than the other sites. Thus, the changes in the Collembola community at family level can be useful to recognize the quality of soil in different land uses.
Keywords: *bioindicators, canonical correspondence analysis, Entomobryidae, Hypogastruridae, Isotomidae*

Introduction

Land-use change is the main factor determining patterns of biodiversity of soil organisms (Bengtsson, 2002; Martins da Silva et al., 2015). Thus, the need to highlight the fact that disturbances caused by human land-use practices can affect biodiversity positively or negatively, usually having a loss of species (Bengtsson et al., 2000; Cuchta et al., 2012) is important to land management. Understanding the impact that changes in land-use practices has on the environment biodiversity is essential for the implementation of effective measures to preserve biodiversity in human-disturbed landscapes (Niemelä, 2000; Gisladottir and Stocking, 2005; Wu and Cho, 2007). Therefore, since Collembola is highly sensitive to changes in soil conditions, they have been used as bioindicators to follow the evolution of several types of edaphic systems

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(Fiera, 2009; Muturi, 2009; Paul et al., 2011; Ponge et al., 2003; Sousa et al., 2004, 2006). Some species, as *Mesaphorura krausbaueri*, have also been proposed as possible indicators of soil fertility; they have been considered pioneer in secondary succession on cultivated soil (Dunger, 1986). Other species, such as *Protaphorura armata*, have been considered in the study of the effects that pollution from human activities have on biological and ecological processes such as reproduction, mortality and population growth (Bengtsson et al., 1983, 1985a, b). Furthermore, the important role that Collembola play in the edaphic process makes them a useful tool in identifying the modification and quality of the soil ecosystems (Rusek, 1998; Culliney, 2013). They can be used at a species and even at a family levels, as has been shown in studies of the subtropical forest floor in Manipur, India (Waikhom et al., 2006) and those carried out in areas with *Araucaria* forest in Brazil (Baretta et al., 2008) have demonstrated. It has been shown that data sets built at the family systematic rank can also detect the effects of disturbance with little loss of information and can be a preliminary tool for describing patterns and successions in human-disturbed soil ecosystems (Caruso and Migliorini, 2006).

Veracruz State is recognized for its high biodiversity, and high rate of endemism as well as its contribution to global diversity, and the ongoing conservation of the tropical forest. The State takes the first place in the species richness of many groups, including Collembola (Palacios-Vargas et al., 2000, 2004). Nevertheless, as most of the State is in the tropics, its territory is experiencing severe and rapid change in land-use, increasing the alarm at the deforestation rate and original vegetation loss (Phillips, 1997; Dalrymple, 2006).

The deforestation of tropical rain forest at Los Tuxtlas region has been particularly high in the last five decades, mainly due to the conversion of forest land in pastures and corn fields, that is the dominant land use at this region (Flint et al., 2000). Adding, the region was transformed severely to gain cattle pastures and only about 21% of the original forest vegetation remains unspoiled (Gutiérrez-García and Ricker, 2011).

The change in use modifies the biogeochemical cycles of the soil at local, regional and global scales, and also produces important effects in the diversity and role of soil organisms. Many studies on this subject have been developed in the tropics (Lemessa et al., 2015; Smith et al., 2015; Peña-Peña and Irmler, 2016). Although Collembola is one of the major components of the soil biota, the evaluation of human activities' effect on Collembola assemblages has not been reviewed recently in Mexico (Lavèlle et al., 1981; Palacios-Vargas, 1985; Villalobos, 1989, 1990; Miranda and Palacios-Vargas, 1992; Mendoza, 1995; Mendoza et al., 1999). Palacios-Vargas and Castaño-Meneses (2014) compiled recently the information about importance of Collembola as bioindicators in different environments. There are evidences that Collembola richness and abundance show modifications in agroecosystems according to the quality of irrigation, moreover some edaphic parameters, such as pH, organic matter and exchangeable cations are altered as well (Cutz-Pool et al., 2007).

The main purpose of this study is to evaluate the effects of four land-use types: corn crop, grassland, agroforestry and forest on the Collembola family abundance patterns in three sites. Besides, this article will explore the relationships of these organisms to some soil variables. We hypothesize that agricultural and livestock practices produce negative effects on the Collembola diversity and abundance, and changes in the composition and dominance of Collembola families, suggest that some families can be as indicators of

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land use and agricultural practices, and can be a useful tool in management and conservation programs.

Materials and methods

Study site

The St. Marta Range (SMR), is located at the East of Veracruz (extreme coordinates: 18° 15′ 18′′ N; 94° 40′ 95′′ W) in Los Tuxtlas Biosphere Reserve (*Fig. 1*), Mexico. SMR comprises 20,000 hs in the Soteapan and Mecayapan municipalities. The highest peaks are the volcanoes Santa Marta (1720 m), San Martín Tuxtla (1680 m), and San Martín Pajapan (1180 m) (Ramírez, 1999). The climate of Los Tuxtlas region is tropical, hot and humid in the lowlands (below 300 m) and medium altitudes (300-700 m). It turns semi-warm at higher elevation (700-1700 m). The rainfall is abundant in the area, close to 5,000 mm in the lowlands though this could increase in elevated places. Overall annual average temperature can reach 26 °C falling to 18 °C (Soto and Gama, 1997). Among the most important types of vegetation are high evergreen tropical forest, medium evergreen tropical forest, cloud forest, pine forest, oak forest and savanna (Castillo-Campos and Laborde, 2004). Unfortunately, the region of Los Tuxtlas has been deforested severely, mainly to gain cattle pastures, and only about 21% of the original forest vegetation remains unspoiled (Gutiérrez-García and Ricker, 2011).

Sample collection

Sampling of fauna was carried out in February and March 2005 during the dry season (which runs from January to April in the area). Three sites with different altitude and vegetal cover percentages were sampled: San Fernando (SF; range 740-1145 m asl; 50% vegetal cover), López Mateos (LM; 191-357 m asl; 75% vegetal cover) and Venustiano Carranza (VC; 160-380 m asl; 25% vegetal cover) on the slopes of the Santa Marta Volcano in the biosphere reserve of Los Tuxtlas. In each site, we sampled four land-use types: forest (Fr), agroforestry (Ag), grassland (Gr) and corn crop (Cc). Sampling methodology and extraction of soil fauna followed that recommended by Franklin and Morais (2008). We chose 5 points in each land-use at random. Beside, we sampled litter (Li) and soil at 0-5 cm (So1) and 5-10 cm (So2) being 15 samples a total: 5 of Li, 5 of So1 and 5 of So2 for each site/land-use. Samples were taken in the same core: first, we removed the litter then we took the first 5 cm, and finally the following 5 cm. In this way, the total of samples per site was 60, being 180 the sample total in all three sites. For sampling, a metal cylinder was used: 5 cm diameter and 5 cm height. Samples of Li, So1 and So2 were taken in a vertical profile fashion in each point.

Fauna extraction was by Berlese funnels with electric light bulb 40 W/funnel for five days. All fauna was first fixed in formalin 5% and then was preserved in 70% alcohol.

In addition to faunal sampling, soil samples were analyzed to obtain some soil parameters; pH was measured in a solution of $CaCl_2$ extract (1:2.5); moisture percentage; particle-size distribution (clay, sand and silt) was determined by hydrometer method (Bouyoucos, 1962); Mg^+ and Ca^+ were extracted by 1 M ammonium acetate pH7 and quantified by EDTA, whereas Na^+ and K^+ were evaluated using a flame photometer (Page et al., 1982); also Cationic Exchange Capacity (CEC) and porosity were recorded.

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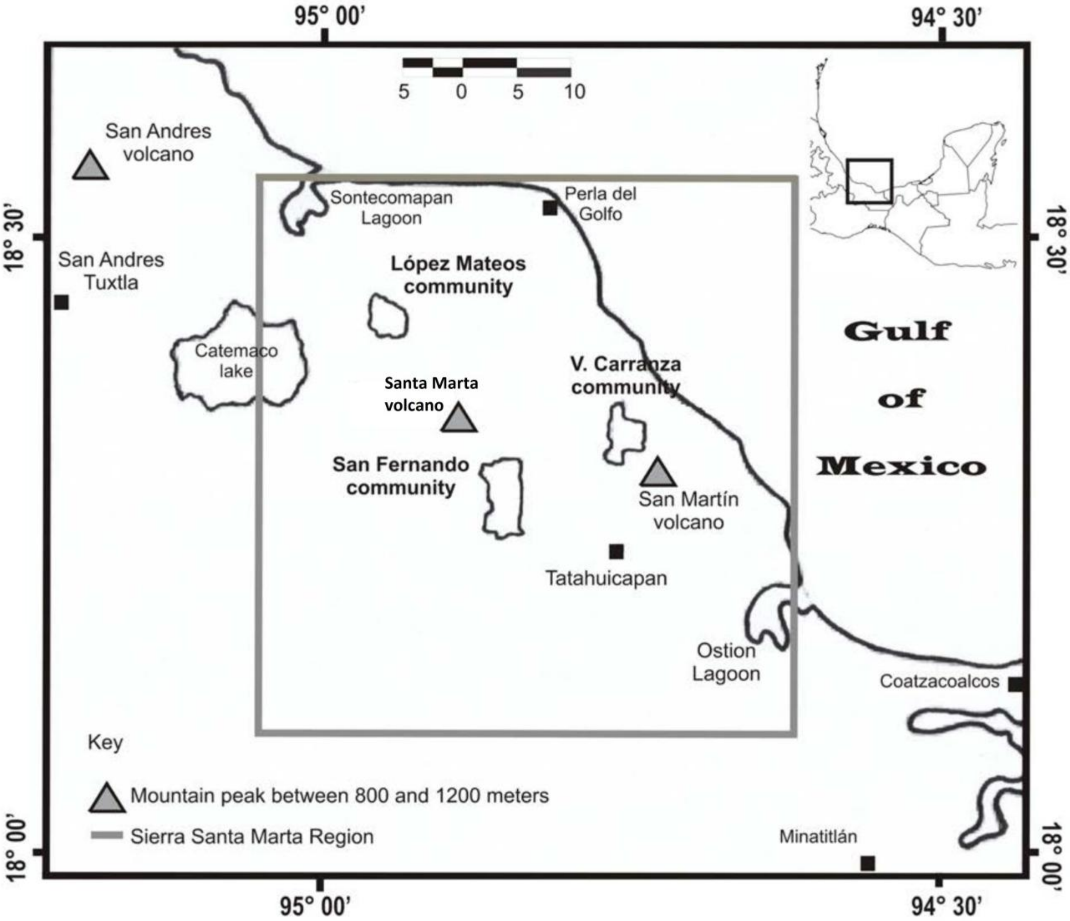

Figure 1. *Location of the three study sites, López Mateos, San Fernando and Venustiano Carranza in St. Marta Range (grey frame), in Los Tuxtlas Biosphere Reserve*

Statistical analysis

A nested multiple analysis of variance (MANOVA) was applied with two hierarchized level, land-use nested within sites, and sites, to evaluate the effect of these factors on all the physical and chemical soil variables. A nested analysis of variance (ANOVA) with three hierarchized levels, depth strata nested within land-uses; land-use nested within sites, and sites, to evaluate their effect on the Collembola abundance transformed as $\log_{10}(x + 1)$ to meet homoscedasticity and normality (Zar, 1999). A post hoc multiple comparisons with Bonferroni paired test (Zar, 1999) was performed when significant effects were found. Statistical analyses were performed using Statistica 6.0 (StatSoft, 2006). A Cluster analysis (CA) on a Bray-Curtis similarity matrix and using the unweighted pair-group average (UPGMA) amalgamation rule was performed on all data including site, land-use type and biotope: litter and soil at two depth levels in order to explore the faunal relationships. The analysis were made using PC-ORD v. 5.0. A Canonical Correspondence Analysis (CCA) was used to explore the influence of soil variables on faunal composition by using CANOCO 4.5 (Ter Braak and Smilauer, 1998). A Montecarlo test with 500 permutations was used to evaluate the significance of axes. Also Shannon diversity index (*H*) was calculated using the abundance of family level of taxa.

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Results

Nested MANOVA showed significant effect of site (λ = 0.04, $F_{18,\ 164}$ = 37.13, p < 0.01) and land use nested in site (λ = 0.11, $F_{81,538.9}$ = 2.66, p < 0.01) on physical and chemical soil parameters. Means and 95% confidence intervals (CI) for all variables are shown in *Table 1*.

Table 1. *Means and 95% confidence intervals for all the physical and chemical soil variables. Moisture to porosity in %, pH to CEC in cmol/Kg^{-1}*

	Moisture	Clay	Sand	Silt	Porosity	pH	Na	K	Mg	Ca	CEC	N
LM	44.09	20.06	57.59	22.34	74.92	5.21	0.47	0.99	3.61	8.62	13.68	32
±95	±2.02	±3.21	±3.90	±2.19	±1.85	±0.08	±0.08	±0.20	±0.72	±1.49	±2.26	
SF	67.35	47.82	30.16	22.01	69.35	5.05	0.15	0.54	3.04	9.18	12.91	37
±95	±5.77	±5.73	±3.31	±4.16	±3.26	±0.16	±0.02	±0.11	±0.50	±1.73	±2.08	
VC	38.27	55.21	25.09	19.70	68.32	4.63	0.12	0.31	1.78	4.35	6.55	33
±95	±4.43	±3.47	±1.93	±3.04	±3.63	±0.13	±0.03	±0.07	±0.43	±1.07	±1.48	
LM-Ag	44.12	15.00	64.50	20.50	76.08	5.13	0.55	0.82	3.04	8.49	12.89	8
±95	±5.18	±5.91	±8.32	±3.66	±5.50	±0.11	±0.16	±0.26	±1.21	±2.14	±3.57	
LM-Cc	39.88	15.88	55.38	28.75	71.80	5.26	0.29	0.75	2.54	6.19	9.77	8
±95	±3.24	±6.68	±10.35	±4.87	±3.2	±0.16	±0.15	±0.59	±1.33	±2.53	±4.23	
LM-Gr	46.91	28.13	51.63	20.25	74.31	5.17	0.44	1.45	3.88	7.86	13.63	8
±95	±2.74	±8.64	±9.33	±2.96	±2.82	±0.14	±0.11	±0.38	±1.65	±2.31	±3.92	
LM-Fr	45.44	21.25	58.88	19.88	77.47	5.28	0.59	0.95	4.96	11.95	18.45	8
±95	±5.54	±1.99	±5.20	±4.90	±4.37	±0.26	±0.20	±0.37	±1.94	±4.72	±6.69	
SF-Ag	64.09	43.79	32.43	23.79	71.08	5.24	0.13	0.25	3.27	11.30	14.95	7
±95	±3.85	±18.77	±13.43	±8.62	±4.33	±0.43	±0.07	±0.10	±1.52	±6.53	±7.82	
SF-Cc	70.05	44.47	26.88	28.65	66.81	5.34	0.09	0.45	4.13	9.78	14.45	8
±95	±4.64	±16.67	±4.36	±15.08	±1.6	±0.26	±0.05	±0.29	±1.32	±2.59	±3.69	
SFGr	72.87	54.81	30.00	15.19	62.93	4.95	0.16	0.78	2.79	7.40	11.13	12
±95	±13.75	±6.93	±4.80	±3.65	±8.03	±0.23	±0.03	±0.21	±0.89	±1.98	±2.83	
SF-Fr	60.85	44.95	31.40	23.65	77.87	4.81	0.19	0.52	2.33	9.36	12.39	10
±95	±15.81	±13.47	±8.68	±9.22	±3.70	±0.41	±0.05	±0.21	±0.73	±4.91	±5.51	
VC-Ag	34.91	51.63	25.31	23.06	66.64	4.53	0.13	0.21	2.33	4.51	7.18	8
±95	±2.64	±7.84	±3.18	±8.94	±3.47	±0.27	±0.10	±0.05	±1.38	±2.29	±3.49	
VC-Cc	32.49	53.38	25.44	21.19	68.69	4.94	0.12	0.45	2.26	7.33	10.17	8
±95	±3.13	±7.64	±5.08	±5.77	±11.94	±0.23	±0.08	±0.19	±1.14	±2.82	±4.06	
VC-Gr	43.49	60.44	25.22	14.33	66.34	4.53	0.11	0.36	1.19	2.38	4.05	9
±95	±17.09	±5.60	±5.31	±5.61	±10.94	±0.20	±0.05	±0.14	±0.32	±1.15	±1.48	
VC-Fr	41.53	54.75	24.38	20.88	71.88	4.54	0.11	0.20	1.41	3.39	5.11	8
±95	±4.34	±10.34	±4.47	±6.40	±1.78	±0.38	±0.07	±0.12	±0.75	±1.66	±2.41	

Moisture content was higher in the four SF land uses than in other localities, with no differences among them. We observed significant differences between SF corn and grass with the four LM land uses, in addition to this, there were differences between SF agroforestry and LM corn. The four SF land uses differ from VC agroforestry and corn

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land use. The SF corn and grass vary form VC grass and forest. The sand and Na contents showed similar patterns, observing the highest averages in the four LM land uses. Both parameters, sand and sodium, were lower in SF and VC than in LM with no significant differences among them. We observed significant differences in sand content among the four LM land uses with the four SF and VC land uses. Sodium showed differences among LM agroforestry, grass and forest compared to the four SF and VC land uses. There were also differences between corn with agroforestry and forest in LM. Porosity was very homogeneous among the four land uses of the three sites. We observed differences only among SF grass compared with LM agroforestry and forest, and between SF grass and forest. The silt content did not show defined patterns between land uses and sites. The pH of VC grass, agroforestry and forest was different to LM corn, grass and forest, so were SF agroforestry and corn; we found differences between LM agroforestry and VC grass as well. Potassium content was higher in LM grass and forest. We identified important differences in the potassium levels among LM grass and the four land uses of SF and VC. Magnesium was also homogeneous among the four land uses of the three sites; we found some differences among LM and SF forests and the VC four land uses. The CEC was also very similar among the four land uses of the three sites. We recorded differences in CEC between LM forest with VC agroforestry, grass and forest, also between LM and VC grasses, SF agroforestry with VC grass and forest, and SF corn and VC grass. Calcium content also showed few important differences in such levels among land uses most of them correlated with CEC. The clay contents were significantly lower in the four LM land uses than those of SF and VC, among these, there were no differences. We observed significant differences in clay levels among LM agroforestry, corn and forest with all land uses of both SF and VC.

The nested ANOVA showed significant effect of site ($F_{2,\,154} = 13.86$, $P < 0.001$), and land-use nested within site ($F_{6,\,154} = 2.54$, $P < 0.05$) but not for depth strata nested by land-use ($F_{8,154} = 1.09$, $P > 0.05$) on Collembola abundance. The Bonferroni paired test detected significant differences among LM with SF and VC. For land-use nested by site, we identified differences among corn crop in LM with corn crop, grassland and forest in SF and VC. Other differences were found among corn crop and agroforestry in LM, grassland in SF and both grassland and forest in LM, and grassland and forest in VC. Means and 95% confidence intervals (CI) for Collembola abundance are shown in *Table 2*.

A total of 1,088 springtails of seven families were obtained from the three sites (LM, SF and VC). The most abundant families were Isotomidae (37%), Entomobryidae (27%) and Hypogastruridae (24%) (*Fig. 2a*). The number of Neanuridae and Odontellidae recorded was far less, under1% each (*Fig. 2*). LM had more than half of the total amount of Collembola (54%), while SF and VC had 25% and 21%, respectively (*Fig. 2b*). Abundance was higher in Gr and lower in Ag (*Fig. 2c*). In soil biotope the abundance was higher in So1 (40% in the first 5 cm deep) than in So2 (34%), with the lowest occurring in litter (26%) (*Fig. 2d*). All families were recorded in LM while Neanuridae was absent in SF and VC (*Fig. 3a*). We found all families in soil in both So1 and So2, however Neanuridae was absent in litter (*Fig. 3b*). In land-use types, all families were found in Gr, although Neanuridae was absent in Ag and Fr; Odontellidae also was absent in Cc (*Fig. 3c*). Shannon index showed a higher diversity in LM, mainly this fact comes from agroforestry and forest; however, we identified the highest diversity in SF forest. In biotopes, the highest diversity was observed in litter and both depth level of soil of forest (*Table 2*).

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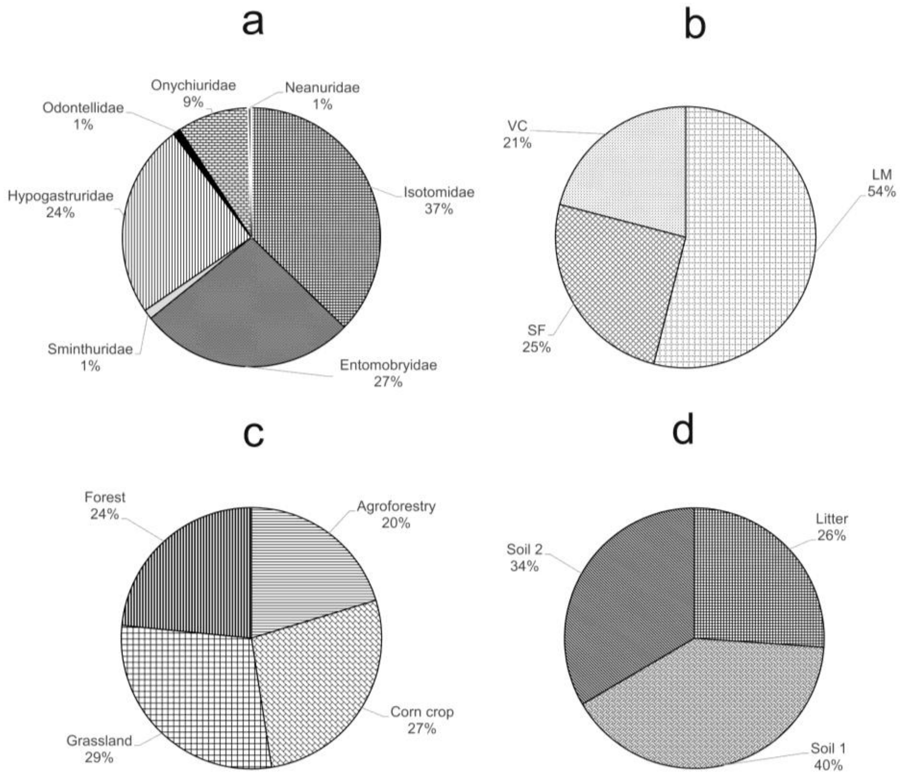

Figure 2. *Distribution of Collembola abundance by families a), sites b), land-use types c) and biotopes d) from the St. Marta Range, Veracruz*

Table 2. *Averages and 95% confidence intervals ((individuals/m^2) for Collembola abundance in sites, land-use in sites and litter, soil at 0-5 cm and soil at 5-10 cm depth*

		Average	±95	N	Shannon index *H´*
López Mateos		4979.63	±1160.90	60	1.37
San Fernando		2398.17	±1130.35	58	1.22
V. Carranza		2072.30	±580.45	56	1.17
López Mateos	Agroforestry	2846.23	±1527.49	17	1.48
López Mateos	Corn crop	8375.76	±3406.31	11	1.28
López Mateos	Grassland	5962.32	±3324.85	14	1.21
López Mateos	Forest	4159.88	±1369.65	18	1.36
San Fernando	Agroforestry	2800.41	±2270.88	10	1.12
San Fernando	Corn crop	2362.53	±1125.25	14	0.82
San Fernando	Grassland	2876.78	±3151.73	20	0.74
San Fernando	Forest	1456.21	±860.49	14	1.51
V. Carranza	Agroforestry	2408.35	±1583.50	15	0.80
V. Carranza	Corn crop	1960.29	±1079.43	13	1.17
V. Carranza	Grassland	1242.36	±560.08	16	0.78
V. Carranza	Forest	2886.97	±1563.14	12	1.27
Agroforestry	Litter	1201.63	±509.16	14	1.32
Agroforestry	Soil 1	3630.35	±2092.67	15	1.32
Agroforestry	Soil 2	3172.10	±1664.97	13	1.21
Corn crop	Litter	3238.29	±2275.97	11	1.33
Corn crop	Soil 1	4073.32	±2683.30	14	1.24
Corn crop	Soil 2	4465.38	±2713.85	13	1.23
Grassland	Litter	3477.60	±3477.6	18	0.98
Grassland	Soil 1	3722.00	±3548.88	13	1.11
Grassland	Soil 2	2627.29	±1690.43	19	1.38
Forest	Litter	2107.94	±921.59	14	1.43
Forest	Soil 1	4276.99	±1848.27	15	1.40
Forest	Soil 2	2408.35	±1069.25	15	1.36

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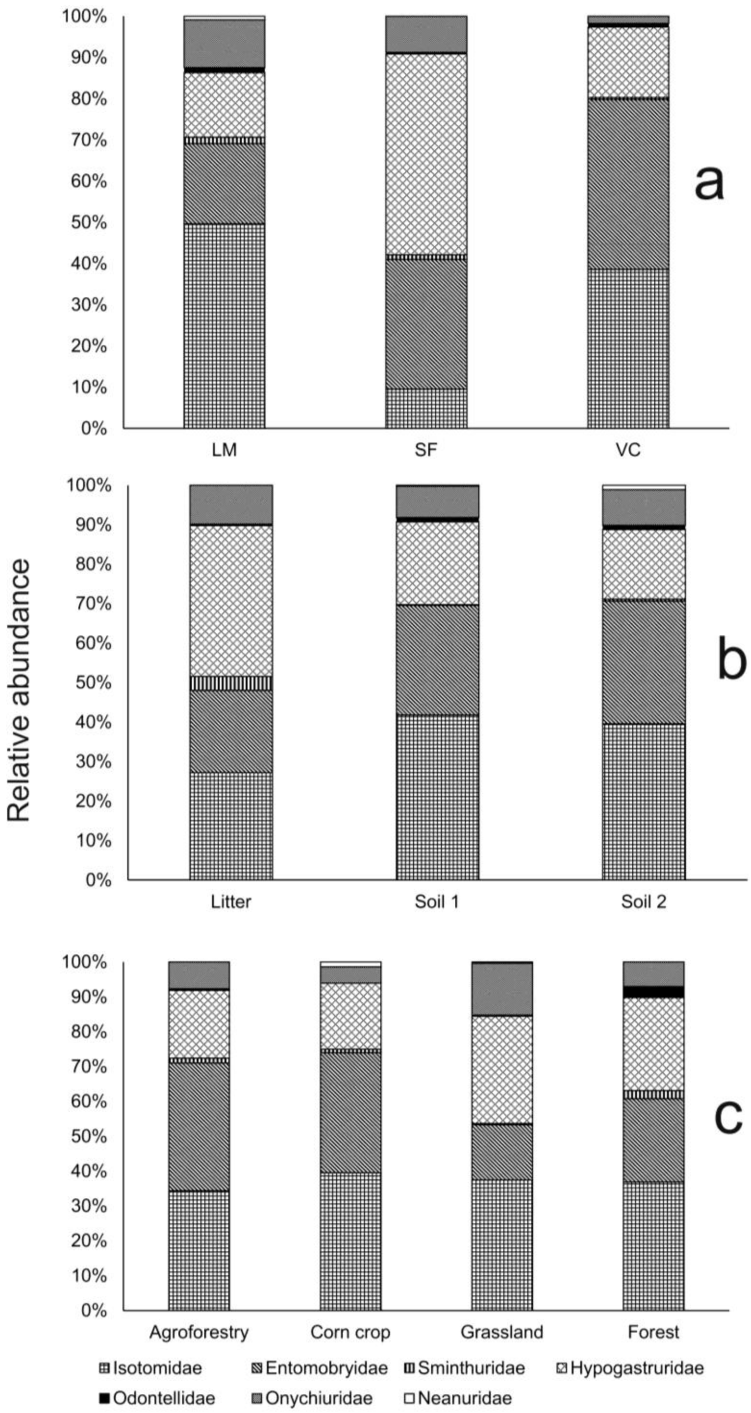

Figure 3. *Relative composition of Collembola assemblages in sites a), biotopes b), and land-use types c). LM, López Mateos; SF, San Francisco and CV, Venustiano Carranza*

In the CA, almost all the samples of LM and VC tended to form one high group (Gr1), while those of SF formed a second high group (Gr2, *Fig. 4*). In the first group, soil samples of all the land-use types from VC tended to be close forming a sub-cluster. The litter samples of all land use of VC had low abundance of Collembola and they were dispersed in Gr2.

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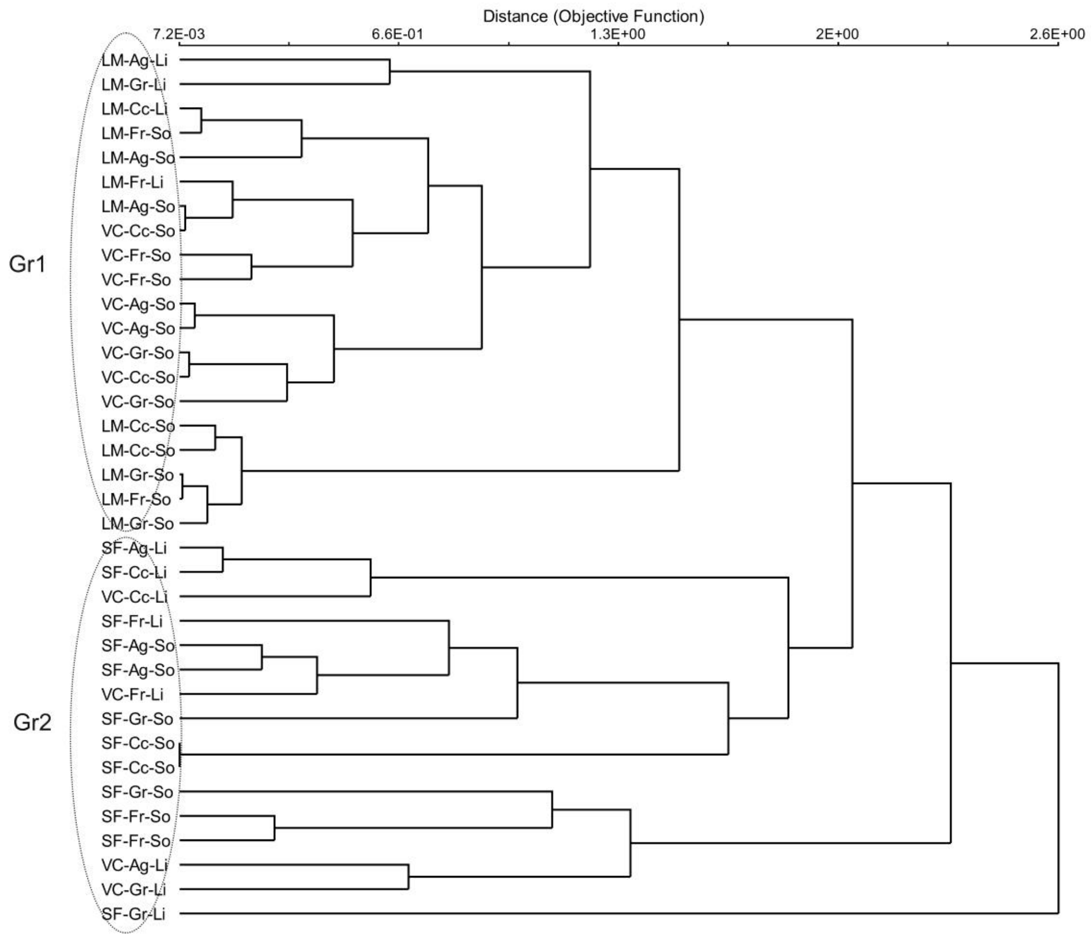

Figure 4. *Classification dendrogram showing faunal relationships of Collembola assemblages based on a Bray-Curtis similarity matrix and UPGMA amalgamation rule using all the samples including sites, biotopes and land-use types*

The CCA analysis (*Fig. 5*) showed that canonical axes 1 and 2 together explain 84% of the variance in family composition of Collembola. Correlation coefficients between ordination axes and environmental variables, rather than canonical coefficients, were used to infer the importance of each parameter in predicting the family composition. More related land uses were Cc and Gr and in our analysis, they were opposite located to Fr (*Fig. 5*). Corn crop and forest axes (although opposed) exerted the strongest influence on Collembola abundance. Odontellidae were more related to forest axis and soil moisture. Sand, porosity and the presence of silt and clay are also important in determining the distribution of Collembola abundance. We represented these variables by longer arrows in *Figure 5*. The first two exerted an opposite effect on each other but sand was an important determinant of abundance of both Sminthuridae and Onychiuridae. We mainly recorded the abundance of these two families in all the land uses of LM except Sminthuridae in Ag, in which higher records of sodium and sand were obtained. Neanuridae seems to be related to the corn crop of LM because only a few specimens of this family were found in this land use. Samples of all the land uses from VC and LM were well separated in the plane generated by the first two axes; however, samples of SF crossed between LM and VC. Samples of Ag and Cc from SF were more related to VC-Cc and samples of Fr and Gr from SF to LM-Fr and LM-Ag.

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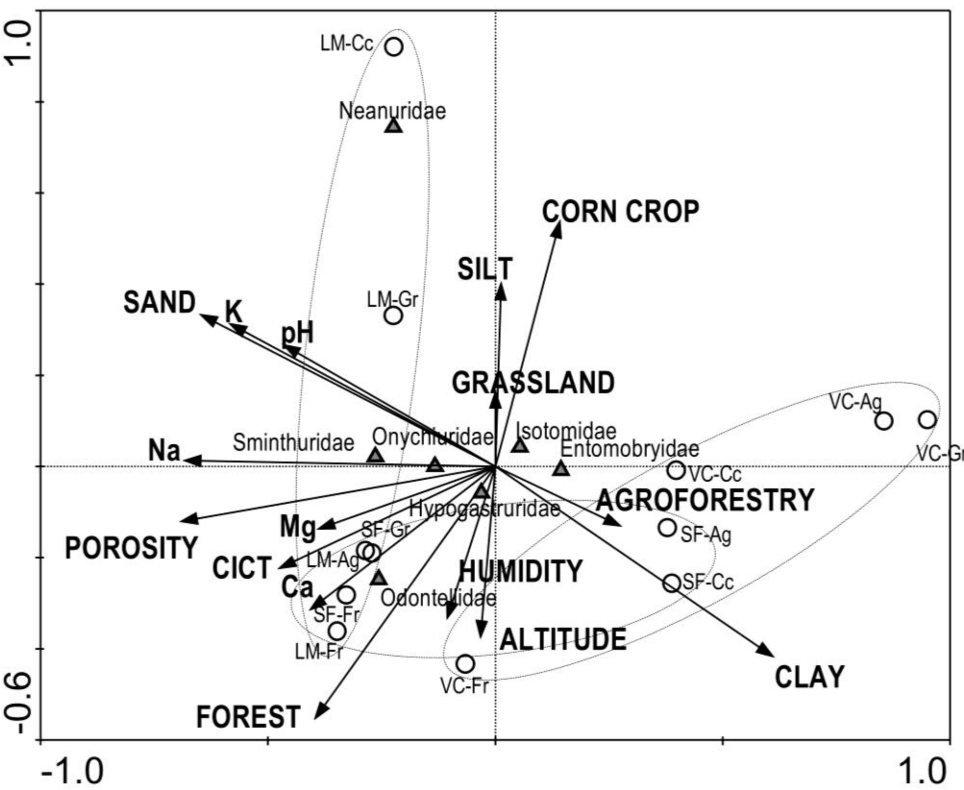

Figure 5. *Triplot of CCA ordination showing environmental variables (large arrows) most strongly correlated with axes CC1 and CC2; families in black triangles and sites-land-use in clear circles. Abbreviations for sites and land use as in Table 1. In terms of predicting family composition, important environmental variables have longer arrows than less important ones*

Discussion

Abundance patterns of the Collembola from SMR correlates with most of those reported in literature. Such correlation lies in the fact that the dominant families in soil and litter are Isotomidae, Entomobryidae and Hypogastruridae, although some types of soils can contain great populations of other families such as Onychiuridae (Lavèlle et al., 1981) or Neanuridae (Garita-Cambronero et al., 2006).

The estimation of Collembola density (individuals/m^2) for the grassland of SMR was 3,361 m^2, similar to that registered by Lavèlle et al. (1981) from the grassland of the Laguna Verde region, Veracruz (3,005 – 4,383 m^2). The estimated density of 2,834 m^2 for the forest of SMR is lower than that reported by the same author from the tropical rain forest of Laguna Verde region (6,257-7,025 m^2). Collembola density for the agroforestry of SMR was 2,685 m^2 and for that of the corn crop was 4,232 m^2. In agricultural zones of tropical regions, densities of 60,600 m^2 have been registered (Culik et al., 2002), which represent important differences within this study. Meanwhile, in the litter of SMR a density of 2,506 m^2 could be expected and for the first 5 cm deep of the soil of 3,925 m^2 and for the next 5 cm deep 3,168 m^2. Isotomidae, accounting for 37%, dominated the Collembolan assemblage in the study area. Particularly, Isotomidae accounted for 65% in the forest, and 40%, 37% and 34% for corn crop, grassland and agroforestry, respectively.

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Luanga-Reyrel and Deconchat (1999) reported similar results for oak coppice forests in France and Muturi et al. (2009) for Embu, Kenia. Dominant Isotomidae species represented high reproductive rate and adaptive ability. The feature has allowed some of the genera to colonize forests and open micro-habitats.

In general, Collembola density was higher in the land uses subjected to a greater impact such as corn crop and grass. It is possible that this fact is related to a higher incidence of individuals from one or a few dominant species of Isotomidae and Entomobryidae.

The land-use practices have an important effect on the soil biodiversity and they are usually associated with the loss of species and a reduction of biodiversity (Bengtsson et al., 2000). The results of this study are compatible with this assertion. The lowest diversity of Collembola was recorded in both grassland and agroforestry of Venustiano Carranza (VC) and corn crop of San Fernando and from litter of grassland use type. Opposite to this, the highest diversity was found in the forest of SF, the most unspoiled site. This fact is consistent with the hypothesis of finding greater diversity in the most conserved sites such as the forest that represents our control use type. The next most diverse land use was agroforestry of López Mateos. It is possible that the intensity and duration of practices in agroforestry of LM produce few fluctuations in the original Collembola assemblage.

On the other hand, the highest abundance of collembolans was recorded (in decreasing order) at corn crop, grassland and forest of LM, most of them pertaining to the Isotomidae family. All families were recorded in the four land uses of LM, except Odontellidae which was absent in corn crop. On the contrary, the lower densities of colembolans were recorded in grassland of VC and forest of SF. In grassland of VC diversity was 0.78 because only three families were present, Isotomidae, Entomobryidae and Hypogastruridae unlike the diversity of forest of SF (1.51) because six families out of seven were present. We discovered Neanuridae only in López Mateos, in corn crop and grassland, but in very low densities. Additionally, Odontellidae was a rare family, only recorded in the three land uses of LM and both forest of SF and VC in very low densities. The low densities of Odontellidae and Neanuridae throughout the land uses did not show a clear pattern, so their role as reliable bioindicators is here uncertain despite the fact that some authors have mentioned them as important bioindicators of edaphic conditions. Mendoza et al. (1999) reported incidence of Odontellidae (*Superodontella*) in a recovery land with corn crop from a tropical site in Balún Canán, Chiapas, Mexico. Perhaps the presence of Odontellidae in a recovery soil could be considered as an indicator of restoration.

About Collembola distribution on biotopes, it seems clear that, with rare exceptions, soil contains a greater abundance and diversity of Collembola than litter as has been reported for other tropical forests (Gómez-Anaya et al., 2010) but in this study the diversity of litter was little higher than that of both soil layers. In LM the highest diversity was recorded in the soil of forest and the lowest in the litter of forest, this could be because this biotope was scarce and variable. In litter the abundance of springtails can be extremely variable, due to the different levels of the accumulation of litter on the ground depending on many factors: slope, soil relief, vegetable cover. Especially, abundance in litter depends largely on precipitation and moisture level. In biotopes of land uses the lowest abundance of Collembola was recorded in the litter of agroforestry and the highest abundance in the deepest layer of soil (So2) of corn crop. The accumulation of litter in some grassland systems can be high because of an increase

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of the number of herbaceous plants (Sánchez et al., 2007). Litter accumulation in grassland of SMR was more variable than in the other land uses and may correlate to the lower Collembola abundance recorded. In relation to the higher Collembola abundance in corn crop, the species composition is likely to be represented only by one or few species of Isotomidae and/or Entomobryidae, the most abundant families recorded, that find by peculiar temporal microhabitat conditions, e.g. moisture, favorable to maintain high populations. Agricultural stubble is mainly represented by cornstalk debris, that can be either highly variable or a very homogeneous medium depending on the type of management used. Under humid conditions stubble can generate a fungal mycelium-rich medium during the decaying cycle which provides abundant food for sustain high populations of Isotomidae (pers. obs. J. A. Gómez). Particularly in the two layers of soil abundance was higher in the deepest layer So2 (0-5 cm deep) in the corn crop and then in the first layer of soil of the forest. This shows a contrast between the site of greatest intensity of use and the control one. Composition of families shows that most of the Collembola collected from So1 of forest soil were epiedaphic forms of the Isotomidae family. Similar dominance of Isotomidae have been reported from other tropical site used in a corn cultivation (Mendoza et al., 1999) in Balún Canal, Chiapas. In corn crop soil, the abundance of Collembola was very similar between the two soil layers, 4073 m^2 and 4465 m^2 for So1 and So2, respectively. This is because the variation in depth between the two layers of soil was minimal and the land preparation promote a more homogeneous edaphic vertical profile in characteristics e.g. moisture, sand, clay, silt, porosity and therefore the vertical distribution of Collembola was similar. However, this was only for abundance distribution since in composition there was no Sminthuridae in So1 and no Odontellidae in either soil layers.

It is a fact that some soil physicochemical properties are modified by different agricultural practices and grazing. For example, silt content has been reported to tend to increase in the grassland and farmland (Evrendilek et al., 2004). Particularly, silt content was higher in cropland and grassland in SRM in which higher Isotomidae and Entomobryidae were registered (see CCA *Fig. 5*). Contrary, porosity is another property that tends to decrease in soils with grassland and agricultural management and increases in preserved soil, like forest, as this study proved. Apparently, the clay content also tends to decrease with soil management even though our study showed to be higher in the grassland (Evrendilek et al., 2004). The highest abundances of Isotomidae and Entomobryidae seem to be correlated mainly to corn crop cultivation practices, agroforestry and grazing but not to the preserved forest. Undoubtedly, the different land-use practices tend to transform the physicochemical properties of soil and thus to modify assemblages structure of wildlife that inhabits it.

Among Poduromorpha some species of Hypogastruridae, Neanuridae, Odontellidae and Onychiuridae have been regarded as reliable indicators of perturbation (Barbercheck et al., 2009; Ponge et al., 2003; Van Straalen, 1998). Nevertheless, contrary to expectations, there was Neanuridae recorded in the corn crop and grassland of LM but absent in the tropical rain forest of the same site. Whereas, Odontellidae was absent in the most land-use types, except in forest, the most unspoiled site, in Santa Marta Range. It is feasible that, Neanuridae and Odontellidae could be more sensitive than other Collembola to human influence.

As a group, Collembola has shown to be an efficient bioindicator of the effects of land-use mainly in aspects of incorporation of Nitrogen and pesticides in soils, as well as in the evaluation of the quality of grassland environments (Hodkinson and Jackson,

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2005). Several families are more or less sensitive to the pollution of the environment. It is of great value to detect the assemblages of microarthropods at family level, those of Collembola, to provide an efficient tool to determining the quality and health of altered soils (Garita-Cambronero et al., 2006). We consider that a family level of quantification can give an effective indication to the different human impacts on soils such as the different land-use types in Santa Marta.

Conclusions

According to our results, it can be concluded that the differential land use by practices such as corn crop, grazing and agroforestry in St. Marta Region (Los Tuxtlas) produces significant changes in abundance and diversity of the Collembola families in leaf litter and soil when compared to a barely modified forest. These changes occurred in the three sites as well as in the two biotopes, leaf litter and soil, of the four land uses. Collembola showed a reduction in diversity and abundance in those land uses subjected to a more intensive use such as corn crop, grazing and agroforestry. On the other hand, as expected, the Collembola were more diverse and abundant in the land use that experiences less intensity as the forest. The higher abundance of Isotomidae and Entomobryidae recorded in corn crop can be probably associated with the soil management in agricultural soils, with more humidity due the crop requirements, and promote mycelial growth, and that is exploited for those Collembola families increasing their abundances. A detailed study of the taxonomy of these two families would, undoubtedly, reveal that relatively a few opportunistic epiedaphic species are dominant in corn crop and that a reduction in diversity is always constant consequence that can be verified in any place where the intensity of land use is increased. Odontellidae and Neanuridae were rare families because of their abundance, however, their incidence/absence patterns are not clear here and their value as bioindicators was not possible to be highlighted. Nonetheless, some authors have proposed that some Collembola species with higher bioindicator value are in these families. Apparently, they tend to disappear with farming practices and grazing. They are probably sensitive to the use of agrochemical and pesticides used in agriculture and cattle farming. Particularly we think that the Odontellidae, which were not registered in the corn crop in St. Marta, could be used as good bioindicators. It is recommended to observe their abundance and/or presence-absence in soils subjected to intense use. Finally, the use of higher taxonomic categories other than species is recommended, as in this case the family level, in rapid assessments to detect coarse quantitative/qualitative changes introduced by human impact. Our results suggest that the changes in dynamic community of Collembola can be used as indicator of modified soil use and quality.

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MANAGEMENT OF SUGARCANE DISTILLERY SPENT WASH FOR IMPROVING THE GROWTH, YIELD AND QUALITY OF WHEAT CROP

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Abstract. Disposal of industrial wastewater is a current issue of urbanization. However, this problem can be sorted out by using wastewater as an alternate source of irrigation after the addition of some amendment. The present study was performed at the Shakarganj Sugar Research Institute (SSRI) Jhang, Pakistan to determine the influence of different combinations of distillery spent wash (DSW) and NPK fertilizers on growth, yield and nutrient uptake of wheat crop. The experiment was composed of different combinations of DSW and NPK (0:0, 0:100, 25:75, 50:50, 75:25, 100:0). The results revealed that the application of different combinations of DSW and NPK significantly improved the growth, yield, and nutrient uptake in wheat crop. The maximum improvement in growth attributes (LAI, LAD, CGR and TDM) was recorded with the application of 25:75% DSW and NPK fertilizers. Similarly, application of 25:75% DSW and NPK improved the plant height, fertile tillers, 1000 grain weight, biological yield grain yield nitrogen uptake, phosphorus uptake and potassium uptake up to 32.88%, 37.5%, 38.87%, 34.61%, 78.42%, 62.42%, 92.55% and 73.25% respectively, over the control. In conclusion, application of 25:75% DSW and NPK was proven superior to other combinations in terms of growth, yield and nutrient uptake.
Keywords: *wheat, spent wash, yield, grain, N. P. K.*

Introduction

The imperious issue of water scarcity has devastating impacts on the economic growth, livelihoods and environmental supremacy around the globe. The rapid increase in industrialization excretes immense amount of waste water, as demands for fresh water is amplifying day by day, therefore, waste water often seen as a valuable asset, thus it can be a prime alternate source to irrigate the crops (Noori et al., 2013). Additionally, the agricultural crops also require a huge quantity of water for their growth and development. Therefore, the use of waste water in agriculture will solve both problems, and will improve the environmental quality and crop yield. Moreover, the use of waste water can improve the soil physical and chemical properties, soil nutrition status (Kiziloglu et al., 2007) and crop growth, yield as well as quality (Nagajyothi et al., 2009; Nath et al., 2009). Therefore, the wise use of waste water would be helpful in water conservation, nutrient recycling, reduction of water pollution and input application (Thapliyal et al., 2009; Vasudevan et al., 2010).

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The sugar industry plays a pivotal part in the economic development of Pakistan. There are 88 sugar mills in Pakistan (PSMA, 2014). These mills have limited capacity to store and to treat this spent wash, therefore, much of the spent wash is disposed into surrounding areas of mills. The poor handling and management of DSW create the problems of water pollution and deterioration of soil health (Chhonkar et al., 2000).

DSW is a by-product of sugar industry, it is purely of plant origin and contains appreciable amount of macro nutrients (N, P, K, Mg, Ca, and S), micronutrients (Fe, Zn and Mn) and organic matter, and therefore it can be a valuable fertilizer when applied to soil along with irrigation water (Vadivel et al., 2014). Likewise, DSW also contains a significant amount of growth promoting substances, i.e. auxins and gibberellins, which further increases its value as a fertilizer. The higher amount of calcium in DSW has the potential to reclaim the sodic soil, similarly to gypsum effect (Suganya and Rajannan, 2009).

The application of DSW not only controls the water pollution, but also improves the crop growth and productivity (Suganya and Rajannan, 2009). The application of DSW substantially improved the yield of sugarcane crop (Haron et al., 2004), rice and wheat yield (Pathak et al., 1998), and ground nut owing to improvements in soil quality and nutrient uptake (Ramana et al., 2001). Moreover, the use of DSW appreciably improved the crop productivity without affecting the soil fertility (Ramana et al., 2001).

The land application of DSW improved the microbial biomass, and enzymatic activities, which increased the mineralization of organic matter, thus improved the uptake and availability of nutrients (Mahimairaja et al., 2004). The judicious application of DSW enhanced the nutrient uptake in root vegetables (Chandraju et al., 2008), top vegetables (Basvaraju and Chandraju, 2008), leafy vegetables (Chandraju et al., 2007) and pulses (Chandraju et al., 2008).

The conjunctive application of synthetic fertilizers along with the organic fertilizers further improves the growth, yield and quality of crops. The combined application of DSW and NPK fertilizers, improved the growth, yield and nutrient uptake in sugar cane crop (Sharma, 2013). Likewise, the application of diluted DSW and NPK fertilizers improved the soil health, growth and yield and quality of sugarcane crop (Kaloi et al., 2017). On the basis of these facts, the present investigation was carried out to determine the suitable combination of spent wash and inorganic fertilizers, which has the best effect on growth, yield and nutrient uptake in wheat crop.

Materials and methods

Study site and experimental design

A Field study was conducted for two consecutive years (2013-14, 2014-15) at the farm of Shakarganj Mills Farms, Jhang, (longitude 73·8°E, latitude 31·8°N, and altitude 184·4 m asl) Pakistan to investigate the influence of different combination of distillery spent wash and NPK fertilizers on growth, yield and quality of wheat crop. The study site comes under sub-tropical region, moreover, the prevailing conditions during both growing seasons are given in *Figure 1*. Composite soil samples were taken from the site in order to determine the different physical and chemical properties of soil. The soil samples were analyzed by standard procedures of Homer and Pratt (1961). The soil was sandy loam, containing 0.74% organic matter, 0.058% nitrogen (N), 4.63 ppm phosphorus (P), 121 ppm potassium (K), and has a pH of 7.94. The experiment was laid out in randomized complete block design having four replications.

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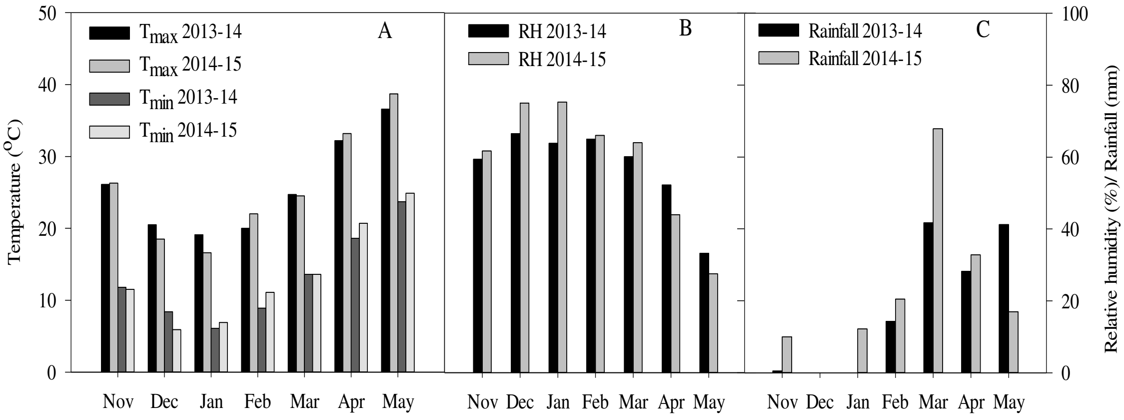

Figure 1. *Prevailed climatic conditions during the growing seasons. A: Maximum and minimum temperature during 2013-14 and 2014-15, B: Relative humidity during 2013-14 and 2014-15, C: Rainfall during 2013-14 and 2014-15*

Collection of spent wash and analysis

Spent wash (SW) was collected from distillery of Shakarganj Mills. The spent wash samples were preserved in polycarbonyl sterilized air-free containers, after that they were transported into the laboratory and kept in refrigerator at 4 °C till use. The different components of spent wash like pH, total solids, N, P, K, Ca, Mg, Na, BOD and COD (*Table 1*) were determined by the standard procedures of AWWA and WEF (1998).

Table 1. *Characteristics of spent wash used in study*

EC (dS m^{-1})	7.7	Total Na (mg L^{-1})	223
pH	4.1	Total P (mg L^{-1})	75
Organic matter (%)	1.25	Total N (mg L^{-1})	410
BOD (mg L^{-1})	232	Total K (mg L^{-1})	2303
COD (mg L^{-1})	6109	HCO3 (mg L^{-1})	101
Total Ca (mg L^{-1})	124	Indole acetic acid (mg L^{-1})	41
Total Mg (mg L^{-1})	63	Gibberellic acid (mg L^{-1})	3119

Treatment and crop husbandry

The study was composed of the following treatments: T_1: 0%DSW+0%NPK, T_2: 0%DSW+100%NPK, T_3: 25%DSW+75%NPK, T_4: 50%DSW+50%NPK, T_5: 75%DSW+25%NPK and T_6: 100%DSW+0%NPK. The seed bed was prepared as recommended by the department of agriculture, Punjab, Pakistan (Govt. of Punjab, 2013-14). The recommended rates of NPK for wheat crop were, 120:90:60 kg ha^{-1} and they were applied in the form of urea, diammonium phosphate and sulphate of potash. All the P and K fertilizers were applied at the time of sowing, while N was applied in two splits, i.e., at sowing and with first irrigation. The spent wash was applied with the first irrigation to the plots according to treatments. The distance between the sugarcane distillery and field was 1387 m. The Punjab-2011 was used as test cultivars and sown in 23 cm apart lines by manual drill. During, both the growing seasons' five irrigations

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were applied to the wheat crop. All the management practices were kept uniform in order to get good production.

Collection of growth and yield data

Leaf area was measured by leaf area meter (Model: Licor 3000), whereas the leaf area index (LAI) was measured by the standard procedures of Watson (1947). Similarly, the leaf area duration (LAD) and crop growth rate (CRG) was measured by standard methods (Hunt, 1947). The unit area was selected to determine the number of fertile and un-fertile tillers, whereas, twenty plants were selected from each plot to determine the different yield and yield components (Chattha et al., 2017). At harvest grain and biological yield of each plot were measured and later on converted to ton per hectare basis mathematically (Chattha et al., 2017).

Chemical analysis

At harvesting, grain samples were collected from each plot, for analysis of grain NPK. The collected grain samples were dried in over at 72 °C until constant weight, after that they were grinded and sieved. The grinded samples were digested by adding di-acid mixture (H_2SO_4 and H_2O_2 in 2:1 ratio). The grain N contents were determined by standard procedure of nitrogen Chapman and Parker (1961), whereas the P contents were measured by the Olsen et al. (1954) methods. Moreover, K was determined by flame photometer by using standard curve (Chapman and Parker, 1961).

Statistical analysis

In combined analysis the interaction of year (2013-14 and 2014-15) and treatments were found non-significant, therefore, the data was pooled for both years (2013-14 and 2014-15) and an average of two years was taken. The collected data regarding growth, yield and chemical analysis were analyzed by using analysis of variance technique and treatment means were compared by least significant difference test (Steel et al., 1997).

Results

Influence of different combinations of spent wash and NPK fertilizers on growth attributes of wheat crop

Different combinations of distillery spent wash (DSW) and NPK fertilizers significantly improved the growth attributes of wheat (*Triticum aestivum* L.) crop as compared to control (No DS and NPK) (*Fig. 1*). Crop attained maximum leaf area index (LAI) 75 days after sowing (DAS) with highest LAI value under the application of 25%DSW+50%NPK fertilizers, that was comparable to the application of 50%DSW+50%NPK, whereas the non-application of both DSW and NPK fertilizers remarkably decreased the LAI. Nonetheless a declining trend in LAI was recorded 75 DAS, whereas this declining trend was more pronounced when no DSW or NPK were applied followed by a 100% DSW application, whereas the minimum declining trend was found with the use of 25% DSW+75% NPK (*Fig. 3*). Likewise, the effect of different combinations of DSW and NPK fertilizers was also significant on LAD, CGR and TDM. Crop attained maximum LAD and CGR 60-75 DAS, and this maximum LAD and CGR was recorded with the use of 25% DSW and 75% NPK, followed

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closely by the application of 50%DSW+50%NPK whereas the lowest LAD and CGR was recorded with no DSW and NPK application (*Fig. 3*). Similarly, TDM also increased with different combination of DSW and NPK, however, the highest TDM was recorded with the use of 25% DSW+75% NPK, whereas no application of DSW and NPK fertilizers remarkably reduced the TDM (*Fig. 3*).

Influence of different combinations of spent wash and NPK fertilizers on root growth and flag leaf length of wheat crop

The influence of different combinations of DSW and NPK on root length and flag leaf length was substantiated (*Table 2*). The maximum root length (13.10 cm) and flag leaf length (18.1 cm) were recorded with application of 25% DSW and 75% NPK fertilizers, however, it was similar to application of 50% DSW and 50% NPK, meanwhile the lowest root length (7.5 cm) and flag leaf length (11.20 cm) were recorded when no DSW or NPK were applied, nonetheless it was also comparable with application of 100% DSW.

Table 2. *Effect of different combinations of DSW and NPK fertilizers on growth and attributes of wheat crop*

Treatments	Root length (cm)	Flag leaf length (cm)	Plant height (cm)	No. of fertile tillers (m^2)
Control	7.50c	11.20d	73.00d	226.67c
0%DSW+100%NPK	10.80b	16.51c	87.66bc	297.33ab
25%DSW+75%NPK	13.10a	17.70ab	95.00a	305.67a
50%DSW+50%NPK	12.93a	18.06a	97.00a	311.67a
75%DSW+25%NPK	11.36b	16.83bc	91.66ab	277.67b
100%DSW+0%NPK	8.23c	12.23d	82.33c	246.33c
LSD ($p \leq 0.05$)	**0.97**	**1.10**	**6.82**	**20.05**

Values sharing same letter(s) in a column do not differ at 5% probability

Influence of different combinations of spent wash and NPK fertilizers on yield attributes of wheat crop

Distillery spent wash and inorganic fertilizers significantly increased the yield attributes of wheat crop as compared to alone application of DSW, whereas the yield attributes considerably decreased when no DSW or NPK were applied (*Tables 2* and *3*). A significant increase in plant height was recorded with the use of DSW and NPK fertilizers compared to control. The application of 25%DSW and 75%NPK exhibited maximum improvement in plant height of wheat crop up to 32.88% over the control, nonetheless, it was comparable with 50%DSW+50%NPK and 100% DSW, whereas a substantial reduction in plant height was recorded under control treatment. Likewise, the effect of DSW and NPK on fertile and unfertile tillers was also significant. Maximum fertile tillers (311.67) was found with 25%DSW and 70% NPK, followed by 50%DSW+50%NPK, similarly, minimum fertile tillers were recorded in the control treatment. Additionally, minimum number of unfertile tiller (13.33) were counted from plot fed with 25%+75%NPK that was the same with the application of

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50%DSW+50%NPK, moreover, the maximum unfertile tillers (26.67) were found in the control treatment. Regarding the 1000 grain weight combination of 50%DSW+50%NPK increased the 1000 grain weight up to 26.24% that was the same with the application of 25%DSW+75%NPK, whereas lowest 1000 grain weight was recorded with no DSW + NPK. Similarly, different levels of DSW and NPK fertilizers also had a significant effect on the biological yield and grain yield (*Table 3*). Similarly, up to 36.18% and 78.42% increase in biological and grain yield was found with the application of 25%DSW+75%NPK, compared to control, however it was at par with the application of 50%DSW+50%NPK.

Table 3. *Effect of different combinations of DSW and NPK fertilizers on yield attributes of wheat crop*

Treatments	**No. of unfertile tillers (m^2)**	**1000 grain weight (g)**	**Biological yield (t ha^{-1})**	**Grain yield (t ha^{-1})**
Control	26.66a	30.01d	7.60d	2.92e
0%DSW+100%NPK	18.66c	35.33c	9.11c	4.44c
25%DSW+75%NPK	14.66d	41.66a	10.23ab	5.07ab
50%DSW+50%NPK	13.33d	40.66ab	10.35a	5.21a
75%DSW+25%NPK	19.33c	37.00bc	9.75b	4.74bc
100%DSW+0%NPK	22.33b	34.33c	8.16d	3.53d
LSD ($p \leq 0.05$)	**1.78**	**4.16**	**0.58**	**0.33**

Values sharing same letter(s) in a column do not differ at 5% probability

Influence of different combinations of spent wash and grain NPK uptake and grain NPK concentration

The results revealed that the application of different combinations of DSW+NPK and alone DSW, NPK and control treatment had considerable effects on the nutrient uptake in wheat crop (*Fig. 2*). The maximum increase in nitrogen uptake was found with 25%+75%NPK application, i.e. 69.18% over the control, and similar trend was found with 50%DSW+50NPK application. Moreover, minimum nitrogen uptake was found in the control treatment. A significant increase in phosphorus uptake was found with the use of 25%DSW+75%NPK treatment, i.e. 92.70% over the respective control (no DSW and no NPK application). Furthermore, K uptake also increased by application of DSW and NPK and maximum increase of 73.26% in K uptake was found by 25%DSW+75%NPK, followed by 50%DSW+50%NPK that was similar to 100%DSW, meanwhile the minimum K uptake was found in control (No DSW+ No NPK). Similarly, different combinations of DSW+NPK fertilizers also had the considerable effect on the grain NPK concentration (*Fig. 2*). Maximum grain nitrogen concentration (2.8%), phosphorus concentration (0.49%) and potassium concentration (1.05%) was recorded with application of 25%DSW+75%NPK, however, it was comparable with 50%DSW+50%NPK, whereas the minimum grain nitrogen (1.10%) phosphorus (0.24%) potassium (0.40%) was observed in the control treatment.

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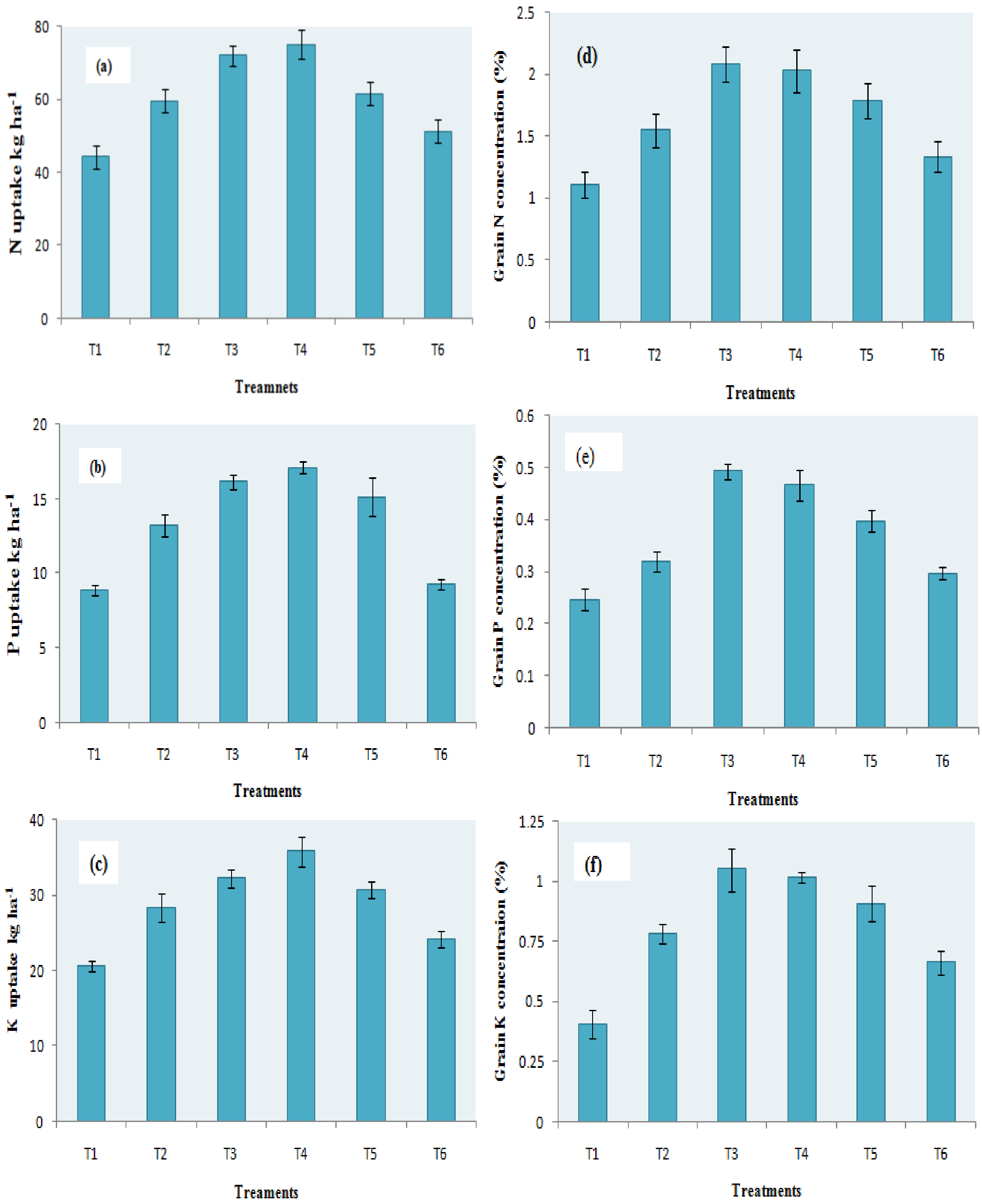

Figure 2. *Influence of different combinations of DSW+NPK fertilizers on grain NPK uptake (a,b,c) and grain NPK concentration (e,f,g)*

Discussion

The rapid growth of the population substantially increased the quantity of industries around the globe. These industries excrete a huge amount of waste water, which is continuously deteriorating our environment. As the fresh water resources are declining and the demand of fresh water is increasing day by day due to blooming population, waste water usage in agriculture for irrigation purposes may offer a solution to this

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problem. Moreover, this waste also contains undesirable substances which can deteriorate the soil health, crop yield and environmental quality. However, the use of this water along with certain amendments can reduce its negative impacts on soil health, crop, environment and microbial activities. The application of NPK fertilizers along with DSW is a viable strategy to reduce the negative impacts of DSW on soil and crop. The present study determines the effectiveness of different combinations of DSW along with NPK fertilizers on the growth, yield, and nutrient uptake in wheat (*Triticum aestivum* L.) crop. The results revealed that the application of different combinations of DSW+NPK significantly improved the growth attributes, i.e. LAI, LAD, CGR, TDM, as compared to alone application of DSW and no application of DSW and NPK (*Fig. 3*).

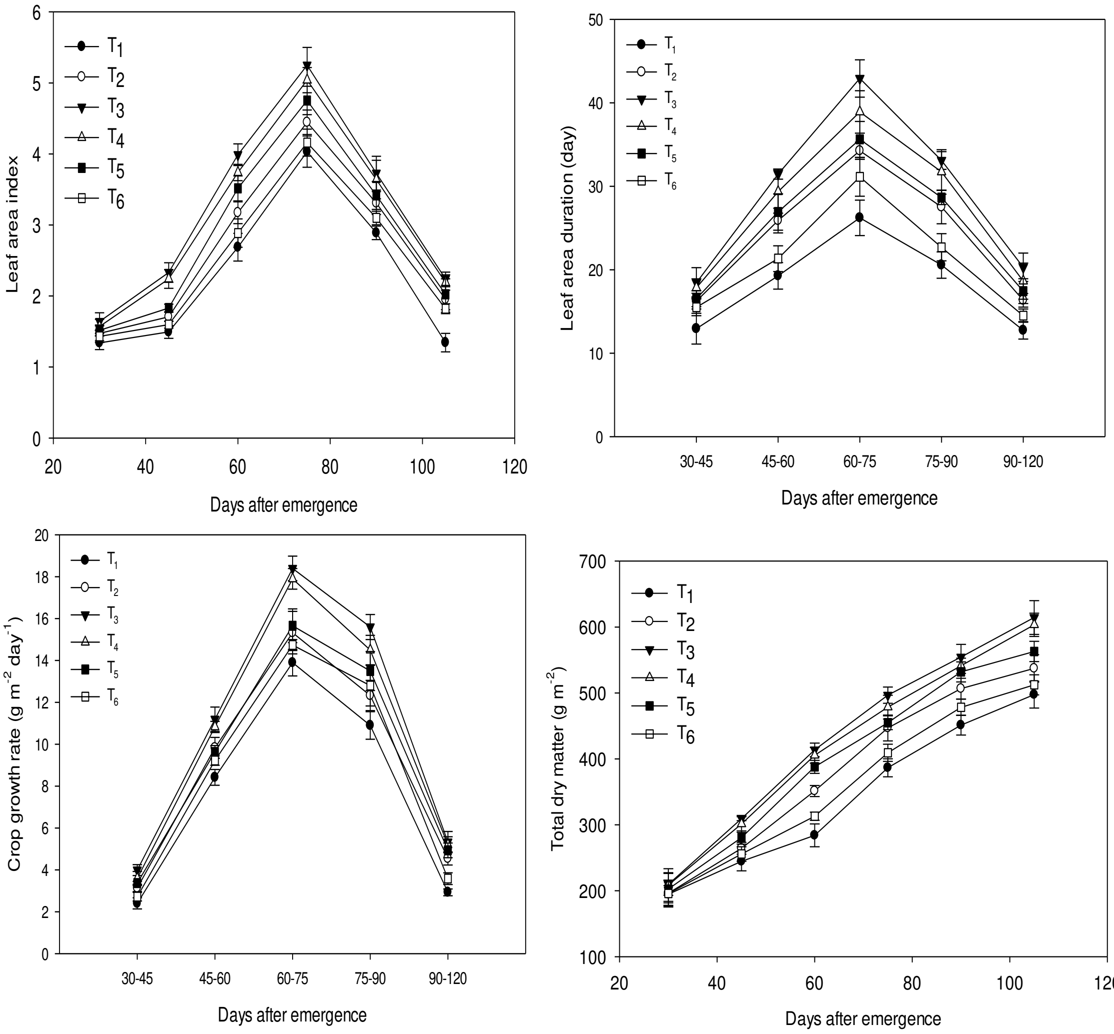

Figure 3. *Influence of different combinations of DSW and NPK fertilizers on LAI, LAD, CGR and TDM*

The increase in growth attributes of wheat crop (LAI, LAD) at lower concentration of DSW might be due to improved root length, which resulted in better absorption of nutrient and water thereby improved the growth attributes. Likewise, the maximum CGR and TDM were found with application of 25%DSW+75%NPK. This improvement in CGR and TDM can be due to increased flag leaf length; therefore, the plants having longer flag leaf absorbed more

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light, that resulted in production of more assimilates; thus produced more CGR and TDM. Similarly, Ramana et al. (2001) and Nandy et al. (2002) reported that application of DSW at lower concentrations significantly improved the growth attributes of rice and maize crop due to better availability of nutrients. Similarly, the reduction in growth attributes of wheat crop at higher concentration of DSW might be due to the presences of higher quantity of organic matter and BOD, which resulted in depletion of soil oxygen and accumulation of carbon dioxide in soil (Rath et al., 2010).

Likewise, the DSW+NPK also improved the root length, flag leaf length and plant height of wheat crop. The improvement in root length, flag leaf length, by application of the lower concentration of DSW, can be due to the presence of decreased amount of salts. Therefore, the application of DSW has substantially improved microbial activities which improved the decomposition of organic matter thus improved the nutrient uptake and therefore the plant growth. These results are supported by previous findings of Rajkishore and Vignesh (2012), who found that application of DSW at lower concentration substantially improved the microbial activities, therefore, increased the nutrient uptake and availability and thereby improved plant growth. Similarly, the various combinations of DSW and inorganic fertilizers had considerable effects on the yield and yield attributes of wheat crop (*Table 3*). The improvement in yield attributes of wheat crop by lower doses of DSW might be due to the improvement of soil properties, which resulted in better availability of nutrient and thereby improved the growth attributes. Moreover, the lower dose of DSW and application of higher dose of NPK might have increased the concentration of nutrients in soil solutions that overcome the toxic effects of heavy metals. Conversely, higher concentration of DSW might have increased the salt accumulation in the root which resulted in higher osmotic pressure within the root zone. This increase in osmotic pressure can substantially reduce the nutrient uptake, thereby, reduced the crop yield and yield components. These results are in line with previous findings of earlier researchers who reported that application of DSW at lower concentration remarkably improved the growth, yield and yield attributes (Mahimairaja and Bolan, 2004; Chandraju et al., 2008; Chidankumar et al., 2009). Similarly, Rath et al. (2010) reported that application of higher concentration of DSW significantly decreased the growth, and yield of sugarcane crop.

The results showed that conjunctive application of DSW+NPK fertilizers considerably improved the nutrient uptake in wheat crop (*Fig. 2*). The increase in nutrient uptake by DSW+NPK might be due to the presence of appreciable amounts of nutrients in DSW and improvement in soil conditions due to DSW application. Similar results were reported by many researchers who reported that application of DSW improved the uptake of nutrients (Chandraju et al., 2008; Chidankumar et al., 2008; Basvaraju and Chandraju, 2008). Moreover, the application of DSW remarkably improved grain NPK concentration. The improvement in grain NPK concentration by lower DSW might be due to the counter effect of beneficial nutrients, reduction in metal toxicity, and improvement of microbial activity that produces chelate and hormones, which improved growth, yield and nutrient uptake in wheat crop.

Conclusion

Distillery spent wash contains many important nutrients; therefore, it can be used to grow the crops along with inorganic fertilizers. This study implies that combination of 25%DSW+75%NPK could be a viable strategy, and it can remarkably improve the growth, yield, and quality of wheat crop.

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RESPONSE OF SWEET CHERRY (*PRUNUS AVIUM* L.) POLLEN GRAINS TO VEHICULAR EXHAUST POLLUTION AT QUETTA, BALOCHISTAN, PAKISTAN

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Abstract. The aim of this study was to assess the effect of air pollution on road side fruit plants (*Prunus avium* L.) palynology. Flower samples were collected from polluted (1-2 m away from the edge of Quetta to Hunna Urak road) and for the comparison from non-polluted sites (250-300 m away from the same collection site). During the investigation different pollen parameters including pollen umbers/production, pollen size, pollen tube growth, pollen viability, pollen regularity and pollen protein contents were used through different methods. Results indicated that pollen grains of *Prunus avium* L. were very sensitive to automobile air pollution. Consequences showed significant reduction in numbers and sizes of pollen grains, pollen tube length and pollen viability of polluted site with respect to the control site. Maximum irregularity in pollen morphology and low pollen protein contents were also recorded from the polluted site. Moreover, results also designated that vehicular pollution increases the resting period of pollen grains for 1 to 3 h. So the different attributes of pollen grains can be used as sensitive bioindicators of adverse factors in botanical environment and this leads to the idea that reduction in the development and potential of pollen grains in response to various poisonous environmental pollutants can be exploited as a good index of air purity.
Keywords: *sweet cherry, road side contamination, flowers, soil and statistical analysis*

Introduction

Growing *human* population and technologies are producing one of the important problems which we are facing today i.e., air pollution. Plant fertility and precise insemination of plant mostly depend on the pollen grains. In severe air pollution conditions, the fertility of plant decreases because of the straight and subsidiary effects on the propagative mechanism. Air pollution due to vehicle exhaust causes irregularity in anthers, decreases the number and masculine infertility (Rezanejad, 2007). The chemical pollutants produced by industries and traffic emissions not only put damaging effects on morphological and physiological parameters of plants but also comprise alterations and chromosomal impairment. According to Pfahler (1981) these irregularities are contingent on ecological and hereditary issues and may eventually go to alteration in propagative capability of plant species. Between several features that add to air contamination,

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locomotive emissions are problems of growing dimensions. Vehicular emissions enhance large quantities of dust particulate matters, smolder, toxic gases, heavy metals and biological molecules in the environment all over the world. All these air contaminants produced adverse effects on the health of humans, animals and vegetation (Rezaei et al., 2010; Atkinson et al., 2012). Majd et al. (2004) reported that air pollution due to automobile emission enhances pollen allergenicity and increases the problem of asthma among people residing along road sides. According to Shannigrahi et al. (2004) outdoor vegetation growing is the main and primary target of all types of air contamination.

Several investigations have indicated vehicular emission effects on plant leaves stomatal apertures, viability of pollen and development of plants (Iannotti et al., 2000; Verma et al., 2006; Leghari et al., 2015). Other researchers such as Iannotti et al. (2000), Duro et al. (2013), Kaur et al. (2016) indicated that air pollution mainly effects the size, shape, viability and exine sculpturing of pollen grains. Kaur et al. (2016) also reported that developed pollen grains when released from the flower buds in the contaminated air absorbed humidity and some pollutants, which influenced their viability henceforth plants reproductive system. The pollutants such as heavy metals, fluorides, pesticides gather on the pollen grains surfaces and make them lively and sensitive bioindicators of atmospheric pollution (Noori et al., 2009; Yousefi et al., 2011). To perceive and assess the poisonous materials in the atmosphere, biomonitoring techniques can be engaged.

In the current ages several researchers of diverse fields give their attention to the application of palynology in various fields. In palynology the development of pollen grains, pollen grain viability and pollen tube evolution are the superficial bio-pointers of adverse atmospheric pollution. Vehicular traffic adds up different kinds of contaminants like heavy metals, polyaromatic hydrocarbons, gaseous toxins etc. to the atmosphere. Pollen grains of different plants have been found to be sensitive to these pollutants, which show varied effects on their viability, and size and hence can be used as bioindicators of air pollution (Kaur and Nagpal, 2017). This research directed to the knowledge that the downcast growth and developmental potential of pollen in reply to many poisonous environmental pollutants released by traffic can be introduced as a good directory of air cleanliness. This current research study, therefore, was carried out to understand the harshness of vehicular exhaust pollution on the fruit plant at Quetta, Balochistan and delivers some reasonable ideas to decrease vehicular contamination. The present study was conducted on *Prunus avium* L. through different attributes of pollen grains.

Materials and methods

Study area

A heavily trafficked road from Quetta city to Urak valley (*Fig. 1*) located in Balochistan, Pakistan was selected in order to evaluate the effect of air pollutants released by vehicles on *Prunus avium* L. fruit crop pollen grains. The road between Quetta city and Urak valley is about 21 km segment and lined on either side with wild roses, medicinally important plants and fruit orchards. Peaches, plums, apricots, cherries and apples of many varieties are grown in this valley. There is no industry on this road and the major source of pollution is from the vehicular emissions. One beautiful water fall is located at the end of Urak valley which makes an interesting picnic spot. The surrounding area of this picnic spot is full of orchards of apple, sweet cherry and apricot, gathering large number of visitors from all over the country to visit the beautiful valley especially during pleasant summer/flowering season. Before sampling in the

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target area, a walk through survey of the study area was made and on the basis of small and large vehicular traffic density, the emissions were expected very high along the road sites.

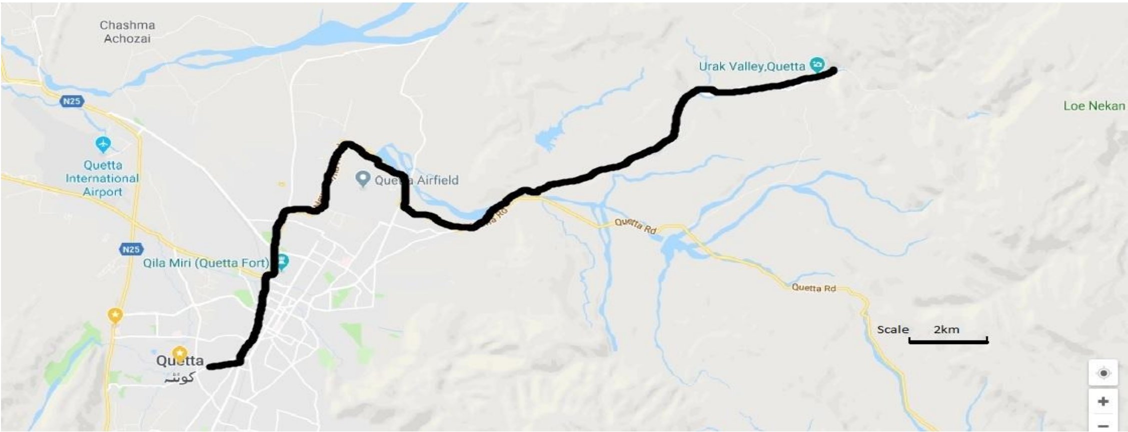

Figure 1. *Google roads map of study area. (Black colour showing road from Quetta city to Urak valley)*

Climate of the study area

Study area (Quetta district) lies between 300-03’ and 300-27’ N and 660-44’ and 670-18’ E. It is situated at an altitude of 1,700 m above sea level. The weather is extremely dry and mean relative humidity of this area mostly remains in range of 15-25% throughout the year. The winter is very cold and the minimum temperature ranges between, -15 to -7 °C and summer is relatively mild and the maximum temperature ranges between 32 to 35 °C, July is generally the hottest month. The district lies outside the range of the monsoon currents and the rainfall is scanty and irregular. The long term 127 year average annual rainfall for Quetta city is 220 mm (BARDC annual report 2017), whereas in the Hanna Urak area, the average is about 312 mm which is mostly recorded in winter months (December –March). (Source: Directorate of Minerals G.O.B Quetta 2017).

Plant material collected

For the investigation of vehicular exhaust emission effects on physiological parameters of pollen grains such as; production, viability and size of pollen along with germination rate and growth of pollen tube, flower samples were collected from polluted (1-2 m away from the edge of the heavily trafficked road) and comparatively non-polluted areas (about 250-300 m away from the same collection site) having the same soil characteristics (*Table 1*) during the year 2017, as was carried out by Ma et al. (2009). Five sampling sites on the heavily trafficked road were designated after a walk through inspection of various sites and on the basis of assessment of traffic density, level of visible auto emissions fumes/smoke, road dust. Collection of flowers for anther was done by random selection of 5 plants from the polluted (P) and control (C) sites separately at each location from the base, middle and upper portion of the plant around all four sides (north, south, east and west) for the accuracy. The average of the results obtained was used for statistical analysis

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and interpretation of results. The flower sample collection was done on the same day and time from both sites (polluted and control).

Table 1. *Summary of polluted and control sites soil samples*

Experimental sites	Soil parameters			
	Soil pH	**Specific conductivity**	**Organic matters**	**Soil texture**
Polluted site	7.7 ±0.06	5.29×10^{-4} ±0.01	1.0 ±0.2	Sandy loam
Control site	7.4 ±0.2	5.26×10^{-4} ±0.02	1.2 ±0.06	Sandy loam

±: standard deviation

Determination of pollen grain count/production per anther

For the investigation of pollen grain count/production 36 normal anthers were taken from each plant species of *Prunus avium* L. and put into a 1.5 ml Eppendorf tube at 25 °C, 1 ml of 50 $g^{\cdot}$ L^{-1} sodium polyphosphate solution was added, and the tube was capped and shaken. Small quantities of the pollen suspension were placed in a hemocytometer counting chamber and the chamber was placed on different microscopic stages to count the number of pollen grains. The number of pollen grains in one anther was calculated as the number of pollen grains per square area × 10 × 36 × 1000)/36, with three replications (Shi et al., 2015).

Analysis of pollen grains regularity and size

Regularity of pollen grains was evaluated on the basis of percentage of well-regulated and normal pollen grains. At least 500 pollen grains were investigated from each site (polluted and control). Pollen size was determined by micrometry method. A visual micrometer was fixed on light microscope and measurements of pollen grain size were determined under the magnification of 400×, as per procedure carried out by (Kaur and Nagpal, 2017). A minimum of 500 pollen grains were examined from each plant species. The investigation was repeated thrice and an average was expressed in µm (Iyer and Bholay, 2015).

Pollen viability

Pollen grain viability were examined by using 2, 3, 5 - triphenyl tetrazolium chloride (TTC) staining technique by small alterations as described by Iannotti et al. (2000). By adding 1% solution of TTC in 60% sucrose solution, TTC strain was made stain and saved in dark bottles for the process (making slides). Ten anthers from ten randomly selected appropriate size flower buds of the respective plant species were removed by a shrill needle and placed on a glass slide for examination adding one drop of TTC. After removing air bubbles, the slides were covered by cover slip and airtight by didutylphathalate xylene (DPX) and hatched in the daylight for 2-3 h. These slides were prepared with-in no time after sampling from both sites (polluted and control). Prepared slides were examined under light microscope at the exaggeration of 400× separately. Pollen grain viability was determined by noting/counting the viable and non-viable pollen grains under following categories: the yellowish pollen grains with shrunken configuration were designated as non-viable, whereas the red marked and deep pink pollen grains,

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which were rounded in shape, were counted as viable pollen grains. At least 500 pollen grains were studied by each plant (separately from polluted and control sites) and the experiment was repeated for three times and an average was calculated (Kaur and Nagpal, 2017). Further the viability of pollen grains was also tested by the germination of pollen grains (Iyer and Bholay, 2015) and percentage of viable pollen grains was calculated by the following formula:

$$\text{Pollen viability}\,(\%) = \frac{\text{Number of viable pollen grains}}{\text{Total number of counted pollen grains}} \times 100$$

Rate of pollen grain germination and pollen tube growth

Pollen grains propagation probability and pollen tube growth was determined by marking the different stages of pollen grain development on flower buds of different sizes. At the same time open flowers were pulled and the succeeding flowers series were marked as; F-0, F-24, F-48 and F-72, where; F-0 was designated for flower bud at the time of dehiscence of anthers, F-24 was for flower buds needed 24 h to open, F-48 was for the flower buds required 48 h to open and F-72 was for the flower buds required 72 h to open. Potential of pollen grains germination, the in-vitro experiment was performed by the method of standing drop in Brewbaker and Kwack's culture medium in the laboratory of Botany department in suitable relative humidity (Brewbaker and Kwack, 1963). Pollen germination and tube growth rate were noted by setting the cultures in one hour intervals that was extended for 12 h. The germination tests were conducted in triplicates and average data were displayed in the results.

Rate of decrease in pollen grain propagation and pollen tube growth

It contracts by the capability of the pollen grains to persist feasible in the detached successive flowers under natural conditions after the dehiscence of the anthers. The decreasing rate of pollen grain germination and pollen tube development was also deliberated time to time throughout storing via setting cultures medium at 3 h intermissions. These cultures were sustained for 15 h (Nair and Rastogi, 1963).

Determination and analysis of protein

Protein contents from *Prunus avium* L. pollen grains were determined by the methods used by Rezanejad (2007). Pollen extracts were made by incubating pollen grains in 0.1 M phosphate buffered saline (PBS) pH 7.4 in 15% quantity through inspiring at 4-8 °C for 8 h. Postponements were centrifuged at 10000 × *g* for 50 min and the supernatants were removed. Protein concentration in pollen grain extracts was recorded in 595 nm as per method of Bradford (1976). Sample protein and standard protein were unglued via SDS-PAGE patterns at 80 V continuous powers for 1-2 h at 15 °C, similar to the modified procedure of Laemmli (1970). Proteins were detected by using a mixture of methanole; acetic; distilled water (1:1:8) with Coomassie brilliant blue R 250.

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Soil analysis

The soil samples were collected from the areas, where under investigated plant species were grown (polluted and control sites) and examined to know the range of soil pH, electrical conductivity (E.C.), contents of organic matters (O.M.) and soil texture. Electrical conductivity and pH were determined by using the conductivity and pH meters respectively. Soil texture and soil organic matter were determined by using Atomic absorption spectrophotometer as the methods described by ICARDA (2001).

Vehicle count

The vehicles passing along the selected road were counted for 12 peak hours from 8 am to 8 pm for three consecutive times of the season at 5 sites each on target study road. Busses, trucks, vans, cars, motor bikes, rickshaws, container and loaders were counted and classified into three groups (2 and 3-wheelers, 4-wheeler and ≥ 6-wheelers (Leghari et al., 2013; Kalid et al., 2017).

Statistical analysis

All the observation of investigated parameters were calculated as an average and standard deviation. T-test of significance was used for the statistical analysis of data by using self-coded software on Microsoft excel 2007. Comparison between two means (polluted and control) was also intended for the determination of relative stimulatory and inhibitory effects of air pollution on the different attributes (Iyer and Bholay, 2015).

Results and discussion

The rapid increase in automobile activities and industrial processes, are the main causes of environmental pollution which have adverse effects on plant growth, fertility and productivity. In several contents it has been recommended that the pollen grains have capacity as bioindicators of environmental pollution. In this study, observations recorded show that the plants growing along the road side exhibited considerable damage to their pollen grains in response to automobile exhaust emission as compared to the control site while having the same soil characteristics (*Table 1*). All results regarding the pollen grains parameters are illustrated in *Figure 2a-j* and *Tables 2 to 5*.

Table 2. *Effect of vehicular exhaust pollution on the rate of decrease (µm) in pollen germination of successive flowers of Prunus avium L.*

Time in hrs.	Successive flowers				
	F-0			F-24	
	Control site	Polluted site	Sig. (p<0.05)	Polluted site	Control site
00	95.3 ±0.8	67.5 ±2.22	**	Ng	Ng
03	85.9 ±1.3	55.1 ±2.13	**	Ng	Ng
06	77.1 ±0.7	47.4 ±0.97	*	Ng	Ng
09	67.2 ±0.9	38.8 ±0.79	*	Ng	Ng
12	60.4 ±0.4	31.3 ±0.41	*	Ng	Ng
15	55.7 ±0.4	21.4 ±0.52	*	Ng	Ng

±: standard deviation, Ns: non-significant, *: slightly significant, **: significant, Ng: no germination

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Table 3. *Effect of vehicular exhaust pollution on the rate of decrease pollen tube length (µm) of successive flowers of Prunus avium L.*

Time in hrs.	**Successive flowers**				
	F			**F-24**	
	Control	**Polluted**	**Sig. (p<0.05)**	**Polluted**	**Control**
00	814.8 ±54.4	710.1 ±18.9	**	Ng	Ng
03	751.8 ±38.3	633.7 ±57.6	*	Ng	Ng
06	667.8 ±40.3	547.4 ±30.9	*	Ng	Ng
09	567.9 ±28.2	440.6 ±21.0	Ns	Ng	Ng
12	524.5 ±23.5	365.5 ±28.1	*	Ng	Ng
15	452.3 ±22.5	215.3 ±24.4	**	Ng	Ng

±: standard deviation, Ns: non-significant, *: slightly significant, **: significant, Ng: no germination

Table 4. *Effect of vehicular exhaust pollution on the rate of pollen tube length (µm) of successive flowers of Prunus avium L.*

Time in hrs.	**Successive flowers**				
	F			**F-24**	
	Polluted	**Control**	**Sig. (p<0.05%)**	**Polluted**	**Control**
01	Ng	152.2 ±23.3	-	Ng	Ng
02	Ng	253.3 ±28.1	-	Ng	Ng
03	Ng	341.3 ±44.5	-	Ng	Ng
04	164.9 ±27.0	453.2 ±57.8	*	Ng	Ng
05	244.3 ±35.1	555.7 ±52.1	*	Ng	Ng
06	323.9 ±43.7	650.8 ±65.7	**	Ng	Ng
07	400.4 ±38.5	736.4 ±60.2	*	Ng	Ng
08	480.2 ±57.1	832.3 ±45.0	*	Ng	Ng
09	550.2 ±75.2	920.5 ±42.8	*	Ng	Ng
10	611.2 ±99.6	1013.4 ±36.9	*	Ng	Ng
11	660.5 ±60.1	1141.7 ±50.4	**	Ng	Ng
12	707.2 ±36.2	1237.4 ±95.3	*	Ng	Ng

±: standard deviation, Ns: non-significant, *: slightly significant, **: significant, Ng: no germination

Table 5. *Average traffic density at 5 study sites*

Average number of vehicles per hour			
2 and 3 wheelers	**4 wheelers**	**≥ 6 wheelers**	**Sum**
530.5 ± 160.31	324.23 ± 140.45	50.2 ± 10.4	904.93

±: standard deviation

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In this study the consequences of pollen production, pollen size, pollen viability and pollen regularity are shown in *Fig. 2a, b, c, d, e,* respectively. Statistics exhibited slightly to significant ($p < 0.05$) decrease in pollen grains production, pollen size (breadth and length), pollen regularity and pollen viability at polluted site as compared to the non-polluted site. The number of pollen grains per flower from polluted site was found 38551.2, whereas pollen numbers were found 40819.2 from control site which is significantly ($p < 0.05$) high (*Fig. 2a*). During the study of pollen grain size; pollen breadth before and after the dehiscence from anther was noted 35 and 47 µm at control site, while at polluted site its breadth decreased by 20 and 31 µm respectively. Similarly pollen length: before and after dehiscence from anther was 45 and 60 µm at control site, whereas it decreased by 32 and 46 µm at the polluted site. Pollen size and length showed slightly to significantly ($p < 0.05$) reduction at polluted site (*Fig. 2b*). Slightly significant ($p < 0.05$) differences in pollen viability were also found between polluted and control sites samples. The percentage of pollen viability at polluted site was recorded 76.2% (T.T.C method) and 78.7% (germination method) with respect to the control site samples 85.6 and 85.5 %, respectively (*Fig. 2c, d*). The data shown in *Figure 2e* indicate that air pollution has significant effect on pollen structure. The air pollution caused a significant ($p < 0.05$) increased number of irregular and breakable pollen grains with respect to control site pollen grains. At control site sample 90% pollen grains were regular, while at polluted site pollen grains regularity reduced with the percentage of 70%. So the data disclosed in *Fig. 2a-e* indicate that the investigated plant species exhibited inhibition in its production of pollen grains, pollen viability, pollen regularity and pollen size due to auto exhaust pollution. Therefore, palynology of any plant can be used as vehicular pollution indicators. Similar results regarding inhibition in pollen production, pollen viability, and pollen size and pollen regularity were also reported by Iyer and Bholay (2015) and Rezanejad (2009). Pollen grain fertility reduction due to vehicular emission was also reported by Bharadwaj and Chauhan (1998) and this effect extends to the heredities development of plants and generative ecology which was highly influenced by the pollen grains propagation (Jain et al., 1997). Rezanejad (2007) also verified that air contamination produced abnormality in anthers, decrease of pollen grains, pollen numbers and male infertility of *Spartium junceum* L. (Spanish broom).

Effects of vehicular emission on pollen grains in the form of reduction in pollen germination and pollen tube length considerably affect the yield of plants. Many other researchers, including Gottardini et al. (2004), Higashitani (2013), Rezanejad (2013), Paupiere et al. (2014), have concluded that the vehicular emission and other atmospheric generated pollutants significantly influence the pollen grain production, pollen size, structure and its viability of different plant species. Kaur and Nagpal (2017) reported inversely proportion between number of traffic and pollen viability for *Alstonia scholaris, Nerium oleander* and *Tabernaemontana divaricata* at selected study sites.

Results presented in *Table 4* indicate that vehicular pollution increases the resting period of pollen grains in examined plant species of F-series by 1, 2 and 3 h, because the pollen grains of *Prunus avium* L. were pretentious due to the perpetuation of the resting time of F-series from 1 to 3 h at polluted site sample. At polluted site the pollen grains started their growth after 3 h and at 4th hour the growth was recorded only 164 µm as compared to control site sample 453.2 µm, while on the other hand pollen grain started its growth at 152.2 µm within one hour at control site. These results indicate that the pollen grains were under the stress of vehicular pollution. Data in

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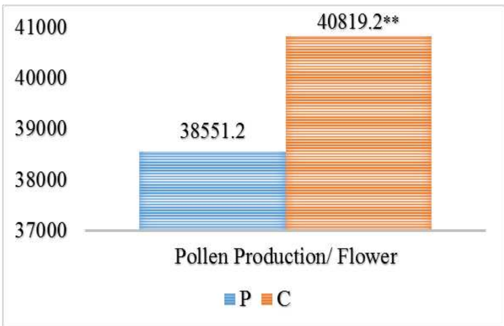
Table 4 also shows that the pollen grains of *Prunus avium* L. exhibited no propagation in F-24 series at all in both sites samples, including polluted and control. The results of this study are in conformity with observation recorded by Iyer and Bholay (2015) who found serious effects of air pollution on pollen grains of *Peltophorum ferrugineum.* They found resting period elongation to 5 h in F-series along with no germination in F-24 series due to air pollution.



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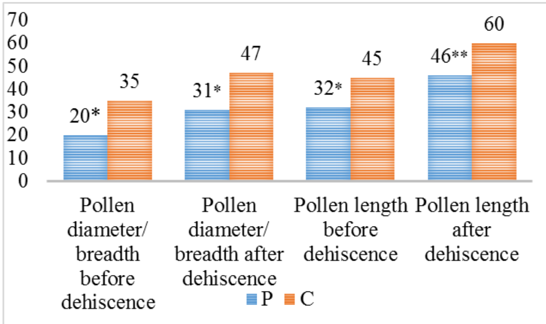

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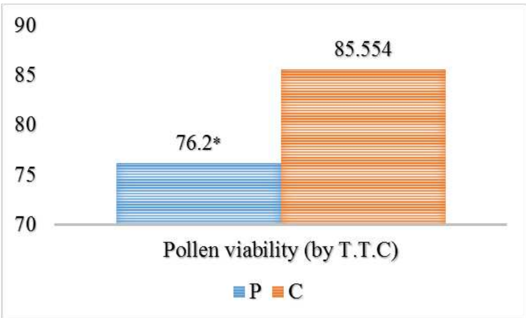

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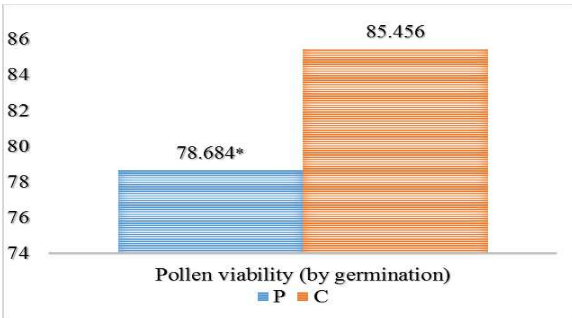

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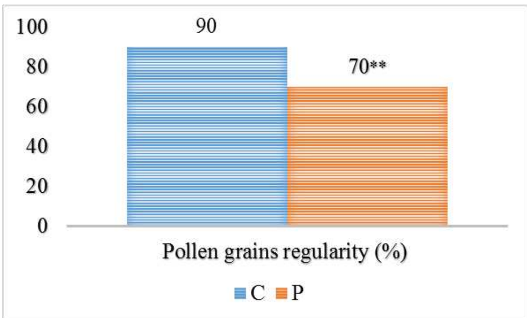

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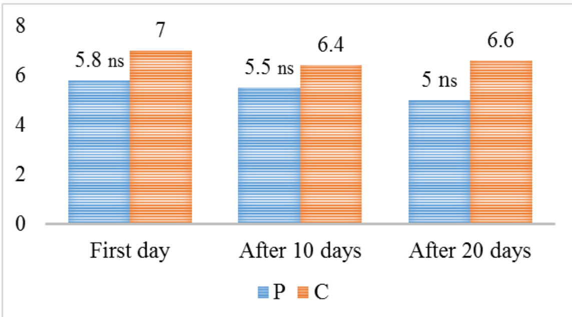

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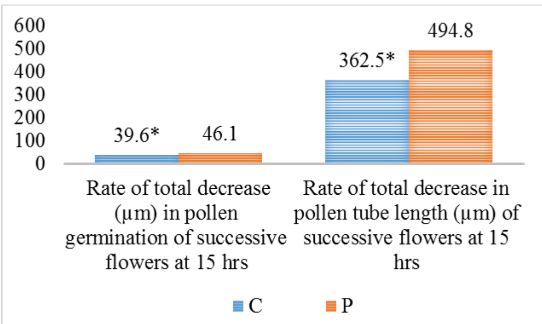

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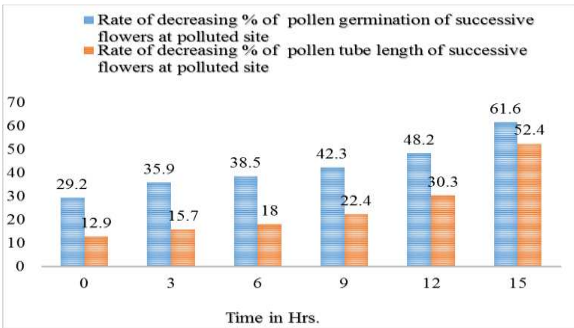

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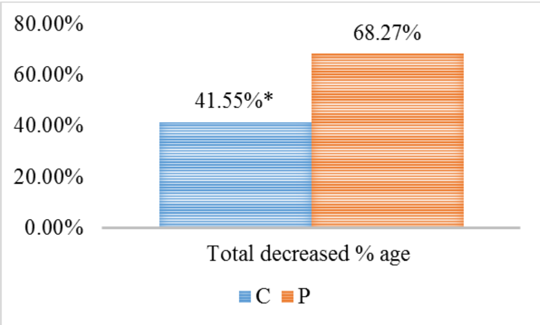

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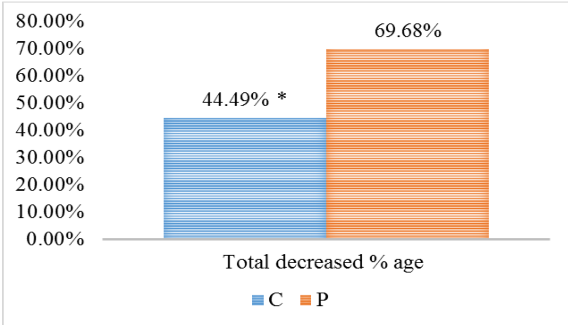

j

Figure 2. ***a*** *Effect of pollution on pollen production.* ***b*** *Effect of air pollution on pollen size (µm).* ***c*** *Effect of pollution on pollen viability (T.T.C).* ***d*** *Effect of pollution on pollen viability (by germination).* ***e*** *Effect of air pollution on pollen grains regularity (%).* ***f*** *Effect of air pollution on protein contents (mg/g dw) of pollen grain in different days.* ***g*** *Effect of vehicular exhaust pollution on the rate of total decrease (µm) in pollen germination and pollen tube length (µm) of successive flowers of Prunus avium L. at 15 h.* ***h*** *Effect of vehicular exhaust pollution on the rate of decreasing % of pollen germination and pollen tube length of successive flowers.* ***i*** *Total decreased % age in the rate of pollen grains germination of successive flowers at 15 h with respect to 0 h due to effect of vehicular exhaust pollution.* ***j*** *Total decreased % age in the rate of pollen tube length in F-series of successive flowers at 15 h with respect to 0 h due to effect of vehicular exhaust pollution. (C: control site or non-polluted site, P: polluted site, ns: non-significant, *: slightly significant, **: significant)*

Observations about the rate of decrease in pollen grains germination and pollen tube length in F-series of successive flowers are displayed in *Tables 2* and *3*. These consequences show reverse proportion to the storage period in F-series of successive flowers. At 0 h, rate of decrease in pollen grain germination was 95.3 and 67.46 µm for control and polluted site respectively, while at 15^{th} hour the rate of decrease was 55.7 and 21.4 µm, respectively (*Table 2*). Total decrease rate of pollen grains germination after 15 h was 39.6 µm (control) and 46.06 µm (polluted site) with respect to the 0 hour (*Fig. 2g*). The total decreasing percentage was 41.55 and 68.27%, at control and polluted site samples respectively (*Fig. 2i*). Similarly the pollen tube length at 0 h was 814.8 µm (control site) and 710.1 µm (polluted site) in successive flowers, while at 15 h it was decreased by 452.3 and 215.3 µm respectively (*Table 3*). The total decreased rate of pollen tube length at 15 h was 362.5 and 494.8 µm for control and polluted sites respectively (*Fig. 2g*) with respect to 0 hour. The total decreasing percentage in pollen tube length at control and polluted sample after 15 h was 44.49 and 69.68 %, respectively (*Fig. 2j*). Observations reported by Iyer and Bholar (2015) in *Peltophorum ferrugineum* are also coinciding with our results. Significant reduction in both parameters was also noted for each interval of time (0 to 15 h), respectively in the samples of polluted site with respect to the control site (*Fig. 2h*). From these results, it is concluded that the low rate of pollen grains propagation and pollen tube length in the plant species was might be due to greater rate of air pollution in city area. The decrease or inhibition in pollen grain germination and pollen tube length through auto exhaust pollution was also reinforced by other researchers, Farkhondeh (2009) who also found reduction in pollen of *Thuja orientalis* L. (Cupressaceae). Similarly destructive properties of automobile pollutants on vegetation have long been documented (Carhart,

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1995; Agrawal and Tiwari, 1997; Singh et al., 2002; Byers et al., 2008; Mashitha and Pise, 2008; Leghari et al., 2013; Laghari et al., 2015) and can be utilized as a bioindicator of air pollution.

Data about soluble protein contents in pollen grain extract is shown in *Figure 2f*. These results conclude that the total contents of protein in polluted site sample was low (5.8 mg/g dw) as compared to the control site sample (7.0 mg/g dw). The results also indicate that as exposing time to the air pollution increased the level of protein contents decrease, which was non-significant. After 20 days at control site sample the pollen protein concentration was recorded at 6.6 mg/g dw but in control site sample it was only 5 mg/g dw (*Fig. 2f*). Similar results were revealed by Hjelmroos et al. (1994) and Parui et al. (1998) who found decrease in Bet v 1 concentration in air contaminated sample. Our results indicate that the difference in protein contents between the sample of polluted and non-polluted sites was non-significant. Similarly, non-significant variation was also noted by Rezanejad (2007) in soluble pollen proteins of *Spartium junceum* L. (Fabaceae) in the samples of polluted and control sites. Observations reported by several other researchers are found in confirmation to our results. Helender et al. (1997) did not perceive any important changes in the bands of protein of control and polluted site samples. Investigation on *lagerstroemia indica* exhibited reduction in total protein concentration and succeeding lower intensity of proteins in air contaminated conditions (Rezanejad et al., 2003).

Conclusion

This study concludes that the pollen grains are sensitive to air pollution. Vehicular exhaust pollutants prolonged the resting period of pollen grain of F-series by 1 to 3 h in the species study. *Prunus avium* L. exhibited potentiality of pollen grain germination only in F-series and showed no germination at all in F-24 series. Air pollution inhibited pollen tube length, pollen structure, viability and growth of pollen tube and their growth was also found inversely proportionate to the storage time. It is concluded that air contamination seems proficient in altering the pollen grain fertility, development, viability and biochemical and physiological properties of the airborne pollen, therefore imperiling fertilization, reproduction ability, seed setting, plant breeding and possibly allergencity. So it may be concluded that pollen grains can give significant data about the organic impact of air impurities and can be used as decent applicants as bioindicators of environmental pollution. This type of research may be supportive in exploration of tolerance or sensitivity level of different roadside plant species against air pollution.

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EXOGENOUS APPLICATION OF 24-EPIBRASSINOLIDE AND NANO-ZINC OXIDE AT FLOWERING IMPROVES OSMOTIC STRESS TOLERANCE IN HARVESTED TOMATO SEEDS

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Abstract. The favorable effects of exogenously applied brassinosteroids and zinc micronutrient on plant growth performance and seed development have been extensively outlined in many studies. Nevertheless, almost no published document is available on the effects of 24-epibrassinolide and nano-zinc oxide on tomato seed quality and osmotic stress tolerance of the resulted seedlings. In this study, the effects of 24-epibrassinolide (0, 15 and 30 mg.ha^{-1} EBL) and nano-zinc oxide (0, 600 and 1200 ppm N-ZnO), which were exogenously applied on tomato mother plants, on seed germination parameters and stress tolerance capability of the produced seedlings under osmotic stress conditions (water potential of 0, -0.1 and -0.2 MPa stimulated by PEG-6000) were investigated. According to obtained results, the water potential of -0.2 MPa resulted in the lowest seed germinability and seedling growth performance with the highest amount of proline, hydrogen peroxide, malondialdehyde and activity of catalase and peroxidase, compared to non-stress control. All the foliar treatments resulted in decreased H_2O_2 and MDA content, especially EBL_2+N-ZnO_2. In addition, foliar application of EBL and N-ZnO enhanced the activity of antioxidative enzymes and ameliorated the inhibitory effects of osmotic stress on tomato seedling growth, except for percentage and rate of germination.
Keywords: *antioxidant system, brassinosteroids, micronutrients, seed quality, water potential*

Introduction

Tomato is the second most important vegetable crop next to potato worldwide (Eevera and Vanangamudi, 2006), and also it is highly popular in Northwest of Iran. According to the Food and Agriculture Organization (FAO), in 2016, around 4.7 million hectares of world farms allocated to tomatoes, which included about 177 million tons of annual production of fresh fruit (Anonymous, 2016). Tomato is mainly cultivated in tropical and sub-tropical countries, preferably in Mediterranean regions, which is mostly encountered by the varying environmental fluctuations. Among these adverse conditions, soil drying in seedbed is one of the critical problems that negatively influence seed germination and subsequent seedling growth (George, 2009; Chandrasekaran et al., 2017). The reduction in soil water potential results in disorder in seed germination by decreasing the imbibition rate (Eisvand et al., 2010). Individual seeds have to cross a threshold water potential in order to develop enzyme activity and lower their puncture force. The endosperm weakening opposite the radicle tip

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determines the threshold water potential for germination, which determines the rate and extent of germination (Finch-Savage, 2013).

In normal conditions, the reactive oxygen species (ROS) and antioxidant enzymes are produced in plant cells in a stable equilibrium (Gill and Tuteja, 2010), while under abiotic stresses, the balance can be overcome, mediating an accumulation of ROS and inducing oxidative damage (Mignolet-Spruyt et al., 2016). Lipid peroxidation as a main destructive event occurs due to increased ROS production and is detected by malondialdehyde accumulation in plant cells (Petrov et al., 2015). The antioxidant defense including the antioxidative enzymes (such as catalase and peroxidase) and metabolites is exploited by plant cells to cope with ROS attack (Gill and Tuteja, 2010). Catalase converts hydrogen peroxide (H_2O_2) into water and molecular oxygen (DeLay, 2017). Peroxidase can also detoxify H_2O_2 using various compounds as electron donor (Puthur, 2016). In addition, some researchers have introduced proline as an antioxidant molecule, proposing its role as ROS scavenger along with compatible osmolyte (Hossain et al., 2014; Rejeb et al., 2014). Under osmotic stress condition, the high capacity of antioxidant system is essential for plants to scavenge ROS and then avoid oxidative stress (Uzilday et al., 2014).

To ensure optimum stand establishment in soils with low water content, high-quality seeds must be used to achieve rapid and uniform seedling emergence, which may improve the resources use efficiency of established seedlings (Copeland and McDonald, 2012; Taiz et al., 2015). High-quality, also known as high-vigor seed lot has a great potential for rapid and uniform seed germination and successful seedling emergence under stressful conditions (Ellis and Roberts, 1981; Rehman et al., 1999; de Figueiredo et al., 2003). In all horticultural plants, including tomato, the high-quality seeds are the most important input in an efficient plant production system. So that, the low-quality seeds result in the poor stand establishment and consequently lower the crop productivity, especially under abiotic stresses (Rashid and Singh, 2000; Elias et al., 2006; George, 2009).

Seed quality consists of the physiological, physical and health attributes and is influenced by the growth conditions of the mother plant, which is well known as ‘maternal effect’ (Sangkram and Noomhorm, 2002; Pfeifer et al., 2011). In tomato plant, the fruit as a surrounding environment directly transfers the external conditions to the developing seeds. In this respect, the phytohormones and the mineral nutrients that reach the seeds play a key role in plant cell metabolism and ultimately improve the quality of produced seeds (Srivastava and Handa, 2005). Overall, the internal concentrations of plant growth regulators such as brassinosteroids and nutritional status of the plant, especially after adequate supply of micronutrients during the formation and development of seeds on the mother plant, are so vital. Some researchers have previously reported the production of high-quality seeds in plants exogenously treated with brassinosteroids and zinc (Biesaga-Koscielniak et al., 2014; Laware and Raskar, 2014).

Brassinosteroids are the sixth group of phytohormones, which have different physiological effects on seed and seedling tissues including the stimulation of cell elongation and division, the regulation of enzymes activity and the promotion of seed germination (Özdemir et al., 2004). Previous researchers have reported that these compounds alleviate the detrimental impacts of environmental hazards such as drought, on plant performance (Prusakova et al., 2000; Vardhini et al., 2010). In this regard, Yuan et al. (2010) demonstrated that the exogenous application of 24-epibrassinolide

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mediated drought tolerance in tomato plants through reducing malondialdehyde and hydrogen peroxide and increasing the activity of antioxidant enzymes. The micronutrient element of Zinc (Zn) plays a fundamental role in plant metabolism (Marschner, 2011) and biosynthesis of RNA, DNA and proteins (Welch, 2001). Although zinc is required in small amounts (5-100 ppm), its complete lack or insufficiency may influence the important physiological aspects of plant growth (Baybordi, 2006) including antioxidative enzymes and some regulatory metabolites (Cakmak, 2000). Supplemental zinc can alleviate the negative impacts of oxidative damage on plant metabolism (Tavallali et al., 2010). For example, zinc can help maintain sulfhydryl groups of cell membrane, which are easily oxidized by ROS (Rengel, 1995; Rengel and Wheal, 1997).

Already, some efforts have been made to produce high-quality seeds by employing certain agronomic techniques. The aim of the present research was to evaluate the osmotic stress tolerance capability (as an indicator of seed quality) of tomato seeds harvested from mother plants subjected to 24-epibrassinolide and nano-zinc oxide at the flowering stage. In the present experiment, seed germinability and seedling growth indices were measured and the antioxidative enzyme activities, hydrogen peroxide, malondialdehyde and proline accumulation in tomato seedlings were determined. This work is important as it demonstrates the collaborative role of brassinosteroid and zinc micro-element in providing osmotic stress tolerance and creating awareness of the potential for tomato plants to be cultivated in arid and semi-arid areas.

Materials and methods

The present experiment was conducted during 2015-2016 growing season in the Department of Agronomyand Plant Breeding, Faculty of Agriculture and Natural Resoursec at the University of Mohaghegh Ardabili, Ardabil, Iran. The seed lot of tomato (*Solanum lycopersicum* L.) cv. Y was obtained from SPCRI (Seed and Plant Certification and Registration Institute, Karaj). The viability and moisture content of prepared seeds were about 98% and 8%, respectively. All chemicals used in this research were purchased from Merck and Sigma-Aldrich.

Tomato seeds were sown in nursery beds of 0.5 m × 0.5 m, rich in loam and provided with shade and regular watering for seed germination. After 25 days of growth in the nursery, tomato plantlets were carefully transferred to the main field. The farm soil characteristics are listed in *Table 1*. The fertilizer application was 180 kg N, 150 kg P_2O_5 and 150 kg K per hectare. Tomato seedlings were transplanted in 3 rows in each plot at spacing of 45 cm × 120 cm, giving a total of 1500 plants per experimental unit. The final crop density was around 18,500 plants per hectare. A factorial experiment was conducted based on randomized complete block design (RCBD) with three replications. Experimental factors were foliar treatment combinations [control (only water), 15 $mg.ha^{-1}$ of 24-epibrassinolide (EBL_1), 30 $mg.ha^{-1}$ of 24-epibrassinolide (EBL_2), 600 ppm of nano-zinc oxide (N-ZnO_1), 1200 ppm of nano-zinc oxide (N-ZnO_2), EBL_1 + N-ZnO_1, EBL_1 + N-ZnO_2, EBL_2 + N-ZnO_1, EBL_2 + N-ZnO_2] and osmotic stress levels (0, -0.1 and -0.2 MPa). After complete vegetative growth in the field, foliar treatments with 24-epibrassinolide (EBL) and nano-zinc oxide (N-ZnO) were imposed at the flowering stage. Tween-20 (0.1%) was used as surfactant to enhance the effectiveness of foliar spray. The foliar applications were made with a portable field sprayer at 150 kPa pressure with 1000 l of liquid applied per hectare.

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Table 1. Soil characteristics of the farm where the experiment was conducted

Characteristics	Values
Soil texture	Loam
pH (1:1 H_2O)	7.4
EC_e ($dS.m^{-1}$)	3.8
Organic carbon (%)	0.9
Total N (%)	0.09
Available P ($mg.kg^{-1}$)	20
Available K ($mg.kg^{-1}$)	230
Zn ($mg.kg^{-1}$)	0.994

After harvesting the fully ripe fruits, the seeds were extracted by fermentation method (at 25 °C for 24 h) and then dried to moisture content of about 8%. The quality of obtained seeds was assayed under osmotic stress condition. In order to create the different water stress levels (0, -0.1 and -0.2 MPa) in seed germination mediums, PEG-6000 was used. The standard germination test of the processed seeds was conducted according to the rules approved by the International Seed Testing Association (ISTA, 2008). In brief, after 10 min of surface sterilization with sodium hypochlorite 1%, four replicates of 50 seeds were incubated in alternating temperatures (25/30 °C) for 14 days. The daily counting of the number of germinated seeds (>2 mm radicle protrusion) continued until the end of the 14th day. In each experimental unit, the standard germination percentage and dry weight and length of seedlings were measured. The germination rate was calculated using the formula of Ellis and Roberts (1981). The vigor index (VI) was calculated using the following equation: VI = seedling length × germination percentage.

Free proline content in tomato seedlings was determined using ninhydrin reagent according to the most used method described by Bates et al. (1973). Malondialdehyde (MDA) content was measured by thiobarbituric acid reaction (Heath and Packer, 1968), with slight modifications. Fresh seedlings (0.1 g each) were homogenized in 1 ml of 0.1% (w/v) trichloroacetic acid (TCA) and the homogenates were centrifuged at 5000 × g for 10 min at 4 °C. In the following, 400 µl of the supernatant was added into1 ml of 0.5% (w/v) thiobarbituric acid. The obtained mixture was incubated at a temperature of 95 °C in a water bath for 30 min. After centrifugation at 10000 × g for 10 min, the absorbance of the supernatant was measured at 532 and 600 nm in a spectrophotometer. To calculate the concentration of MDA, the related extinction coefficient (0.155 $\mu M^{-1}.cm^{-1}$) was used and the values were expressed as $\mu mol.g^{-1}$ F.W. Hydrogen peroxide was quantified by the method of Hung et al. (2005). Fresh seedlings (0.1 g each) were homogenized in 1 ml of 0.1% (w/v) TCA. The obtained mixture was centrifuged at 10000 × g for 10 min, at 4 °C, then 0.5 ml of supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH = 7) and 1 ml of 1 mM potassium iodide. The absorbance was read at a wavelength of 390 nm. The amount of hydrogen peroxide in each sample was calculated using the specific extinction coefficient (0.28 $\mu M^{-1}.cm^{-1}$) and the values were expressed in units of $\mu mol.g^{-1}$ F.W. Catalase (CAT) and peroxidase (POD) activities in tomato seedlings were assayed using the methods of Aebi (1984) and MacAdam et al. (1992), respectively.

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After conducting the normality test of obtained data, two-way analysis of variance (ANOVA) for randomized complete block design was done using SAS 9.4 software and the means were compared by least significant difference (LSD) test at 5% probability level and the figures were created with Microsoft Excel 2013.

Results

In the present study, tomato plants were exogenously treated with different combinations of 24-epibrassinolide (EBL) and nano-zinc oxide (N-ZnO) at the flowering stage and then the harvested seed lots were subjected to osmotic stress (OS). Finally, germination percentage and rate, seedling length, dry weight and vigor index, proline, malondialdehyde and H_2O_2 content and catalase and peroxidase activity in the seedlings were estimated. In general, the main effects and interaction of two experimental factors: (1) foliar application of EBL+N-ZnO and (2) osmotic stress were statistically significant ($P \leq 5\%$) in all measured characteristics, but germination percentage and rate which had the main effects, were significant (*Table 2*).

Table 2. *Analysis of variance of EBL+N-ZnO effects on some characteristics related to seed germination and seedling growth performance of tomato under drought stress condition*

S.O.V.	d.f.	Mean squares				
		Germination percentage	**Germination rate**	**Seedling length**	**Seedling weight**	**Vigour index**
Block	2	1.148^{ns}	0.0066^{**}	0.031^{**}	0.0002^{ns}	0.13^{ns}
EBL+N-ZnO	8	170.527^{**}	0.0094^{**}	4.714^{**}	0.0538^{**}	11.126^{**}
Drought stress	2	148.111^{**}	0.453^{**}	96.123^{**}	1.09^{**}	176.553^{**}
EBL+N-ZnO × drought stress	16	1.486^{ns}	0.00001^{ns}	1.008^{**}	0.0113^{**}	0.973^{**}
Error	52	5.827	0.0005	0.0038	0.00006	0.135
C.V. (%)	-	*2.360*	*5.522*	*1.403*	*1.486*	*2.625*

Continuation

S.O.V.	d.f.	Mean squares				
		Proline content	**Hydrogen peroxide content**	**Malondialdehyde content**	**Catalase activity**	**Peroxidase activity**
Block	2	4733.405^{ns}	0.0009^{ns}	0.0008^{**}	0.000002^{*}	0.00001^{ns}
EBL+N-ZnO	8	152822.922^{**}	0.157^{**}	0.287^{**}	0.00008^{**}	0.0003^{**}
Drought stress	2	2794500.611^{**}	3.344^{**}	1.216^{**}	0.0012^{**}	0.0150^{**}
EBL+N-ZnO × drought stress	16	27339.950^{**}	0.033^{**}	0.075^{**}	0.0002^{**}	0.0001^{**}
Error	52	5188.522	0.0004	0.050	0.000001	0.0002
C.V. (%)	-	*1.713*	*3.032*	*7.609*	*1.575*	*2.870*

The effect of osmotic stress on the germination percentage and rate of seeds produced from tomato mother plants exogenously treated with EBL and N-ZnO are shown in *Table 3*. Overall, the promotive influence of foliar application of EBL and N-ZnO on the germination percentage and rate were observed. The treatment, EBL_1 (15 $mg.ha^{-1}$), had no significant difference from control ($P > 5\%$) in percentage germination. The sole applications of EBL and N-ZnO were statistically the same in the rate of germination. The highest germination percentage and rate were recorded when EBL_2 + N-ZnO_2 was applied in the mother plants, compared to control in which only the

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distilled water was sprayed. The germination percentage and rate was significantly decreased with increased levels of osmotic stress. So that, the maximum reduction in germination percentage (about 5%) and rate (approximately 72%) were observed in water potential of -0.2 MPa compared with control.

Table 3. *Germination percentage and rate of seeds harvested from tomato plants subjected to exogenous EBL and N-ZnO in response to osmotic stress*

Experimental factors		Germination percentage	Germination rate (d^{-1})
Foliar application of EBL and N-ZnO	Control	84.0 ± 1.756 f	0.164 ± 0.039 e
	EBL_1 (15 $mg.ha^{-1}$)	85.8 ± 1.839 fe	0.178 ± 0.038 d
	EBL_2 (30 $mg.ha^{-1}$)	92.4 ± 1.732 c	0.185 ± 0.037 d
	$N\text{-}ZnO_1$ (600 ppm)	87.3 ±1.849 e	0.182 ± 0.037 d
	$N\text{-}ZnO_2$ (1200 ppm)	90.0 ± 1.636 d	0.181 ± 0.041 d
	EBL_1+$N\text{-}ZnO_1$	89.8 ± 1.632 d	0.197 ± 0.038 dc
	EBL_1+$N\text{-}ZnO_2$	93.7 ± 1.563 bc	0.221 ± 0.037 c
	EBL_2+$N\text{-}ZnO_1$	92.1 ± 1.501 b	0.244 ± 0.041 ab
	EBL_2+$N\text{-}ZnO_2$	97.1 ± 1.444 a	0.263 ± 0.038 a
LSD (5%)		**2.283**	**0.0346**
Osmotic stress	Control	93.1 ± 0.590 a	0.347 ± 0.009 a
	-0.1 MPa	89.9 ± 0.773 b	0.165 ± 0.008 b
	-0.2 MPa	88.1 ± 0.735 c	0.097 ± 0.008 c
LSD (5%)		**1.318**	**0.02**

Similar letters indicate no significant difference at $p \leq 5\%$

Changes in the length, dry weight and vigor index of tomato seedlings in response to treatment compounds almost followed the same trend (*Fig. 1 A-C*). Overall, the foliar treatments of EBL and N-ZnO significantly increased the seedling growth parameters, compared to untreated control (only water spray). On the other hand, the inhibitory effect of osmotic stress on the growth performance of tomato seedlings was intensified by decreasing the water potential. The highest values of seedling length (*Fig. 1A*), dry weight (*Fig. 1B*) and vigor index (*Fig. 1C*) were recorded in treatment combination of EBL_2+$N\text{-}ZnO_2$ under non-stress condition. The lowest length, dry weight and vigor index of tomato seedlings belonged to both $N\text{-}ZnO_1$ (600 ppm of nano-zinc oxide) and untreated control, when -0.2 MPa water potential was created in the growth medium. Notably, when the osmotic stress was amplified in seedling growth medium (-0.2 MPa water potential), the effectiveness of exogenously applied brassinosteroid and zinc was relatively maintained in comparison with the mild water deficit condition (-0.1 MPa water potential).

The free proline content in tomato seedlings was significantly influenced by osmotic stress levels ($P \leq 0.05$). In an osmotic potential of -0.2 MPa, the accumulation of proline was increased approximately by 15% compared to non-stress control. In general, the foliar treatments had negligible but significant positive effects on the proline amount ($P > 0.05$). On the other hand, the interaction between the two experimental factors was statistically significant. The proline content was considerably elevated in response to concomitant application of 24-epibrassinolide and nano-zinc oxide under both osmotic stress levels, so that the highest proline accumulation occurred in treatment combination of EBL_2+$N\text{-}ZnO_2$ under water potential of -0.2 MPa (*Fig. 2*).

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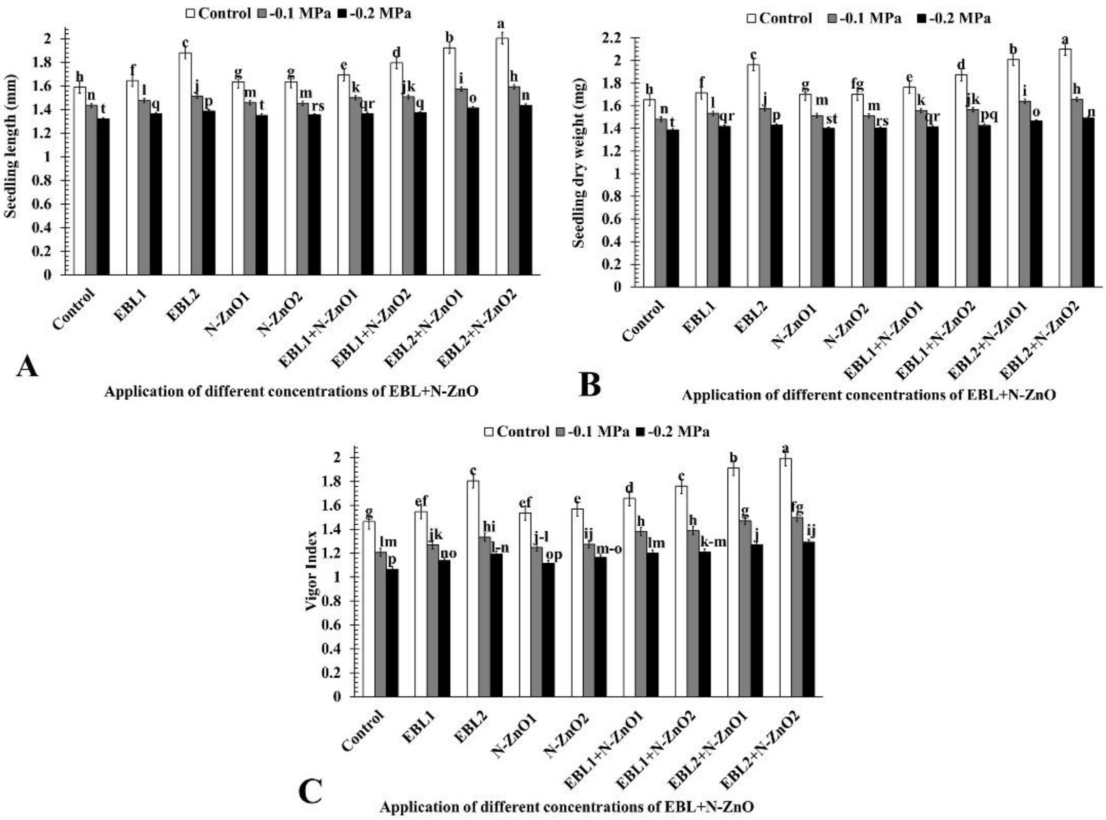

Figure 1. *Changes in the seedling length (A), dry weight (B) and vigor index (C) in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at $P \leq 5\%$. LSD values for the seedling length, dry weight and vigor index were 0.01, 0.0134 and 0.06, respectively*

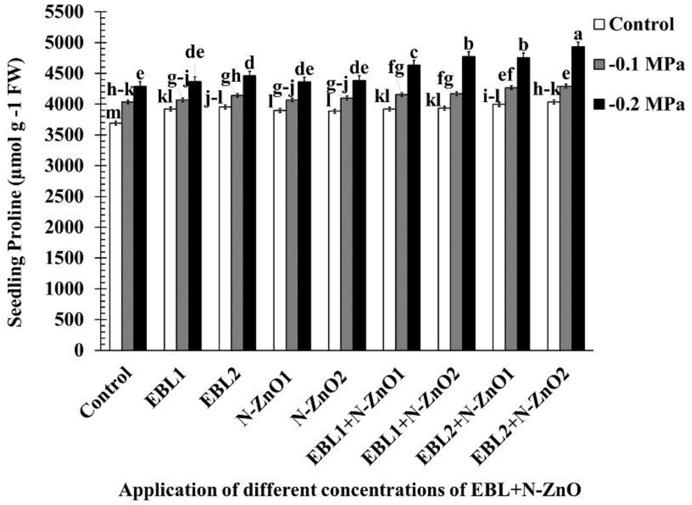

Figure 2. *Changes in the free proline content of seedling in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at $P \leq 5\%$. LSD value was 118.02*

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The tomato seedlings showed the higher H_2O_2 accumulation under osmotic stress compared to the corresponding control, especially when the osmotic potential of the culture medium was -0.2 MPa (*Fig. 3*). Under both non-stress and osmotic stress conditions, foliar sprayed EBL+N-ZnO combination had more significant reduction in the H_2O_2 accumulation compared to sole application of EBL and/or N-ZnO. The lowest H_2O_2 content was clearly observed in EBL_2+N-ZnO_2 (30 mg.ha^{-1} EBL and 1200 ppm N-ZnO) under non-stress condition. Among the sole applications of 24-epibrassinolide (EBL_1 and EBL_2) and nano-zinc oxide (N-ZnO_1 and N-ZnO_2), 30 mg.ha^{-1} brassinosteroid (EBL_2) showed more decrease in hydrogen peroxide amount.

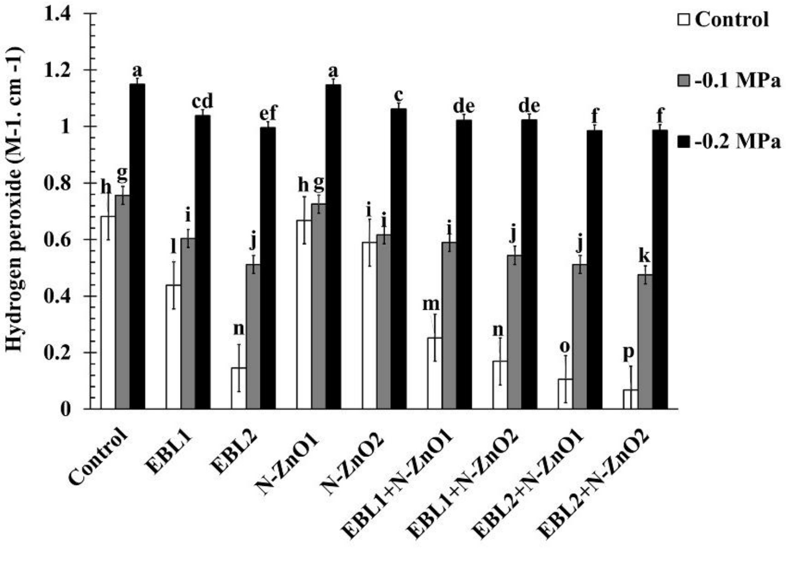

Figure 3. *Changes in the H_2O_2 content of seedling in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at $P \leq 5\%$. LSD value was 0.0328*

The osmotic stress led to a significant increase in malondialdehyde (MDA) content in tomato seedlings compared to corresponding control (*Fig. 4*). The greatest accumulation of MDA was recorded in tomato seedlings untreated with EBL and N-ZnO under osmotic stress level of -0.2 MPa. Among the sole applications of brassinosteroid and zinc, EBL_2 (30 mg.ha^{-1}) indicated more reduction in MDA content. Overall, the osmotic stress-induced MDA production was further restricted when EBL+N-ZnO combinations were exogenously applied, instead of using them alone. The lowest accumulation of malondialdehyde was observed in EBL_2 + N-ZnO_2 treatment under all three levels of osmotic stress.

As shown in *Figure 5,* the catalase activity in tomato seedlings was significantly increased under osmotic stress condition (-0.1 and -0.2 MPa) compared to control, and the foliar treatments relatively enhanced the CAT activity. The highest activity of catalase (about 3-fold of control) was observed in water potential of -0.2 MPa when 30 mg.ha^{-1} EBL and 1200 ppm N-ZnO were concomitantly applied.

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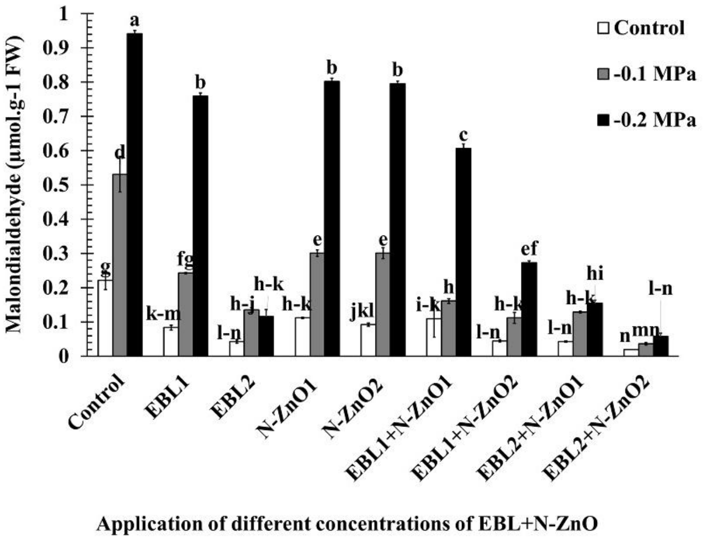

Figure 4. *Changes in the MDA content of seedling in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at P ≤ 5%. LSD value was 0.0509*

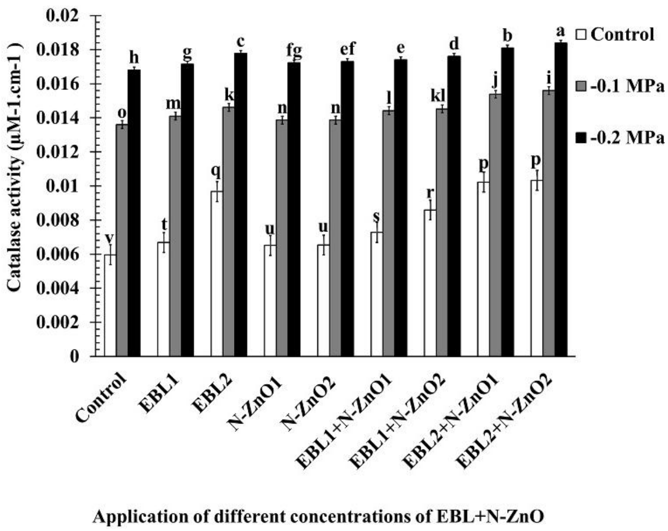

Figure 5. *Changes in the activity of CAT in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at P ≤ 5%. LSD value was 0.0001*

Under non-stress conditions, foliar sprayed EBL and N-ZnO did not have any significant effect on the peroxidase activities (*Fig. 6*). Under osmotic stress, the activities of POD in tomato seedlings were obviously increased, whereas the application of 24-epibrassinolide and nano-zinc oxide negligibly but significantly promoted the increase. Like the CAT enzyme, the highest activity of POD (more than two fold of control) was recorded in treatment combination of EBL_2 + N-ZnO_2 under water potential of -0.2 MPa.

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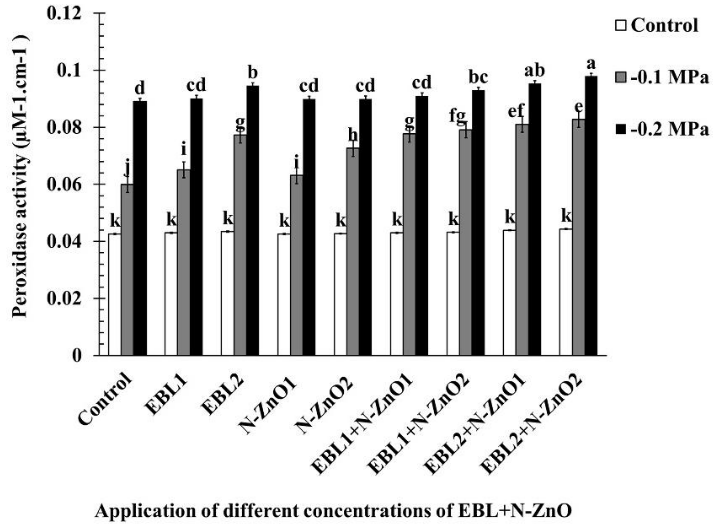

Figure 6. *Changes in the activity of POD in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at P ≤ 5%. LSD value was 0.0033*

Discussion

Promotive effects of brassinosteroid and zinc micronutrient on seed quantity and quality of field crops has already been mentioned by some authors (Hayat et al., 2001; Biesaga-Koscielniak et al., 2014; Laware and Raskar, 2014). According to previous studies, exogenous application of 24-epibrassinolide (Özdemir et al., 2004) and nano-zinc oxide (Prasad et al., 2012) at germination stage can lead to improved seed germinability and subsequent seedling growth. To the best of our knowledge, almost no research has been conducted to evaluate the effects of foliar spray of EBL and N-ZnO on seed germination and subsequent seedling performance under osmotic stress condition in seed lots produced from tomato mother plants. In the present research, the results showed that osmotic stress resulted in a delayed and decreased germination, which was not improve by foliar application of EBL and N-ZnO in tomato plants (*Table 2*). The results are in good agreement with previous investigations which reported osmotic stress-induced reduction in the seed germinability (PENG et al., 2013; Bhatt et al., 2015). Rajabi et al. (2013) stated that interruption in water absorption by seed could affect the metabolic activities related to germination process under osmotic stress condition. On the other hand, foliar spray of EBL and N-ZnO combinations at the flowering stage could enhance the percentage and rate of germination in the obtained seeds (*Table 2*). Brassinosteroids have a similar role as gibberellin in the germination process and enhances the germinability of seeds by reducing and/or increasing their biosynthesis and sensitivity to abscisic acid and/or gibberellin, respectively (Erik et al., 1996; Steber and McCourt, 2001; Zhang et al., 2009; Divi and Krishna, 2010). Similar results have reported that exogenous application of zinc in safflower (Movahhedy-Dehnavy et al., 2009) and soybean (Sedghi et al., 2013) increased the germination of produced seeds under drought stress conditions. Zinc micro-element is required for the biosynthesis of auxin (IAA) through the tryptamine pathway (Haslett et al., 2001), and has a promotive effect on the cell division (Alloway, 2004). Therefore, it can be

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concluded that Zn-mediated increase in the seed germination parameters can be originated from the signaling of IAA and the proliferation of cells.

According to the present results, the tomato seedling performance (measured as seedling length, dry weight and vigor index) was adversly affected when osmotic stress was intensified in the culture medium of harvested seeds. As seen in *Figure 1,* 30 $mg.ha^{-1}$ EBL along with 1200 ppm N-ZnO (treatment combination of EBL_2 + N-ZnO_2) may to a great extent compensate for the drought-induced inhibition in tomato seedling growth. The results of the current study are consistent with the recent study (Khripach et al., 1998) in which the growth performance of seedlings was improved when the mother plants was treated with 24-epibrassinolide and the harvested seeds were exposed to low water potentials. Prasad et al. (2012) demonstrated that the foliar spray of nano-zinc oxide caused a significant increase in seedling length and dry weight, compared to the control. Boonchuay et al. (2013) found that foliar application of zinc resulted in higher seedling vigor index in seed lots obtained from exogenously treated mother plants. Overall, nanoparticles provide an efficient means to distribute agro-chemicals including fertilizers, in a controlled fashion (Agrawal and Rathore, 2014). So, plants can rapidly absorb slow-release nano-fertilizers and surely cause the saving of fertilizer consumption and minimize environmental pollution (Mura et al., 2013; Bindraban et al., 2015). It should be noted that the hormone (24-epibrassinolide) application was more efficient than the micronutrient (nano-zinc oxide), when only one of them was used alone, compared to EBL+N-ZnO co-application.

In consistency with the results of this experiment, many researchers reported that osmotic stress increases the accumulation of free proline in tomato seedlings (Calvo-Polanco et al., 2016; Sun et al., 2016). Kishor et al. (2014) stated that the proline accumulation has a positive and direct relationship with increasing the tolerance to water deficit stress in plants, as was evident in the current study (*Fig. 2*). Further increase in proline amount leads to preventing ROS attack and subsequently reducing the cell membrane damage. In addition, drought tolerance is increased by proline-induced osmotic adjustment (Szabados and Savouré, 2010). On the other hand, exogenous EBL and N-ZnO, exogenously applied in tomato mother plants during anthesis, promotes the production of free proline in seedlings under low water potential (*Fig. 2*). In this regard, Behnamnia et al. (2009) reported that 24-epibrassinolide increased the proline content in tomato leaves under limited water access. It has commonly been assumed that, EBL can promote free proline biosynthesis through inducing the expression of genes responsible for the related enzymes (Talaat and Shawky, 2013) and regulating the synthesis of nucleic acids (Bajgaz, 2000). As an important cofactor, Zinc has a key role in the production and activity of several enzymes (Bagci et al., 2007), particularly those involved in the main pathway of proline biosynthesis, i.e. glutamate pathway which is dominant in higher plants (Delaney et al., 1993). So, it seems that great increase in the proline content of tomato seedlings in response to exogenous application of EBL and N-ZnO at the flowering stage can help maintain an efficient osmotic homeostasis into the cell (by increasing proline and other compatible osmolytes), and therefore induce the osmotic stress tolerance in seedlings.

In this study, the hydrogen peroxide (*Fig. 3*) and malondialdehyde (*Fig. 4*) accumulation was clearly observed in leaves in response to low water potential imposed to seedlings from harvested tomato seeds. There is strong evidence that osmotic stress can result in the accumulation of ROS including hydrogen peroxide and MDA, as an indicator of lipid peroxidation, in different plant tissues (Todorova et al., 2016). As previous studies

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have emphasized, the overproduction of toxic ROS in plant cells under stress can mainly damage the lipid-containing components of cell (Tavallali et al., 2010) and then cause the plasma membrane disintegrity that increases the permeabilization (Anjum, 2015). As found in the present experiment, osmotic stress-induced accumulation of H_2O_2 and MDA in seedlings was significantly declined as a result of foliar treatment of tomato mother plants with 24-epibrassinolide and nano-zinc oxide (*Figs. 3* and *4*). Several researchers reported the decline in H_2O_2 and MDA content of leaves due to 24-epibrassinolide application (Yuan et al., 2010; Talaat and Shawky, 2013). Brassinosteroid hormones may prevent the membrane damage caused by lipid peroxidation through influencing fatty acid composition and regulating the expression of genes (Khripach et al., 1998; Cao et al., 2005). In addition, Zn reduced ROS generation by indirect activation of antioxidant system (Grewal and Wiliams, 2000; Bagci et al., 2007).

Higher plants evolutionary have an antioxidant defense mechanism for scavenging reactive oxygen species, and efficient modulation of this defense system is essential for plants to tolerate the stress-iduced oxidative damage (Mittova et al., 2015). In the present study, antioxidative enzymes including catalase (*Fig. 5*) and peroxidase (*Fig. 6*) showed a significant increase in tomato seedlings under osmotic stress conditions, which were effective in alleviating the adverse impact of ROS and subsequently preventing lipid peroxidation. Foliar application of EBL and N-ZnO resulted in increased activity of both CAT and POD, especially when no osmotic stress was imposed on tomato seedlings. This may also imply that CAT plays more important role than POD in scavenging excessive hydrogen peroxide in osmotic stress condition. This is in consistency with the same report in silicon-supplemented tomato seedlings exposed to water deficit stress (Shi et al., 2014). Yuan et al. (2010) reported that the use of epibrassinolide induced tolerance to drought in tomato via reducing malondialdehyde and hydrogen peroxide and increasing the activity of antioxidant enzymes. Researchers believe that brassinosteroids increase the resistance of plants to ROS-mediated oxidative damage through the modulation of expression of genes encoding antioxidative enzymes (Goda et al., 2002; Cao et al., 2005). Zinc is effective in the induction of gene expression responsible for the synthesis of proteins and antioxidant enzymes and in some cases, is also considred a cofactor for these enzymes (Grewal and Wiliams, 2000; Bagci et al., 2007; Alharby et al., 2016).

Conclusion

Finally, it could be concluded that seeds produced from tomato plants, which were treated with 24-epibrassinolide and nano-zinc oxide, showed higher tolerance to osmotic stress. So that seed germination indices, seedling growth performance and activity of antioxidative enzymes were significantly increased by preventing the accumulation of toxic ROS and MDA as the main peroxidative products. Although, both two applied concentrations of 24-epibrassinolide and nano-zinc oxide showed the promotive effects on osmotic stress tolerance in tomato seedlings, the combined treatment of 30 $mg.ha^{-1}$ 24-epibrassinolide and 1200 ppm nano-zinc oxide (EBL_2+N-ZnO_2), had higher tolerance capability. Based on the current findings, foliar application of 24-epibrassinolide and nano-zinc oxide with the optimal concentrations at the flowering stage is highly recommended. On the other hand, it is recommended that researchers investigate the effects of optimal concentrations of 24-epibrassinolide and nano-zinc oxide on different developmental stages of tomato seeds and report their results in order to complete the results of current study.

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LARIX GENUS GROWING RATE AND ASSOCIATION WITH METEOROLOGICAL CONDITIONS IN THE BOTANICAL GARDEN OF KLAIPĖDA UNIVERSITY, LITHUANIA

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Abstract. The most important climate parameters in dendrochronological investigation are air temperature and dynamics of precipitation in certain periods. In the research time from 2005 to 2016 3 species and 2 hybrids: *Larix sibirica, L. decidua, L. kaempheri, L. x gmelinii* and *L. x eurolepis* were evaluated in the Botanical Garden of Klaipėda University, that is situated in the western part of Lithuania. This region can be characterized with high precipitation in the summer season, warm air temperature in winter and the lowest temperature values are in the summer season. According to research data the precipitation in autumn season influenced the highest radial growths of all observed Larix (r from 0,33 to 0,78), but average air temperature in this season had no influence (r from -0,19 to 0,50), similarly temperature had no influence in the spring season either (r from -0,12 to -0,76, $p<0.001$). 62 % of *L. sibirica* was influenced by precipitation but 22 % of L. *decidua* by air temperature in the autumn season. The average radial growth of hibrid larches, namely *L. x gmelinii* and *L. x eurolepis* was 1.3 – 1.5 times lower compared with *L. sibirica* and *L. decidua* . *L. x gmelinii* and *L. x eurolepis* were also positively influenced by precipitation in the autumn season.
Keywords: *botanic garden, Larix, average temperature, precipitation, season, radial growth*

Introduction

Tree rings analysis is widely used in modern forest lumbering, climate change research and in forest monitoring studies (Worbes, 2004). Most important climate parameters in dendrochronological investigation are air temperature and dynamics of precipitation content in certain periods (Xue et al., 2016). In Lithuanian conditions the formation of annual tree rings is mostly influenced by air temperature in active vegetation period (April – September) and precipitation in the summer time (Stravinskienė, 2002). Climate is a major environmental factor affecting the phenotype of trees and a critical agent of natural selection that has molded among-population genetic variation. Population response functions describe the environmental effect of planting site climate on the performance of a single population, whereas transfer functions describe among-population genetic variation molded by natural selection for climate. Although these approaches are widely used to predict the responses of trees to climate change, both have limitations (Wang et al., 2010). The rapid rate of change coupled with the large absolute amount of change are expected to have profound effects on terrestrial ecosystems at all hierarchical levels: from vegetation zones (Tchebakova et al., 2005), to ecosystems (Guisan et al., 1995), species (Fujiwara and Box, 1999), and to populations within species (Rehfeldt et al., 1999, 2002; Tchebakova et al., 2005).

Larch (*Larix*) belongs to the Pinaceae family and is one of the most abundant coniferous genera of the northern hemisphere. The larches - Larix P. Mill. - of the world are usually grouped into 10 species that are widely distributed in Eurasia and North America (Hora, 1981; Krüssmann, 1985; Schmidt, 1995; Farjon, 2010; Ide et al., 2016). Some species

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dominate at the northern limits of boreal forests and others occur above subalpine forests (Gower and Richards, 1990). Larch cannot grow in the shade. It prefers moist soil. The plant can tolerate maritime exposure, they cannot tolerate atmospheric pollution (http://www.pfaf.org). Geographic races have developed in many widely distributed larch species, and these often exhibit marked differences in growth rates and other characteristics (Rudolf, 1974; Bednarova et al., 2013). The European larch includes at least 5 geographic races (often considered to be subspecies or varieties) that roughly coincide with major distributional groups of the species. European larch (*Larix decidua*, Miller, 1768) is endemic to Europe and is characterized by a disjunctive distribution (Wagner, 2013). European seed sources perform similarly in northeastern United States as in Great Britain, Germany and Italy (Dylis, 1981). Some varieties of Dahurian larch that is confined to definite areas appear to be geographic races. The native western larch specimens from more continental climates with lower humidity are doing poorly. European larch compared with other species can be characterized with faster growth. Natural habitat of European larch is relatively small and covers mainly the European mountainous regions (Busetto et al., 2010). The rapid growth of larch, high productivity and sustainability are achieved when soil and climatic conditions are in accordance with the biological requirements. The dry weather, hot summers and cold winters are important conditions for larch (Тимофеев, 1961). *Larix sibirica* is common in the lowland taiga of West Siberia, where it forms the northern limit of trees alternately with *Picea obovata* and with *Pinus sylvestris*. Larrix also occurs in the mountains (from 500 m to 2,400 m a.s.l.). It grows on a great variety of soils, from peat bogs to well drained, sandy or rocky soils, where it has its optimum, that is in very cold climate (min. temp. -55 °C), continental or subarctic, dry, with very long winters. There are stands on peat or on mountains above the steppe (Altai Mts.), but more common it is mixed with *Pinus sylvestris*, *P. sibirica*, *Picea obovata*, *Abies sibirica* and broad-leaved trees such as *Betula pendula* and *Populus* spp (Farjon, 2013). Japanese larch (*Larix kaempferi*) is one of the most important timber species in the sub-Alpine region and northern area of Japan (Zhu et al., 1998). *Larix gmelinii* (Rupr.) is one of the most economically important species in boreal forests (Zyryanova et al., 2007). It is a deciduous needleleaf conifer that is adapted to growing in very harsh climates and is widely distributed over a range from 40° N to 72° N and 110° E to 130° E (Zhu et al., 1998). It is the dominant tree species in the Greater Khingan Mountains forest ecosystem in northern China (Farjon, 1990; Fu et al., 1999).

Introduction of European larch (*Larix decidua* Mill.) in Lithuania launched in the middle of the XIX century as a valuable industrial and decorative species. In forestries of the country larch began to grow 160 years ago, and now grow such forest stand in south part of Lithuania in Alytus district (Navasaitis, 2004). As in natural habitats, in Lithuania larches resistant to cold, and not affected by winter (Pūkienė and Bitvinskas, 2000). In Lithuania European larch mainly grows in mixed stands associated with Norway spruce (*Picea abies*), stone pine (*Pinus cembra*), Swiss pine (*Pinus mugo*), European beech (*Fagus sylvatica*) or silver fir (*Abies alba*) but can also occur in pure stands (Navasaitis, 2004).

The stem radial growth of trees in any year integrates the effects of the climate during the previous and current year (Zhang and Chen, 2015; Jiang et al., 2016). Tree radial growth is influenced by environmental factors (e.g., temperature, precipitation, and competition) and conditions within the individual tree (Zhang and Chen, 2015). Tree-ring records are often used to investigate the responses of tree growth to historical climate variations, to predict the effects of future climate change on tree growth, and to understand the spatial and temporal patterns of tree-growth variability of forest ecosystems (Thuiller et al., 2005; Tardif et al., 2006; Metsaranta et al., 2008). Stem sensitivity growth rate is an important indicator for the

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assessment to increasing annual radial gain and their connection with environmental conditions (Karpavičius, 2004). Reasearch of Larix genus growing intensity is important to climate changes context in botanical gardens, because there ussualy carry out evaluating of plants acclimatization. This is important to preserve biological diversity and educate the society. Many people may have a conceptual grasp of climate change, but may not know what climate effects to expect in the region, and what impacts climate change may have on their day-to-day environment. As the urgency and significance of climate change continues to mount, new approaches are needed for interpreting and visualizing climate change for the public that is tangible and approachable. Botanical gardens have made longstanding contributions to climate change research, particularly with respect to temperature and its effects on the timing of plant flowering and leaf out by participating in phenological networks of botanical gardens, monitoring standardized plantings in phenological gardens, and studying and examining herbarium specimens and historical photographs (Primack and Miller-Rushing, 2009).

The aim of this work is to evaluate *Larix* Mill genus plant radial growth in the context of climate parameter changes in the Botanical Garden of Klaipėda University located in the west part of Lithuania (coastal zone).

Material and methods

Study area and object

The Botanical Garden of Klaipėda University is located in Western Lithuania, in the northern part of the city of Klaipėda, in the valley of the Dane River. It was established in 1993, since 2002 it has dendrological park status. In general, the width of the area is 9.3 ha, and collections of ornamental plants are established here. The most important purpose of the Botanical Garden is the conservation of the genetic resources of herbaceous and woody ornamental and medicinal plants in the collections. It is a unique plant introduction centre, where climatic conditions differ from other parts of Lithuania, because of having higher air temperature, more precipitation, and a longer plant-vegetation period. Therefore, these are favourable conditions for plant adaptation. Since 2005, Botanical Garden is a member of the International Phenological Gardens (IPG).

In the Dendrological section of the Botanical Garden 10 taxa of Larix P. Mill. are growing. For research 3 species and 2 hybrids of larches were evaluated: *Larix sibirica, L. decidua, L. kaempheri, L. x gmelinii* and *L. x eurolepis* (3 individuals from every taxa). The age of the evaluated larix were different: *L. sibirica* was 19, *L. decidua* was 14, *L. kaempheri* was 13, *L. x gmelinii* was 14, *L. x eurolepis* was 10 years old. In this research radial growth of stems were compared during the period of 2005-2016.

All plants were growing in the same soil type, namely FLc-ar (Fluvisol Areni-Calcicaric). The texture of soil at 0.30 m: 84.8% sand, 9.4% dust, 5.8% clay. The soil pH is 6.2, mobile phosphorus concentration is 581 mg/kg, mobile potassium concentration is 135 mg/kg, total nitrogen – 0.114 mg/L, biohumus content is 2.05%.

Dendrochronological investigation

Dendrochronological investigation of larch tree stems was done by the methodological recommendation of Stravinskienė, 2005. Experimental bore was carried out in March from 2015-2017 with Pressler borer. 1.30 m height was bored from tree root mouth, to the same north-east direction. In total 15 trees were bored. For the

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evaluation measuring system LINTAB was used with computer programme WinTSAP 0.30 (F. Rinn Engineering Office and Distribution, Heidelberg, Germany), the accuracy of the measurement was 0.001 mm. The quality and synchronity of dating between lines of radial growth was measured by COFECHA 3.00 programme. Non synchronic examples remained undetermined.

Meteorological data

The average monthly and annual data of climate factors (air temperature and precipitation) was supplied by the Lithuanian Hydrometeorological Service (LHS) under the Ministry of Environment, Department of Klaipėda. Distance between LHS and Klaipėda University Botanical Garden is approximately 3 km. Data were presented from 2005 to 2016. In this period in January –June precipitation content was from 30 to 50 mm per month in average, but in July – December 100 mm and more per month (*Fig. 1*). During the period of 2005-2016 negative air temperatures usually are fixed in January and February (from -1.5 to -1.8 °C respectively), the highest annual air temperature in this period was in June – September months (from 14.6 to 18.9 °C) (*Fig. 2*).

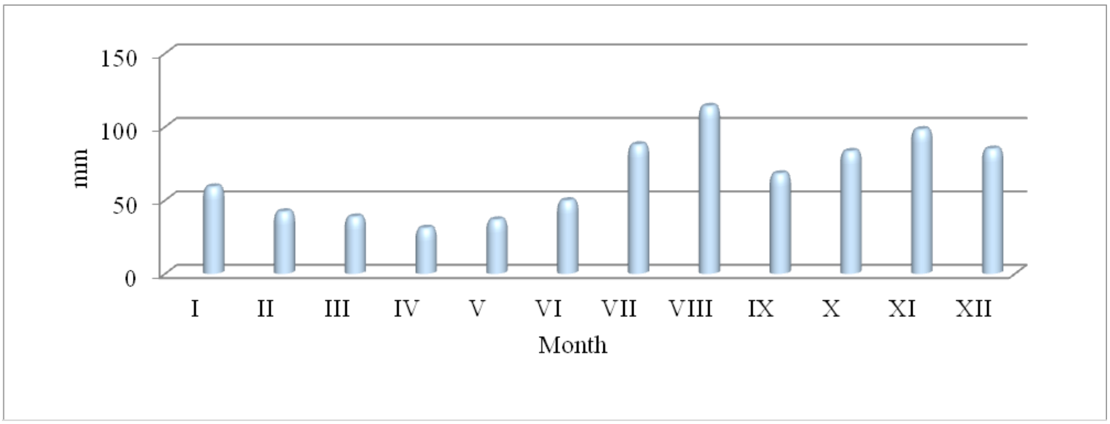

Figure 1. *Distribution of precipitation (mm), average in 2005-2016. Data of Klaipėda meteorological station*

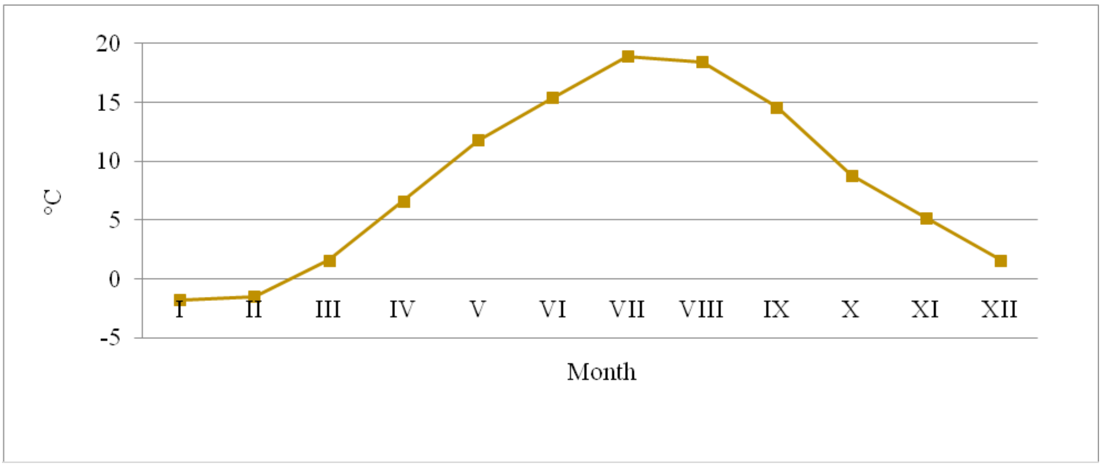

Figure 2. *Average air temperature (°C) in 2005-2016. (Data of Klaipėda meteorological station)*

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Statistical analysis

The experimental data were processed using statistical package SPSS version 17.0. Interrelationship between radial growth (width of tree – ring) and precipitation and air temperature were estimated. Correlation (r) and regression (R) was considered significant when $P < 0.05$.

Results

Annual air temperature and precipitation and humidity are important factors in plants growing rate and productivity. The values and linear trend of means of annual precipitation and air temperature in four seasons are presented in *Figure 3* and *4*. The total precipitation was the highest in the summer time (3014.5 mm). In other seasons this indicator was 1273.5 mm, 2890 mm and 2145.9 mm in the spring, in the autumn and in the winter, respectively.

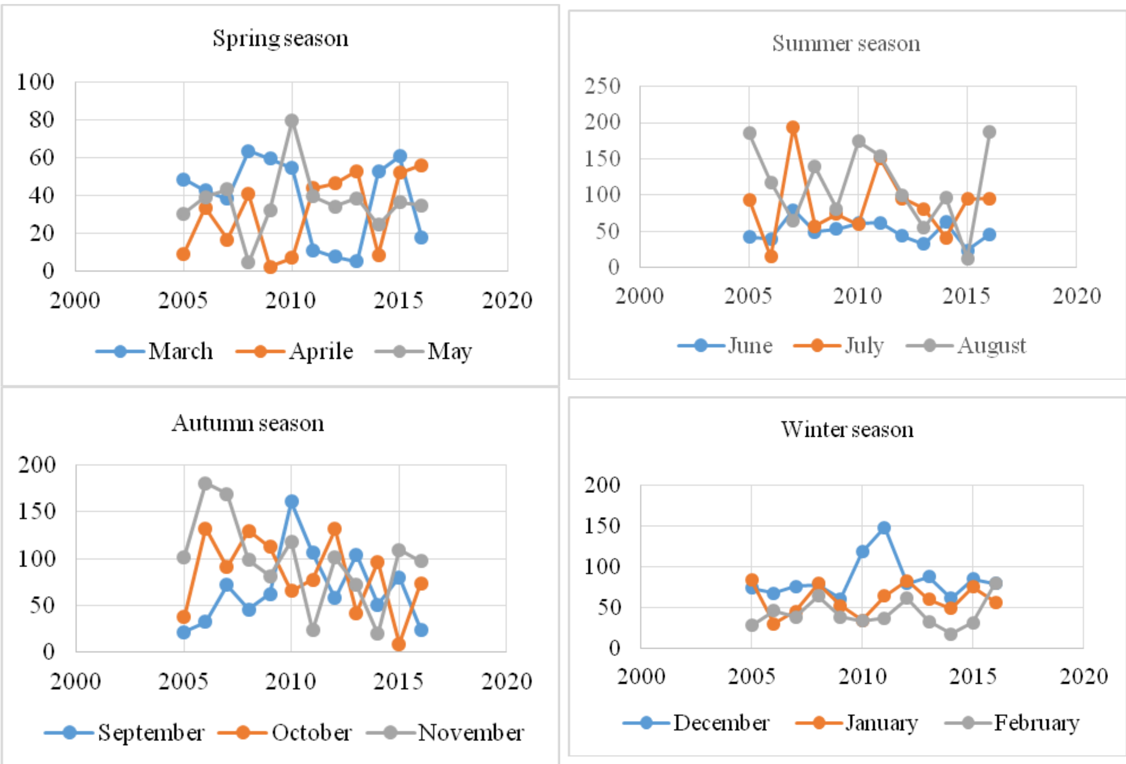

Figure 3. *Distribution of precipitation (mm) in seasons. (Klaipėda, 2005-2016)*

In the period of 2005 – 2016 the highest precipitation in spring was in 2010 and 2015 years (respectively 141.6 mm and 149. 4 mm). The lowest amount of precipitation was in 2014 (86.1 mm). The exclusive summer season was in 2015, when precipitation was only 130.5 mm and in 2011 when it was over 366.5 mm. In the autumn season the highest precipitation was 345.4 mm (2006) and the lowest - 97.6 mm (2016), in the winter season the highest content 248.5 mm (2011) and the lowest 135.9 mm (2016) .

If range of temperatures per season (*Fig. 4*) are compared, the highest temperature in spring was in 2014 (8.1 °C) and the lowest in 2005 (4.9 °C). The summer and autumn

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seasons haven't important fluctuations per period. Temperature in the summer is in average 17.5 °C (from 16.8 °C in 2012 to 18.4 °C in 2010) and in the autumn it was the lowest in 2010 (8 °C) and the highest in 2011 (10.3 °C). Winter season was mutable per period: temperature ranged from - 5.4 (2010) to 2.3 °C (2015), but mostly was about 0 °C, in average it was -0.7 °C per period.

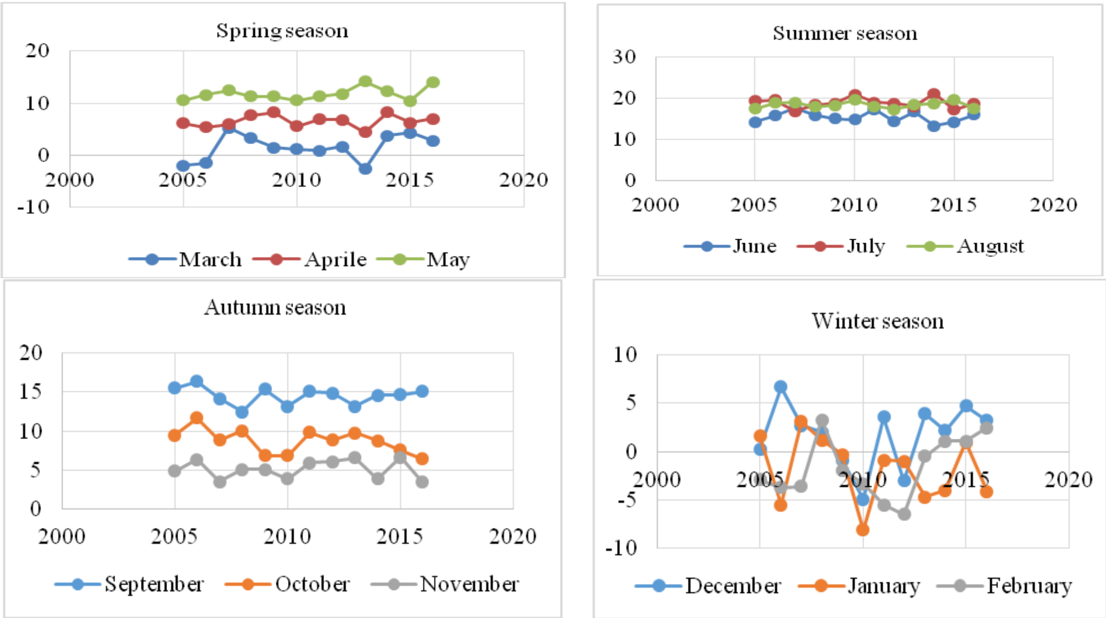

Figure 4. *Distribution of average air temperature (°C) in seasons. (Klaipėda, 2005-2016)*

All larches growing rapidly are lush and vital. *L. sibirica* is the oldest in the Botanical Garden, it is 19 years old and the diameter of its stem is 40 cm. *L. decidua* and *L. x gmelinii* are 14 years old and their average stem diameter is 30 cm. The meteorological conditions (precipitation and air temperature) individually influenced the radial growth rate of different Larix species in separate years (*Table 1*).

For observing plants per period was investigate, unequal growth of stem rings (*Fig. 5*). Per period the highest radial growth of *L. sibirica* was in 2006 and 2012 years – 11.5 mm, but the lowest in 2014 only 4.5 mm. Very similar tendencies can be observed in L. *decidua*: the highest radial growth (11.5 mm) was in 2006 year and the lowest (3.7 mm) in 2013. *L. kaempferi* growth rate in 2014 was 10.00 mm (its highest increase) and the lowest – 5 mm determine in 2013, 2015 and 2016 as well. In *L. x gmelinii and L. x eurolepis* the highest value of this indicator was 9.3 mm in 2008 and 7.3 mm in 2011, but the lowest in 2016 to both species, 4.3 mm and 3.0 mm, respectively. Statisticaly significant and negative correlation (*Table 2*) between radial growth and precipitation was found mostly in the spring season related to *L. sibirica, L. kaempferi, L. x gmelinii* and *L. x eurolepis,* but in the summer and winter seasons it can be related to *L. decidua.* Positive, but statistically unreliable correlation between mentioned parameters in all species of larch was determined only in the autumn season. Statistically significant and negative correlation between radial growth and air temperature in *L. sibirica* was established in the spring, summer and winter seasons, in *L. decidua* only in the spring season, in *L. kaempferi* in the autumn and winter seasons, in hybrids of *L. x gmelinii* and *L. x eurolepis* in the spring and autumn seasons. Very similar and statistically significant dependences

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are also shown regression coefficients (*Table 3*). To be noted, that statistically more significant relationships (r and R) in the investigated larches were determined between radial growth and temperature than between radial growth and precipitation. Thus, a tendency that annual air temperature makes more influence on the width of tree rings than annual precipitation can be observed. However, according to research data, in autumn season on the radial growth of *L. sibirica* precipitation positive affected by 62% (y = 0.0245x + 2.6459; R^2 = 0.6216), of *L. x eurolepis* – by 53% (y = 0.0148x + 1.7703; R^2 = 0.5256) and of *L. x gmelinii* – by 39% (y = 0.0119x + 3.6817; R^2 = 0.3925).

Table 1. *Characteristics of meteorological conditions influencing the radial growth rate of different Larix species. (Klaipėda, 2005-2016)*

***Larix* species**	**Years with fixed possitive radial growth rate (higher than average)**		**Years with fixed negative radial growth rate (lower than average)**	
	Years	**Characteristic of meteorological conditions**	**Years**	**Characteristic of meteorological conditions**
L. sibirica	2006-2008, 2010-2012	The warm and wet autumn, warm beginning winter season	2005, 2009, 2013, 2014-2016	Air temperature and precipitation satisfy with the multi-annual averages, the excess humidity in August
L. decidua	2006, 2009, 2014, 2015	Humidity November, positive average temperature in March, warm December	2007, 2008, 2010-2013, 2016	Warm and humidity autumn, deficiency precipitation in summer season
L. kaempferi	2007-2011, 2014	The warm and wet autumn, warm beginning winter season	2012, 2013, 2015, 2016	Dry autumn season
Larix x gmelinii	2006-2008, 2011	The warm and wet autumn, warm beginning winter season	2009, 2010, 2012 – 2016	High air temperature and dry summer season
Larix x eurolepis	2010-2013	Humidity spring and autumn season	2014 – 2016	High air temperature and dry summer season

Table 2. *Correlation between tree-ring width and precipitation and air temperature*

***Larix* name**	**Precipitation in seasons, mm**				**Temperature in seasons, °C**			
	Spring	**Summer**	**Autumn**	**Winter**	**Spring**	**Summer**	**Autumn**	**Winter**
L. sibirica	-0,02**	0.27	0.78	0.35	-0.23**	-0.46**	0.13	-0.38**
L. decidua	0.07	-0.32**	0.33	-0.42**	-0.20**	0.10	0.50	0.06
L. kaempferi	-0.42**	0.43	0.37	0.02*	0.27	0.47	-0.45**	0.13**
L. x gmelinii	-0.05**	0.09	0.64	0.41	-0.12**	0.14	-0.20*	0.37
L. x eurolepis	-0.01**	0.44	0.72	0.79	-0.76**	0.44	-0.29**	-0.58

Note: *P < 0.05; **P < 0.001

Table 3. *Regression between tree-ring width and precipitation and air temperature*

Larix name	Precipitation in seasons, mm				Temperature in seasons, °C			
	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter
L. sibirica	-0.13**	9.14	27.05	5.81	-0.11**	0.05	0.05	-0.36**
L. decidua	0.72	-12.40**	13.21	-8.36**	-0.11**	0.02*	0.25	0.07
L. kaempferi	-4.7**	17.67	14.38	0.49	0.13	0.12	-0.19**	-0.16*
L.x gmelinii	-0.79**	4.99	35.63	11.65	-0.09**	0.04*	-0.13**	0.58
L.x eurolepis	-0.21**	23.29	35.40	20.84	-0.48**	23.29	-0.16**	-0.88**

Note: *P < 0.05; **P < 0.001

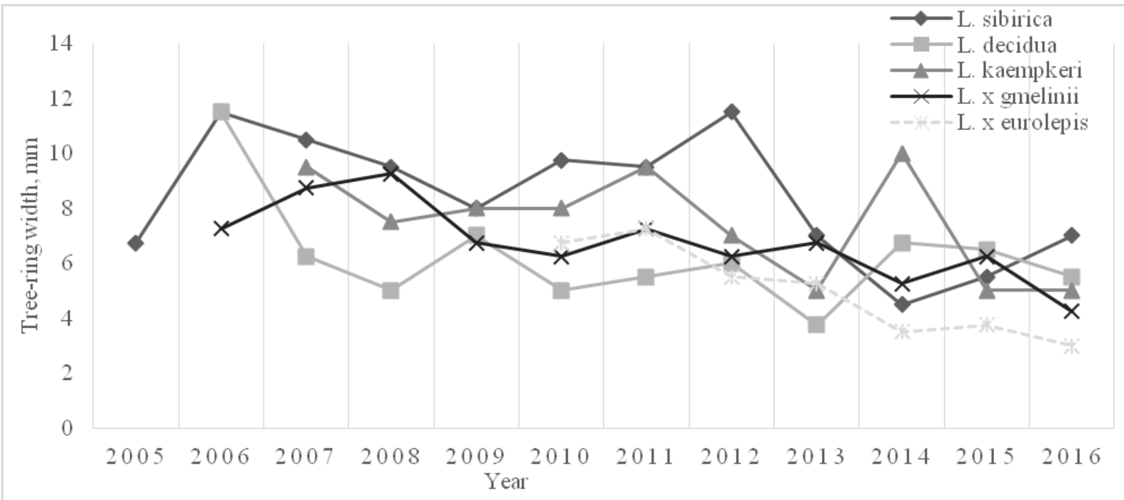

Figure 5. *Average width of tree-ring of different larix species. (Klaipėda, 2005-2016)*

Discussion

Larch cannot grow in the shade. It prefers moist soil. The plant can tolerate maritime exposure, they cannot tolerate atmospheric pollution (http://www.pfaf.org). In the Klaipėda University Botanical Garden are favourable conditions for larches to grow and cultivate. In the optimal conditions the climatic factors correlation with annual rings of trees radial gains are negligible, however in worse growing conditions a higher increasing effect on the formation of annual radial rings is noticed (Läänelaid, 1996). In the research period from 2005 – 2016 in the Botanical Garden of Klaipėda University the radial growth ranged from 3 to 11.5 mm in average (*Fig. 5*). The highest precipitation (*Fig. 3*) was in 2007 (925 mm), 2010 (966 mm) and 2011 (917.5 mm) and the lowest in 2014 (581.6 mm), the temperature (*Fig. 4*) had its highest value in 2008 and 2015, 9.1 and 9.0 °C, respectively and the lowest in 2010, only 6.7 °C. For larches in Lithuanian conditions active vegetation begins in the middle of April and ends in the middle of October, in this period air temperature usually ranges from 12.0 to 15.8 °C, in average perennial air temperature was 12,8 °C. Winter and spring seasons air temperature for Larix radial growth is very important too (Karpavičius and Kaselytė, 2000). In Klaipėda, costal area it is noticed, that in spring and winter seasons air temperature and precipitation are unequal, but in summer season they are smooth. Average temperature in summer season is 17.5 °C (in the spring, autumn and winter seasons were 6.7°C; 9.7°C and -0.7 °C, respectively). By the evaluation of data of larches in

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the spring season statistically significant correlation between tree-ring width and precipitation (*Table 2*) be found for all Larix except for *L. decidua.* The correlation and regression between tree-ring and precipitation in autumn season was statistically insignificant for all Larix (*Table 2, 3*). If compared in the winter season, the statistically significant correlation between tree-ring width and precipitation were only true for *L. decidua* (P<0.001) and *L. kaempferi* (P<0.05)*,* but tree-ring width and air temperature only for *L. sibirica* (P<0.001) and *L. kaempferi* (P<0.001). In the spring and winter seasons precipitation was 1273.5 mm and 2145.9 mm, respectively.

The annual hybrid larix radial growth in Estonia was 3.5 mm, the highest value was 13 mm. The high rings width of young age of larix show that it is hybrid and had good environmental conditions (Sander and Läänelaid, 2007). In Poland, the annual radial growth showed more dependence from the amount of precipitation in summer season (Oleksyn and Fritts, 1991). Evaluating all larches in the Botanical Garden, only *L. decidua* in the summer season showed significant negative correlation (*Table 2*). In the summer season height regression between tree-ring width and precipitation for all larix too, but statistically significant value was only for *L. decidua* (P<0.001). The influence of precipitation on the radial growth of larch across Lithuania is usually positive but more variable than the relationships with air temperature (Karpavičius and Kaselytė, 2000).

Temperature requirements of European larch regarding the onset of individual phenological stages were evaluated based on the sums of air temperatures above 0 °C and of effective air temperatures higher than +5 °C (Bednarova et al., 2013). Efficient growth and good adaptation of European larch and *Larix deciduas* subsp. *Polonica* in Lithuania can be associated with the northern habitat border. The increased air moisture, relatively warm winters and cool summers are factors that European larch can much better endure. In addition, European larch grows very rapidly during the growing season, it takes up much more water than other conifers and larch is one of the most sun-loving tree species (Karpavičius, 2004). In Lithuanian climate conditions the L. *decidue* radial growth is mostly influenced by the warm and dry summer season (Karpavičius, 2004). Sensitivity of growth rate of *L. decidua* was 0.35 ± 0.01 (Karpavičius, 2004). European larch grows very rapidly during the growing season, it takes up much more water than other conifers and larch is one of the most sun-loving tree species. Average radial growth of L. *decidua* pursue 2.18 ± 0.04 mm. The period of 1850 – 2007 was a distinguished time when 70 – 90% from all evaluating trees average radial growth was bigger compared with multi – annual average. It is observed that more wood gained in years with warm spring and warm winter (Stravinskienė and Erlickytė, 2003; Vitas and Žeimavičius, 2006; Vitas, 2011). In west Lithuania due the research period the radial growth fluctuate from 6.25 ± 0.02 mm of *L. decidua*, 8.46 ± 0.02 mm – L. *sibirica*, 7.45 ± 0.02 mm – *L. kaempferi,* 6.75 ± 0.01 mm – *L. x gmelinii* and 5 ± 0.01 mm – L. *x eurolepis.* The Botanical Garden is good environmental condition for Larix to grow and in west Lithuania climate parameters as temperature and precipitation are favorable too.

Conclusions

1. According to research data in the west Lithuania (costal area) the highest precipitation per evaluating period was in the summer time 3014.5 mm, the lowest in the spring, 1273.5 mm. The mutable temperature per period was in the winter season from -5.4 to 2.3 °C. The air conditions for growing of *Larix* genus is good but for some species is a too big amount of precipitation.

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2. After the evaluation of 5 taxa of larches, it was established, that on the radial growth the climate parameters (precipitation and air temperature) in separate seasons influenced the larches individually. Hybrid larches *L. x gmelinii* and *L. x eurolepis* average radial growth was 1.3 – 1.5 times lower compared with *L. sibirica* and *L. decidua*. It means, that lower radial width are for pure species rather than for hybrids.
3. Positive, but statistically unreliable correlation between radial growth and precipitation in all species of larch was only in the autumn season. Usually autumn precipitation can more influencing on the growing quality in next year.
4. Noted, that more statistically significant relationships (r and R) in the investigated larches was determined between radial growth and temperature than between radial growth and precipitation. Thus, there is a tendency that annual air temperature makes more influence on the width of tree – rings than annual precipitation.

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MAPPING QTLS FOR YIELD AND YIELD COMPONENTS UNDER DROUGHT STRESS IN BREAD WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract. The aim of this study was to discover the geneic basis of drought tolerance, a double haploid mapping population, Drought Mapping Population 5 (DR. M.P. 5) was assessed for drought tolerance in hexaploid wheat (*Triticum aestivum* L.) under control and drought stress condition. The germplasm was planted under control and stress conditions. The yield and yield components were recorded. QTLs were detected by linking morphological data with genotypic data. Five drought tolerant wheat lines were found based on a thousand-grain weight, ranging from 49 to 62 g. Novel Quantitative Trait Loci (QTLs) for spike length and grain per spike were identified during the present study. The novel QTLs are of great importance as these may be helpful in finding such regions in genome, which are responsible for drought tolerance contributing characteristics. The D genome is the main allele which denote drought tolerance to hexapoloid wheat while A and B genome in durum contribute the stress tolerant characteristics.
Keywords: *SSR, DNA polymorphism, interval mapping, multiple QTL mapping, clustering of QTL*

Introduction

Abiotic stress, such as drought not only interferes with the growth of plants (Tuberosa and Salvi, 2006) but also triggers several changes even at cellular level (Holmberg and Bulow, 1998). Further, it reduces nodes and internodes number; which reduces the height of plant (Ahmed et al., 2007). Drought prevails in Asian countries, and its severity can be observed in the arid and semi-arid areas, creating massive losses to their agricultural economics (Huaqi et al., 2002). Drought stress affects the rate of transpiration and ultimately plants may start wilting. Particularly in wheat, the developmental stages are also affected by drought (Wahid et al., 2007). Usually plants grow normally under favorable conditions, but if plants are exposed to any kind of stress, then it is natural that they complete their life cycle rapidly and escape the stress (Fischer, 1985; Hossain et al., 2009, 2011, 2012a, b, c, Hakim et al., 2012; Nahar et al., 2010). Further in moisture-affected areas, late sowing results in yield reduction of wheat and as a result, early sowing

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is the common practice in drought prone areas such as Pakistan, India and Bangladesh to avoid drought and heat stress (Mahboob et al., 2005; Din and Singh, 2005).

Common wheat (*Triticum aestivum* L.) is originated from three different genomes. Each genome has three groups of chromosomes: A, B and D. The genome size of the hexplaoid is 16×10^9 bp per chromosome (Bennett and Smith, 1976). As wheat is a major staple food and its intake is growing day by day, there will be approximately a double quantity of wheat grain required by the next fifty years (Rajaram, 2001; Mujeeb-Kazi and Rajaram, 2002). To meet the increasing demand, genetic resources can play a vital role both locally as well as globally, ultimately supporting agricultural economies worldwide (Rajaram, 2001). Significant work was accomplished in the past by synthesizing the bread wheat by crossing durum and *Ae. tauschii* (2n = 14) and then duplicating its chromosome Mujeeb-Kazi (2000). By crossing the *T. turgidum* (2n = 28) with a variety of *Ae. tauschii,* stress tolerance was ultimately enhanced in synthetic wheat. *Ae. tauschii* is the D genome donor and enhances drought tolerance in bread wheat (Del Blanco et al., 2000, 2001; Schmidt et al., 2005). Hexaploid wheat is the most essential cereal crop in the world and the chief staple food of Pakistan. It is paramount to increase the production of wheat by 70% globally to meet the nutritional demands by 2050 (Ray et al., 2013). As wheat is the staple of Pakistan, it occupies the central position in the agricultural policies of the country. Wheat contains 55% carbohydrate, which equals to 20% food calories. Pakistan is ranked ninth in wheat production. The bread wheat production was noted in 2010 to 2012 as 23.3, 25 and 23.5 million tons respectively (GOP, 2012). According to the Pakistan Bureau of Statistics, the world wheat production in 2016-17 was 749 million tons. Pakistan's statistical data of wheat reveal that the amount of cultivated land is 9.05 m ha, the total production is 25 million tons and the yield is 2.78 tons/ha (GOP, 2017).

DNA polymorphisms play a crucial role in unveiling Simple Sequence Repeats (SSRs) derived from Triticeae. The genomic relationship among various traits in wheat was identified by using SSR (Dreisigacker et al., 2004; Sun et al., 1998). For QTL analysis, a linkage map is required and by linking the genotypic and phenotypic data, QTLs can be find out (McCouch and Doerge, 1995). SSR markers could be used to measure agronomic traits of concern, which minimize the cost and time of quantitative trait loci analyses (Young, 1996; Ijaz and Khan, 2009). The current research was planned for QTL mapping in drought mapping population 5 (DR MP 5) by linking the genotypic and phenotypic data.

Material and methods

Plant material

The mapping population consists of two parents that is Opata and SH349 and eighty-four individuals of doubled haploids. The pedigree is Opata // Decoy (DOY) / *Ae. tauschii* (*Ae. squarrosa*) {458}. *Ae. tauschii* was assigned 458 accession number. Opata (bread wheat) is a high yielding cultivar and is drought sensitive while SH349 is synthetic hexaploid wheat and has drought tolerance properties.

Soil type and agriculture practices

These eighty-four individuals with parents were planted at the Wheat Wide Crosses Program in the National Agriculture Research Center (NARC) in Islamabad, Pakistan. Two treatments were control (fully irrigated) and a drought stress treatment was in a tunnel. To protect it from precipitation, plot for stress treatment was sheltered with plastic

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sheets sustained on iron rods of the tunnel. Water was suspended at flowering in the middle of March. The site of experiment originates from the upland part of potohar, confined in Gujranwala type soil series (Location 6; Rashid et al., 1994). This soil is comprised of well-drained, deep and temperately fine-textured units. It is non-saline, somewhat calcareous; its pH is 8.1 with 0.24 dS/m electrical conductivity (EC). In order to provide ample nutrition, standard agronomical practices were carried out.

Healthy seeds were selected for sowing. Seeds were surface sterilized by using 1% mercuric chloride for 5-7 minutes and, then washed carefully with distilled water. Petri plates were used for seeds germination in the dark for two days on wet filter paper while monitoring temperature. The healthy seedlings were shifted to jiffies containing humus for auxiliary growth. The seedlings of equal height (six days old) were transferred to the field and tunnel. Drought stress was enforced at pre-anthesis period, where water was withheld for 70-90 days. For control treatment, regular mode of irrigation was maintained. Soil humidity was checked by using the Time-Domain Reflectometer (TDR), a soil moisture meter. The control showed a 25% soil moisture, which dropped to 13% under the drought-stressed conditions.

Experimental design

RCBD (Randomized Complete Block Design) was applied in tunnel as well as in field for control and drought treatment observations. Each row was two meters long. A distance of 30 cm was maintained for inter row spacing. After sowing, the yield and yield components were recorded during the experiment such as Plant height (measured in cm) (PH), Days to heading (DH), (measured in numbers), Days to physiological maturity (DPM), (measured in numbers), Spike length (measured in cm) (SL), Grain per spike (G/S) and Thousand Grain weight (measured in gm) (TGW). Statistical analysis was performed through the software Statistica.

Extraction, polymerization and electrophoresis of DNA

Fatima et al. (2014) published the map under consideration; it was of a double haploid mapping population (DR. M.P.5). According to that published data, the DNA of parental lines (Opata and SH349) and the whole mapping population were extracted (Sharp et al., 1988). Fresh leaves were used for this purpose. The extracted DNA was stored at -20 °C. The florimeter was used for the quantification of DNA. (The Qubit fluorometer is a small instrument used for quantification of DNA, RNA, and protein and used in many different applications.) The Qubit fluorometer uses fluorescent dyes to determine the concentration of nucleic acids and proteins in a sample. The robotic station was used for the normalization of DNA at 20 ng/µl. The robotic station is a fully automatic system, which is used to dilute the DNA samples with more accuracy and speed.

The SSR markers are used for the genotyping of parental lines and for the whole mapping population. The SSR markers were selected based on the literature cited. The SSR were selected in such a way that they cover the entire genome of the wheat. 174 SSRs were used during the present study. By using PCR, the parental line genomes were amplified and after this, parental peaks were identified by using capillary sequencer. The selected polymorphic markers were used to detect the polymorphism in the entire mapping population. Seventy-nine polymorphic SSRs genomic loci were applied overall mapping population to detect the QTLs. The following markers were used, WMC (Gupta et al., 2002); GWM (Roder et al., 1998); MGBE and TaPGAM (Xue et al., 2008), STS-

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PSR (Xue et al., 2008), SWES (Peng and Lapitan, 2005), BARC (Song et al., 2005), MAG (Xue et al., 2008), CFD (Guyomarc'h et al., 2002) and CFA (Sourdille et al., 2003). The mixture, which was used for PCR, consisted of DNA, dNTPs, buffer, forward and reverse primer, DMSO, water and DNA taq polymerase. Roder et al. (1998) prescribed conditions were followed for PCR reaction. Alleles having difference of at least two base pairs with reference to amplified product were selected for polymorphic marker. The difference up to one base pair is difficult to predict so it is ignored during analysis (Jones et al., 1997). The Capillary electrophoresis is an analytical technique that separates ions based on their electrophoretic mobility with the use of an applied voltage, is a better instrument to find out polymorphism than the gel electrophoresis. Highest quality data can be obtained at low cast per sample. Fluorescent primers such as FAM, HEX, NED and TET were used to see the peaks and peaks were recorded at 500 nm absorbance maximum. The GeneMapper 4 was used to separate the electropherograms. To save time and money, primers were run simultaneously, which have different colors of electropherograms.

The JoinMap 4 software (Van Ooijen and Voorips, 2004) was used to construct the map with a minimum LOD value 4 by using the Kosambi mapping function. This map was developed by taking Opata and SH349 as having contrasting characteristics of interest. This map was constructed by using 79 PCR based polymorphic genomic loci. Chi-square test was used for all loci for verification of goodness of fit to an estimated value 1:1. Sixteen linkage groups were obtained. Previous published wheat maps were used to associate the linkage groups to the chromosomes of wheat (Somers et al., 2004).

Statistical analysis and QTL detection

Analysis of variance, frequency distribution and Pearson correlation were determined by using Statistix. Genotypes, which have α0.5 values, were differentially different from each other and significant interaction was found between the treatments and the genotypes. The JoinMap4 and the MapQTL 5 were used to analyze the molecular diagnostics (Van Ooijen, 2004). The generated data were entered on an Excel sheet as As (parent A type) and Bs (parent B type). To construct the linkage map, JoinMap4 was used and data was analyzed (Van Ooijen and Voorips, 2004). The computer program MapQTL 5 was used to link the morphho-physiological and genotypic data to find out QTLs. Corel draw 4 and Map chart were used to draw the QTLs on chromosomes. Permutation test was used to check the LOD value. Threshold levels were determined by using $P < 0.05$ for the comparison of 1000 data permutations which is suitable for assessing critical thresholds at $\alpha = 0.10$, and $\alpha = 0.05$ (Churchill and Doerge, 1994). First, Interval Mapping (IM) was used to find the major QTLs. After finding the major QTLs, Multiple QTL Mapping (MQM) was applied to find more precise results.

Results

Phenotypic analysis of drought tolerance

The phenotypic data for yield and yield components was recorded (*Fig. 1*). Opata, SH349 and the DHs displayed high phenotypic variation for these traits. Opata and SH349 performed distinctly in all observations. Throughout the study, certain characters reduced under water stress e.g. the PH reduced by 15.26%, the SL reduced by 15%, the number of G/S reduced by 62.34% and the TGW reduced by 35.80% respectively.

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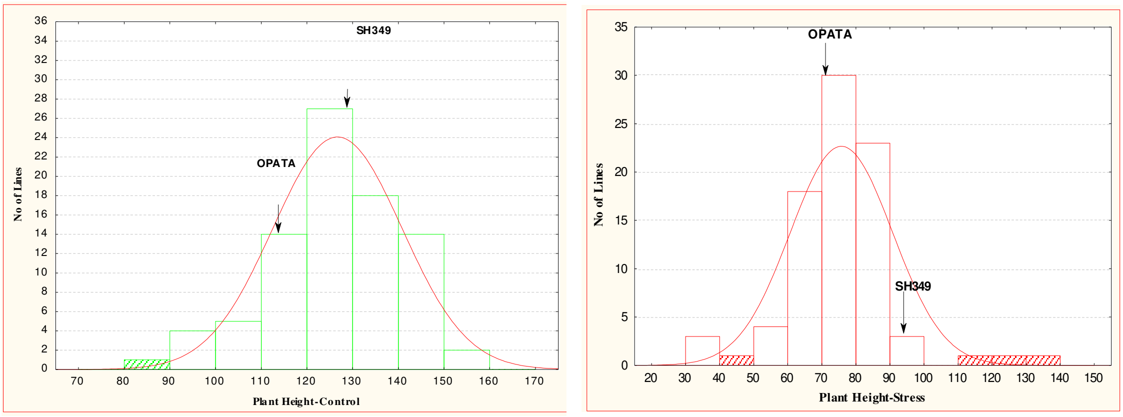

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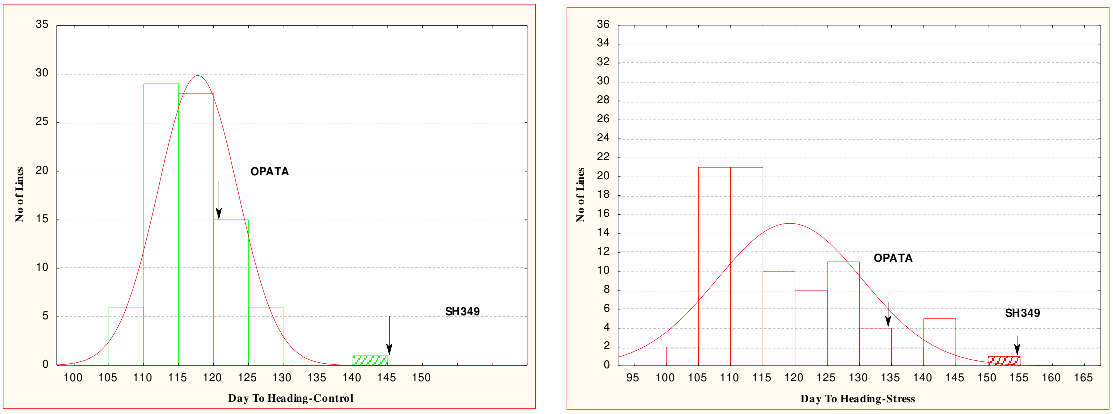

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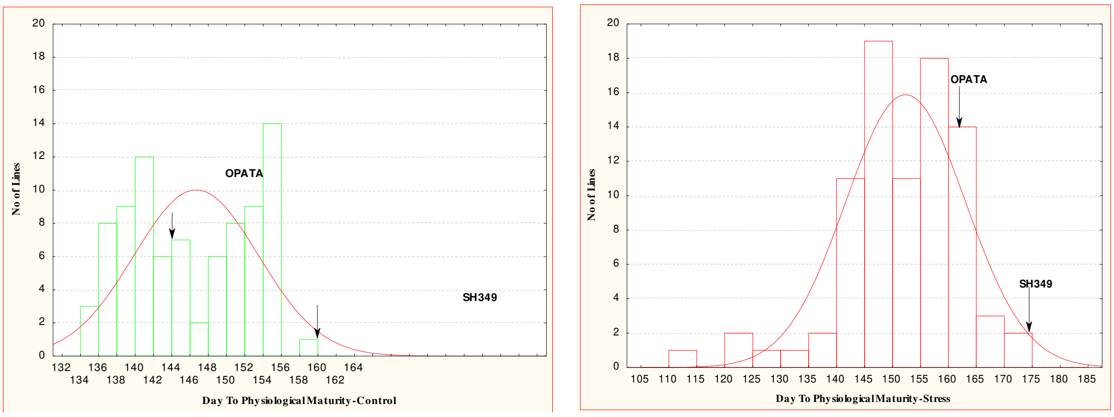

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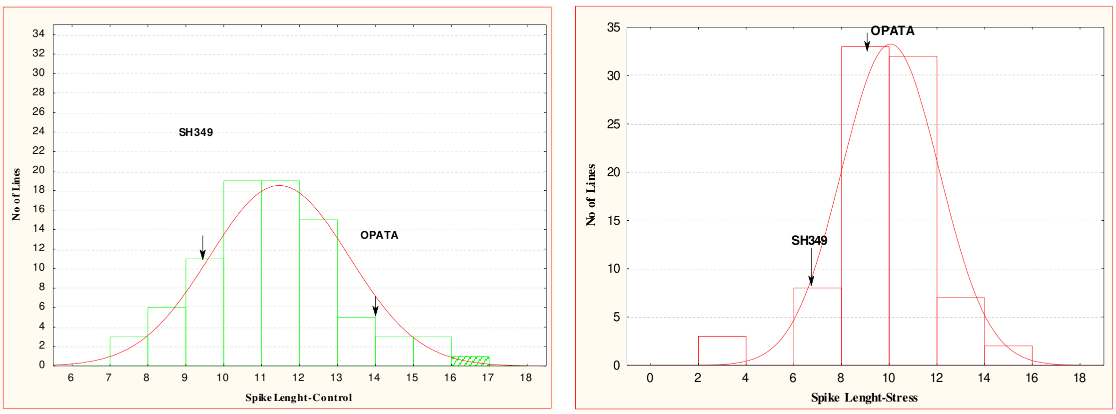

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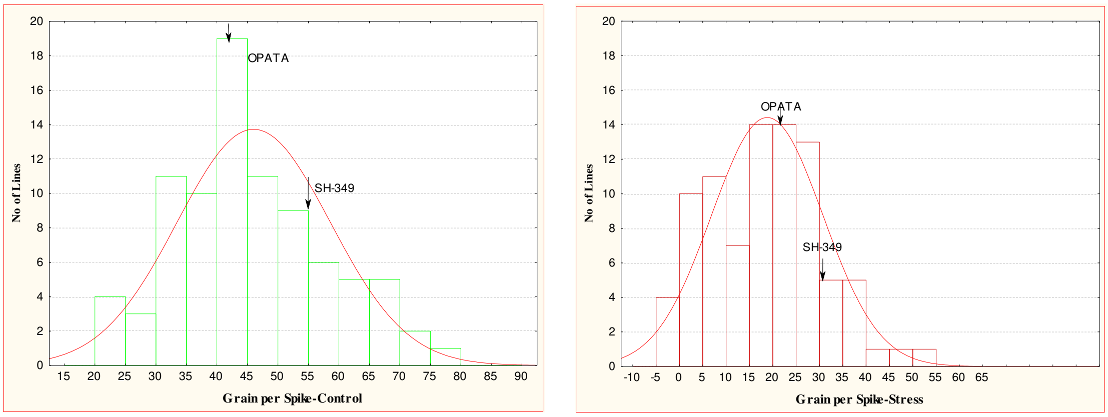

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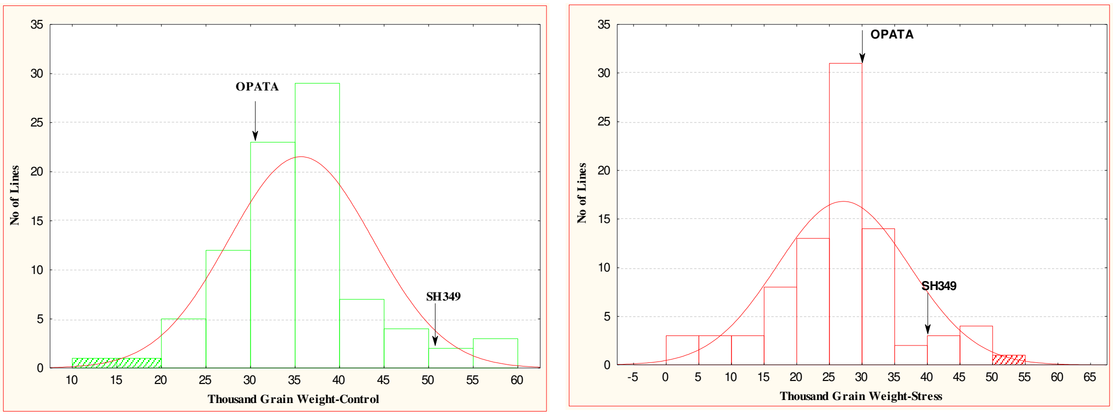

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Figure 1. *(a) Histogram of phenological attributes of plant height (cm) in field and in tunnel (PH-C and PH-S). (b) Histogram of phenological attributes of days to heading (n) in field and in tunnel (DH-C and DH-S). (c) Histogram of phenological attributes of days to physiological maturity (n) in field and in tunnel (DPM-C and DPM-S). (d) Histogram of phenological attributes of spike length (cm) in field and in tunnel (Sp.L-C and Sp.L-S). (e) Histogram of phenological attributes of grain per spike (n) in field and in tunnel (G/S-C and G/S-S). (f) Histogram of phenological attributes of thousand grain weight (gm) in field and in tunnel (TGW-C and TGW-S)*

Correlations

Significant correlation was found between (DH) and (DPM) with r-value = 0.7613 (*Table 1*). Further significant correlation was found between (DH) and (DPM) with r = 0.8210 value under water stress condition that indicates delay in heading that leads to delay in days to physiological maturity. The PH is a genetically controlled character and varies with environmental conditions and genotype. Under water stress condition PH has significant and positive correlation with DH and DPM with values r = 0.2276 and 0.3766. The SL has positive and significant correlation with DPM with a value r = 0.2570. Values r = 0.2644, 0.4340 and 0.5608 were found for spike length, DH and DPM under drought stress. Values for correlation, r = 0.4688 and 0.2149 were recorded for G/S, PH and SL respectively. Values r = 0.2389, 0.4330, 0.3791 and 0.5354 were found for DPM, PH, SL and G/S.

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Table 1. *Correlation of yield and yield components under control and stress conditions*

Trait	Treatment	DH	DPM	PH	SL	G/S	TGW
DH	Control	1.0000					
		p= ---					
	Stress	1.0000					
		p= ---					
DPM	Control	.7613	1.0000				
		p=.000	p= ---				
	Stress	.8210	1.0000				
		p=0.00	p= ---				
PH	Control	-.2319	-.1351	1.0000			
		p=.032	p=.215	p= ---			
	Stress	.2276	.3766	1.0000			
		p=.035	p=.000	p= ---			
Sp.L	Control	.1910	.2570	.1990	1.0000		
		p=.078	p=.017	p=.066	p= ---		
	Stress	.2644	.4340	.5608	1.0000		
		p=.014	p=.000	p=.000	p= ---		
G/S	Control	.0071	-.0739	-.0544	.0274	1.0000	
		p=.948	p=.499	p=.619	p=.802	p= ---	
	Stress	-.0932	.0836	.4688	.2149	1.0000	
		p=.394	p=.444	p=.000	p=.047	p= ---	
TGW	Control	-.1272	-.1388	.2954	.1962	.1684	1.0000
		p=.243	p=.203	p=.006	p=.070	p=.121	p= ---
	Stress	-.0349	.2389	.4330	.3791	.5354	1.0000
		p=.750	p=.027	p=.000	p=.000	p=.000	p= ---

DH (Days to Heading), DPM (Days to Physiological Maturity), PH (cm) (Plant Height), Sp.L.(cm) (Spike length), G/S (n) (Grain per Spike), TGW (gm) (Thousand Grain Weight)

QTLs detected by interval mapping (IM)

Interval mapping was used to detect QTL and data are shown in *Table 2* and *Figure 2*. Four QTLs for DH were detected in the field experiments with LOD values 7.69, 3.89, 1.91 and 2.00 respectively on the 6A chromosome. A QTL for DPM was detected by interval mapping in field experiments, located on 1B having a LOD score 3.06 with very high phenotypic variation i.e. 80.4% and allele contributed by SH349 for this QTL. LOD values 2.31 and 2.43 were found for two QTLs located on the 6A and the 7B chromosomes for G/S. A QTL was detected by interval mapping having a LOD value 2.14 under drought stress condition on 5A chromosome. Under control conditions, a QTL having LOD value 2.02 was detected on 6A chromosome for SL.

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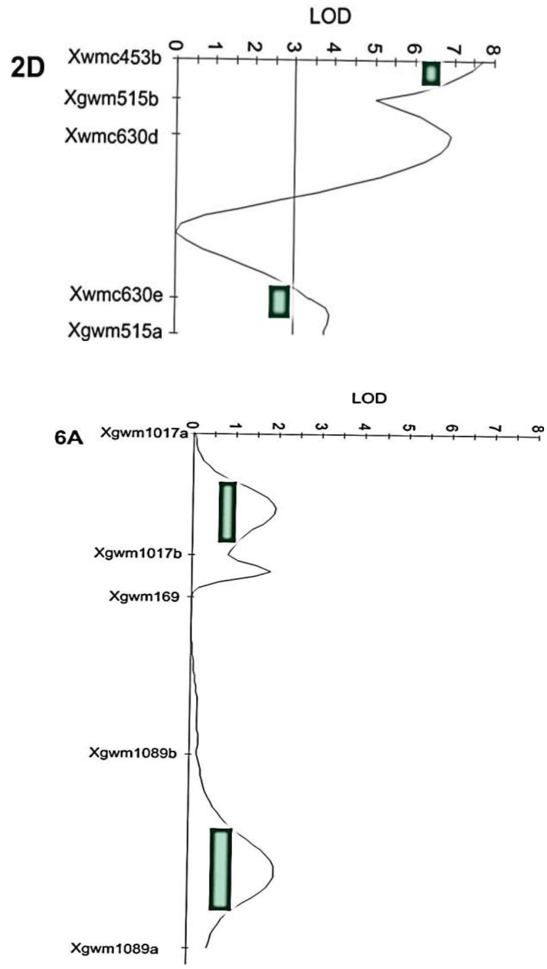
Table 2. *QTLs detected by interval mapping of a DH population Opata × SH349. QTL were identified by interval mapping and significance was recognized at an LOD threshold following 1,000 permutations (in brackets) (different threshold for each trait). Size of the effect and phenotypic variation explained (R^2) are also presented*

Sr #	Name of QTL	QTL interval[a]	Peak marker	Chr[b]	Trait	Env	LOD	Adtv eft[c]	R^2 (%)[d]
1	*QDH.C.IM.wwc-2D.1*	wmc453b-gwm515b	wmc453b	2D	DH	Field	7.69 (3.4)	2.88	34.8
2	*QDH.C.IM.wwc-2D.2*	wmc630e-gwm515a	wmc630e	2D	DH	Field	3.89	-2.4	21.4
3	*QDH.C.IM.wwc-6A.3*	gwm1017a-gwm1017b	gwm1017a	6A	DH	Field	1.91	2.99	37.1
4	*QDH.C.IM.wwc-6A.4*	gwm1089b-gwm1089a	gwm1089b	6A	DH	Field	2.00	-3.41	44.6
5	*QDPM.C.IM.wwc-1B.1*	gwm153e-wmc611c	gwm153e	1B	DPM	Field	3.06 (3.56)	-5.98	80.4
6	*QG/S.C.IM.wwc-6A.1*	gwm1017a-gwm169	gwm1017b	6A	G/S	Field	2.31 (2.89)	-4.81	13.9
7	*QG/S.C.IM.wwc-7B.2*	gwm146c-gwm146b	gwm146a	7B	G/S	Field	2.43	-4.55	13.1
8	*QTGW.S.IM.wwc-5A*	wmc630c-wmc630b	wmc630c	5A	TGW	Tunnel	2.14 (2.2)	7.97	59.5
9	*QSp.L.C.IM.wwc-6A.1*	gwm1017a-gwm1017b	gwm1017a	6A	Sp-L	Field	2.02 (2.56)	-0.68	14

a: Marker interval where the QTL has been detected. b: Chr Chromosome. c: Effects on the examined characters of the alleles from the 'Opata'. d: R2 (%) is the quantity of phenotypic variation clarified by the QTL



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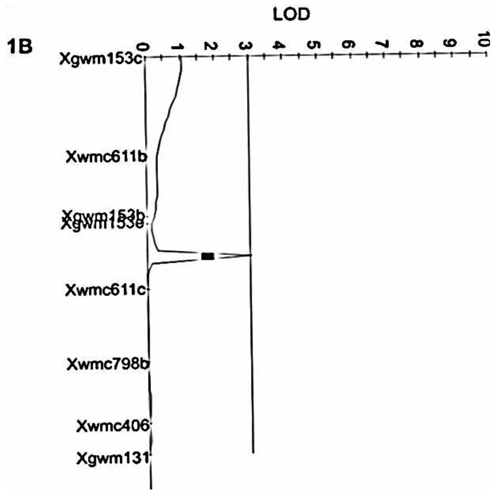

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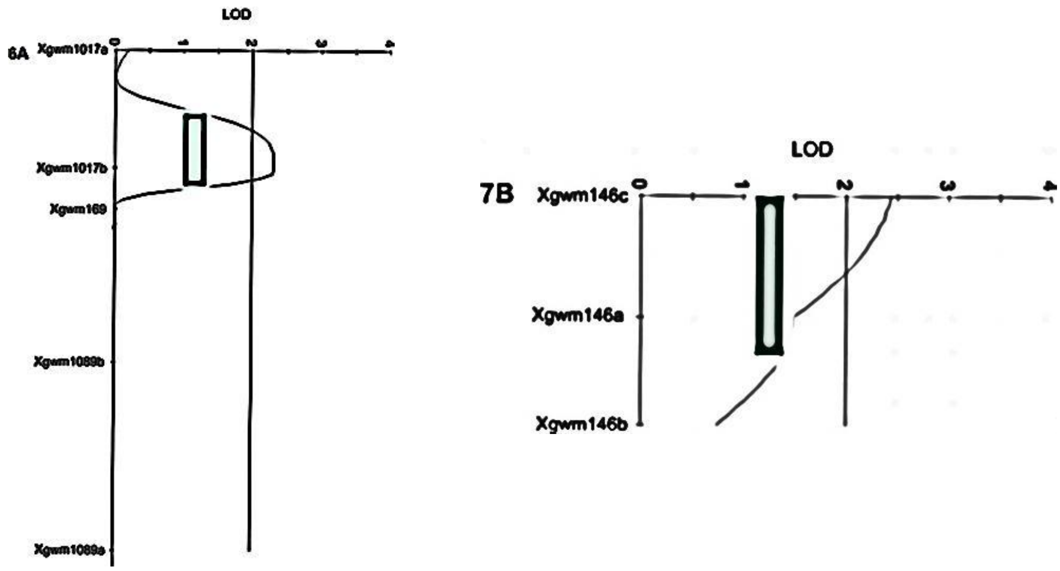

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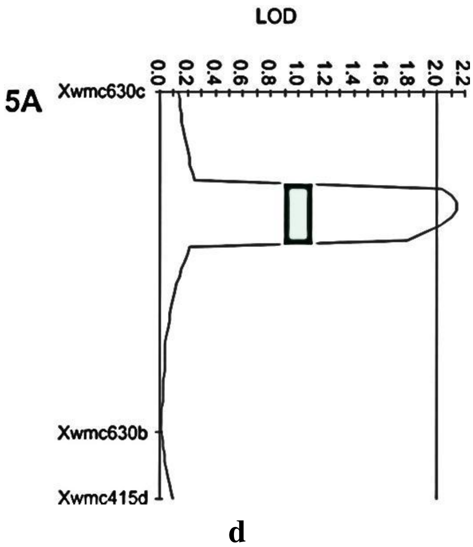

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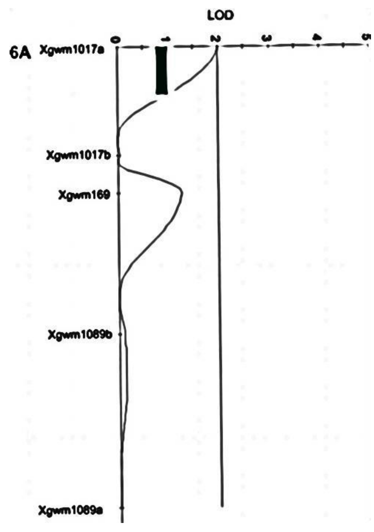

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Figure 2. *Interval Mapping (IM) for the 84 DHs population. (a) Days to heading-field. (b) QTLs for days to physiological maturity-field. (c) Grain per spike-field. (d) Thousand grain weight-stress. (e) Spike length-field*

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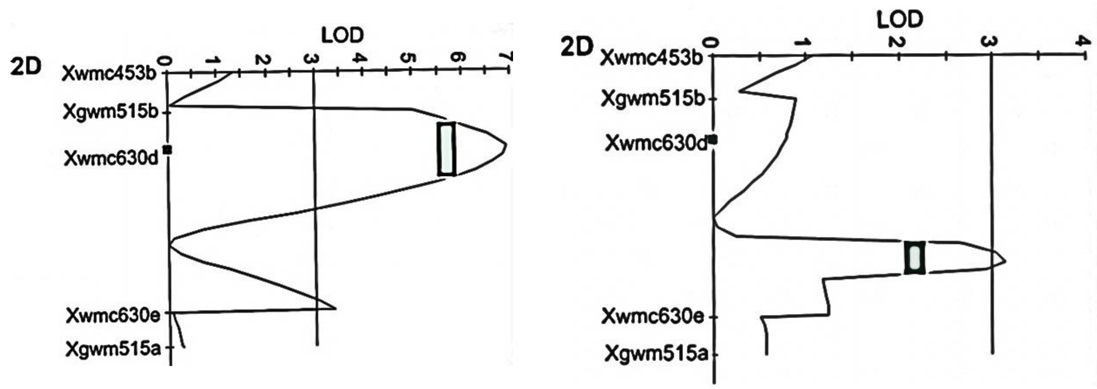
QTLs detected by multiple QTL mapping (MQM)

Data detected by Multiple QTL Mapping is shown in *Table 5* and *Figure 3*. Two QTLs for days to heading were detected by MQM in field (control) and tunnel (drought stress) trials, on a 2D chromosome, with LOD values 6.93 and 3.14. A major QTL for DPM was found by MQM mapping under control conditions, on 2D chromosome with LOD value 9.39. For SL, a major QTL was found on 4A chromosome under control conditions. For SL, a second QTL was detected on 7A chromosome under water stress with 4.93 LOD value. A LOD value 2.15 was found for TGW under water stress condition. Two QTLs with LOD values 3.98 and 2.41 were found under control and stress conditions on 6A and 1B chromosomes.



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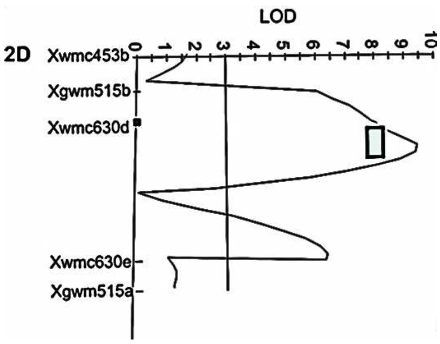

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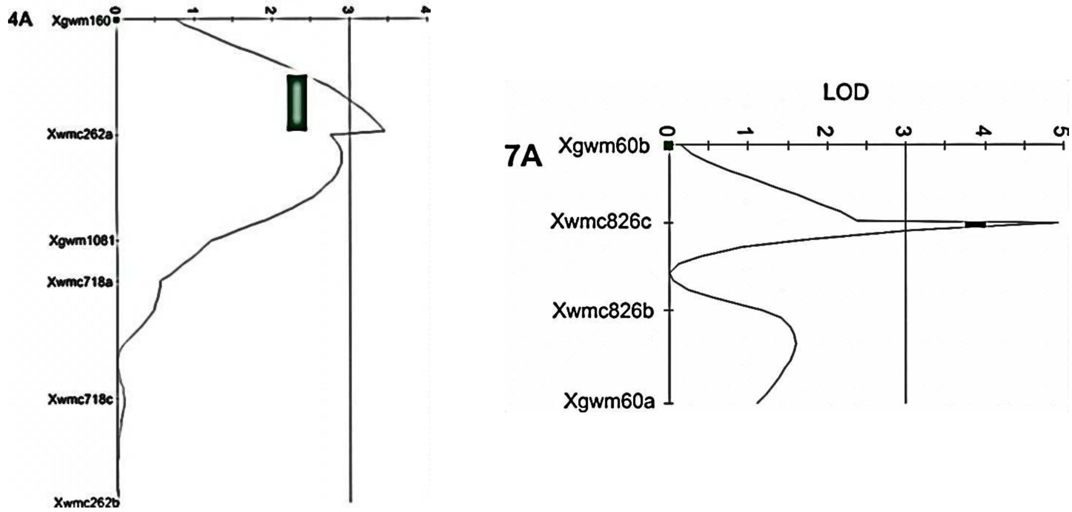

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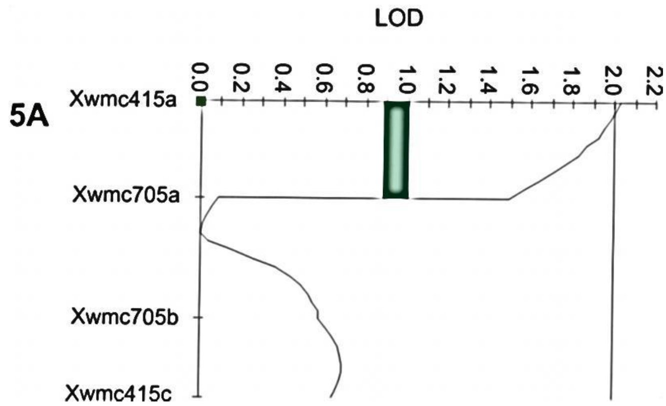

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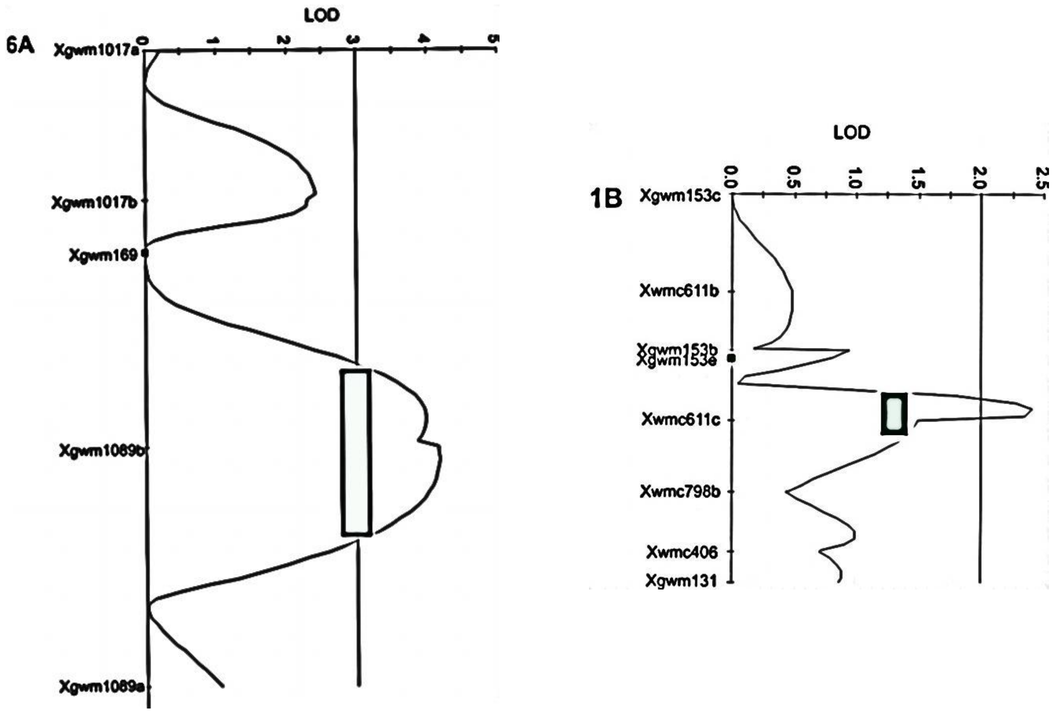

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Figure 3. *Multiple QTL Mapping (MQM) for the 84 DHs population. (a) Days to heading-field-tunnel. (b) Days to physiological maturity-field. (c) Spike length -field and tunnel. (d) Thousand grain weight-tunnel. (e) Grain per spike-field and tunnel*

Clustering of QTLs for interval mapping

During current study, the different QTLs were found (*Fig. 4*). Two clusters of QTLs were detected on 6A chromosome, which covered almost all part of it. First group contained QTLs for (DH) under field conditions, (DPM) under field conditions, Spike length under field conditions, covered the short arm of chromosome. The second group contained QTLs for (DH) under field conditions and (DPM) under field conditions, which covered the long arm of chromosome. On 2D chromosome, two groups of QTLs were found. First group of QTLs consisted of (DH) under field conditions and DPM under field conditions. The second group consisted of four QTLs for DPM under field conditions and DH under field conditions.

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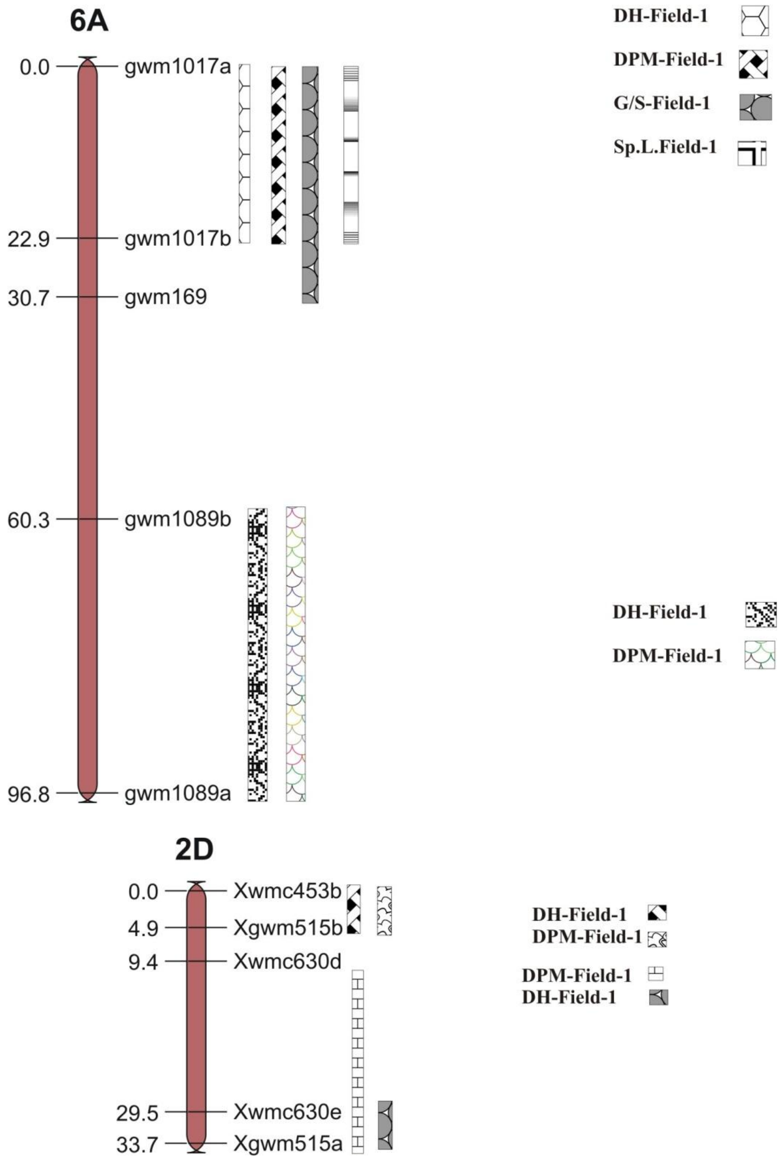

Figure 4. *Clustering of QTLs for the 84 DHs population (IM - interval mapping)*

Clustering of QTLs for MQM mapping

A group of QTLs was detected on 2D chromosome for different traits. This cluster consisted of QTLs for DH under control and stress conditions and DPM under control conditions (*Fig. 5*).

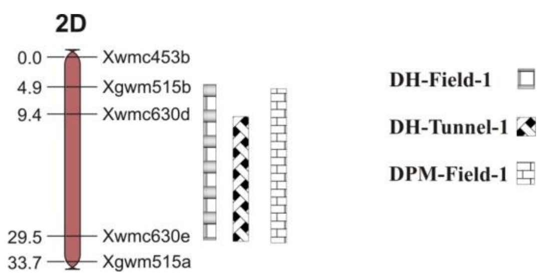

Figure 5. *Clustering of QTLs for the 84 DHs population (MQM- Multiple QTL Mapping)*

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Discussion

These are molecular advances, which made it possible to detect chromosomal regions containing genes for quantitative traits. Bread wheat (*Triticum aestivum* L.) is the staple food of our country and most part of the country is drought prone area so the yield of wheat is badly affected by environmental stress. According to International Water Management Institute (IWMI), wheat production of South Asia will reduce by 50% by 2050 (de Fraiture et al., 2007). The main objective of the study was to trace such lines which are drought tolerant and can produce improved yield in drought prone areas of the country. Hexaploid wheat has complex genome with three types of genome A, B and D. At the time of wheat evoluation three types of plants contribute A, B and D genomes. At that time, *Ae tauschii* is D genome donar and diversity of this plant was very less as compared to two genome contributing plants (Dubcovsky and Dvorak, 2007) limiting its ability for its own evolutionary progress. Few genes of *Ae tauschii* were found for the development of new wheat cultivars, which forced the breeders to collect the accession of *Ae. tauschii* from different parts of the world and used these accessions to produce genetic diversity in wheat genome (Sohail et al., 2011). *Ae. tauschii* is the specie which contributes characteristics of biotic and abiotic to wheat genome. *Ae. tauschii* accessions have D-genome diversity. CIMMYT (International Center for Maize and Wheat improvement) incorporated this diversity of *Ae. tauschii* in elite durum lines and resynthesized the wheat by crossing the *Ae. tauschii* and durum lines (Trethowan and Mujeeb-Kazi, 2008). Synthetic wheat lines are stress tolerant so these lines have ability to overcome stress and better yield under drought stress conditions. During field and tunnel experiments, drought-mapping populations presented extensive segregation as compared to the SH 349 and Opata for most traits under consideration. Recombination events were evidenced by improved offspring as compared to their parents (Zhuang et al., 1997). The mapping population displayed a usual dissemination for drought tolerance of maximum yield components with a great extent of transgressive segregation (*Fig. 1*). All traits showed Normal distribution during the experiment. Transgressive segregation was displayed by all traits in both directions during the analysis of frequency distribution. Polygenic inheritance was observed as the individual lines showed lower and higher values as compare to the parental lines (Kearsey and Pooni, 1996). Transgressive segregation and continuous variation are the two evident attributes of polygenic inheritance (Poehlman and Sleper, 1995). From *Figure 1,* it is cleared that DH and DPM are polygenic inheritance traits and showed normal distribution with transgressive segregation. It is necessary to detect QTLs that the parents should be of contrasting characters under consideration so parents were selected on contrasting characters of interest. For determination of QTLs, it is necessary that major variation exist between the descendant lines.

Table 3 shows the exact parental positions and ranges. Two-factor factorial analysis of variance was used to identify the interaction between the genotypes and treatments. Significant interactions occurred among all genotypes and treatments (*Table 4*). The Pearson coefficient was used to find out significant correlation between traits of interest. They showed different increasing or decreasing values according to control and stress conditions (*Table 4*). The major interaction may be because of environment and genotype interaction, which results in obvious alterations. Significant interactions were observed in genotypes and treatments at α 0.05. Supporting results were found during the El-Feki (2010) experiment, who found out significant ($P \leq 0.05$) correlations in four environmental conditions over two years between grain yield and plant height and

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average kernel weight. A research also carried out by Narasimhamoorthy et al. (2006) who found a significant ($P \leq 0.05$) correlation between plant height, average kernel weight and test weight under two environmental conditions. Same results were also found during the experiment of Butler et al. (2005) for correlations of these traits under two environmental conditions. During the recent study, few yield components showed significant correlation with each other and rest of traits showed non-significant results.

Table 3. *Basic statistics for each yield component trait, (field and tunnel experiment) from parents and DHs between individual for control and drought treatments*

Trait	Mean	Minimum	Maximum	Range	Variance	Std. Dev.	Coef. Var.	Skew	Kurtosis	Opata	SH-349
DH-C	117.66	108.00	145.00	37.00	32.25	5.68	4.83	1.3164	4.4003	121	145
DH-S	119.04	104.00	155.00	51.00	126.58	11.25	9.45	-3.5053	4.878	134	155
DPM-C	146.60	135.00	160.00	26.00	46.91	7	5.01	0.0311	-1.3952	145	161
DPM-S	152.11	113.00	175.00	63.00	116.95	11.67	7.02	-5.4634	3.154	162	174
PH-C	126.37	81.00	156.30	75.30	198.36	14.08	11.15	-0.5067	0.5626	114.5	129
PH-S	75.46	36.75	131.00	94.25	223.71	14.96	19.82	-0.5453	3.1250	71.25	95
Sp.L-C	11.44	7.30	16.20	8.90	3.35	1.83	16.01	0.2281	0.0363	14	9.5
Sp.L-S	10.00	2.50	15.25	12.75	4.16	2.04	20.39	-1.4520	3.6083	9	6.75
G/S-C	45.78	21.00	79.30	58.30	157.45	12.55	27.41	0.3713	-0.1349	55	43
G/S-S	19.56	1.20	55.00	53.80	130.44	11.42	58.39	0.3707	-0.1032	31	22
TGW-C	34.13	1.50	51.00	49.50	69.62	8.34	24.45	-1.0098	2.0317	35	51
TGW-S	23.47	0.58	36.77	36.19	75.55	8.69	37.04	-1.0047	0.2014	25	35

DH (n) (Days to Heading), DPM (n) (Days to Physiological Maturity), PH (cm) (Plant Height), Sp. L.(cm) (Spike length), G/S (n) (Grain per Spike), TGW (gm) (Thousand Grain Weight), C (Control) and S (Stress)

Table 4. *Two factor factorial analysis of variance for each yield component trait (field and tunnel experiment) from parents and DHs between individual for control and drought treatments*

Trait	Source	DF	SS	MS	P
DH	LINE	85	38492.20	452.85	0.00
	TREAT*LINE	85	37344.60	439.35	0.00
	TREAT	1	1466.86	1466.86	0.00
DPM	LINE	85	57022.4	670.852	0.00
	TREAT*LINE	85	51678.1	607.978	0.00
	TREAT	1	1466.86	1466.86	0.00
PH	LINE	85	86334.80	1015.70	0.00
	TREAT*LINE	85	37127.60	436.80	0.00
	TREAT	1	95472.00	95472.00	0.00
G/S	LINE	85	42410.40	498.95	0.00
	TREAT*LINE	85	33496.60	394.08	0.00
	TREAT	1	300.23	300.23	0.00
Sp. L	LINE	85	1552.49	18.27	0.00
	TREAT*LINE	85	653.95	7.69	0.00
	TREAT	1	15484.50	15484.50	0.00
TGW	LINE	85	21346.40	251.13	0.00
	TREAT*LINE	85	17406.80	204.79	0.00

DH (n) (Days to Heading), DPM (n) (Days to Physiological Maturity), PH (cm) (Plant Height), Sp. L. (cm) (Spike length), G/S (n) (Grain per Spike), TGW (gm) (Thousand Grain Weight), C (Control) and S (Stress)

For the growth and development of plants, climatic factors play a very important role. Under stress conditions, morphological, physiological and biochemical changes occurred in plants, which ultimately affect growth and yield of plants. QTLs detected by Interval Mapping are mentioned in *Table 5*. Days to heading, is a very crucial stage in wheat growth and development as it is a complex trait and is controlled by many genes at one time. There is variation in day to heading time, which enables plants to grow under diverse environmental conditions. Four QTLs that affected days to heading were detected under field experiment with LOD values 7.69, 3.89, 1.91 and 2.00 on 2D and 6A chromosome respectively. Alleles for two QTLs contributed by Opata and alleles for two QTLs contributed by SH349. First two QTLs were very important, having values 7.69 and 3.89, which were major QTLs. Narasimhamoorthy et al. (2006) reported a QTL for DH on 2D chromosome, while recent findings were also supported by the study and QTLs for DH were reported on 2D and 6A chromosome and results for 6A were supported by Huang et al. (2003).

Table 5. *QTLs detected by multiple QTL mapping of a DH population Opata × SH349. QTL were identified by interval mapping and significance was recognized at an LOD threshold following* 1,000 *permutations (in brackets) (different threshold for each trait). Size of the effect and phenotypic variation explained (R^2) are also presented*

Sr #	Name of QTL	QTL interval[a]	Peak marker	Cr[b]	Trait	Env	LOD	Adtv eft[c]	$R^{2\,d}$ (%)
1	*QDH.C.MQ.wwc-2D*	gwm515b-wmc630e	wmc630d	2D	DH	Field	6.93 (3.4)	2.91	35.7
2	*QDH.S.MQ.wwc-2D*	wmc630d-wmc630e	wmc630d	2D	DH	Tunnel	3.14	-9.7	72.1
3	*QDPM.C.MQ.wwc-2D*	gwm515b-wmc630e	wmc630d	2D	DPM	Field	9.39 (3.56)	5.94	79.4
4	*QSp.L.C.MQ.wwc-4A*	gwm160-gwm1081	wmc262	4A	Sp-L	Field	3.45 (2.56)	0.76	17.7
5	*QSp.L.S.MQ.wwc-7A*	gwm60b-wmc826b	wmc826c	7A	Sp-L	Tunnel	4.93	-1.86	31.9
6	*QTGW.S.MQ.wwc-5A*	wmc415a-wmc705a	wmc415a	5A	TGW	Tunnel	2.03 (2.2)	-2.94	10.8
7	*QG/S.C.MQ.wwc-6A*	gwm169-gwm1089a	gwm1089b	6A	G/S	Field	3.98 (2.89)	-6.75	28.8
8	*QG/S.S.MQ.wwc-1B*	gwm153e-wmc611c	gwm153e	1B	G/S	Tunnel	2.41	-5.99	27.2

a: Marker interval where the QTL has been detected. b: Chr Chromosome. c: Effects on the examined characters of the alleles from the 'Opata'. d: R2 (%) is the quantity of phenotypic variation clarified by the QTL

The duration of maturity of any crop is decreased by drought stress and differs with genotype, because of their inherent nature. A QTL on chromosomes 1B affected days to physiological maturity having LOD value 3.06, which was a major QTL, and the allele for this QTL was contributed by SH349 and 80.4% R^2 values. Results found during the previous study by Peleg et al. (2009) were in agreement with the present study as QTL for DPM was reported on 1B chromosome. Two QTLs for G/S were found by interval mapping under control conditions having values 2.31 and 2.43 on 6A and 7B chromosomes respectively. A QTL was found on 6A chromosome by Peleg et al. (2011). No supporting reference was found for QTL discovered on 7B, therefore, it is a novel QTL found during our research. The allele for this QTL was contributed by SH349 with 13.1 R^2 value. Under drought conditions, a minor QTL was found on 5A

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chromosome with a LOD value 2.03 for thousand-grain weight by interval mapping. Peleg et al. (2011) and Dashti et al. (2007) found same results for thousand-grain weight on 5A chromosome under stress conditions. A QTL was found on 6A chromosome under control conditions having a LOD value 2.02. No supporting reference was found for QTL found for Spike length so it is also a novel QTL.

Multiple QTL Mapping (MQM) was used to get more refine results for QTLs obtained by Interval mapping. Two QTLs were found on 2D chromosome under control and stress conditions for days to heading. These two QTLs were more reliable and consistent as these were found under control and stress conditions and peak marker and chromosome were same. A very little work has been done on yield and yield components of wheat, as wheat genome is very complex and mainly under water stress conditions (Quarrie et al., 2005). They reported QTLs for days to heading on 2D chromosome. A major QTL for days to physiological maturity was detected on 2D chromosome with LOD 9.39 value during the recent study by MQM mapping. Same results were found during the previous study by Huang et al. (2006). Wheat spike grows from the axils of main shoot leaves. The number and length of spike differs from genotype to genotype and mainly depends on ecological situations. For spike length, a major QTL was found on 4A chromosome under control conditions with 3.45 LOD value. A second QTL for spike length was found under stress conditions on 7A chromosome with a LOD value 4.93. Previous results of Chu et al. (2008) and Jantasuriyarat et al. (2004) were in agreement with the recent study.

The main aim of the present study is to improve the existing high yielding drought sensitive wheat cultivars. During the last twenty years, QTL analysis largely used to detect QTLs associated with complex traits, such as yield and yield components under drought stress (Ain et al., 2015). These traits are polygenic traits and are controlled by many genes at a time so it is very difficult to clone such QTL of the traits under drought stress. Only a few QTLs have been utilized in plant molecular breeding for wheat and none QTL is clone yet (Fleury et al., 2010). As discussed above, the major QTLs found during the study can be used for molecular breeding. Such lines can be grown under water stress conditions. Two major QTLs for DH were found by interval mapping under control conditions having LOD values 7.69 and 3.89 respectively. These QTLs are of great importance as these were located on 2D chromosome as it is difficult to find polymorphism for D genome so we can use these QTLs in marker-assisted selection. Additive effect showed that the allele for the second QTL for DH was contributed by SH349 (drought tolerant parent) with 21.4% phenotypic variation. Again, drought tolerant parent (SH349) contributed alleles for third major QTL with 3.06 LOD value and 80.4 % phenotypic variation. QTLs found by the MQM are more reliable and precise as these were obtained after the interval mapping. Eight QTLs were found during the present study and out of which six were major QTLs. Highest LOD value 9.39 was noted for DPM with 79.4 % phenotypic variation under control conditions on 2D chromosome. After that, second major QTL was reported on 2D chromosome for DH with 6.93 LOD value with 35.7% phenotypic variation. Third major QTL was found on 7A chromosome with 4.93 LOD value and allele for this QTL was contributed by SH349. 7A chromosome is considered as important chromosome for yield and yield component (Quarrie et al., 2006). Fourth QTL was found on 6A chromosome with 3.98 LOD value for G/S and again allele for this QTL was contributed by SH349. Same results were found during previous study by Peleg et al. (2011) who reported QTL for G/S on 6A chromosome. Last two major QTLs were found on 4A and 2D chromosomes

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with 3.45 and 3.14 LOD values respectively. These QTLs are very important as we can exploit in future for molecular breeding especially the QTLs, which were located on 2D chromosome with high LOD value. A minor QTL for thousand-grain weight was found during the study on 5A chromosome with 2.15 LOD values. QTL for TGW on 5A chromosome was reported during the previous study by Wang et al. (2009) and Peleg et al. (2011). Another minor QTL for G/S was found on 1B chromosome with 2.41 LOD value and additive effect -5.99, means alleles contributed by SH349. In previous study, QTL was reported on 1B chromosome by Dashti et al. (2007). Five drought tolerant wheat lines were identified based on a thousand-grain weight, ranging from 49 to 62 g (*Table 6*).

Table 6. *Drought tolerant lines*

Sr no	Line	Thousand Grain Weight (gm)	Grain per spike (n)
1	54	62.0	26
2	56	62.0	45
3	34	59.0	24
4	59	55.6	55
5	18	49.0	26
Parent 1	Opata	22.0	31
Parent 2	SH349	45.0	23

Conclusion

The present study was targeted to dissect the complex quantitative inheritance of yield component of wheat under control and drought stress conditions. Genotyping was executed using SSR markers. Major and minor QTLs were recognized for different yield components. One QTL for thousand-grain weight was found by interval mapping on 5A chromosome while four QTLs were found by multiple QTL mapping under drought stress conditions on 2D, 7A, 5A and 1B chromosome. These identified QTLs are of primary importance for high resolution mapping in synthetic hexaploid wheat. These identified genomic loci were considered auxiliary as they were saturated with molecular markers for accurate localization of QTLs leading to the inheritance of these polygenic traits. Further, genetic and transcriptome characterization of this mapping population could be supportive for the identification of genomic regions closely associated with drought stress resistance. Introgression of these resistant regions into adapted genetic backgrounds through marker-assisted selection is a promising tool for plant evolution, gene cloning and transgenic crop improvement.

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APPENDIX

Chi-square test outcome

Sr	SSR	A/B	Chi-square ($p < 0.05$)
1	wmc415a-5A-5B	1.80	0.02
2	wmc415b-5A-5B	0.87	0.02
3	wmc415c-5A-5B	0.95	0.02
4	wmc415d-5A-5B	1.21	0.02
5	wmc415e-5A-5B	0.84	0.02
6	wmc705a-5A	1.19	0.02
7	wmc705b-5A	0.86	0.02
8	wmc611a-1A-1B-7B	1.29	0.03
9	wmc611b-1A-1B-7B	1.22	0.02
10	wmc611c-1A-1B-7B	1.58	0.03
11	wmc606a-7B-7D	0.88	0.02
12	wmc606b-7B-7D	1.13	0.02
13	wmc606c-7B-7D	0.84	0.02
14	wmc606d-7B-7D	1.38	0.02
15	wmc235a-5B	1.45	0.02
16	wmc235b-5B	0.57	0.03

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17	gwm1040-6A	6.64	0.02
18	gwm160-4A	0.95	0.02
19	gwm210a-2A-2B-2D-1B	0.95	0.02
20	gwm210b-2A-2B-2D-1B	4.25	0.02
21	gwm210c-2A-2B-2D-1B	1.15	0.02
22	gwm1089a-6A	3.37	0.02
23	gwm1089b-6A	1.24	0.02
24	wmc453a-2A-2B-2D	1.48	0.02
25	wmc453b-2A-2B-2D	1.08	0.02
26	wmc453c-2A-2B-2D	2.07	0.02
27	wmc453d-2A-2B-2D	1.31	0.02
28	wmc718a-4A	1.67	0.03
29	wmc718b-4A	0.01	0.02
30	wmc718c-4A	0.93	0.02
31	gwm60a-7A-7B	4.06	0.02
32	gwm60b-7A-7B	1.66	0.03
33	wmc826a-1A-4B-7A	0.16	0.03
34	wmc826b-1A-4B-7A	2.81	0.03
35	wmc826c-1A-4B-7A	1.79	0.02
36	wmc826d-1A-4B-7A	1.11	0.03
37	wmc262a-4A	0.93	0.02
38	wmc262b-4A	0.98	0.02
39	wmc406-1B	0.70	0.03
40	w630a2A7D2D5D5B1A5A	0.60	0.03
41	w630b2A7D2D5D5B1A5A	1.21	0.03
42	w630c2A7D2D5D5B1A5A	6.20	0.03
43	w630d2A7D2D5D5B1A5A	1.06	0.03
44	w630e2A7D2D5D5B1A5A	2.04	0.03
45	w630f2A7D2D5D5B1A5A	0.24	0.03
46	w630g2A7D2D5D5B1A5A	2.04	0.03
47	w630h2A7D2D5D5B1A5A	0.27	0.03
48	gwm126-5A	0.85	0.03
49	wmc798a-1B	2.32	0.03
50	wmc798b-1B	1.12	0.03
51	gwm122a-2A	3.11	0.03
52	gwm122b-2A	1.24	0.03
53	gwm195a-7B	0.51	0.03
54	gwm195b-7B	1.35	0.03
55	gwm148-3B-2B	1.96	0.03
56	gwm108-3B	1.38	0.03
57	wmc398-6A-6B	0.67	0.03
58	gwm698a-7A	1.12	0.04
59	gwm698b-7A	0.69	0.03
60	gwm1017a-6A	1.13	0.03
61	gwm1017b-6A	0.49	0.03

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62	gwm131-3B-6B-1B	0.69	0.02
63	gwm169-6A	0.59	0.03
64	gwm146a-7B	1.06	0.03
65	gwm146b-7B	2.13	0.03
66	gwm146c-7B	0.77	0.03
67	gwm1081-4A	2.71	0.03
68	gwm153a-1B	0.05	0.02
69	gwm153b-1B	1.80	0.02
70	gwm153c-1B	3.94	0.02
71	gwm153d-1B	26.33	0.02
72	gwm153e-1B	1.71	0.02
73	gwm153f-1B	19.50	0.02
74	gwm515a-2A-2D	1.90	0.02
75	gwm515b-2A-2D	0.89	0.02
76	gwm515c-2A-2D	1.71	0.03
77	gwm515d-2A-2D	0.08	0.02
78	gwm285-3B	0.82	0.03
79	gwm495-4B	0.67	0.03

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NEGATIVE EFFECT OF PHOSPHOGYPSUM OVER PHYSIOLOGICAL ACTIVITY OF EARTHWORM *EISENIA FETIDA*

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Abstract. The study aimed to assess the genuine impact of phosphogypsum on the growth, feeding, respiration and regeneration of earthworm *Eisenia fetida*. In laboratory condition the earthworms were cultured under 0% (control), 4%, 8% and 10% concentration of phosphogypsum for 30 days. After completion of every 10 day changes in the above parameters were observed to track the impact of phosphogypsum. With increasing exposure duration and concentration of phosphogypsum lower growth rate, declined feeding habit, maximum respiration rate and deprived regeneration power were noticed. The highest and lowest growths were 1.39 gm at 0% and 0.05 gm at 10%, respectively. Maximum feeding rate was 32.65 with a minimum rate 16.20 g g^{-1} live tissue. Respiration rate was highest at 10% i.e. 0.0578 g^{-1} live worm tissue hr^{-1} kg^{-1} soil, as most of the energy used to respire to sustain in such diverse condition and 0.575 g^{-1} live worm tissue hr^{-1} kg^{-1} soil recorded as lowest in 0%. The rate of regeneration was deeply hampered and there was no viable worms left at 8% and 10% concentration to assess. Regeneration was only observed at 0% and 4%.
Keywords: *growth, feeding, respiration, regeneration, earthworm, concentration*

Introduction

Anthropogenic activity and environmental changes are the major sources which directly affect the integrity of terrestrial ecosystems. Industrial activities, waste disposal and agricultural practices are sources of soil pollution. Soils are complex associations containing living organisms, mineral particles and organic matter. The clay fractions of the minerals and the humus of organic matter are colloids of a very small size with a large surface area to volume ratios, and consequently with a high binding capacity to inorganic and organic molecules. The binding of pollutants to soil colloids reduces their mobility and their bioavailability and modulates their biological effects. Deposition of different contaminants on soil through anthropogenic sources are very much prone to alter the basic composition and this might be hazardous to inhabiting organisms. The macrofaunal groups of soil are the integral part of the soil ecosystem and have a significant role in soil fertility and functioning. Earthworms are the one of the macrofaunal inhabitant of the soil and hold the key role in soil formation, alternation and function.

The casts of earthworms boost soil fertility and help various organisms to inhabit the soil and to complete their life cycle. Macro- and micro nutrients and their cycling process solely depend upon earthworms and it has been shown that macronutrients are abundant around earthworm casts and burrows which supports root growth (Edwards and Bater, 1992). Earthworm casts have been found to contain elevated amounts of NH:, NO;, Mg, K and P relative to the surrounding soil (Lunt and Jacobson, 1944;

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Parle, 1963; Gupta and Sakal, 1967; Syers et al., 1979; Syers and Springett, 1984; Tiwari et al., 1989). Earthworms influence the soil structure by forming macropores, which allow oxygen to enter the soil, whereas micropores between the aggregates give a good water-holding capacity (Lavelle, 1988; Willems et al., 1996). In addition to forming macropores and increasing water infiltration, earthworms have been shown to increase soil the aggregate stability (Li and Ghodrati, 1995) and the water-holding capacity (Stockdill, 1982).

Generally organisms are known to avoid areas polluted with contaminants above tolerable levels (Amorim et al., 2005; Fenoglio et al., 2007). Earthworms serve as indicators of soil status such as the level of pollutants e.g. agrochemicals, heavy metals, toxic substances, industrial effluents and human-induced activities e.g. land-management practices and forest degradation (Radha and Natchimuthu, 2010). Any changes in soil properties have great impact on earthworms and hence they serve as indicator organisms for ecotoxicological studies (OECD, 1984).

The human impact on the environment can be scaled by the measurements of heavy metals in the soil, in plants and in animals because metal pollution adversely affects the density and diversity of biotic communities including humans (Bengtsson et al., 1981; Mountouris et al., 2002). the abundance and distribution of earthworm species (*Pontoscolex corethrurus, Perionex excavatus, Dichogaster modigliani* and *Polypheretima elongate*) (Ching et al., 2006), is primarily based on soil the type, soil moisture, texture and the soil constituent. Usually, the abundance and distribution of earthworms in undisturbed soil are greater than those, that soils that have been cultivated, burnt or undergone various crop practices like the use of fertilizers, fallowing etc. Some researchers have focused on the role of earthworm species in their tolerance and absorption of metals, petroleum hydrocarbons and polynuclear aromatic hydrocarbons (PAHs) (Zachary and Reid, 2008; Owojori et al., 2009).

Generally, the potential hazards of various environmental toxicants to soil invertebrates are assessed by bioassays with the keystone species - earthworm. Earthworms are one of the first receptors affected by soil contamination. They are more susceptible to metal pollution than any other groups of soil invertebrates and toxicity data on earthworms are important in determining “Safe levels” for metals and other contaminants in soil.

Heavy metals have been shown to cause lysosomal membrane instability, changes in gene expression, oxidative stress (Spurgeon et al., 2004a; Berthelot et al., 2008; van Gestel et al., 2009), reduced growth (Spurgeon et al., 1994), slower sexual development (Spurgeon and Hopkin, 1996; Spurgeon et al., 2004b), depleted cocoon production, hatchability (Reinecke et al., 2001; Davies et al., 2003; Spurgeon et al., 2004a) and juvenile viability (Bengtsson et al., 1986; van Gestel et al., 1992), increased mortality (Neuhauser et al., 1985; Spurgeon et al., 1994; Davies et al., 2003) and also they affect the population size, abundance and species diversity of earthworms (Spurgeon et al., 2005).

Earthworms, especially the compost worm *Eisenia fetida*, are model organisms for assessing the effects of various chemicals on terrestrial invertebrates (Spurgeon et al., 2003; Nahmani et al., 2007). *Eisenia fetida* (*andrei*), which is a manure species living in organic matter-rich substratum is recommended by the international guidelines (OECD, 1984; ISO, 1998) as a test species and has been mainly used in genotoxicity studies. It has a short life cycle, hatching from cocoons in 3 to 4 weeks, and reaching maturity in seven to eight weeks. It is a prolific species and its rearing in laboratory is

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simple. It is a representative component of the terrestrial fauna, but is mostly used as a compost worm because of its great potential towards decomposing waste. It is an epigeic species and is sensitivity to soil pollutants compared to other field earthworms.

Leachate is a liquid formed from the percolation of rain water through the disposed wastes. The dissolution of contaminants such as heavy metals in the leachate can pose serious pollution problems. Species richness may be reduced due to metal pollution (Nahmani et al., 2003) and this indirectly alters species interaction (Grzes, 2009). From ecotoxicity point of view, earthworms are likely to be sensitive to tillage, inputs of organic matter and the application of chemicals (Edwards et al., 1995 and Smith et al., 2008). Due to their beneficial role in terrestrial ecosystems, earthworms are often used as an indicator species in ecotoxicological evaluation (Abdul Rida and Bouche, 1995). Previously few attempts have been made to study the effect of industrial discharge on earthworms (Goats and Edwards, 1982; Mishra and Sahu, 1989, 1997; Callahan et al., 1991).

Heavy metal can be defined as any metal with a specific gravity higher than 4.00 and which is toxic and poisonous even at a low concentration (Agbaire and Emoyan, 2012). Heavy metal concentrations in the soil are associated with biological and geochemical activities and are influenced by anthropogenic activities (Agbaire and Emoyan, 2012). Heavy metals are considered serious pollutants because they are toxic and non-degradable (Agbaire and Emoyan, 2012). The accumulation of heavy metals in the soil poses many risks to humans and the ecosystems (Odoh et al., 2011). Most importantly pollution by heavy metals in terrestrial ecosystems is a serious environmental concern due to their non-biodegradability and tendency to accumulate in plants and animal tissues (Otitoloju et al., 2009).

As a by-product phosphogypsum is produced in phosphoric acid plants. Phosphogypsum is generated through a filtration process in phosphoric acid plants where insoluble gypsum is separated from the product i.e. phosphoric acid as efficiently as possible. About 4.5 -5 tons of phosphogypsum as by-product is being generated per ton of phosphoric acid recovered. Phosphogypsum generation in India is about 11 million tons per annum and primarily consists of calcium sulphate dihydrate with small amounts of silica, usually as quartz and un-reacted phosphate rock, radioactive material (like radium, uranium) and heavy metals namely arsenic, cadmium, chromium, mercury, nickel, lead and zinc (HAZWAMS/2012-2013). There was very considerable volume of work and research regarding the positive roles of earthworms in agroecosystems, environmental monitoring and sustainability (Dada et al., 2013) but the concern here is to evaluate the influence of phosphogypsum over various physiological parameters of *Eisenia fetida* under laboratory conditions.

Review of literature

Lee et al. (2009) determined the effect of alkalized phosphogypsum on soil chemical and biological properties. Their study came into the conclusion that by mixing alkalizing phosphogypsum with an alkaline material such as Ca(OH)2 can turn it into a useful material for agricultural utilization. Pure phosphogypsum could damage the soil biological properties.

Mishra et al. (2011) assessed the impact of phosphogypsum amendment on soil physic-chemical properties, microbial load and enzyme activities. They carried out

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their experiment with 0, 5, 10 and 15% of Phosphogypsum to track the impact and found that the optimal results were recorded in 10%. The experiment summarized with a conclusion that Phosphogypsum amendment will be fruitful at a particular range and can be fatal for the microbial and soil enzyme activities if it exceeded its threshold range.

Chen et al. (2014) investigated the effects of gypsum on trace metals in soil and earthworms. For the study they used FGD (Flue Gas Desulfurization) gypsum which was produced by the removal of SO2 from flue gas streams. The application rates of gypsum ranged from 2.2 Mg ha^{-1} to 20 Mg ha^{-1}. These rates were 2 to 10 times higher than typically recommended. It was observed that the earthworm numbers and biomass were decreased significantly and with the higher rates of gypsum application was found to be hazardous to earthworms and soil integrity.

Rakhimova et al. (2017) evaluated the impact of coal ash and phosphogypsum on soil fertility of Chernozem soils of north Kazakhstan. Soil samples were collected (three times a year) and the initial content of humus, pH, plant nutrient elements and heavy metal content of the soil were tested in laboratory and recorded. The application of Phosphogypsym and coal ashes for fertilization of the soils influenced the growth of soil nutrient elements such as nitrogen, phosphorous, copper and zinc. It also impacted the on neutralization of the soil environment and further investigation is needed to find out actual content of heavy metals (Cu, Zn, Cd and Pb).

Materials and methods

Material

Phosphogypsum

Phosphogypsum was collected from Paradeep Phosphate Limited (PPL), Paradeep, Jgatsinghpur, Odisha, India.

Earthworm used

Eisenia fetida used as experimental organisms. For regeneration adult earthworms with prominent clitellum were used.

Soil

Soil was sieved using 2 mm sieve and packets were made containing 0 g%, 4 g%, 8 g% and 10 g% gypsum with 25 g% water.

Method

500 gm 2 mm sieved, air dried soil was taken in polythene packets with moisture maintained at 25 g % by addition of distilled water in all packets. The control consisted of 500 g soil only and PG was added to soil in experimental sets i.e. 4%, 8% and 10%. Ten replicates of each concentration were taken for each parameter (growth, feeding, respiration and regeneration). After moisture addition the packets were left for five days for stabilization. Culture was maintained with 25 g% moisture and 25 °C temperature (Initially we went for 25 g%, 50 g% and 75 g% concentration of phosphogypsum with 25 g% water for the experiment but it was found that worms unable to tolerate such high amount of phosphogypsum percentage and found dead).

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Experimental set up for growth

After five days of moisture addition and stabilization of soil, up to 1.70 g of earthworms were inoculated to each packet. Weight of earthworms in each packet was determined and change in weight over initial weight was observed after on 10^{th}, 20th and 30th days. Percentage change in weight of earthworms over zero day culture was estimated.

Experimental set up for feeding

2.5 g of earthworms were inoculated to each packet and estimation of stable aggregates was done at an interval of 10 days by sieving culture sets with water. Carbon content of stable aggregate formed was estimated by Walkley and Black titration method (Walkley and Black, 1934). Then the amount of carbon was converted to energy according to available standard values energy conversion for carbon is 41.44 kJ g^{-1} dry wt. (Remmert, 1980).

Experimental set up for respiration

Out of 10 replica of each concentration, 5 were maintained without earthworms which accounted for microbial respiration. To each packet, an average of 1.5 g of earthworms were added. The respiratory metabolism was estimated on 10th, 20th and 30th day by Alkali Absorption Method (Witkamp, 1966). Carbon dioxide evolution was measured and expressed as mg of $CO_{2.}$ g^{-1} live worm tissue hr^{1} kg^{1} soil.

Experimental set up for regeneration

For regeneration, the guts of the matured earthworm were cleaned and about 50% of post clitellar region was cut with sharp blade. Five earthworms were inoculated into each packet after amputation. Thus twenty five earthworms each were cultured in control, 4%, 8% and 10% of phosphogypsum. The amputed earthworms could not survive in 8% and 10% phosphogypsum. So the regeneration experiment was performed with 4% PG only.

Statistical analysis

Statistical analysis were performed to infer the results (Microsoft excel version 2007). For growth, feeding and respiration, ANOVA test was performed. T-test was performed for regeneration. Comparisons of values were made and values at $p \leq 0.01$ are said to be significant.

Results

Growth

Table 1 and *Figure 1* indicate the changes in the growth i.e. changes in weight pattern of *Eisenia fetida* earthworm under the impact of phosphogypsum in laboratory cultures. In the first phase of estimation (initial 10 days), a positive increase of about 1.53% in weight in control set ups was observed in comparison to experimental set ups. On exposure to 4%, 8%, and 10% phosphogypsum, there was significant decrease (at 0.01 level) of about 23.07%, 23.07% and 75% respectively over zero day. At 20 days,

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the weight percentage reached up to 5.03 over zero day in control but a continuous decrease in weight by 33.84%, 68.46% and 93.84% was observed when exposed to 4%, 8% and 10% of phosphogypsum. After the end of final phase (last 10 days), 96.40% decrease in weight over initial weight at 10% phosphogypsum. In at 4% and 8%, weight was decreased by 30.93 and 74.82% over initial weight respectively. But in Control sets, there was a remarkable growth noticed (21.53%). Analysis of variance showed significant impact of phosphogypsum treatment on earthworm weight at 0.01 level of significance.

Table 1. *Weight of Eisenia fetida earthworm under impact of phosphogypsum in laboratory culture*

Culture in days	Parameter analysed	Conc. of phosphogypsum in %			
		0	4	8	10
0	Weight % change over '0' day	1.01 0	1.03 0	1.05 0	1.02 0
10	Weight % change over '0' day	1.04 + 1.53	0.8 -23.07	0.8 -23.07	0.26 -75.0
20	Weight % change over '0' day	1.3 +5.3	0.86 -33.84	0.41 -68.46	0.08 -93.84
30	Weight % change over '0' day	1.39 +21.53	0.96 -30.93	0.35 -74.82	0.05 -96.40

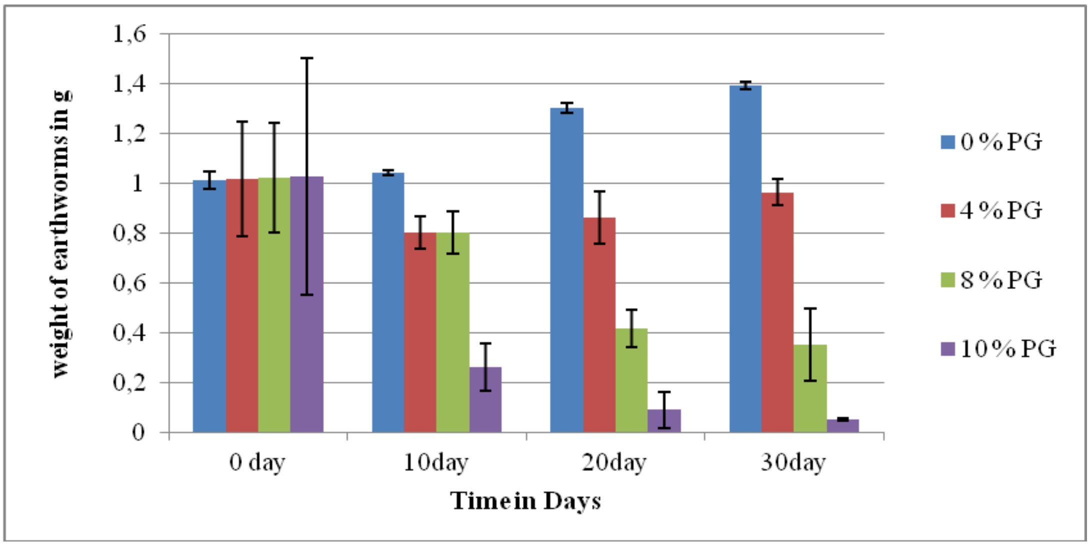

Figure 1. *Change in weight of Eisenia fetida earthworm under the impact of phosphogypsum in laboratory culture. (Mean ± standard deviation)*

Feeding

After 10 days of exposure to 0, 4, 8, 10% of phosphogypsum, the weight of stable aggregates formed was 12.67, 11.72, 12.40 and 9.42 g g^{-1} live tissue (*Fig. 2; Table 2*) and a visible decrease of about 0.009%, 97.66% and 99.20% in stable aggregate formation as comparison to zero day in 4, 8 and 10% of phosphogypsum respectively (*Fig. 3*). On completion of 30 days there was decrease of about 99.7%, 99.8% and

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99.93% respectively was observed (*Table 2*). When it comes to the matter of carbon content of stable aggregate formed by the earthworms, a consistent and significant decline was noticed by the increase of concentration dose and exposure duration (*Fig. 4*). Highest energy content of stable aggregate formed was 784.74 kJ kg^{-1} soil g^{-1} live tissue at 0% and lowest was 349.09 kJ, kg^{-1} soil, g^{-1} live tissue at 10% on 30th day. A remarkable significance difference noticed in stable aggregate formed at the end of 30 days in comparison to 10th day. After 30 days at 0%, the weight of stable aggregate formed by the earthworms was 32.65 g g^{-1} live tissue which was much higher when it compared to 4, 8 and 10% (22.45, 18.16 and 16.20 g g^{-1} live tissue respectively). Analysis of variance showed significant decrease in the stable aggregate formation by the worms (at 0.01 level of significance) on application of phosphogypsum. Significant decrease in energy content of stable aggregate was also found at 0.01 level of significance.

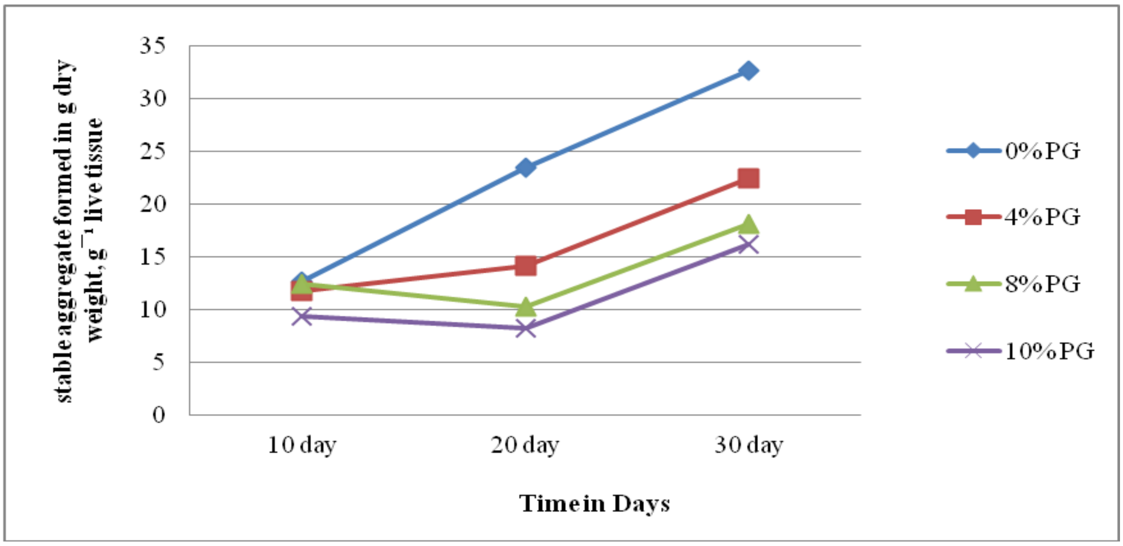

Figure 2. *Stable aggregate formed by Earthworm Eisenia fetida under the impact of phosphogypsum in laboratory condition*

Table 2. *Stable aggregate formed by Eisenia fetida under the impact of phosphogypsum*

Culture time in days	Parameters analysed	Concentration of phosphogypsum in percentage			
		0	**4**	**8**	**10**
10	Weight of stable aggregate	12.67	11.72	12.40	9.42
	% change over “0” percent	0	-0.009	-97.66	-99.20
	Energy content	283.52	237.98	226.09	160.04
20	Weight stable aggregate	23.40	14.16	10.32	8.25
	% change over “0” percent	0	-99.12	-99.57	-97.81
	Energy content	552.72	332.73	209.55	164.44
30	Weight stable aggregate	32.65	22.45	18.16	16.20
	% change over “0” percent	0	-99.7	-99.8	-99.93
	Energy content	784.74	530.28	414.65	349.09

Weight: g dry weight stable aggregates, g^{-1} live tissue
% change: gain or loss
Energy content: kJ kg soil g^{-1} live tissue

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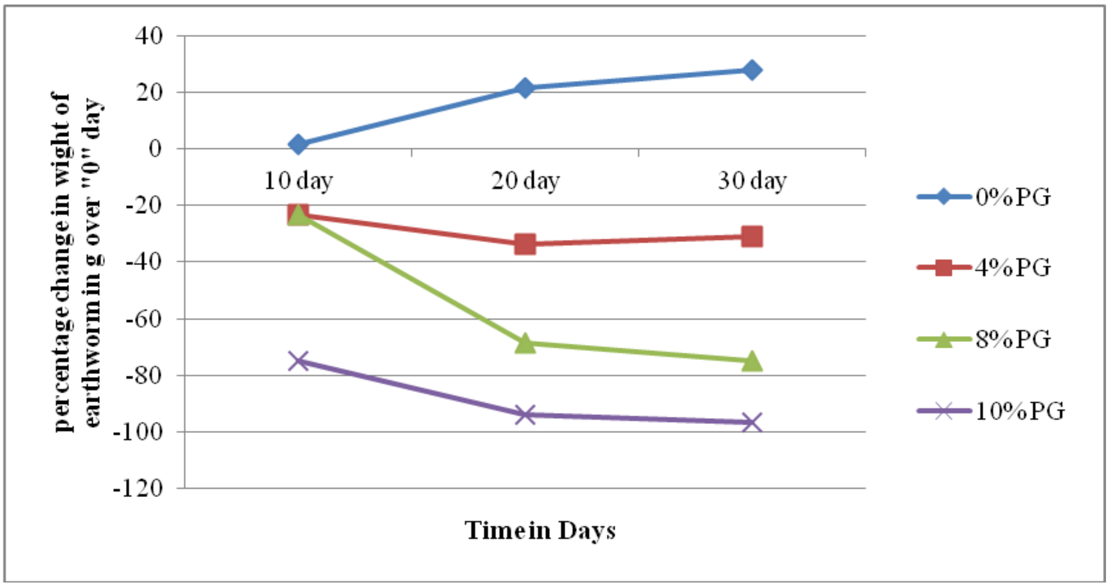

Figure 3. *Percentage change in weight of earthworm Eisenia fetida under the impact of phosphogypsum in laboratory cultures*

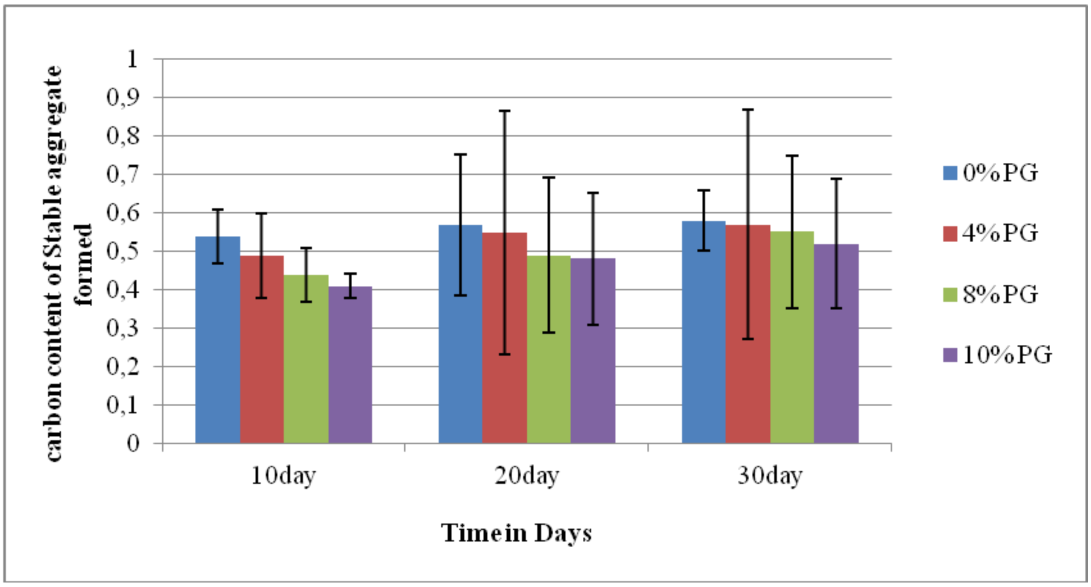

Figure 4. *Carbon content of stable aggregate formed by earthworm Eisenia fetida under the impact of phosphogypsum in laboratory cultures. (Mean ± standard deviation)*

Respiration

The rate of respiration was found to increase with duration and treatment. After 10 days of exposure the rate of respiration was found to be 0.0564, 0.0565, 0.0567 and 0.0568 mg of CO_2 evolved, g^{-1} live worm tissue hr^{-1} kg^{-1} soil at 0%, 4%, 8% and 10% of phophogypsum, respectively (*Fig. 5; Table 3*). At the end of the last phase (30 days), there was increase of about 0.17%, 0.34% and 0.52% in rate of respiration on exposure to 4%, 8% and 10% phosphogypsum over control respectively. The mg of CO_2 i.e. evolved, g^{-1} live tissue hr^{-1} kg^{-1} soil increased from lower concentrations to higher

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concentrations of phosphogypsum as the days increased. Analysis of variance showed significant increase in respiratory metabolism of earthworm at 0.01 level of significance under the impact of phosphogypsum.

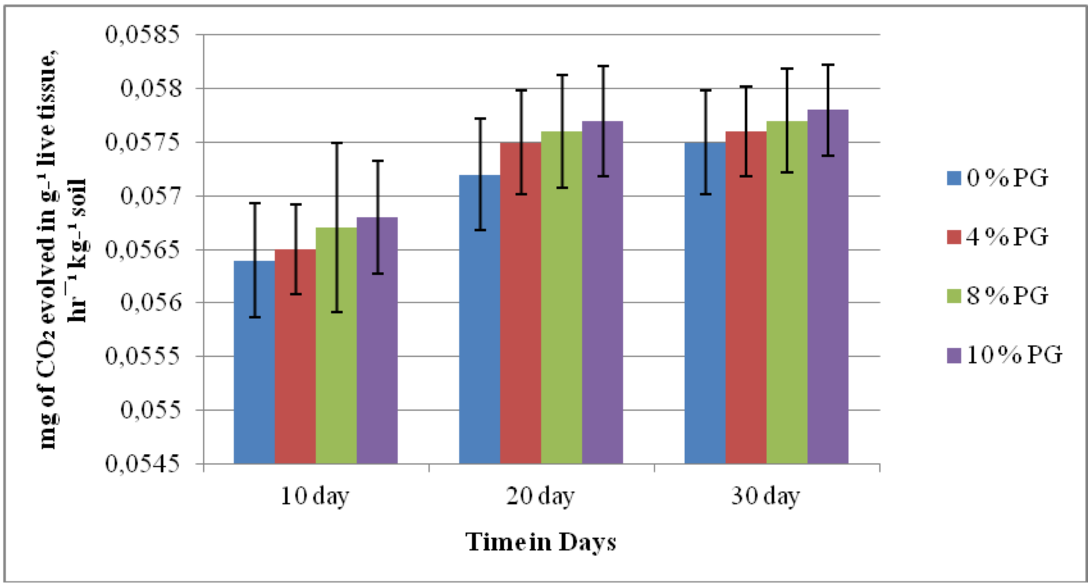

Figure 5. *Respiratory metabolic rate of earthworm Eisenia fetida under the impact of phosphogypsum. (Mean ± standard deviation)*

Table 3. *Respiratory metabolic rate of Eisenia fetida earthworm under the impact of phosphogypsum*

Culture time (in days)	Rate of respiration (mg of CO_2 evolved, g^{-1} live worm tissue hr^{-1} kg^{-1} soil) at different concentrations of phosphogypsum in percentage			
	0%	4%	8%	10%
0-10	0.0564 ± 0.000516	0.0565 ± 0.000527	0.0567 ± 0.000527	0.0568 ± 0.000422
10-20	0.0572 ± 0.000789	0.0575 ± 0.000527	0.0576 ± 0.000516	0.0577 ± 0.000483
20-30	0.0575 ± 0.000527	0.0576 ± 0.000516	0.0577 ± 0.000483	0.0578 ± 0.000422

Mean ± standard deviation

Regeneration

The amputated earthworms could not survive beyond two days in 8% and 10% phosphogypsum. So the experiment was continued with control and 4% phosphogypsum. On completion of 10 days of exposure to 0% and 4% of phosphogypsum, the percentage increase in regenerated segments was found to be 6.28% and 2.68% respectively (*Figs. 6* and *Table 4*). After 20 days of exposure to 0% and 4%, the percentage of regenerated segments was found to be 10.64 and 5.72% respectively. At the end of 30 days, the percentage increase in regenerated segments was found to be 13.4% in 0% and 8.4% in 4%. The regenerated parts of the worm grown in 4 % phosphogypsum can be marked in the *Fig. 7* after the completion of 30 days. T-test reveals that impact of phosphogypsum has a significant effect on *Eisenia fetida* at 0.01 level of significance.

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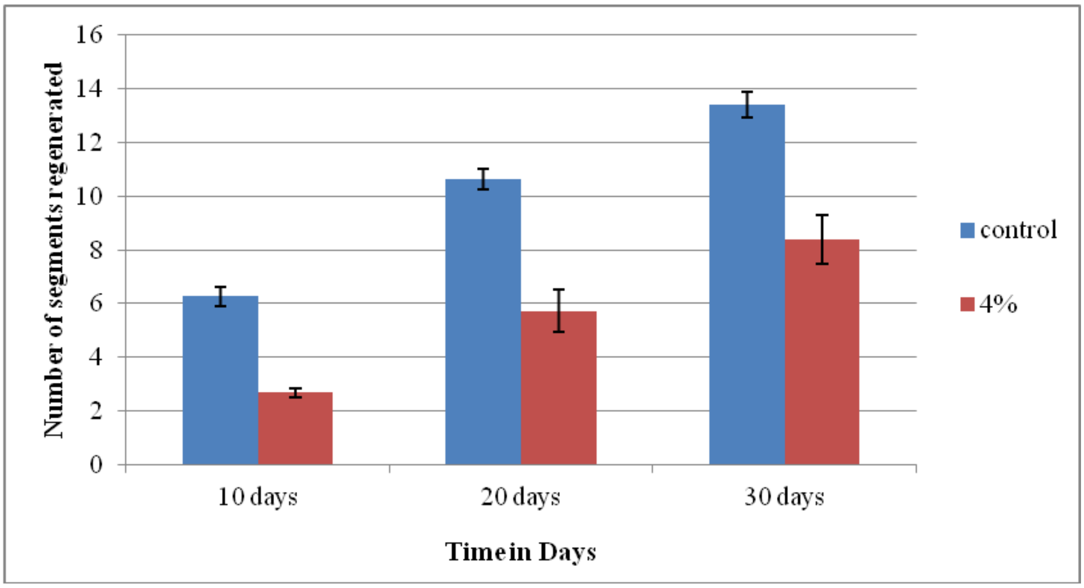

Figure 6. *Percentage change in regeneration of earthworm Eisenia fetida under the impact of phosphogypsum in laboratory culture. (Mean ± standard deviation)*

Figure 7. *Regenerated segments of earthworm Eisenia fetida at 4% phosphogypsum*

Table 4. *Regeneration of Eisenia fetida earthworm under the impact of phosphogypsum in laboratory culture*

Culture in days	Parameters analysed	Conc. of PG in g %	
		Control	4
10	Total no. of segments regenerated	6.28	2.68
	%age change over control	0.0	57.32
20	Total no. of segments regenerated	10.64	5.72
	%age change over control	0.0	46.24
30	Total no. of segments regenerated	13.4	8.4
	%age change over control	0.0	37.31

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Discussion

Earthworms ingest organic matter of the soil particle and excrete this in the form of casts, which get deposited on the soil surface and this cast acts as a natural fertiliser for the soil and boosts the soil fertility. Loss of fertility is directly proportional to stable aggregate formed by earthworms. Heavy metallic factor present in the soil directly affects the various physiological activities of *E. fetida*. Majority of heavy metals are toxic to living organisms when retained in the soil and interfere with the biochemical processes and alter the ecological balance (Nwuche and Ugoji, 2008). These heavy metals do not biodegrade rather they bioaccumulate and this may have adverse effects on the biodiversity of the area if the organisms do not develop a mechanism of adaptation to it. Nickel and lead accumulated significantly in worm bodies during the 3-weeks exposure whereas zinc accumulation was efficiently regulated. Nevertheless zinc exposure significantly inhibited the weight gain. Inhibition of weight gain in zinc-exposed worms may be putatively explained by the high energetic costs of efficient zinc regulation (Podolak et al., 2011).

In many other case studies, *D. veneta* (Kwadrans et al., 2008) and *Aporrectodea caliginosa* (Dutkiewicz et al., 2009) were maintained for 4 and 8 weeks, respectively, in soil samples soaked with Cd, Cu, Pb, or Ni chlorides. Body weights of *D. veneta* were unaffected by 4-weeks metal exposure. In a study by Spurgeon et al. (1992), control worms and those on the lowest concentration of metals had slightly increased in weight after 1 week. However, in subsequent weeks their weight declined. Malecki et al. (1982) tested the effect of Cd, Cu, Pb, Ni and Zn on the physiological activity of *Eisenia fetida* in laboratory cultures. They concluded that cadmium was the most toxic metal, as significant decreases in growth noticed. Here it is important to mention that Cadmium is one of the heavy metal present in the phosphogypsum beyond the recommended range. It was observed (Khalil, 2013) that earthworms *Allolobophora caliginosa* feed less and *Pheretima hawayana* escaped into their burrows when exposed to arsenate. Miguel et al. (2012) revealed in a study that weight and mortality of the worms were significantly affected by high levels of heavy metals.

Metal exposure can imbalance the host-bacteria relationship, as evidenced in *D. veneta* after 3-days exposure to filter paper soaked with water (controls) or metal (Zn, Cu, or Cd) chlorides (Podolak et al., 2011). It may assumed that effects of metal exposure on immunity are rather associated with the disrupted balance between the worm immune system and microbial impact from surrounding metal-polluted soil (Salice and Roesijadi, 2002; Wieczorek-Olchawa et al., 2003; Olchawa et al., 2006). Various results indicated that earthworm activity increases the mobility and bioavailability of heavy metals in soil (Wen et al., 2004; Sizmur and Hodson, 2009). But the matter is how far and how much worms can accumulate and able to rotate the mobility of heavy metals. Although concentrated heavy metals in the earthworm's body demonstrate the ability of *E. fetida* to accumulate the heavy metals in their body still it needs to reveal the limit or range which is non-deleterious.

Fordsmand et al. (1998) investigated the toxic effects of nickel on survival, growth, and reproduction of *Eisenia veneta* for 4 weeks of exposure to a nickel chloride spiked loamy sand soil. Nickel caused a significant toxic effect on *E. veneta* at soil concentrations above 85 mg Ni/kg. Survival of adults was only reduced at concentrations above 245 mg Ni/kg, while adult and cocoon wet weight were not affected by soil nickel concentrations up to 700 mg Ni/kg. The results of our study are in close agreement with Ma (1983), Neuhauser et al. (1985), Bengtsson et al. (1986),

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Gestel and Dis (1988), Gestel et al. (1989, 1991, 1992). The results of the present study clearly correlate with the work of Zaltauskaite (2010) who reported that *E. fetida* exposed to different concentrations of lead in the soil tended to lose more weight than those in control.

Single metal such as Cu can affect the physiology and growth of *E. fetida* may hampered due to increased tissue concentration of Cu., while the cocoon hatchability and number of juveniles increased as the tissue Cu concentration increased (Leduc et al., 2008). So it was a combine impact of heavy metals which might be more hazardous and has more pronounced toxicity than the individual metals (Olaniya et al., 1991). In some situations *E. fetida* can allocate more energy to growth than reproduction, delaying other physiological development (Leduc et al., 2008). A combination of As, Cr and Cu can impair growth, affect reproduction and cause death also (Leduc et al., 2008).

Sivakumar and Subbhuraam (2005) evaluated the toxic effects of chromium exposure on *E. fetida* and found 14-day LC50 values ranging from 1656 to 1902 mg kg^{-1} for Cr (III) and from 222 to 257 mg kg^{-1} for Cr (VI) in ten soils. Cocoon production by *E. fetida* was reduced by 50% after exposure to a range between 679 and 1110 mg Cr (III) kg^{-1} (Lock and Janssen, 2002b). In a contradictory study Shahmansowri et al. (2005) revealed that though heavy metals such as Cr, Cd, Pb, Cu and Zn were bioaccumulated by *E. fetida,* but a significant reduction of body weight only marked when exposed to higher concentration. Similarly pattern of findings also observed in weight *Pheretima guillelmi* at high concentration of lead in (Rongquan and Canyang, 2009). Among *E. albidus, E. crypticus* and *E. fetida* the most sensitive species towards industrial waste material was *E. fetida* (Kobeticova et al., 2010). The weight of worms was significantly affected by high levels of heavy metals in the study of Miguel et al. (2012).

Impact can vary due to the different contact times during which the earthworm exposed to contaminants (Haghparast; 2013; Golchin et al., 2013). Concentration of the metals in the body of earthworms can reach the toxic level if the contact time of earthworm is a prolonged duration (Hagparast et al., 2013). Jamshidi and Golchin (2013) also confirmed same. Thus, the potential effects of metals may be overlooked or underestimated in short term experiments (Bengtsson et al., 1986), which may explain the truth that sometimes despite the high levels of metal contamination in the soil, no significant impact may not noticed like Kennett et al. (2002). In a such kind of study, Honsi et al. (2003) reported no effect of heavy metals (Cu, Zn, Cd and Pb) on survival of earthworm *E. fetida* exposed to contaminated soils in Norway despite the fact that the concentrations of metals were high (max Pb – 8750 μg g^{-1}, Cd – 110 μg g^{-1} soil). Kaur and Hundal (2016) observed a very sound impact of heavy metals in the body weight of earthworms. Berthelot et al. (2008), Matuseviciute et al. (2005) and van Gestel et al. (2009) findings also support our results. Spurgeon and Hopkin (1996) also argued that the worms living in metal polluted soils reached the lower weight or needed more time to reach the maximum weight than in non-polluted sites.

Trace elements added to the soil with gypsum or any soil amendment may be concentrated in food chains as the elements are consumed and passed from one trophic (feeding) level to another level (Duffy and Gulledge, 2011). Garg et al. (2009) found a pattern of heavy metals accumulation in *E. fetida* after 45 days i.e. Cr>Cd>Pb>Zn and Cd had the greatest detrimental effect on cocoon production and matter of concern is these heavy metals are one of the major part of phosphogypsum.

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During their study, Avila et al. (2009) identified that increasing the organic material can reduce the toxicity of heavy metals in the body of earthworms. Irizar et al. (2015) concluded during their study that, if the organic material in the soil is low, earthworms are not able to digest the soil and, as a result, the toxicity of cadmium increases in them, and the mortality and disorder in reproduction rise. Haghparast et al. (2013) showed that organic material is a source of energy for *Eisnia Fetida* earthworms and increases the percentage of their survival. Avila et al. (2009) showed that adding 5% of organic matter to soil contaminated with chromium at a concentration of 0.06 mg/g after 21 days gave no bioremediation, but after 42 days the efficiency of bioremediation (18.33%) increased. At the concentration of 0.1 mg/g the bioremediation efficiency of 30% after 21 days reached 53% after 42 days.

Heavy metallic cations are said to be more mobile or dynamic under acid conditions (Alloway, 1996) as the correlation between soil pH and micronutrients availability has been one of the major aspect of soil (Brady, 1990; Joshi et al., 1983, Sharma et al., 2003; Akinrinde et al., 2005). Although organisms like earthworm can accumulate a high concentration of heavy metals in the body (Shahmansouri et al., 2005; Li et al., 2010; Brewer and Barrett, 1995; Bamgbose et al., 2000) but mineralization of dead earthworms releases accumulated heavy metals back to the soil (Morgan and Morgan, 1988a). The amount of metals accumulated within earthworm tissues is partly dependent on the absolute concentration of metal within a given soil and physiochemical interactions (Ma et al., 1983). When a contaminant is not in equilibrium with the soil, it becomes more bioavailable to living organisms (Davies et al., 2003). Phosphogypsum is one of the industrial waste materials which is acidic in nature and contains various heavy metals. Another major concern here is *E. fetida* has already been reported as very sensitive species towards industrial waste material exposure by Kobeticova et al. (2010).

Earthworms are well capable of regenerating lost segments, but different species have different regeneration ability. After studying regeneration in different kinds of species for 20 years, Gates (1949) published some of his findings that show for certain species there is a possibility of growing into two intact ones from a bisected specimen. *Eisenia fetida* with head regeneration, in an anterior direction, possible at each inter-segmental level back to and including 23/24, while tails were regenerated at any levels behind 20/21, i.e., two worms may grow from one.

Three potential pathways exist for the removal of chemicals from tissues; elements can be regulated by excretion from the body, bound within the matrix of inorganic granules and attached to proteins or other ligands (Tessier et al., 1994). If metals are detoxified primarily by excretion, body concentrations should decrease when previously exposed individuals were transferred to a clean environment. However, if an element is bound in an inorganic matrix or to organic ligands, metal levels may remain constant, even after the exposure has ceased (Spurgeon and Hopkin, 1999). Animals exposed to high concentrations of metals over a long period, the storage capacity of the hepatopancreas may become saturated, allowing metals to pass into the haemolymph and interfere with sensitive biochemical processes (Spurgeon and Hopkin, 1999).

Heavy metals are strongly bound to soils rich in organic matter or clay (Sauve, 2002) and simultaneously earthworm developed specific trafficking, storage pathways and redistribution capacity to regulate the heavy metals, especially essential trace metals such as Cu and Zn, in their bodies that may lead to balance between uptake and excretion (Dallinger, 1993; Morgan and Morgan, 1999). The regulatory capacity of

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metals can partly explain the ability of some earthworm species, like *D. octaedra*, to live even in a highly metal contaminated areas. Thus, the metal regulation may also have contributed to the development of metal resistance observed in some of the earthworm populations (Bengtsson et al., 1992; Langdon et al., 2001a, b; Reinecke et al., 1999). One additional factor that may reduce harmful effects of metals on the earthworms and also increase intraspecific variation in body burdens is heterogeneous distribution of metals in the soil (Lukkari et al., 2004).

Conclusion

Earthworms form the base of various food chains because they are preyed on by many species of snakes, mammals, and invertebrates (Edwards and Bohlen, 1996) and could increase biomagnification in various trophic levels of the food chain. This whole concept can upset the food chain as well as the food web. Increasing pollution and spontaneous addition of pollutant to soil through anthropogenic sources can alter growth, feeding, regeneration and respiration of *E. fetida* by both direct and indirect effects. These physiological activities of worm can be impaired by direct toxic effects of metals or by bringing changes in the energy budget as an individual attempts to prevent accumulation in sensitive tissues. In earthworms, cadmium, lead, and some zinc are detoxified by binding in granules (chloragocytes) or metallothionein like proteins in the chloragogenous tissue (Morgan and Morgan, 1988b; Morgan et al., 1989). In contrast, copper and the remaining zinc are eliminated by an excretion mechanism (Morgan and Morgan, 1990, 1991). The mechanisms of metal sequestration and elimination have metabolic costs in both development of the system and for the maintenance and repair. This increased the requirement for maintenance energy will ultimately result in a reduction in the energy available for growth and development (Donker et al., 1993a). If there is an increased energy demand for metal sequestration and elimination, this will decrease the energy available for other physiological activity and ultimately affects the organism. Present findings very much evident and transparent about the sensitivity of the worm *E. fetida* towards metallic contamination and indicates juvenile *E. fetida* is more sensitive to metal-contaminated soils than that of mature worms (Spurgeon and Hopkin, 1996). After 4-5 weeks of culture significant reduction was marked in the weight, feeding activity and regeneration capacity of the worm. In contradiction, rate of respiration clearly increases by the increasing of exposure duration with concentration dose. Number of studies on other soil dwelling invertebrates also suggests a clear reduction in growth on exposure to metal-contaminated diets due to avoidance of contaminated food (Drobne and Hopkin, 1995; Laskowski and Hopkin, 1996). In contradiction to our study Podolak et al. (2011) stated that metals may be either regulated (Zn) or accumulated (Ni, Pb) in worm bodies, with or without deleterious effects on body weights, immune competent cells and physiological activity putatively due to their differential impact on soil and coelom-inhabiting microbes. For last few years, phosphogypsum is being used in agricultural soil as a calcium supplement to enhance the crop production (Nayak et al., 2011). Mullino and Mitchell (1990) have reported the use of phosphogypsum to increase yield and quality of forages in Florida. But respiration of soil was deeply affected by phosphogypsum application (Delaune et al., 2006) which directly hampers the physiological activities of soil dwelling organisms including earthworms. However, the chemical and biological response of soil to the waste amendment needs to be thoroughly investigated before recommending its large

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scale field application. When it comes to statistical part to verify the significance of phosphogypsum on *Eisenia fetida* (at 0.01 significance level), it was clearly significant. All the physiological activity of the earthworm was significantly hampered due to phosphogypsum and it was almost lethal. The heavy metallic constituents of phosphogypsum adversely affect the growth, feeding, respiration and regeneration activity of the worm.

Nevertheless earthworm based assessment is complicated by the fact that earthworms can develop tolerance to various pollutants, as documented in several studies of populations that have been in contact with high polluting sources over long periods (Bengtsson et al., 1992; Morgan and Morgan, 1992). The direct measurement of heavy metal concentrations in earthworm tissues could provide a means of assessing environmental pollution levels, given the demonstrated correlation between soil contamination and earthworm metal bioaccumulation (Motalib et al., 1997). But here the matter of concern is to generalize these vital facts regarding impact of heavy metals upon soil macrofauna in particular earthworms and standardize the accumulation range. How much and how far earthworms are able to tolerate such wide range and hazardous impact of heavy metals with varying composition of soil. This is a big question to answer. It becomes more necessary to generalize these facts and must be much more aware and watchful towards soil contamination.

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EFFECT OF BED PLANTING ON THE QUANTITY AND QUALITY OF HAY IN DIFFERENT MIXING RATIOS OF VETCH AND BARLEY IN THE NORTH OF IRAN

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Abstract. This study was conducted to investigate the effect of bed planting on the quantity and quality of forage yield of vetch and barley in different crop mixing ratios. The field experiment was conducted in 2014-2015 and 2015-2016 on the paddy fields of the North of Iran (Rasht, Guilan Province) as a factorial analysis in randomized complete block design (RCBD) with three replications. The experimental treatments included bed planting at 2 levels (1. rice bran with bed planting and 2. without bed planting as control) and crop mixing at 6 levels (1. Sole vetch, 2. Sole barley, 3. 20% vetch to 80% of barley, 4. 40% vetch to 60% of barley, 5. 60% vetch to 40 % of barley, 6.25% vetch to75% barley as replacement ratio of crop mixing). The recorded data were the followings: forage yield, crude protein yield and quality traits like crude protein (CP), dry matter digestibility (DMD), and acid detergent fiber (ADF), neutral detergent fiber (NDF). Mixed cropping indices were calculated by means of land equivalent ratio (LER) and Money advantages index (MAI). Results showed that rice bran with bed planting affects some quantity and quality traits of forage. Among mixing ratios, planting of 40% barley and 60% additive vetch had highest forage yield. Additive mixing ratios had the highest crude protein yield among mixing ratios. According to the results, in the North of Iran the highest LER and forage yield was obtained from rice bran with bed planting and 40% additive of vetch to barley.
Keywords: *forage yield, crude protein yield, money advantage index, dry matter digestibility, land equivalent ratio*

Introduction

During the post rainy season (October–March) in the sub-humid region of northern Iran, average cumulative evapotranspiration and runoff are high. Therefore, the low status of soil moisture in the root zone usually limits productivity of rainfed winter crops in this region. Conserving moisture that has accumulated in the root zone during the previous rainy season can increase productivity of rainfed winter crops in the dry season. Soil management practices can influence conservation and efficiency of stored water (Sarkar and Singh, 2007). Applying mulch on the soil surface to reduce evaporation rate and discourage weeds is another water conservation practice in India (Sarkar and Singh, 2007).

Mixed cropping as method crop intensification is commonly practiced in densely populated countries to provide more food. Mixed cropping of annual cereals and pulse crop is commonly practiced in humid topic regions because total seed production generally enhanced compared with sole cropping (Ciftci and Ulker, 2005). The legume-grass combination has been used in several mixed cropping systems including forage

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and cover crops (Ramos et al., 2011). Generally, grasses in mixed cropping with legumes take more N than in sole cropping, because of transfer from rhizodeposit-N of legume to the associated grass (Rasmussen et al., 2007).

Vetch is one of popular pulse crops of Iran and is an annual leguminous crop which produces high-quality forage and can be cultivated in most climates as rainfed or irrigated, but grows best in temperate and cold-temperate conditions. In recent years, vetch and grass pea cultivation has drawn much attention as a crop well-adapted to harsh conditions and arid and semi-arid regions, with high protein content (Habibi et al., 2010). The different *Vicia* species are used as direct grazing and also for their green forage, hay and seed (Lanyasunya et al., 2007). In studies of Lanyasunya et al. (2007), the CP, Ca and P contents of common vetch (*Vicia sativa* L.) has shown to be 18.4, 0.132 and 0.34% on a dry weight basis, respectively, with 59% total digestible nutrients. In north of Iran, farmers prefer planting of vetch with cereals like barley over pure planting of them. Their mixture can be considered as feed for animals during winter or early spring.

Sowing a forage crop in rotation with cereals, is a highly effective method for, for example, soil properties reclamation (MeVay et al., 1989) and increasing rainwater infiltration in soils (Daniel et al., 2006). The high ability of forage legumes for nitrogen fixation is among their most important characters that can reduce need for applying chemical nitrogen fertilizers with implementing them in farming rotations (Rao et al., 2005). Kusmiyati et al. (2013) reported that application of 3 and 6 ton/ha mulch increased plant growth, forage yield and nutritional quality of *guinea grass* but Plant growth, forage yield and nutritional quality of *guinea grass* were not affected by monoculture or mixed cropping with *Sesbania*, however the highest yield was obtained from 6 ton/ha mulch and mix cropping.

Forage grass quality is affected by many factors such as the species, cutting interval, phase of plant development, soil fertility, fertilizer, disease and climatic conditions. It is usually found that the most important factor affecting protein content in herbage dry matter is phase of plant development. Forage quality will decrease, in term of decreasing crude protein and increasing levels of cell wall constituents, with advancing maturity (Kusmiyati et al., 2013).

Various indices such as land equivalent ratio (LER) and monetary advantage index (MAI), have been developed to describe the competition and possible economic advantage in intercropping (Banik et al., 2000; Ghosh, 2004). Mathematical indices can help researchers summarize, interpret, and display the results from plant competition trials (Weigelt and Jolliffe, 2003). Indices can express various attributes of competition in plant communities, including competition intensity, competitive effects, and the outcome of competition. They help in the interpretation of complex data and allow comparison of results from different studies with the use of the same index. Among indices being used for assessing competition between intercrops, land equivalent ratio is the most commonly used for intercrop versus sole crop comparisons (Agegnehu et al., 2006).

One the most important reason for mixed cropping is increasing production per area (Ghosh, 2004). Land Equivalent Ratio (LER) and monetary advantage index (MAI) is used by researchers as an indicator to assess effect of intercropping on land productivity (Mead and Willey, 1980).

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In this study we aim to quantify the effects of different bed planting and mixing ratio treatments on the quantity and quality yield of barley and vetch under rainfed conditions.

Material and methods

This study was conducted as a factorial experiment in a randomized complete block design with 3 replications in Rice Research Institute of Iran (RRII), located in Rasht (Guilan Province), Iran, during two years, 2014-2015 and 2015-2016. The area is N 37° 16’ and E 041° 36’ and -7 m above sea level.

Experimental factors included bed planting at 2 levels (1. Rice bran as bed planting and 2. Without bed planting as control) and mix cropping at 6 levels (1. Sole vetch, 2. Sole barley, 3. 20% Additive of vetch to barley, 4. 40% Additive of vetch to barley, 5. 60% Additive of vetch to barley, 6. 25% vetch and 75% barley as substitutive ratio of mix cropping).

The experimental site was ploughed, harrowed and a basal dose of 100 kg NPK ha^{-1} was broadcast. Seed planting of an Iranian native cultivar of barley (cv. Behrokh) and annual common vetch (cv. Lameie), were evaluated in an intercropping system. Plots consisted of various row numbers depending on intercropping ratios. Planting rows were 0.2 m wide for both barley and vetch and 5.5 m long. Plots were seeded on October by hand and two extra rows on the border of each cropping pattern were considered as guard rows. Four meters of all rows within each plot were hand-harvested on 4th of May. Rice bran as bed planting was applied by hand-spreading at the rate of 50 kg per hectare to form an approximately 20 mm thick layer between crop rows. Bed planting treatments were imposed before sowing.

After oven drying at 72° for 48 h, herbage dry yield was determined. To determine the influences of N and seeding rate on forage quality, a 200-g sample was taken and grounded to pass through a 1-mm sieve. Near-infrared spectroscopy (NIR) (Inframatic 8600, Perten Instruments) was used to determine forage quality indices, including crude protein (CP), dry matter digestibility (DMD), and acid detergent fiber (ADF) neutral detergent fiber (NDF). Before using NIR, forage quality indices of one replication (using three subsamples) of each treatment was determined using wet chemistry methods as described below. These were further used to calibrate NIR (Jafari et al., 2003).

Relative feed value (RFV) was identified and formulated by Van Dyke and Anderson (2002). All formulas are indicated below:

$$DDM = 88.9 - (0.77 \times ADF\%)$$

$$DMI = (120 / NDF\%)$$

$$RFV = DDM\% \times DMI\% \times 0.775$$

$$NE1 = ((1.044 - (0.0119) \times ADF\%)) \times 2.205$$

where DDM was digestible dry matter as percentage (%) of dry matter, and DMI was dry matter intake as percentage (%) of body weight.

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The measure used to estimate effectiveness and profitability of inter-or mixed cropping is Land Equivalent Ratio (LER), which is calculated as (Mead and Willey, 1980):

$$LER = Y_{ij} / Y_{ii} + Y_{ji} / Y_{jj}$$

where:

Yii and Yjj = Yields of i and j species in their sole cropping,

Yij and Yji = Their yield in mixed cropping.

When LER measures 1.0, it indicates that the mixed cropping and sole cropping have yield equivalence, LERs above 1.0 indicate advantages of mixed cropping and LERs below 1.0 show no real yield advantages from mixed cropping.

The Monetary Advantage Index (MAI) which gives an indication of the economic advantage of the intercropping system was calculated according to Ghosh (2004) as follows:

$$MAI = (\text{monetary value of combined intercrops})\ (LER - 1) / LER.$$

Economic values of grain and stover produced were estimated based on the average forage yield of barley (70$ per ton) and vetch (110$ per ton).

Data analysis for two years was done using SAS (*Ver* 9.1). For collation of additive and substitutive mixed cropping we used orthogonal analysis. Analysis of combined experiments was done at the end of two years and means were compared using LSD test at 0.05 probability level. Before statistical analysis.

Results and discussion

Ash

There is no difference between additive and replacement of treatment (*Table 1*). Results showed that without bed planting and 60% additive to barley (7.65%) had higher ash and sole vetch on without bed planting (6.64%) had lowest ash (*Table 2*). Among mixtures, the highest Ash values obtained from 25% vetch and 75% barley treatment with 7.48% and sole barley with 7.09% had lowest ash (*Table 2*). Alizadeh and Teixeira da silva (2013) reported that pure vetch obtained the lowest ash values and they did not find any differences between of mixture ratios.

Water soluble carbohydrates (WSC)

There were no significant differences in the two-year and orthogonal average values of WSC (*Table 2*). The results showed that bed planting and mixing ratios had significant effect on WSC and without rice bran treatment had higher amount of WSC. Among mixing ratios, sole vetch had higher WSC and sole barley had the lowest WSC (*Table 2*). Dahmardeh et al (2009) noted that WSC concentration increased in intercrops compared with in the sole cowpea.

Neutral detergent fiber (NDF) and acid detergent fiber (ADF)

Other important quality characteristics for forages are the concentrations of NDF and ADF (Assefa and Ledin, 2001). In this experiment a similar trend to that of CP content

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was observed for NDF, it increased as the common vetch seeding proportion increased in mixtures (*Table 2*). The 25% vetch and 75% barley (33.49%) as well as vetch monoculture (32.90%) had the lowest value of NDF, whereas the highest value was observed in monoculture barley (36.97%). This result like most other studies (Caballero et al., 1995; Assefa and Ledin, 2001).

Table 1. *Results of analysis of variance and F values of some evaluate traits of barley and vetch in pure stand and its mixtures at different bed planting in two years*

S.O.V.	df	Ash	WSC	ADF	NDF	DMD	TDN
Orthogonal	1	0.001 ns	0.21 ns	4.31 ns	9.88**	10.53**	7.14 ns
Year	1	1.37**	0.43 ns	5.55 ns	0.49 ns	15.7*	9.22 ns
R (Year)	4	3.74	54.76	0.03	52.5	0.94	0.05
Bed Planting (BP)	1	0.11 ns	104.1**	0.08 ns	14.7 ns	1.08 ns	0.13 ns
Mixing Ratios (MR)	5	0.38*	11.07**	6.37**	37.6**	22.8**	10.6**
BP * MR	5	0.89**	43.73**	4.62*	16.0*	3.44 ns	7.71*
Year * BP	1	0.40 ns	1.82 ns	0.02 ns	0.21 ns	0.37 ns	0.02 ns
Year * MR	5	0.04 ns	0.21 ns	0.02 ns	0.20 ns	0.24 ns	0.04 ns
Year * BP * MR	5	0.06 ns	0.94 ns	0.03 ns	0.17 ns	0.30 ns	0.0-3 ns
Error	44	0.14	1.27	1.67	4.79	2.27	2.78
CV (%)		5.12	6.48	3.11	6.23	2.66	3.50

** and *, respectively, significant in 1% and 5% level

The concentrations of acid detergent fibers were not affected by year and orthogonal (*Table 1*). Among mixing ratio treatments, 60% additive vetch to barley produced statistically maximum ADF (42.23 g.kg^{-1}) and the minimum ADF values obtained from 25% vetch to 75% barley with average of 40.54 g.kg^{-1} (*Table 2*).

Kocer and Albayrak (2012) noted that sole pea had lowest ADF and NDF concentration. Aasen et al (2004) reported that increasing the legume proportion resulted in decreased ADF and NDF concentrations for the legume-grass mixtures. Carr et al. (2004) found that pea, barley, oat, pea-barley and pea-oat mixtures of ADF values 38.2%, 38.5%, 34.4% and 36.5%, respectively. Strydhorst et al. (2008) reported that barley and pea-barley mixtures of NDF values were 55.2% and 41.8%, respectively. Van Soest (1996) indicated that under similar growth conditions, legumes have low NDF values whereas cereals have high values which are in agreement with the present study.

Dry matter digestibility (DMD)

There were no significant differences in DMD among the Bed planting factors. However, there were significant differences between orthogonal and mixture ratios (*Table 1*). Without bed planting had higher DMD (*Table 2*). Among mixture ratios, the highest DMD was obtained from sole vetch (58.49 g.kg^{-1}) and lower DMD was shown from monoculture barley (55.16 g.kg^{-1}). Sadeghpour et al. (2014) reported that annual medic sole culture had higher DMD than barley and mixture of barley with annual medic therefore decreased DMD of the mixed forage.

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***Table 2.** Mean of some evaluate traits of barley and vetch in pure stand and its mixtures at different bed planting in two years*

Year	Ash	WSC	ADF (g kg^{-1})	NDF (g kg^{-1})	DMD (g kg^{-1})	TDN (g kg^{-1})
2014-2015	7.48 a	17.32 a	41.87 a	35.05 a	57.05 a	47.34 a
2015-2016	7.21 b	17.47 a	41.31 a	35.21 a	56.12 b	48.06 a
Orthogonal						
Additive ratio	7.45 a	17.12 a	42.14 a	35.81 a	56.0 b	47.0 a
Replacement ratio	7.48 a	16.61 a	40.54 a	33.49 b	57.41 a	49.06 a
Bed planting (BP)						
Without rice bran (control) (BP1)	7.38 a	18.60 a	41.56 a	35.58 a	56.71 a	47.74 a
Rice bran bed planting (BP2)	7.30 a	16.19 b	41.62 a	34.68 a	56.46 a	47.66 a
Mixing ratios (MR)						
Sole vetch (MR1)	7.14 bc	18.60 a	40.82 bc	32.90 c	58.49 a	48.69 ab
Sole barley (MR2)	7.09 c	17.82 a	41.77 ab	36.97 a	55.16 c	47.47 bc
20% additive vetch to barley (MR3)	7.47 a	17.71 a	42.18 a	36.03 ab	55.86 c	46.94 c
40% additive vetch to barley (MR4)	7.46 a	17.72 a	42.0 a	36.96 a	56.34 bc	47.17 c
60% additive vetch to barley (MR5)	7.42 ab	15.93 b	42.23 a	34.44 bc	55.80 c	46.88 c
25% vetch and 75% barley (MR6)	7.48 a	16.61 b	40.54 c	33.49 c	57.41 c	49.06 a
BP * MR						
BP1 * MR1	6.64 d	18.82 b	40.44 de	33.70 cd	59.42 a	49.18 ab
BP1 * MR2	7.30 abc	16.75 d	41.46 bcd	38.60 a	55.57 def	47.86 bcd
BP1 * MR3	7.61 a	17.10 cd	42.70 ab	36.47 ab	55.29 def	46.26 de
BP1 * MR4	7.52 ab	21.04 a	42.35 abc	37.43 ab	55.91 cdef	46.72 cde
BP1 * MR5	7.65 a	18.71 b	41.26 cde	35.53 bc	56.58 cde	48.13 abc
BP1 * B6	7.58 ab	19.17 b	41.13 cde	31.77 d	57.46 bc	48.30 abc
BP2 * B1	7.64 a	18.39 b	41.20 cde	32.10 d	58.46 ab	48.20 abc
BP2 * B2	6.88 cd	18.88 b	42.07 abc	35.33 bc	54.73 f	47.08 cde
BP2 * B3	7.32 ab	18.31 bc	41.66 bcd	35.60 bc	56.43 cde	47.61 bcd
BP2 * B4	7.41 ab	14.40 e	41.64 bcd	36.48 ab	56.76 cd	47.63 bcd
BP2 * B5	7.19 bc	13.14 f	43.20 a	33.35 cd	55.03 ef	45.62 e
BP2 * B6	7.39 ab	14.05 ef	39.95 e	35.22 bc	57.36 dc	49.82 a

*Values within a column followed by the same letter are not significantly different at LSD ($P \leq 0.05$)

Total digestible nutrients (TDN)

The TDN refers to the nutrients that are available for livestock and are related to the ADF concentration of the forage and, as percentage of ADF increases, TDN declines (Albayrak et al., 2011). The results showed that mixing ratios and interaction of mixing

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ratios and bed planting had effects on TDN. Among mixing ratios, 75% barley and 25% vetch (49.06 $g.kg^{-1}$) and 20% additive vetch to barley (46.94 $g.kg^{-1}$) had the highest and lowest TDN values respectively. Kocer and Albayrak (2012) reported that Pea-oat and pea-barley mixtures ratios had more TDN values than monoculture oat and barley monoculture. They noted that the TDN refers to the nutrients that are available for livestock and are related to the ADF concentration of the forage.

Relative feed value (RFV)

Forage nutritive value, as indicated by the RFV, was improved in all legume–barley mix cropping relative to the sole barley crop (*Table 3*). The lower barley sole crop RFV was attributed to the higher NDF and ADF concentrations in the sole crop barley. Carr et al (2004) reported higher NDF concentrations in sole crop barley relative to pea–barley intercrops of the legume–barley intercrops, Sole vetch had the highest RFV (168.7%), while sole barley had the lowest RFV (139.3%). Differences in RFV of legume and barley were attributed to differences in NDF, as ADF was similar between legume and barley.

Table 3. *Results of analysis of variance and F values of some evaluate traits of barley and vetch in pure stand and its mixtures at different bed planting in two years*

S.O.V.	df	RFV	NEl	CP	Forage yield	Crude protein yield (CPY)
Orthogonal	1	2871.3**	0.32*	13.35 ns	3555111 ns	4552.6 ns
Year	1	199.4**	0.466*	39.26**	151617 ns	4545417.5*
R (Year)	4	933.8	0.03	6.30	1377456	59088.6
Bed planting (BP)	1	163.7 ns	0.03 ns	2.89 ns	14311250**	260184.9 ns
Mixing ratios (MR)	5	1593.8**	0.66**	75.33**	62953463**	1088928**
BP * MR	5	250.4*	0.1 ns	4.36 ns	1271474 ns	70233.2 ns
Year * BP	1	0.39 ns	0.009 ns	1.0 ns	4966 ns	7474.6 ns
Year* MR	5	2.36 ns	0.007 ns	0.46 ns	192575 ns	25394.6 ns
Year * BP * MR	5	8.1 ns	0.009 ns	1.54 ns	270275 ns	25669. 6 ns
Error	44	106.9	0.066	5.50	1157305	10599.2
CV (%)		6.84	3.37	14.26	11.47	20.91

** and *, respectively, significant in 1% and 5% level

As expected, RFV values are positively correlated with NDF and ADF contents since they are functions of each other. The highest RFV was determined in the pure stand of common vetch while the lowest RFV was observed in the pure stand of barley (*Table 4*).

Van Soest (1996) reported that the RFV is not a direct measure of the nutritional content of forage, but that it is important for estimating the value of the forage. The RFV is an index that is used to predict the intake and energy value of the forages and it is derived from the DDM and DMI. Forage with an RFV value >151 is considered prime (Horrocks and Vallentine, 1999). In the present experiments, the RFV values of most treatments was higher than 151 and it showed high quality of forage production in north of Iran.

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Table 4. *Mean of some evaluate traits of barley and vetch in pure stand and its mixtures at different bed planting in two years*

Year	RFV (%)	NEl (Mcal.kg^{-1})	CP (%)	Forage yield (kg.ha^{-1})	Crude protein yield (CPY) (kg.ha^{-1})
2014-2015	152.8 a	7.70 a	17.18 a	9717 a	1633.9 a
2015-2016	149.5 b	7.54 b	15.70 b	9625 a	1475.0 b
Orthogonal					
Additive ratio	146.0 b	7.52 b	16.04 a	10934 a	1756.6 a
Replacement ratio	160.9 a	7.76 a	16.25 a	10275 a	1680.8 a
Bed planting (BP)					
Without rice bran (control) (BP1)	149.6 a	7.64 a	16.63 a	9225 b	1494.3 a
Rice bran bed planting (BP2)	152.6 a	7.60 a	16.24 a	10117 a	1614.5 a
Mixing ratios (MR)					
Sole Vetch (MR1)	168.7 a	8.02 a	20.97 a	5129 c	1075.1 b
Sole Barley (MR2)	139.3 c	7.38 c	13.29 c	9820 b	1301.0 b
20% additive vetch to barley (MR3)	144.6 bc	7.50 c	15.80 b	10400 ab	1644.1 a
40% additive vetch to barley (MR4)	142.4 c	7.58 bc	16.57 b	11189 a	1844.6 a
60% additive vetch to barley (MR5)	150.9 b	7.49 c	15.76 b	11214 a	1781.3 a
25% vetch and 75% barley (MR6)	160.9 a	7.76 b	16.25 b	10275 b	1680.8 a
BP * MR					
BP1 * MR1	165.6 a	8.10 a	21.20 a	4956 g	1051.8 f
BP1 * MR2	134.2 d	7.45 def	13.68 de	9753 ef	1416.0 cde
BP1 * MR3	141.3 cd	7.40 def	15.46 bcd	9911 def	1534.4 cd
BP1 * MR4	139.8 cd	7.51 cdef	16.26 bc	10322 cdef	1665.8 bc
BP1 * MR5	148.5 bc	7.62 cde	15.70 bcd	10917 bcd	1710.1 bc
BP1 * MR6	168.4 a	7.77 bc	17.53 bc	9494 f	1668.6 bc
BP2 * MR1	171.9 a	7.94 ab	20.74 a	5303 g	1098.3 f
BP2 * MR2	144.4 bcd	7.31 f	12.89 e	9889 def	1268.0 def
BP2 * MR3	148.0 bc	7.59 cde	16.14 bc	10889 bcde	1753.7 abc
BP2 * MR4	145.0 bcd	7.65 cd	16.88 bc	12055 a	2078.5 a
BP2 * MR5	153.4 b	7.35 ef	15.82 bcd	11511 ab	1964.1 ab
BP2 * MR6	153.2 b	7.75 bc	14.98 cde	11055 abc	1693.0 bc

*Values within a column followed by the same letter are not significantly different at LSD ($P \leq 0.05$)

Net energy for lactation (NE_l)

Our results showed that orthogonal, year and mixing ratios had significant effect on NE_l (*Table 2*). Additive mixing ratios had more NE_l than substitutive mixing ratio. Among mixture ratios, the highest NE_l was obtained from sole vetch averaged 8.02 mgcal.ha^{-1} (*Table 4*). These results are similar to the results of other researchers who reported that sole common vetch had highest NE_l (Yilmaz et al., 2015). On the other hand, Lauriault and Kirksey (2004) found that mixtures of pea with rye and barley had no effect on NE_l.

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Crude protein (CP)

Crude protein content of produced forage is one of the most important criteria to measure forage quality (Assefa and Ledin, 2001). Therefore, concerning the relative low protein content of cereals (Mpairwe et al., 2002) and animal requirements for balanced feed, the importance of mixed cereal-legume cropping would increasingly appear to supply a nutritional diet (Lanyasunya et al., 2007). The results evidently showed that the mixture ratios had effect on crude protein and Monoculture common vetch had the highest CP content (average of 20.97%) and sole barley had the lowest CP content (*Table 4*). In our results in all mixtures, the CP content increased as common vetch seeding proportion increased (*Table 4*). These results are in agreement with those reported by Giacomini et al. (2003). Also, Lithourgidis et al. (2006) reported that crude protein values of mixed of cereals with common vetch (*Vicia sativa* L.) had higher amount than the cereal monocultures. Similar to our results, Osman and Osman (1982) also found the highest and the lowest crude protein percentage in legume and cereal sole cropping, respectively and as legume ratio increased in mixture, protein`s percentage of mixture forage was improved.

Forage yield

Application of bed planting significantly increased ($P < 0.05$) forage yield. Rice bran as bed planting treatments (BP2) had higher forage yield than without rice bran (BP1). Kusmiyati et al (2015) reported that the effect of bed planting significantly increased plant growth and forage yield of guinea grass.

According to the results, among mixing ratios, the highest forage yield was obtained from 40% additive vetch to barley with the average value of 1844 kg ha^{-1} that it was not significantly other mixture ratio treatments (*Table 4*). Also, the lowest forage production was harvested from sole vetch with the average value of 1075 kg ha^{-1} (*Table 4*). In average of two years, Forage yield showed significant difference among mixing ratios. The highest yield was obtained with 40% additive vetch to barley and sole cropping of vetch produced the lowest yield. Agegnehu et al. (2008) reported that additive mix cropping increased forage yield between 3% to 22% over sole cropping.

Crude protein yield (CPY)

Crude protein yield is a measure that relates CP concentration to the dry matter yield per unit area and gives a better estimate of total CP available in the season. One of the main advantages of legume–cereal forage intercrops has been increased protein yield, relative to cereal sole crops (Aasen et al., 2004; Carr et al., 2004). There was a significant effect for protein yield by years (*Table 3*) from 40% additive vetch to barley with the average value of 1844 kg ha^{-1} that it was not significantly other mixture ratio treatments (*Table 4*). Also, the lowest forage production was harvested from sole vetch with the average value of 1075 kg ha^{-1} (*Table 4*). The maximum crude protein yields of 2078.5 kg ha^{-1} were obtained from rice bran as bed planting and 40% additive vetch to barley and the minimum of protein yield of forage obtained from sole vetch with both of BP1 and BP2 (*Table 4*). These results suggest that relying on sole vetch as a source of on-farm protein is not sufficient to satisfy the farmers’ need. It could be concluded that planting of sole vetch might reduce the total forage yield but will increase the overall crude protein yield of the forage which is a more desirable feed for animals.

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Carr et al (1998) noted that increasing intercrop protein yield required a significant proportion of legume DM in the intercrop mixture. Crude protein yield of forage is one of the main criteria for forage quality. In all mixtures, an increase in the rate of vetch resulted in higher crude protein yield (Karagic et al., 2011). This was expected since legume establishment was greatly enhanced by the barley support, resulting in a higher protein-rich legume proportion in the mixture. An increase in the rate of dry matter and crude protein content apparently resulted to the increase in the rate of vetch in the mixtures. Strydhorst et al (2008) reported that barley intercrops with legumes improve forage quality compared to pure stand barley. Furthermore, lupin–barley, faba bean–barley, and pea–barley intercroppings had higher protein yield compared to pure barley.

Land equivalent ratio (LER)

The LERs in all mixed treatments were more than one (*Table 5*). Between additive and replacement ratios, the highest LER was belonged to additive ratio (*Table 5*). Results, showed that rice bran as bed planting, had better LER than without rice bran treatments (*Table 5*).

Table 5. *LER and MAI measured for mixing ratios and bed planting of vetch and barley in two years*

Orthogonal	LER	MAI
Additive ratio	1.62	340.6
Replacement ratio	1.52	281.2
Bed planting (BP)		
Without rice bran (control) (BP1)	1.57	291.2
Rice bran bed planting (BP2)	1.67	369.8
Mixing ratios (MR)		
20% (MR1)	1.54	280.6
40% (MR2)	1.66	362.2
60% (MR3)	1.66	378.9
25 vetch and 75% barley (MR4)	1.52	281.2
BP * MR		
BP1 * MR1	1.51	256.0
BP1 * MR2	1.57	305.5
BP1 * MR3	1.66	369.3
BP1 * MR4	1.44	233.9
BP2 * MR1	1.58	305.4
BP2 * MR2	1.75	419.4
BP2 * MR3	1.66	422.5
BP2 * MR4	1.60	332.0

The highest LERs of dry forage production were obtained with mixed cropping 40% and 60% additive vetch to barley, which means 66% more land were needed in sole cropping. The lowest yield-LER and was belonged to 25:75 vetch-barley ratio.

Land Equivalent Ratio (LER) was used to assess profitability of mixtures relative to sole cropping of two crops in respect of dry forage). LERs of >1 were obtained in all

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mixed treatments, showing higher advantage and land use efficiency in mixed compared with sole cropping. For most mixtures, the LER values of vetch were below 0.5, indicating that vetch has disadvantages in terms of land use efficiency (Rakeih et al., 2010). In our study, land use efficiency appeared to be higher in common vetch. This could be explained by the fact that Hungarian vetch is more sensitive to warm climate conditions, which affects its competitiveness with barley in terms of land use efficiency. Similar results were reported for legume–cereal intercropping (Hauggaard-Nielsen et al., 2006; Dhima et al., 2007; Yılmaz et al., 2015). These results again confirm that mixed cropping generally produce more yield per area than its related pure stands (Park et al., 2002). Similar reports on yield enhancement from intercropping of legume-nonlegume crops have been reported (Banik et al., 2006; Ghosh, 2006; Agegnehu et al., 2006; Vasilakoglu and Dhima, 2008).

Monetary advantage index (MAI)

Results showed that additive ratios had higher MAI (*Table 5*). The highest MAI (422.5) was obtained in the BP2:MR3, which implied that the planting pattern was highly economical and advantageous for the mixtures. Dhima et al (2007) reported that if LER value was higher, then there was an economic benefit expressed with MAI values such as obtained in the present study.

Conclusions

Mix cropping is an important practice in the North of Iran and is considered as part of the subsistence farming designed to meet increase domestic food requirements and investigations to finding a better bed planting that is important to find better production. The results of this study clearly indicate that mixing vetch with barley and bed planting affected on the forage yield and quality. The greater benefit for forage quality was found when common vetch was grown in a monoculture or in mixtures with barley. The results indicated that usage of rice bran as bed planting had better effect on some quantity and quality of hay and mixture of common vetch with barley at the 40 and 60% additive vetch to barley gave higher forage yield and crude protein yield of all mixtures is higher than monoculture and can recommended in the North of Iran. Moreover, further studies are required to validate the positive role of other legumes in mixture with barley and to more comprehensively elucidate the effects of other bed planting on plants and their nutritional quality.

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SPATIAL EFFECT AND INFLUENCING FACTORS OF AGRICULTURAL WATER ENVIRONMENTAL EFFICIENCY IN CHINA

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Abstract. Within measurement model from the perspective of strong disposability, the paper measures the Chinese agricultural water environmental efficiency in 2013, by using the spatial econometric model to analyze spatial effect and influencing factors of agricultural water environmental efficiency in China. The results showed that: (1) Agricultural water environmental efficiency of thirty-one provinces in China is spatially correlated, and its distribution characteristics are: on the one hand, agricultural water environmental efficiency is unevenly distributed in space, which characteristics that gradually reduces from east to west; on the other hand, agricultural water environmental efficiency varies in regions, characteristics—the eastern coastal region is high-value agglomeration area, and the central and western regions are low-value agglomeration areas. (2) Agricultural water environmental efficiency can be influenced by many factors, such as high development level of the rural economy, reasonable changes of agricultural structure, good agricultural environment infrastructure, pertinence environmental regulation. However, the extensive mode of rural industrial growth has reduced agricultural water environmental efficiency.
Keywords: *water pollution, directional distance function, spatial autocorrelation, strong disposability, spatial econometric model*

Introduction

"UN-Water Global Analysis and Assessment of Sanitation and Drinking-Water (GLAAS) 2017 report" points out that nearly 2 billion people around the world are still using faecal contaminated sources of drinking water and are therefore at risk of contracting cholera, dysentery, typhoid and polio. It is estimated that contaminated drinking water causes more than 500,000 deaths per year due to diarrhoea. With the rapid development of rural economy, the problems of agricultural water environment in China gradually appear. "China's environmental state bulletin in 2014" shows that agricultural ammonia and nitrogen emissions accounted for 31.4% of total emissions in the national wastewater discharge in 2014, while agriculture chemical oxygen demand (COD) emission accounted for 48%. In order to control the environmental pollution of rural water, the relevant laws, regulations and policies of the central government and the local government all involve issues relating to rural water environmental governance.

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The initial stage of rural water environment management is mainly focused on the formulation of relevant standards and the implementation of regulatory responsibilities. With the continuous intensification of rural water pollution, the source governance strategy that integrates environmental protection policies with industrial policies has been proposed. Due to the randomness and decentralization of rural non-point source pollution, comprehensive environmental supervision is difficult to achieve. It is also more difficult for farmers and township enterprises to afford taxes and penalties for water pollution. Therefore, more effective governance measures are to strengthen publicity, education, and guidance so that farmers can understand the damages of water environmental pollution, master clean production technologies, and participate in water environment management. On the one hand, the rapid growth in the number of policies reflects the increasing importance of our country's water environment issues. On the other hand, policy measures have been constantly changing from "command control type" to "voluntary participation type"; policy objectives have also gradually changed from macroscopic guidance to microcosmic operations. To achieve the goal of coordinated development of economic growth and water environmental protection, more and more people pay attention to the improvement of the efficiency of resources and environment. On this basis, many scholars began to study environmental efficiency and proved the existence of spatial agglomeration effect of agricultural water resources in China (Li et al., 2008; Sun and Liu, 2009; Sun et al., 2010). Due to agricultural water pollution along with the rainfall and runoff in space migration, does agricultural water environmental efficiency have the effect of spatial agglomeration as well as agricultural water resources efficiency? What are the factors that influence agricultural water environmental efficiency? Through the study of the question above, the agricultural water environmental efficiency can be better improved. Thus the coordinated development of economy and environment can be ensured. Therefore, many scholars have researched agricultural water environmental improvement from the perspective of environmental efficiency.

Literature review

For better coordination of economic development and environmental protection, the World Business Council on Sustainable Development (WBCSD) put forward the concept of environmental efficiency to measure the environmental cost during economic development process, and thus to estimate the sustainability of the economy. According to the WBCSD definition, environmental efficiency is achieved through the delivery of "competitively priced goods and services that satisfy human needs and bring quality of life while progressively reducing environmental impacts of goods and resource intensity throughout the entire life-cycle to a level at least in line with the Earth's estimated carrying capacity". Based on this concept, many scholars began to do research in the field of agriculture environment efficiency. Fulginiti et al. (1998) used the Malmquist Production Index and the Cobb-Douglas Production Function to study the agricultural environmental productivity in 25 developing countries over 25 years. The results showed that the production efficiency of the agricultural environment in the least developed countries decreased most significantly to more than half. Countries have experienced a decline in agricultural production efficiency. Regardless of the environment, Ruttan (2002) found that the world's agricultural productivity had dramatically increased in the past half-century. Galanopoulos (2004) used DEA-based

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Malmquist Productivity Index method to evaluate the agricultural environmental efficiency of EU countries and 13 other candidate countries. The results showed that European countries generally have low agricultural environmental efficiency. Nanere et al. (2007) revised the efficiency of agricultural production and considered that the level of environmental damage has a significant impact on the efficiency measurement model. Restuccia et al. (2008) compared agricultural production efficiency and total factor productivity in different countries and regions and found that the correlation between agricultural environmental productivity and labor productivity was not significant in the same period. but compared to agricultural total factor productivity in developed and developing countries, the gap continues to grow. Barnes et al. (2009) believes that measuring the environmental efficiency can achieve a win-win situation, which can not only reduce the investment of farmers but also reduce the environmental externalities. Li (2014), Li et al. (2011) and Min et al. (2012) made an empirical analysis of agricultural environment efficiency of several provinces in China from the perspective of agricultural economic development, resource utilization and environmental coordinated development. Yang and Chen (2011) measured the growth of agricultural environmental technical efficiency of several provinces in China, and the empirical results show that the agricultural environment efficiency in the east is significantly higher than that in the central and western regions. The difference between the central region and the western region is very small. Ignoring environmental factors will overestimate China's agricultural productivity growth. Pan and Ying (2013a) investigated agricultural productivity under the restriction on resources and environment in China. The results show that without consideration of environmental pollution constraints, China's overall agricultural productivity level will be overestimated, and the state of agricultural productivity diversification in each province will continue. Zhang and Feng (2016) studied measurement model of agricultural environment efficiency in China and its dynamic evolution under the strong disposable perspective. The above research on environmental efficiency measurement laid the foundation for follow-up study.

In addition, scholars at home and abroad have further studied the influencing factors of environmental efficiency. Monchuk (2010) studied the influencing factors of agricultural environment efficiency in China. It proved that agricultural structure changes and agricultural economic development level significantly improved the efficiency of the agricultural environment while the industrialization lowered the efficiency of agricultural environment. Through the research on the influencing factors of Spanish agriculture ecological efficiency, Picazo-Tadeo (2011) found out that environmental regulation, education level, and agricultural training can improve the efficiency of agricultural ecological while agricultural economic development level had the opposite effect. According to the study of Gadanakis (2015), the British agricultural technical efficiency was mainly influenced by farm scale, farmers' level of education and experience, agricultural environmental payment, and cost, environmental regulation, etc. Liang et al. (2012) found that agricultural environmental technology efficiency could be better promoted by improving the level of economic development and agricultural infrastructure investment while the agricultural environment management policy affected little on it. Through empirical research, Li (2014) concluded that China's agricultural green productivity was influenced by factors such as the household contract responsibility system, agricultural price system reform, rural industrialization, public agricultural investment, agricultural taxes and fees reform and

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agricultural openness variables, etc. Yang and Liu (2015) found that high abundance of water resource, farmland water conservancy construction and environment regulation could be obviously beneficial to agricultural water resources efficiency.

In conclusion, the existing research has made abundant achievements in measurement and influencing factors of environmental efficiency, which provides a reference for the rural water environment management and relevant policy making. However, the spatial effect of agricultural water environmental efficiency was taken little consideration.

Moreover, the current efficiency measurement model and agricultural water environmental characteristics at present stage have some differences. Therefore, a more reasonable efficiency measurement model should be used to study the spatial heterogeneity of agricultural water environmental efficiency in China, and to analyze the influencing factors of these spatial differences.

Research method

Efficiency measurement model

The existing efficiency measurement models are mainly based on foreign agricultural environmental efficiency and the domestic industrial environmental efficiency such as joint weak disposable distance function and the SBM model. However, agricultural water environmental efficiency in China now could not meet the demand for pollution (undesirable outputs) characteristics including null associatively and joint weak disposability. First, in regards to characteristics of agricultural pollution, the agricultural environmental pollution in China at this stage is not inevitable, and the agricultural environmental governance can increase production to some extent. Therefore, it does not meet the requirements for combined weak disposal and zero integration. Second, as for the pollution control costs, agricultural environmental pollution control yields benefits to economy to some degree and does not satisfy the economic significance of the joint weak disposition. Third, from the perspective of environmental regulation, the existing agricultural environmental regulation is not sufficient to achieve the transformation of agricultural environmental pollution from strong disposability to joint weak disposability. Based on the above analysis, the pollution characteristics of China's agricultural environment are different from those of industrial environmental pollution and agricultural pollution in developed countries. The combination of zero-integration and weak disposal is still unsatisfactory. Therefore, another way must be found to measure the agricultural environmental efficiency in China. The strong disposability thought provides a solution for measuring China's agricultural environmental efficiency (Zhang and Feng, 2016). Here is the definition (*Eq. 1*):

$$AWEE=\frac{1}{1+\vec{D}\left(x,y,b;g\right)} \tag{Eq.1}$$

where x is input variable; y is the expected output variable; b is the unexpected output variable;

$$D\left(x,y,b;g\right)=\sup\left\{\beta:\left(y,b\right)+\beta g\in p^{s}\left(x\right)\ \right\}$$

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$$p^{s}\left(x\right)=\left\{\left(y,b\right)\middle|\sum_{j=1}^{n}z_{j}x_{j}\leq x,\sum_{j=1}^{n}z_{j}y_{j}\geq y,\sum_{j=1}^{n}z_{j}b_{j}\leq b,z_{j}\geq 0\right\}$$

Spatial econometric model

(1) Spatial autocorrelation model

Global Moran's *I* index describes the cluster state of the distribution of regional economic activities from the whole regional space view. Here is the definition (*Eq. 2*):

$$I=\frac{\sum_{i=1}^{n}\sum_{j\neq i}^{n}W_{ij}z_{i}z_{j}}{\sigma^{2}\sum_{i=1}^{n}\sum_{j\neq i}^{n}W_{ij}} \tag{Eq.2}$$

Where n is the number of observations; X_i is the observation in position i; Z_i is the standardized transformation of X_i;

$$z_{i}=\frac{x_{i}-\bar{x}}{\sigma},\bar{x}=\frac{1}{n}\sum_{i=1}^{n}x_{i},\sigma^{2}=\frac{1}{n}\sum_{i=1}^{n}\left(x_{i}-\bar{x}\right)^{2}$$

Global Moran's *I* index involves only the whole regional space, whereas the spatial association patterns of separate regions in the global geographic range are not included. Here we use the local Moran's *I* index to analyze local characteristics of spatial association. Here is the definition of local Moran's *I* index (*Eq. 3*):

$$I=\frac{I_{i}-E\left(I_{i}\right)}{\sqrt{Var\left(I_{i}\right)}} \tag{Eq.3}$$

where $I_{i}=\sum_{j=1}^{n}w_{ij}\left(x_{i}-\bar{x}\right)\left(x_{j}-\bar{x}\right)$.

(2) Spatial lag model

Spatial lag model (SLM) is used to study whether the variables have a spillover effect in the space aspect. The mathematical expression of SLM is as follows (*Eq. 4*):

$$y=\rho Wy+X\beta+\varepsilon \tag{Eq.4}$$

Where x is independent variable; y is dependent variable; β reflects the effect of x on y; ρ is the spatial autoregressive coefficient; Wy is endogenous variable.

(3) Spatial error model

Spatial error model (SEM) can be used to determine whether an error term is dependent on space level. The definition of SEM is as follows (*Eq. 5*):

$$\begin{aligned}y&=X\beta+\varepsilon\\ \varepsilon&=\lambda W\varepsilon+\mu\end{aligned} \tag{Eq.5}$$

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where x is independent variable; y is dependent variable; β reflects the effect of x on y; ε represents the random error term vector; λ is autoregressive parameters; μ is random error vector of standard normal distribution; W is spatial weight matrix; λ stands for the error impact degree which adjacent area has in the local area.

Efficiency measure and its spatial characteristics

Variable definition and data processing

After full consideration of the existing research achievements and the characteristics of agricultural water environment (Li et al., 2014; Han, 2013; Pan and Ying, 2013b; Liang et al., 2012), the indexes in this paper are selected as listed in *Table 1.*

Table 1. *Measurement index of agricultural water environmental efficiency*

First-grade indexes	Second-grade indexes	Variables interpretation
Input	Land input	Cultivated land area
	Irrigation investment	Effective irrigation area
	Fertilizer input	The net amount of fertilizer applied in agricultural production, including nitrogen, phosphorus, potassium and compound fertilizer
	Agricultural machinery investment	Agricultural machinery total power
	Labor input	Gross labor in agriculture, forestry, animal husbandry and fishery
Output	Expected output	Total output value in agriculture, forestry, animal husbandry and fishery (economic benefit) Grain yield (social benefit)
	Unexpected output	Emission of COD Emission of nitrogen and phosphorus

The sources of nitrogen and phosphorus emissions, as well as COD emissions, come from two periods. The first period is after 2012, and the data can be found in “China Environmental Statistics Yearbook”. The second period is between 1990 and 2012, and the statistics can be measured by the way of unit investigation and evaluation of related literature (Chen et al., 2006; Lai et al., 2004). The formula is as follows (*Eq. 6*):

$$E = \sum_i SU_i \times \rho_i \times LC_i \qquad \text{(Eq.6)}$$

where E is agricultural pollutant emission; SU_i is pollutant production base; ρ_i is intensity coefficient of pollutants; LC_i is pollutant discharge coefficient.

Due to the difficulty of obtaining data on water environmental pollution, the research only uses the data before 2013 for analysis. This study will examine the agricultural water environmental efficiency of 31 provinces in mainland China. Based on China’s development and policy factors, these provinces and autonomous regions are divided into three areas: east, central, and west China. The data above are from the annual “China Statistical Yearbook”, “China Rural Statistical Yearbook”, “Handbook of

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Agricultural Pollution Fertilizer Loss Coefficient” and “Poultry Breeding Industry Pollution Discharge Coefficient Handbook”, etc.

Measurement result

The measurement result of China’s agricultural water environmental efficiency in 2013 can be obtained through *Equation 1* and *Figure 1* is the sub-bitmap. The sub-bitmap shows that the spatial distribution characteristics of agricultural water environmental efficiency in China is uneven and the distribution pattern can be described to be gradually decreased from east to west. The average efficiency values of eastern, middle and western regions are 0.89, 0.80 and 0.75.

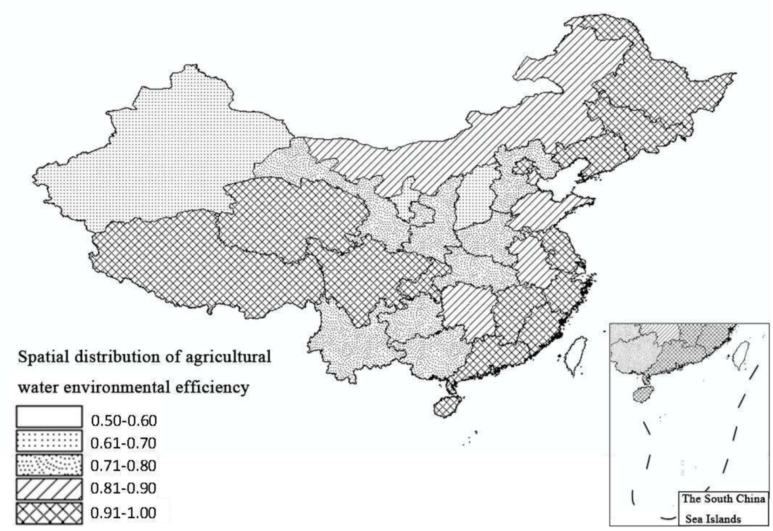

Figure 1. *Spatial distribution of agricultural water environmental efficiency*

Spatial autocorrelation test

By using global Moran’s *I* method to test the measurement results of agricultural water environmental efficiency of China in 2013, the research showed that all the results managed to pass the test under 5% confidence level. The result of this test can support the conclusion that the agricultural water environmental efficiency of thirty-one provinces in China is spatially correlated and its distribution has certain accumulation characteristics, and therefore it is easy to form neighborhood imitation effect of agricultural water environmental efficiency.

To further study the spatial dependence situation of agricultural water environmental efficiency in different regions, the local Moran’s *I* test and Local indicators of spatial association (LISA) was carried out in 2013 (*Fig. 2*). LISA is used to test whether there is agglomeration in the local area. The LISA agglomeration area reflects the closeness of the observation values in adjacent areas to characterize regional differences. From *Figure 2* it turns out that most of the eastern coastal provinces present a High-High accumulation model while some of the mid-western regions still carry a Low-Low accumulation pattern. The former area usually holds high agricultural water environmental efficiency value and is more prone to cooperate with each other to improve that efficiency. The regional economic development level can partly explain

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this phenomenon. The natural characteristics of the more developed river system and more intertwined river network also play an important role. On the contrary, poorer water resources sharing system and a rather slow economic development level have formed a less cooperated environment in the mid-western area, and this has caused low agricultural water environmental efficiency value.

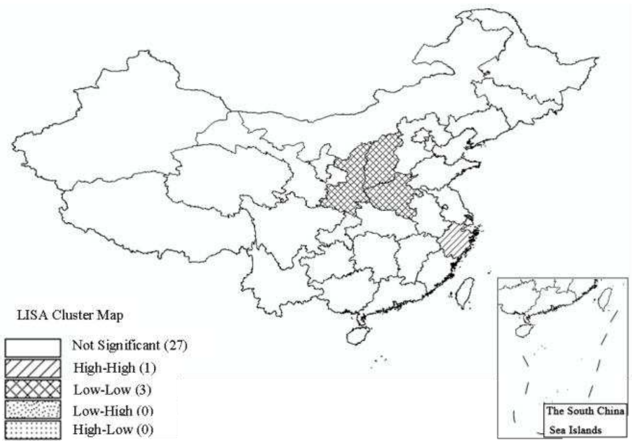

Figure 2. *Local spatial auto-correlated LISA cluster map of agricultural water environmental efficiency in China*

Analysis of the factors influencing spatial heterogeneity of agricultural water environment

Variable selection and data sources

The efficiency of agricultural water environment is influenced by many factors, such as the economy, resources, environment, etc. The influence factors here were chose out of two considerations. The existing research papers listed in *Table 2* and the characteristics of agricultural water environment itself. Based on this, the following influence indexes are selected.

(1) Rural Economy Development Level (REDL). This index is based on per capita net income, which defines the year 2000 as the base year, in rural areas to exclude the impact of price change.

(2) Agricultural structure change (ASCP&ASCG). ASCP represents the proportion of poultry industry output in total agriculture output value, and ASCG shows the proportion of grain crops and economic crops.

(3) Rural industrialization (RI). The total output value of township enterprises which make up the gross rural output value is the measure of this indicator.

(4) Agricultural environmental infrastructure level (AEIL). The investment quota of agricultural infrastructure is the standard.

(5) Environmental regulation (ERCOD&ERNH). Environmental regulation can be reflected in concrete measures like environmental policy making, environmental law enforcement and environmental governance Investment, etc. Considering this, the indexes measuring environmental regulation could relatively be the quantity of environmental policy, the number of pollution control law enforcement and pollution control costs, etc. However, these indexes have their limitations on both index attributes and data sources which might cause deviant results. Therefore, in this paper, the effect

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of pollutant control is used to measure environmental regulation precisely. For example, Cole and Elliott (2003) used pollutant emission density as an indicator to measure environmental regulation intensity. Changes in pollutant emissions and pollution emission intensity coefficient were respectively used as the indicator by Fu and Li (2010) and Li and Tao (2012) to measure environmental regulation better. Based on this idea, agricultural COD and ammonia nitrogen emission intensity were chosen as the proxy variable of environmental regulation in this research. The relationship between environmental regulation strength and pollutant emissions intensity is that the former is directly proportional to the latter.

The data above are drawn from "China Statistical Yearbook", "China Rural Statistical Yearbook" and "China Environmental Statistics Yearbook".

Table 2. *Influence factors of representative researches*

Author	Year	Research contents	Selection of influence factors
Yang Qian	2015	Factors affecting the efficiency of agricultural water resources	Agricultural economic development level; Degree of Water resource abundance; Construction of water conservancy works; Water saving agriculture development level, Planting structure; Environmental regulation
Li Gucheng	2014	Analysis of the institutional factors of the growth of green agricultural productivity	Environmental regulation
Liang Liutao	2012	Factors affecting the agricultural environmental technology efficiency	Agricultural structure; Regional economic development level; Agricultural resource abundance degree; Agricultural infrastructure conditions; Environmental regulation
Picazo-Tadeo	2011	Agricultural ecological benefit evaluation and analysis of its influencing factors	Environmental regulation; Agricultural economic development level; Education level; Agriculture training
Monchuk	2010	Analysis on the influencing factors of agricultural productivity	Agricultural structure; Industrialization; Agricultural economic development level

Interpretation of estimated results

The estimated results of OLS, SLM, and SEM are summarized in *Table 3*. According to the values in *Table 3,* the R-squared value of each respectively is 0.6606, 0.6683 and 0.7468. The value of SEM, as well as SLM, is higher than that of OLS, which means that the assumption of the agricultural water environmental efficiency is independent of provinces in the least squares regression analysis. Thus the conclusion that the agricultural water environmental efficiency of thirty-one provinces in China is spatially related in section 3.3 is proved.

Comparing the LogL, AIC and SC values between SLM and SEM, it proves that SEM model is better than SLM model. This result shows that the efficiency of agricultural water environment in China is not only affected by the agricultural water environmental efficiency of neighboring provinces but also affected by the structural differences between regions. The differences include development level of the rural

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economy, agricultural structure change, rural industrialization, agricultural environmental infrastructure, environmental regulation and other factors not included in the model. Since the spatial error coefficient of SEM has passed the 1% significance level test, the existence of significant spatial dependence of agricultural water environmental efficiency in different provinces is tested.

Table 3. *Regression results of the model*

Variable	Ordinary least square (OLS)	Spatial lag model (SLM)	spatial error model (SEM)
CONSTANT	-0.7513*** (-3.2943)	-0.8283*** (-4.0895)	-0.8025*** (-6.2615)
REDL	0.0336 (1.2138)	0.0401* (1.7602)	0.0507** (2.4834)
ASCP	0.5479*** (4.3034)	0.5835*** (5.4939)	0.6358*** (7.8867)
ASCG	0.0078* (1.8012)	0.0078** (2.2238)	0.0077** (2.3521)
RI	-0.0420 (-1.3450)	-0.0454* (-1.7824)	-0.0445* (-1.7995)
AEIL	0.0121 (0.9185)	0.0107 (0.9816)	0.0136 (1.5101)
ERCOD	-0.1667** (-2.1245)	-0.1810*** (-2.8122)	-0.2225*** (-4.3067)
ERNH	0.0833** (2.1340)	0.0938*** (2.8570)	0.1034*** (4.4473)
ρ	—	-0.1898 (-0.8532)	—
λ	—	—	-0.8208*** (-3.5484)
R^2	0.6606	0.6683	0.7468
LogL	24.8163	25.0475	27.0628
AIC	-29.6326	-28.095	-34.1256
SC	-15.2928	-12.3211	-19.7858

Notes: ***, **, *, respectively, stands for the significant level of 1%, 5%, and 10%. The values below OLS are T-Statistics, and the values below SLM & SEM are Z-Statistics

It can be seen from the above results that the efficiency of agricultural water environment influenced by the structural errors impact shows as follows:

(1) The positive estimated coefficient of rural economy development level (REDL) means that the improvement of rural economic development level can increase the efficiency of the agricultural water environment. With the improvement of rural economic development level, people's demand for water environment quality has also improved. Both farmers and government are paying more attention to the agricultural water environment, and this can improve the pollution problem thus can help to improve the efficiency of the agricultural water environment. Besides, agricultural environment-friendly production technology has been improved; and the resource

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consumption, as well as pollution generation, have been decreased. All these efforts promote the efficiency of the agricultural water environment.

(2) Agricultural structure change causes two kinds of influence on the efficiency of the agricultural water environment. On the one hand, the positive estimated coefficient of ASCP shows that the proportion of animal husbandry in agricultural output helps to the proportion of animal husbandry in agricultural output. The high added value of animal husbandry explains for that. On the other hand, the estimated coefficient of ASCG is also positive. It means that the proportion of grain crops in planting also counts. Rather stable fertilizing amount of grain crops and increasing fertilizing amount of cash crops can help to solve water environment problem because the fertilizer using does great harm to the water environment.

(3) The negative estimated coefficient of rural industrialization (RI) means that rural industrialization decreases the efficiency of the agricultural water environment. Firstly, the overdevelopment and overuse of resources in the process of rural industrialization pollute the rural water environment. Secondly, urban and rural industrial pollution transfer, unreasonable rural industrial distribution, as well as the extensive growth of the rural industry, also influence rural water environment efficiency.

(4) Positive agricultural environmental infrastructure level (AEIL) shows that the improvement of agricultural environmental infrastructure level has an impact on rural water environment efficiency, but with insignificant effect. Projects like irrigation and water conservancy, ecological engineering, water saving irrigation improve comprehensive agricultural production capacity and output level while decreasing pollution discharge and ecological destruction. However, problems like insufficient investment, unreasonable investment project and unmanned management after completion decrease the influence AEIL on rural water environment efficiency.

(5) The influence of environmental regulation on the efficiency of agricultural water environment can be divided into two parts. For the first part, the negative estimated coefficient of ERCOD shows that environmental regulation decreases the agricultural water environmental efficiency on the agricultural COD emission reduction side. The possible explanation is that the livestock and poultry breeding industry, which accounts for 90% of agricultural COD emission, is too dispersed for the specialized management system to be made. Moreover, the randomly caused pollution also adds to the difficulty. For the second part, if the estimated coefficient of ERCOD is positive, it means that the environmental regulation increases the agricultural water environmental efficiency on the agricultural ammonia and nitrogen emission side. This can be attributed to the fertilizer and pesticide management system since these two are the main sources of ammonia and nitrogen emission, and a series of such regulations have been released in China recently. Regulations like “Water Pollution Prevention and Control Law”, “Pesticide Management Regulations” and “Regulations on the Protection of Basic Farmland” are among these effective measures.

Conclusions and policy implications

The strong disposable direction distance function was applied to measure the efficiency of agricultural water environment in 2013, and the spatial econometric model was further used to analyze the spatial effect of agricultural water environmental efficiency as well as its influencing factors. As a result, two main conclusions were drawn from the research. Firstly, agricultural water environmental efficiency in thirty-

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one provinces of China is spatially related. The two spatial distribution features are an unbalanced spatial distribution which presents gradually reduced distribution pattern from east to west and spatial aggregation where eastern coastal areas are mostly high with agricultural water environmental efficiency while the majority of mid-western regions stay in the low part. Secondly, regional structural differences among neighboring provinces also influence the agricultural water environmental efficiency apart from the mutual impact factor. Regional structural differences behave in many aspects such as economic development level of the rural area, agricultural structure changes, industrialization in the rural region, agricultural environmental infrastructure, environmental regulation, etc. These spatial influence factors perform their influences to agricultural water environmental efficiency in their way. For instance, the coordination of the above elements can be a benefit to the improvement of agricultural water environmental efficiency while the extensive rural industrial growth feature will do otherwise.

Based on the conclusions above, the following policy recommendations are drawn:(1) In treatment of agricultural water environment pollution, inter-regional cooperation in economic and environmental protection, spatial linkage of policy measures, cross-regional agricultural water ecological compensation fund and coordination of water environment management among different provinces (municipalities and autonomous regions) are all essential and should be taken into consideration. Specifically, the eastern area should set an example for and cooperate with the mid-western area in agricultural water environment management. For the mid-western area, the “scientific guidance, point to an area” principle should be followed and the detailed steps should be implemented gradually. In this process, the mid-western area should not just imitate from eastern area, adjustment of the policies are necessary when facing problems with specific characteristics of their own. (2) Here are some points every region should follow: Firstly, Improvement of their own economy level and cooperation with other parts should be important tasks, and integration of regional economy should be the target. Secondly, cleaner production and environmentally friendly technology should be advocated to optimize the agricultural structure. Thirdly, rural industrialization should be promoted to more intensive growth mode instead of the existing extensive growth mode. The fourth point suggests that investment in agricultural infrastructure should be increased to form a complete agricultural infrastructure system. Last but not least, policy tools like prevention and control measures, industrial adjustment, and financial support should be comprehensively applied as the inspiriting mechanism to help to improve the agricultural water environmental policy system.

The countries participating in the “Belt and Road” initiative, represented by China, mostly use agriculture as the main industry. There are problems in measuring and improving agricultural water environmental efficiency in these countries. Studying on the evaluation and influencing factors of China’s agricultural water environmental efficiency has certain reference significance for the countries participating in the “Belt and Road” initiative.

This study only compares agricultural water environmental efficiency among the Chinese provinces. If the data includes other advanced countries, for instance, countries participating in the “Belt and Road” initiative, it may provide more information on the level of China’s agricultural sector. Also, this research can be combined with

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Malmquist Productivity Index to investigate the technical efficiency change of China's agricultural sectors. All these remain avenues for future research.

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NESTING SITE SELECTION AND POPULATION NUMBERS OF THE BREEDING BIRD SPECIES IN HOYNAT ISLAND, TURKEY

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Abstract. In this study, bird species of Hoynat Island, which is one of the two biggest islands on and around the Black Sea coast, and population trends of these species in a year and nesting site selection of breeding bird species, were examined. Hoynat Island is also the largest know area to show breeding behaviour for European shag in Turkey. Observations were conducted between 2011 and 2017 years. It was found three bird species preferred for nesting Hoynat Island throughout the year. These species are great cormorant (*Phalacrocorax carbo* Linnaeus 1758), European shag (*Phalacrocorax aristotelis* Linnaeus 1761) and yellow-legged gull (*Larus michahellis* J. F. Naumann 1840). These species show breeding and incubation behaviours on the island and surrounding rocks. While *P. aristotelis* uses the island as a short-term stopover when it first comes to the area, it stays in the rocks on the tunnel then and during the incubation process. *P. carbo* and *L. michahellis* do not use the rocks on the tunnel and only *P. carbo* stays for a while on the top of trees in the upper part of the tunnel. *P. carbo* and *L. michahellis* use the island as a stopover and incubation area. Using the island and surrounding as a nesting site, *P. aristotelis*, *P. carbo* and *L. michahellis* have reached the highest population in June. During this month, 170 *P. aristotelis*, 670 *P. carbo* and 475 *L. michahellis* were counted. It was found that breeding preparations of the birds in the study area started in February and hatching continues until June.
Keywords: *habitat use, great cormorant, European shag, yellow-legged gull, Hoynat Island, Turkey*

Introduction

Turkey is between two continents (Europe and Asia); therefore, it is a wealthy country in terms of fauna, flora and habitat diversity thanks to its location. Habitat loss and degradation on islands are key processes leading to population decline of birds. Habitat generalism is an important attribute in determining the ability to persist in heterogeneous and human-perturbed landscapes (Sol et al., 1997; Marvier, et al., 2004; Vergara and Armesto, 2009). Habitat generalist species have broad habitat amplitudes (occupying several habitat types) and usually they behave as opportunistic species, preferring the habitats offering more resources (Medel and Jaksic, 1988; Magura et al., 2003). Unlike specialist species, which are constrained to use a small habitat spectrum, habitat generalists may switch their habitat selection pattern over time as expected from habitat selection theory (Fretwell and Lucas, 1970; Rosenzweig, 1985; Latta and Faaborg, 2002; Chen et al., 2008). Consequently, changes in population size and in the spatial distribution of their resources over time may result in a wide temporal variability in habitat use by generalist species (Diamond, 1975; Holt, 1993; Mobæk et al., 2009; Hahn et al., 2011).

Great cormorant, European shag and yellow-legged gull are nested in Hoynat Island. In North America, great cormorants are strongly associated with marine coastlines, in contrast to their smaller cousins, double-crested cormorants. In Europe, great cormorants are also found in inland, freshwater areas and in coastal estuaries. Nesting habits may vary among subspecies. North American great cormorants (*P. c. carbo*) nest mainly along coasts. Eurasian subspecies (*P. c. sinensis*) nest in inland areas, but the two subspecies sometimes occur in nesting colonies together in areas of recent overlap (British Isles) (Hatch et al., 2000).

European shags are found throughout western Europe, from Iceland, the British Isles, Portugal, Gibraltar, and northern Africa east to Greece and north into the Ukraine and as far north as Norway. European shags are found along rocky, marine coastlines and islands and are never found very far from land or very far inland. Preferred foraging grounds are in clear, protected waters over sand or rocky substrates, such as in bays or coastal channels. They avoid fresh, brackish, or muddy water (Birdlife International, 2009; Del Hoyo et al., 1992). European shags build nests of sticks, seaweed, and other marine debris on rocky ledges, cliffs, or stacks. Nests have been found from just above the high water level to 100 m above the sea. Nesting areas host large concentrations of these birds, who nest in close proximity. Breeding season varies regionally, Black Sea populations breeding from January to March. (Del Hoyo et al., 1992; Snow, 2008; RSPB, 2009; Birdlife International, 2009; Arkive, 2018).

Yellow-legged Gull can be found in Europe, the Middle East and north Africa. It is resident in much of southern Europe, on the coasts of the Mediterranean, Black Sea and Caspian Sea, on the Azores and Madeira, Portugal, and on the Canary Islands, Spain. The nest is constructed of nearby vegetation, feathers, debris and old carcasses, and is preferably positioned close to or under bushes, or on rocky and sandy islands, beaches, spits, sea cliffs, grassy or shrubby river islands, and occasionally on high ground hundreds of meters from water (Del Hoyo et al., 2013).

Turkey, which is highly important for especially birds, hosts various bird species that are threatened with extinction worldwide. Hoynat Island is one of the 305 Key Biodiversity Areas (KBA) and 184 Important Bird Areas (IBA) of Turkey (Kılıç and Eken, 2004). The island is one of the two biggest islands of Black Sea. Hoynat Island is also the only area to show breeding behavior for European shag in Turkey. There is not any other comprehensive scientific study conducted for bird species in the study area. Only midwinter water bird count was observed during some years. But no data on the island has been revealed during these counts.

Materials and methods

Study area

Eastern Black Sea Region, where Hoynat Island is located, is situated in Caucasus which is one of the most important regions of the world in terms of nature conservation (*Fig. 1*). Caucasus Ecological Region, which is located between Black Sea and Caspian Sea and which covers lands of Azerbaijan, Armenia and Georgia and some parts of Russia, Iran and Turkey, is acknowledged as one of the wealthiest places of the world concerning biodiversity. Eastern Black Sea coasts located in this region host many bird species. The island is 100 meter away from the coast. There is not any settlement area on Hoynat Island. It is estimated that Hoynat Island was once used by sailors as a storage and shelter.

Hoynat (Akkuş) Island and its surroundings selected as study area in this project are located in Bolaman-Efirli route in Ordu province which constitutes the only natural coast line out of the highway influence area of 542 km Black Sea coast road completed

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in 2007. Therefore, this area is also one of the last natural habitats of the bird species in the Black Sea. There is not any other comprehensive study conducted for bird species in the study area. Depending on the observations carried out in different dates, Nature Association of Turkey identified Akkuş Island in the Eastern Black Sea as an Important Bird Area (IBA) (Kılıç and Eken, 2004). So, Akkuş Island IBA was registered as IBA with the code of DKD001-TR059 because of 'island under full protection and necessary to be monitored, area including important populations of bird species threatened with extinction' status, and the island located in 18 km west of Ordu and 100 m off the coast of land and region surrounded by high cliffs that are perpendicular to the sea are identified as 'necessary to be monitored' status. Under this study, *Phalacrocorax aristotelis* and *Larus michahellis* are the bird species determined in the area. Especially the island and its surroundings determined as breeding area of *P. aristotelis* which is threatened with extinction were acknowledged as important bird area with their natural and undisturbed structure. The project area was also identified as 'Culture and Tourism Protection and Development Region' by the Ministry of Culture and Tourism in 2009. Accordingly, Hoynat (Akkuş) Island was accepted by the Ministry of Culture and Tourism as an area necessary to be monitored under the categories of B1(i) and B3 with its 90 double *P. aristotelis* population in the Eastern Black Sea and Çoruh Basin.

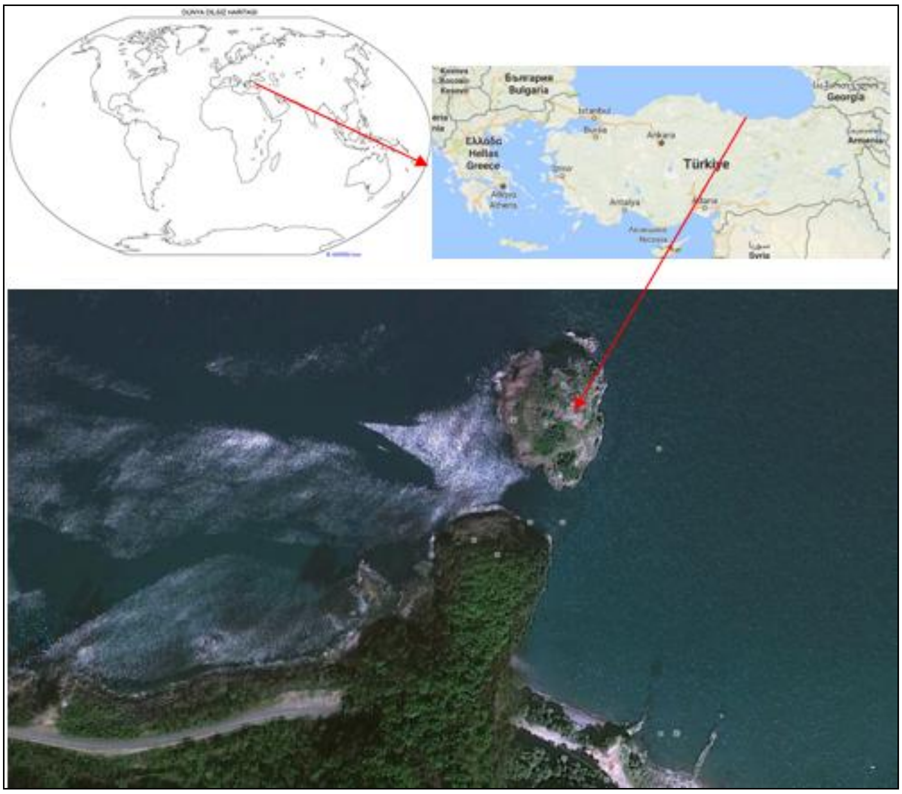

Figure 1. *Location of study area (Google Earth)*

Bird sampling

Direct observations were carried out to the study area every month periodically between June 2011 and January 2013 under the project (Bibby et al., 1992; Oğurlu, 2003). Observations continued from 2013 to 2017 after project during breeding seasons.

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Bird species which have high population spreading the area, habitat use and breeding situation of the species were tried to be identified by benefiting from Kiziroğlu (1989); Heinzel et al. (1992); Kasparek (1992); Jonsson (1993); Roselaar (1995); Kirwan et al. (1998) resources.

Habitat classification

We used the criteria of Hahn et al. (2005, 2010) as a guide for grouping vegetation units (hereafter called "habitat types"). This habitat classification is focused on habitat features important for birds, such as vegetation structure and dominant plant species, as well as geographical factors like altitude, exposition and incline. Three habitats, i.e. bushes on the island, meadows and tunnel bluffs, were classified as breeding habitats in the area.

Results and discussion

As a result of the studies carried out during the project, It was found three bird species preferred for nesting Hoynat Island throughout the year. These species are great cormorant, European shag and yellow-legged gull and showed breeding behaviours on the island and surrounding cliffs (*Fig. 2; Table 1*). Additionally, 1-3 individuals from *Ardea cinerea, Egretta alba, Egretta garzetta, Larus canus, Larus ridibundus, Podiceps cristatus* and *Corvus corone* species were also observed in Hoynat Island. Observations continued from 2013 to 2017 and no significant changes were observed in population numbers. Among these species, there is no endangered species on global scale. But Hoynat Island is the largest known area to show breeding behaviour for European shag in Turkey.

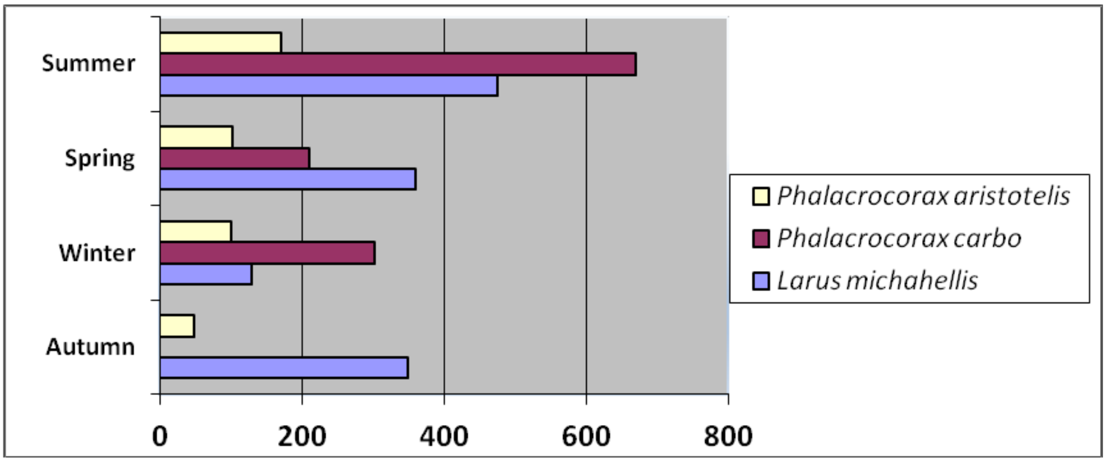

Figure 2. *Seasonal numbers of the breeding bird species (P. aristotelis, P. carbo and L. michahellis) in Hoynat Island*

It was found in the project area that breeding behaviors started in February and hatching continued until June. At the end of January, *P. aristotelis* and *P. carbo* started preparations for nesting at the end of February. Eggs started to open and hatching started in April. Therefore, *P. aristotelis* and *P. carbo* were observed as fully covered with their breeding coverage. On the other hand, *L. michahellis* incubated one month

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later and young birds exited from eggs around at the end of May or the beginning of June.

Table 1. *The breeding bird species in the island and surrounding*

Observation date	Species	Habitat use			Total number of individuals
		Rock balconies on the tunnel	Grassland on the island	Bushland on the island	
6 June 2011	*Phalacrocorax aristotelis*	155		15	170
	Phalacrocorax carbo	25		265	290
	Larus michahellis	10	465		475
27 July 2011	*Phalacrocorax carbo*			25	25
	Larus michahellis		30		30
07 November 2011	*Phalacrocorax aristotelis*	23			23
	Larus michahellis		250	100	350
29 January 2012	*Phalacrocorax aristotelis*	100			100
	Phalacrocorax carbo			120	120
	Larus michahellis		30		30
30 March 2012	*Phalacrocorax aristotelis*	88		15	103
	Phalacrocorax carbo			210	210
	Larus michahellis		360		360
11 May 2012	*Phalacrocorax aristotelis*	70			70
	Phalacrocorax carbo			185	185
	Larus michahellis		275		275
17 June 2012	*Phalacrocorax aristotelis*	97			97
	Phalacrocorax carbo			670	670
	Larus michahellis		450		450
14 July 2012	*Phalacrocorax carbo*			32	32
	Larus michahellis		47		47
15 August 2012	*Phalacrocorax carbo*			36	36
	Larus michahellis		42		42
28 October 2012	*Phalacrocorax aristotelis*	18			18
	Larus michahellis		225		225
30 November 2012	*Phalacrocorax aristotelis*	49			49
	Larus michahellis		320		320
04 February 2013	*Phalacrocorax aristotelis*	48			48
	Phalacrocorax carbo			302	302
	Larus michahellis		130		130

During breeding period, bird species were found in the different habitat types. *P. aristotelis* used rock balconies on the tunnel, *P. carbo* used bushland and *L. michahellis* used grassland on the Hoynat Island. However, none of the species has interfered with each other’s breeding areas during the breeding season (*Table 2*; *Fig. 3*).

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Table 2. *Habitat types for breeding birds in Hoynat Island and surrounding*

Bird species	Habitat types	Area (m^2)
Phalacrocorax aristotelis	Rocky area	7565
Phalacrocorax carbo	Grassland	7294
Larus michahellis	Bushland	1840

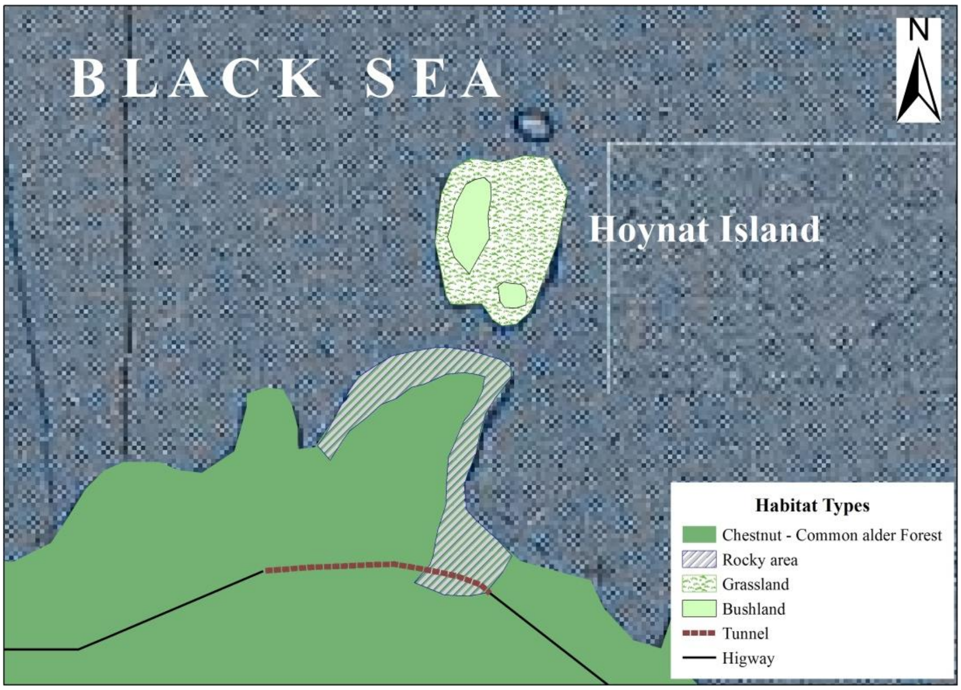

Figure 3. *Habitat types for breeding birds in Hoynat Island and surrounding*

It was also observed that there were differences between the nesting site selections of the identified bird species. Thus, rocks on the island and rocks on the tunnel were addressed as two habitats in terms of area usage of the birds. The island defines the area on which rocks, bushes and small trees exist and which does not have any connection with the land; on the other hand, rocks on the tunnel cover the top of the tunnel on the highway located in 200 meter of the island and cliffs located between tunnel and the island and including some balconies. While *P. aristotelis* uses the island as a short-term stopover when it first comes to the area, it stays in the rocks on the tunnel then and during the incubation process. *P. carbo* and *L. michahellis* do not use the rocks on the tunnel and only *P. carbo* stays for a while on the top of trees in the upper part of the tunnel. *P. carbo* and *L. michahellis* use the island as a stopover and incubation area. Using the island as a temporary stopover, *A. cinerea* never prefers the rocks on the tunnel (*Fig. 4*).

P. aristotelis makes a nest on the rock balconies on the tunnel and rock region near the tunnel by using brushwood; *P. carbo* makes a nest on the top of the bushes in the island or on the high grasses on the ground by using brushwood; *L. michahellis* makes a nest within the grasses in the island or on the ground and stone. In case of *P. aristotelis* and *P. carbo* 3-5 young birds hatch, whereas 2-3 young birds hatch in case of *L. michahellis* (*Fig. 5*).

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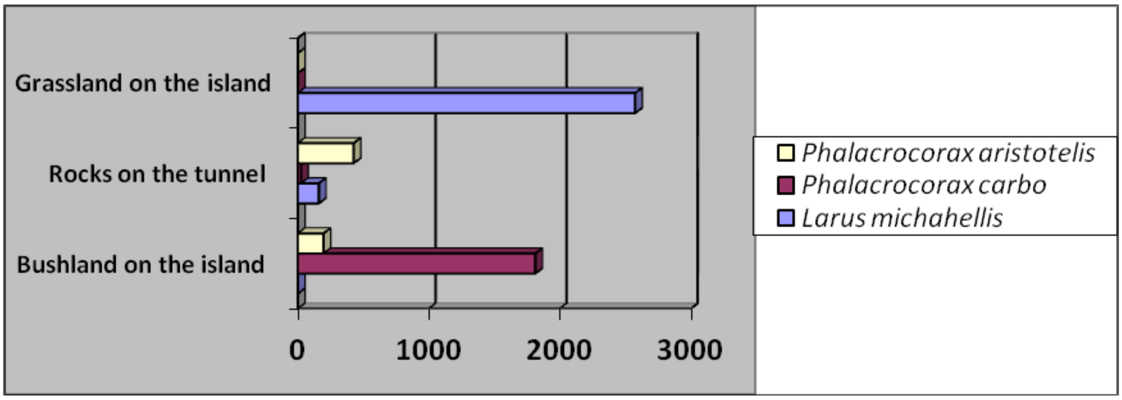

Figure 4. *Nesting site selection of breeding birds in Hoynat Island and surrounding*

Phalacrocorax aristotelis

Phalocrocorax carbo

Phalacrocorax carbo

Larus michahellis

Figure 5. *Nesting sites of the birds in Hoynat Island*

Breeding habitat usage findings obtained in the study are consistent with the literature data. *P. aristotelis* prefers cliffs as nest; *P. carbo* prefers on the top of the bushes and *L. michahellis* prefers meadows. Therefore, rocks on the tunnel are also areas that should be protected as much as the island. That's why, it is recommended to give a 'Natural conservation area' status to the island and rocky area covering the area from the tunnel-top which is the most important area for *P. aristotelis* to the island in order to protect this area and hand down the next generations.

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Many different names are used for the area. For example, in the IBA book it is named as Akkuş Island; however, on the highway signs it is written as Hoynat Island. Local people use Hoynat Island and also Çaka Island. Therefore, it is important to use a single name for resources.

Conclusions

As a result of the studies carried out to date, the occurrences of three nesting bird species in Hoynat Island have been determined. Considering the number of bird populations observed in Hoynat Island, it can be seen how an important nesting area it is for the region and Turkey. Measurements must be taken against threat risks to protect the important bird nesting area. Further urbanization towards Hoynat Island must be stopped, and new buildings must not be allowed in this direction anymore. Study area is not within the scope of hunting ban. The field should immediately be registered into the hunting-ban areas.

Provincial Directorate of Culture and Tourism put a sign with the name of 'Hoynat Bird Paradise' in the project area and opened it ecotourism partly. However, no supportive activity has been implemented in the area. During the field work, many tourist groups who came to the island to make an observation during the summertime were encountered. So, it will be useful to build 'Birdwatching Terraces' in the appropriate places on the west and east exit of the tunnel across the island in order to attract attention and facilitate birdwatching. Hoynat Island should be closed to national and international tourism activities during breeding seasons.

Construction work should not be conducted between February - June which are the breeding months of the birds, and rocks on the tunnel and near the tunnel should not be damaged during the work.

Acknowledgements. This research supported by the Scientific Research Projects Commission of Karadeniz Technical University (project no: BAP03/1100) is fully acknowledged.

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ORF VIRUS INFECTION IN WILD GOATS (*CAPRA AEGAGRUS* ERXLEBEN 1777) OF SARICICEK MOUNTAIN

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Abstract. Orf virus occurs where sheep and goats are raised, and it also infects a broad range of wild artiodactyls. The rates of morbidity and mortality are higher, particularly in lambs and kids experiencing the disease for the first time. Orf virus has been reported in domestic animals and humans in Turkey, so far. In this study, findings on the presence of Orf virus on wild goats were researched for the first time. Field studies were carried out between 2016 and 2017 years. Direct observation methods were used in field studies. In this study, a dead six months of age male wild goat (*Capra aegagrus*) and 3 diseased individuals in poor condition with bloody lesions covering the muzzle area were examined in Sarıçiçek Mountain in Eastern Black Sea Region, Turkey. The gross and microscopic lesions confirmed the diagnosis of contagious ecthyma (CE). This is the first documented report of CE in wild goat from Turkey.
Keywords: *contagious ecthyma, bezoar goat, skin lesions, disease, Turkey*

Introduction

Contagious ecthyma is a parapox virus which affects domestic sheep and goats around the world (Samuel et al., 1975; Yirrell et al., 1989; Wilson and McFarlane, 2012; Peralta et al., 2018; Tedla et al., 2018). It is characterized to have a benign nature, and it can cause a large tumor like vascularized lesions which can be treated using antiviral drugs or removed surgically. It is transmitted through direct contact with infected animals and environmental contamination (Tedla et al., 2018). CE is also a zoonosis of global distribution that affects humans, particularly animal workers, like slaughtermen, veterinarians, farmers, and animal caretakers, after direct or indirect contact with infected animals (Peralta et al., 2018). It has a linear double-stranded DNA genome, of ~135 kbp in size and exhibits an unusually high GC content (~64%). The central region of the genome contains 88 genes which are present in the subfamily *Chordopoxvirinae* and mostly occur in a common order and orientation. The terminal regions of the genome are variable and there are genes associated with virulence, pathogenesis, tropism, and/or immune response modulators (Fleming and Mercer, 2007; Lateef et al., 2010; Peralta et al., 2018). Clinical CE is generally named as contagious pustular dermatitis, orf, soremouth, or scabby mouth. The illness leads to pustules followed by scabbing on the muzzle, face, and lips of ruminants (Wilson and McFarlane, 2012; Tedla et al., 2018). Mortality and morbidity is changeable (Housawi et al., 1992; Elzein and Hausawi, 1997; Haig and Mercer, 1998; Nandi et al., 2011; McInnes, 2014). The reason of CE is Orf virus which is type species of the genus *Parapoxvirus* (Webster, 1958; Delhon et al., 2004; Mercer et al., 2006; McGuire et al., 2012; Li et al., 2012). The virus in dried scabs survived from 8 months to more than a

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year at room temperature (Manley, 1934; Newsom and Cross, 1934). Crusts exposed to external conditions lost their infectivity quickly during summer, but infectivity persisted for at least 6 months during winter (Boughton and Hardy, 1935). The disease may be caused by direct or indirect contact with the infected animals especially with dried crusts fallen on the pastures during grazing (Harriss, 1948; Kummeneje and Krogsrud, 1979). The disease was also transmitted from wild to domestic goats when infected herds had prolonged contact with salt blocks in the mutual habitat. The disease from infected sheep and goats has been transmitted to other species of animals by feeding them raw sheep carcasses (Wilkinson et al., 1970). Contagious pustular dermatitis of sheep and goats was reported initially by Zellor in 1920 from South West Africa. Since then, it has been reported from almost all parts of world involving not only sheep and goats but cattle, dogs, camel and both free living wild and captive animals also. Lesions can also form inside the mouth, on the coronary band above the hooves, on the udder, or on the genitalia. The incubation period from exposure to the virus until development of clinical signs is 1 to 2 weeks (Merck Veterinary Manual, 2016). CE is not usually lethal, and lesions typically disappear within 2 to 4 weeks, but death may occur if secondary complications, such as bacterial infections or myiasis, develop. Additionally, lesions can be quite painful and hinder feeding in adults or nursing in lambs and kids, leading to emaciation and death, depending on the severity of the infection (Davis et al., 1970). CE is infectious to humans and is considered an occupational hazard for shepherds, with lesions typically found on the hands as a result of direct contact with animal lesions (Uzel et al., 2005; CDCP, 2006). However, not all CE cases in humans are confined to those in contact with sheep or goats (Ballanger, 2006). CE viruses spread from one region to other region which has been occurred by free movements of animals (Mayenga, 2015). The diagnosis of CPD is mainly based on benign nature of the disease and characteristic skin lesions (Rodrignez et al., 1983; Coates and Hoff, 1990). Vaccination coupled with biosecurity measures is an effective method in the prevention and control of CE. Suspected CE can be diagnosed based on the characteristic clinical signs, followed by laboratory tests such as electron microscopy, serum neutralization tests (SNT), histopathology of affected tissues and nucleic acid assay like polymerase chain reaction (PCR) (Nandi et al., 2011; Adeleji et al., 2018).

Materials and methods

Sarıçiçek Mountain is located within the borders of Şebinkarahisar and Çamoluk districts (40°19’50.18” N 38°29’55.53” E) in Giresun province part of Eastern Black Sea Region (*Fig. 1*). The study area is 21765 ha and between 1000 and 1800 m elevations on average. On majority of lands of Sarıçiçek Mountain, the dominant climate is continental. Winters are long and harsh, and summers short and hot. Annual mean temperature is between -15 and +32 °C. Annual mean precipitation is between 500 and 700 mm. Pine (*Pinus sylvestris*) and oak (*Quercus* sp.) forests and alpine steppes have a wide spread in the study area. The study area is one of the most important habitats of about 1500 wild goat in the Eastern Black Sea Region.

This study started as a result of reporting the Orf virus symptoms in wild goat individual by wildlife officers in Sarıçiçek Mountain, on February, 2016. Field studies were carried out between 2016 and 2017 years. Direct observation methods were used in the field studies. Camera traps, binoculars and spotting scopes were utilized along with direct observation. Camera trapping was applied to the wild goat population

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density areas during the study period in order to determine diseased wild goats. Also, we investigated the carcasses of wild goats perished due to CE in the study area.

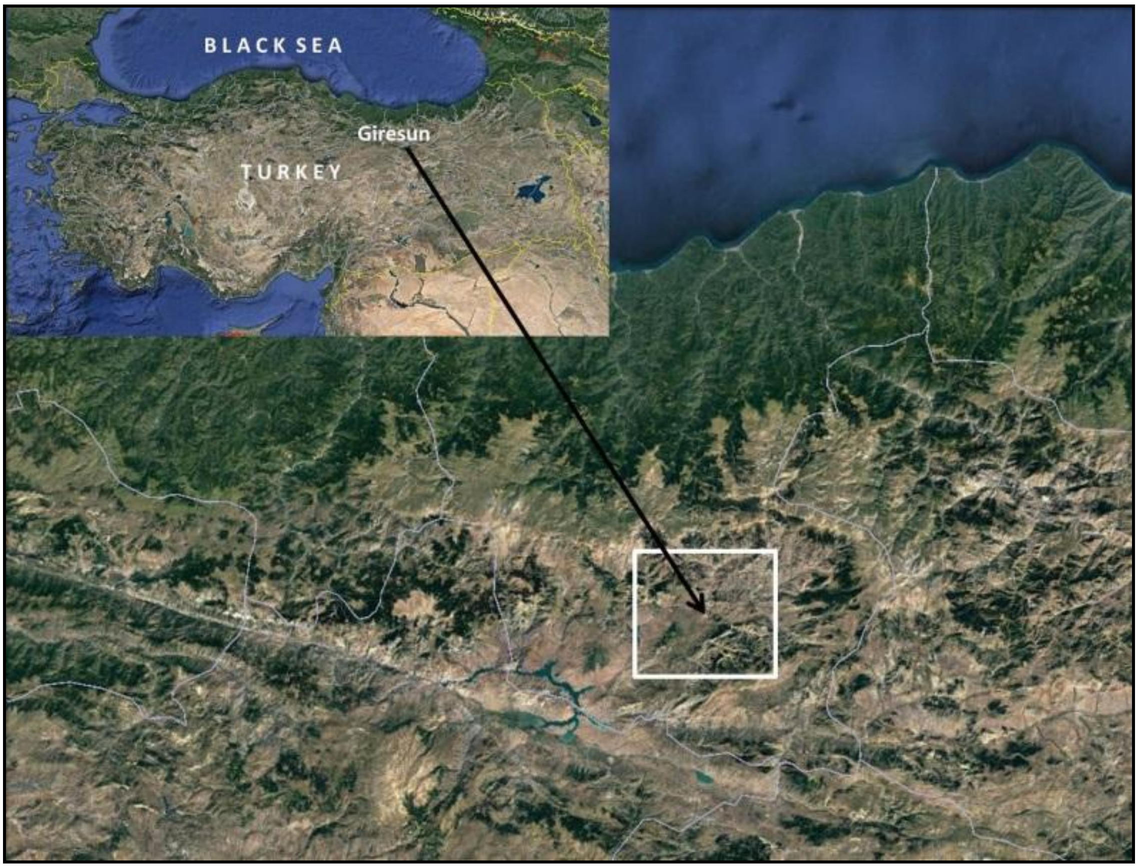

Figure 1. *Location of the study area (GoogleEarth, 2018)*

Results and discussion

As a result of this study, one wild goat individual observed ulcerated papular lesions, including frank red blood on the lips and muzzle with marked swelling and crusting around all edges of the lesions (*Fig. 2*). Histopathological sections were made of the junction between normal skin and the lesions on the lips and muzzle. The remainders of the carcasses were removed from the research area and were buried.

At the end of the study, 442 camera-trap photos belonging to wild goats were obtained and on two wild goat individuals were observed with ulcerated papular lesions, including frank red blood on the lips and muzzle with marked swelling and crusting around all edges of the lesions. Also a dead wild goat was found. This carcass were removed from the research area and were buried (Fig. 2).

Skin lesions of the dead lambs were determined as characteristic CE lesions (poor body condition with bloody lesions covering the muzzle area and warts in the mouth periphery) in our observations. Typical CE viral particles were seen by electron microscopy. Sections of haired skin from the sample had diffuse acanthosis and hyperkeratosis, with numerous intracorneal vesicles and pustules. Moderate numbers of bacterial colonies were observed among superficial keratinized epithelial cells. There was multifocal intracellular edema of keratinocytes of the stratum spinosum. The

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superficial dermis was infiltrated with numerous lymphocytes, plasma cells, neutrophils, and macrophages. The history, gross lesions, and microscopic lesions histopathologically confirmed the diagnosis of CE. The lesions were considered pathognomonic, based on the findings reported literature (Ishii, 1953; Merwin and Brundige, 1982; Housawi et al., 2012).

Figure 2. *Orf virus infection is evident on the muzzle of wild goat in Sarıçiçek Mountain*

It has been reported that lambs may suffer the most from CE infections (Blood, 1971; Samuel et al., 1975; Lance et al., 1981; Merwin and Brundige, 1982; Zarnke et al., 1983) and no signs of CE were reported in previous observations and counts of lambs in Sarıçiçek Mountain. Because this report represents the first documented case of CE about wild goat in Turkey, it is unknown whether the previous low lamb production in this population may have been associated with CE. It is unlikely that the original population was the source of this outbreak of CE, given the short incubation period of 1 to 2 weeks for orf (Merck Veterinary Manual, 2006) and that no diagnoses of CE were made and no clinical signs of orf were reported by wildlife officers or the public for 30 years. It also has been reported that CE may be an important factor for mountain goat

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known to be infected with high numbers of helminths, bacterial infections, or to be in states of poor nutrition (Samuel et al., 1975).

Conclusion

The studies on CE in Turkey are mainly case reports in the form of single or small groups reported in humans and domestic animals. There is no information about the presence of Orf virus in wild goats due to limited studies about wild animal diseases in Turkey.

The Orf virus is an important contagious disease that can lead to outbreaks and deaths, especially in young and low-immune wild goats. For this reason, detection, isolation, vaccination and separation of wild goats, which are the source of infection, will prevent both new cases and economic losses.

The study area is one of the most important wild goat hunting tourism areas in Turkey. The area is also used extensively by the villagers and shepherds. As the disease is transmitted from infected animals, products or contaminating materials, people who are in contact with these animals or products should be informed about the personal preventive measures. Also, identifying infectious diseases and vaccinations of diseased animals will prevent the spread of the disease.

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DETERMINATION OF BIO-VARIATION AMONG DIFFERENT MULBERRY SPECIES GROWN IN TOKAT REGION OF TURKEY BY MOLECULAR MARKERS

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Abstract. The aim of this research is to determinate diversity of mulberries grown in North Anatolia between or within species by ISSR markers. In the study, 38 different mulberry genotypes collected from Tokat region in Turkey were used. ISSR-PCR analyses were carried out on the DNA of genotypes isolated using mini-CTAB extraction method. The levels of polymorphism between genotypes were determined using the UBC-ISSR primers. A total of 96 bands were obtained from 15 UBC ISSR primers. Out of 96 bands, 80 bands were polymorphic. The number of bands obtained per primer ranged between 4 and 11, the average number of bands were determined as 6.4. The average number of polymorphic bands per primer was 5.33. Similarities and differences between genotypes have been studied at the molecular level. The data used for statistical analysis were obtained by the evaluation of ISSR bands. Similarity coefficient and UPGMA dendrogram were built using the Basic Coordinates Analysis. According to the dendrogram, the genotypes have been divided in two main groups, one small and one large group. While the small group was only comprised of black mulberry genotypes, the large group included white mulberry, weeping mulberry and wild white mulberry genotypes. Different mulberry species have been divided into different subgroups within the large group. The polimorfism level within the species was lowest in black mulberry genotypes, and this was followed by weeping mulberry, white mulberry and wild white mulberry genotypes, respectively.
Keywords: *similarity, diversity, dendrogram, North Anatolia, mulberry*

Introduction

Mulberry is a perennial fruit species belonging to the genus *Morus* of the family Moraceae (Datta, 2002; Anonymous, 2006). *Morus*, a genus of flowering plants in the family Moraceae, deciduous trees commonly known as mulberries, grow wild and under cultivation in many temperate world regions. The origin of mulberry is Asia (Awasthi et al., 2004). Over 150 species names have been published, and although differing sources may cite different selections of accepted names, only 10–16 are generally cited as being accepted by the vast majority of botanical authorities. *Morus* classification is even further complicated by widespread hybridisation, wherein the hybrids are fertile. Mulberry trees are either dioecious or monoecious and sometimes will transform from one sex to another. The flowers are held on short, green, pendulous, nondescript catkins that emerge in the axils of the current season's growth and on spurs on older wood. They are wind pollinated and some cultivars will set fruit without any pollination (Anonymous, 2009).

Black, red, and white mulberry are widespread in southern Europe, the Middle East, northern Africa and Indian subcontinent, where the tree and the fruit have names under regional dialects. The most important mulberry species are *Morus alba* (white

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mulberry), *Morus australis* (Chinese mulberry), *Morus indica* (Indian mulberry), *Morus microphylla* (Texas mulberry), *Morus nigra* (Black mulberry), *Morus rubra* (Red-purple mulberry) and *Morus serrata* (Himalian mulberry) (Tutin, 1996; Vijayan et al., 2004). It is sometimes difficult to distinguish mulberry species morphologically and pomologically from each other, especially the fruits of *Morus nigra, Morus rubra* and *Morus pendula* species, those with black fruits. In fact, cheaper black fruits are sometimes sold in markets instead of *Morus nigra* fruits in high prices (Günes and Cekic, 2004; Erdogan and Pirlak, 2005). Therefore, determining the difference in the species and between species at the molecular level is important in terms of consumers as well as in terms of scientific literature.

The mulberry plants are deciduous and are produced for their fruit and leaves in all parts of Turkey (Anonymous, 2009). The species *M. alba*, *M. nigra* and *M. rubra* are common in Turkey (Ercisli, 2004; Ercisli and Orhan, 2007; Özgen et al., 2009). The main mulberry production areas of Turkey are Black Sea Region and Eastern and Central Anatolian Regions. Turkey has very old mulberry cultivation, and mulberries are one of the main fruits grown by Turkish farmers. The four mulberry species (*Morus alba, Morus nigra, Morus rubra*, *Morus laevigata*) can be seen in different agro-climatic regions in Turkey. There is no registered mulberry cultivar in Turkey but each region has its own local genotypes which are propagated by budding or grafting over many years (Ercisli and Orhan, 2007).

Tokat Province of Turkey, at the mid Black Sea region of Anatolia, has a hot-summer Mediterranean climate with considerable maritime and continental influences. Most parts of province are suitable for mulberry production, and some species of mulberry spread over the province. Although there is no intensive mulberry production by producers, one or two mulberry trees can be seen almost in every orchard, especially near by homes.

The aim of this research is to determinate diversity of mulberries grown in North Anatolia (Tokat Province) between or within species by ISSR markers.

Material and methods

Plant material

For research purposes, a total of 38 different mulberry genotypes from Tokat region in Turkey were used (*Table 1; Fig. 1*). Out of total number 15 belongs to black (*Morus nigra*), 13 to white (*Morus alba*), 5 to pendulous (*Morus pendula*), 2 to everbearing mulberry (*Morus alba*) and 3 to wild mulberry (*Morus alba*).

Figure 1. *The localization of Tokat province in Turkey, where the samples were collected*

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Table 1. *The latitudes, longitudes and elevations of the samples taken*

	Sample code	Elevation (m)	Latitude	Longitude
1	K1	608 m	40° 20.014N	36° 31.065E
2	K2	730 m	40° 18.863N	36° 32.191E
3	K3	679 m	40° 18.964N	36° 32.925E
4	K4	700 m	40° 18.307N	36° 33.092E
5	K5	671 m	40° 17.390N	36° 33.017E
6	K6	689 m	40° 17.860N	36° 33.454E
7	K7	677 m	40° 18.229N	36° 33.682E
8	K8	589 m	40° 20.551N	36° 31.812E
9	K9	667 m	40° 21.387N	36° 31.565E
10	K10	658 m	40° 21.336N	36° 31.582E
11	K11	613 m	40° 20.356N	36° 33.272E
12	K12	615 m	40° 20.317N	36° 33.321E
13	K13	649 m	40° 20.497N	36° 34.120E
14	K14	2211 m	40° 17.269N	36° 33.065E
15	K15	2214 m	40° 17.266N	36° 33.064E
16	B1	730 m	40° 18.804N	36° 32.169E
17	B2	676 m	40° 18.506N	36° 32.925E
18	B3	659 m	40° 18.024N	36° 33.086E
19	B4	740 m	40° 17.423N	36° 33.136E
20	B5	589 m	40° 20.551N	36° 31.812E
21	B6	617 m	40° 20.442N	36° 32.796E
22	B7	615 m	40° 20.391N	36° 33.175E
23	B8	611 m	40° 20.225N	36° 33.341E
24	B9	618 m	40° 20.219N	36° 34.123E
25	B10	2214 m	40° 17.266N	36° 33.063E
26	B11	2185 m	40° 17.293N	36° 33.016E
27	B12	2211 m	40° 17.269N	36° 33.065E
28	B13	2270 m	40° 17.504N	36° 33.539E
29	S1	2270 m	40° 17.504N	36° 33.539E
30	S2	589 m	40° 18.804N	36° 32.169E
31	S3	658 m	40° 18.506N	36° 32.925E
32	S4	619 m	40° 18.024N	36° 33.086E
33	S5	626 m	40° 17.423N	36° 33.136E
34	Y1	679 m	40° 18.964N	36° 32.925E
35	Y2	667 m	40° 21.387N	36° 31.565E
36	YB1	659 m	40° 18.024N	36° 33.086E
37	YB2	619 m	40° 20.456N	36° 32.363E
38	YB3	618 m	40° 18.024N	36° 33.086E

DNA extraction

Total genomic DNA was extracted according to the CTAB method (Doyle and Doyle, 1987). DNA sample concentration was determined using a fluorometer employing a Hoechst dye (Hoefer Inc., San Francisco, CA, USA), and the DNA

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samples were diluted to a final concentration of 10 ng/µl with 1 × TE buffer and stored at −20 °C prior to polymerase chain reaction (PCR) amplification.

ISSR amplification

15 primers that produced clear and reproducible fragments were selected out of a hundred UBC primers, which were previously tested for further analyses (*Table 2*). ISSR amplification was performed in a 20 µl volume containing 20 ng genome DNA, 1 × Taq buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 µM primer, 0.5 units of Taq DNA polymerase. The amplification reaction consisted of an initial denaturation step at 94 °C for 5 min, followed by 45 cycles of 45 s at 94 °C, annealing at 50–56 °C for 45 s, extension at 72 °C for 90 s, and ended with extension at 72 °C for 7 min. The amplified products were resolved electrophoretically on 2.0% agarose gels run at 100 V in 1.0 × TBE buffer, visualized by staining with ethidium bromide (0.5 µg/ml), and photographed under ultraviolet light (Charters et al., 1996; Rafalski et al., 1996; Cekic et al., 2001) The amplifications were repeated twice and only clear repetitive bands were used in data analysis, and molecular weights were estimated using a 100 bp DNA marker (Vivantis).

Table 2. *Band number, polymorphic band number and polymorphic band ratio (%) in mulberry genotypes*

	ISSR primer code	Primer sequence and anchors	Band number	Polymorphic band number	Polymorphic band ratio (%)
1	807	AGAGAGAGAGAGAGAG**T**	11	9	81.81
2	808	AGAGAGAGAGAGAGAG**C**	5	5	100.00
3	810	GAGAGAGAGAGAGAGA**T**	8	6	75.00
4	811	GAGAGAGAGAGAGAGA**C**	7	6	85.71
5	826	ACACACACACACACAC**C**	6	6	100.00
6	835	AGAGAGAGAGAGAGAG**YC**	7	6	85.71
7	841	GAGAGAGAGAGAGAGA**YC**	6	4	66.67
8	842	GAGAGAGAGAGAGAGA**YG**	10	9	90.00
9	844	CTCTCTCTCTCTCTCT**RC**	4	3	75.00
10	856	ACACACACACACACAC**YA**	4	3	75.00
11	881	GGGTGGGTGGGTGGGT	5	4	80.00
12	888	**BDB**CACACACACACACA	7	6	85.71
13	889	**DBD**ACACACACACACAC	4	3	75.00
14	890	**VHV**GTGTGTGTGTGTGT	7	6	85.71
15	891	**HVH**TGTGTGTGTGTGTG	5	4	80.00
Average			6.4	5.33	82.76
Total			**96**	**80**	

Data analysis

The amplified fragments were scored for band presence (1) or absence (0) with all the accessions studied. Similarities and differences between genotypes have been studied at the molecular level. The data used for statistical analysis were obtained by the

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evaluation of ISSR bands. Similarity coefficient and UPGMA dendrogram were built using the Basic Coordinates Analysis (Rolf, 1992).

Results and discussion

On the basis of the number, intensity and reproducibility of ISSR bands 15 primers were selected out of a hundred UBC primers, which were previously tested (Cekic et al., 2001). Bands with the same mobility were treated as identical fragments. Weak bands with negligible intensity and smear bands were both excluded from final analysis. Each primer was evaluated for total band number (TBN), polymorphic band number (PBN) and the ratio of polymorphism (PR= PBN/TBN X 100).

The number of scored bands varied from four to eleven with an average of 6.4 bands per primer and an average of 5.33 polymorphic bands per primer. In total, 80 bands out of 96 derived from 15 primers were polymorphic (*Table 2; Fig. 2*).

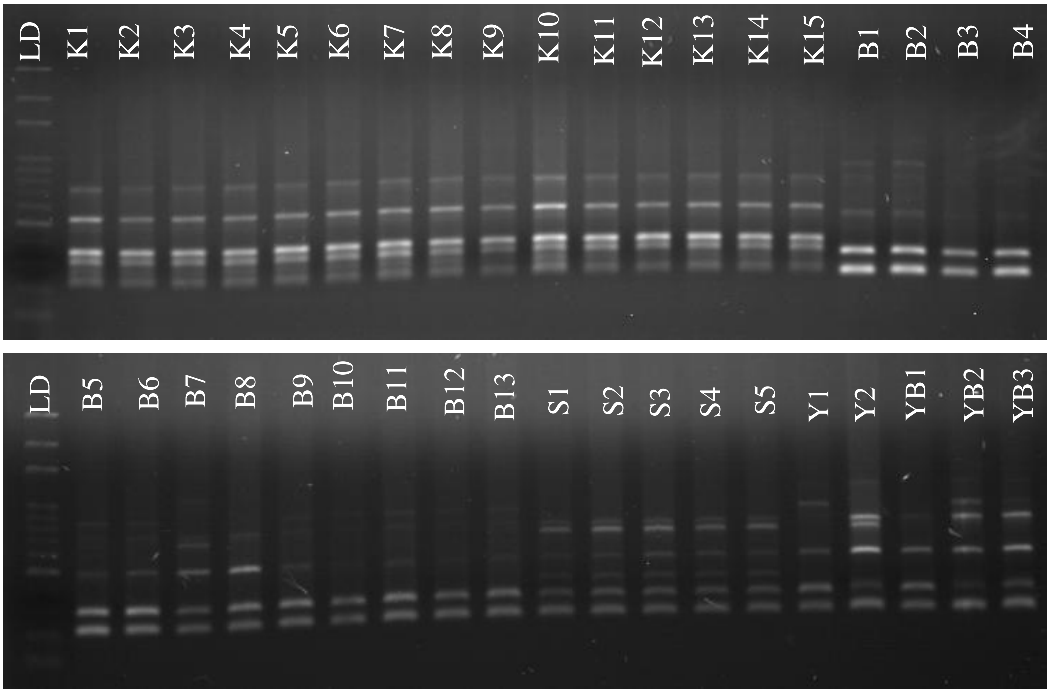

Figure 2. *Agarose gel of polymorphic DNA amplification profiles of different mulberry genotypes (K: Black mulberry, B: White mulberry, S: Pendulous mulberry, Y: Everbearing mulberry (long season harvesting), YB: Wild mulberry) obtained with UBC ISSR primer 811. (LD: 100 bp LADDER -Vivantis)*

Cluster analysis

Genetic similarity among varieties was estimated using dissimilarity coefficient matrix based on ISSR bands scored. Pairwise values of dissimilarity coefficients ranged from 0.27 for genotypes with the same scored bands to 1.00 for the most similar genotypes. The dendrogram was constructed based on the similarity matrix, using UPGMA method (Rohlf, 1992). The 38 mulberry genotypes were divided into two main clusters (*Fig. 3*), in which black mulberry genotypes in one group and the rest of the genotypes falling under the other group. While the first major group contained only

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black mulberries, the second major cluster was further separated into subgroups. The first subgroup contained black mulberry genotypes, respectively which showed very close ISSR profile exhibited the highest genetic similarity ranges (0.95-1.00). On the other hand, the similarity of the second varied 0.75 to 0.96, in which different mulberry species (White mulberry, Pendulous mulberry, Everbearing mulberry, Wild mulberry) separated in different clusters at various stages (*Fig. 2*).

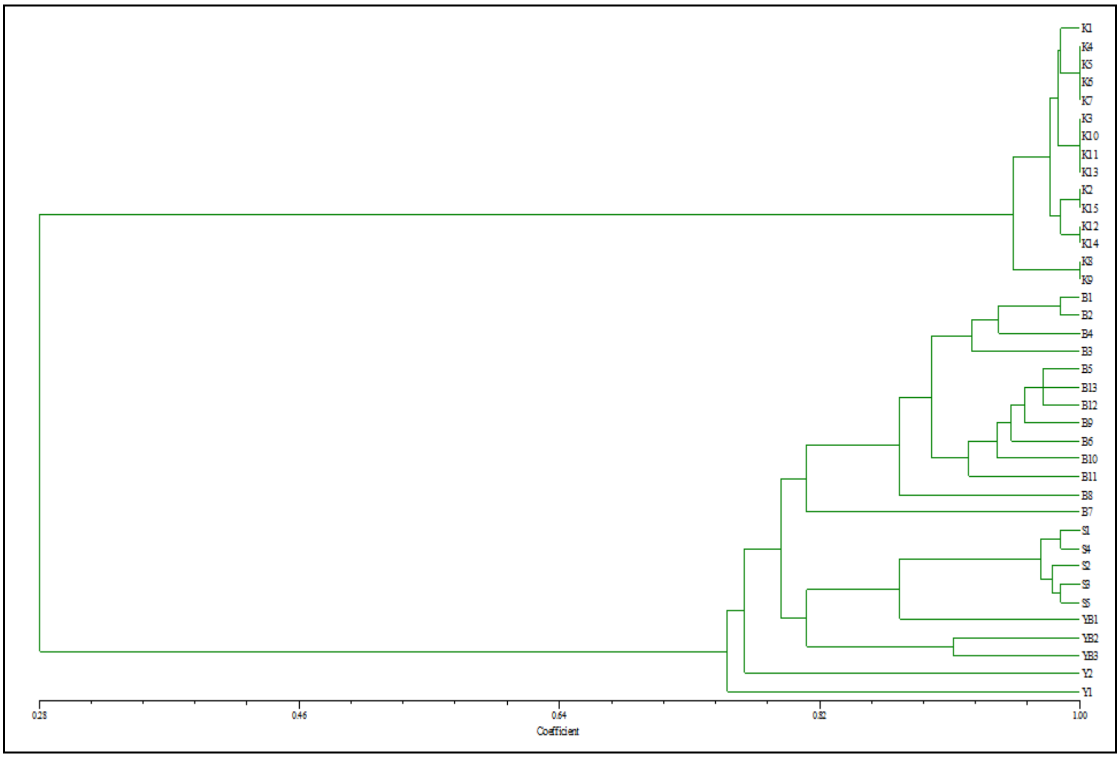

Figure 3. *Dendrogram showing genetic relationships among value of ISSR markers for mulberry genotypes (K: Black mulberry, B: White mulberry, S: Weeping (Pendulous) mulberry, Y: Everbearing mulberry (long season harvesting), YB: Wild mulberry)*

While the small main group in the dendrogram only consists of black mulberry genotypes, the major groups include white mulberry, weeping mulberry, everbearing and wild white mulberry genotypes. However, in the major group, mulberry genotypes belonging to different species constituted their subgroups. The level of polymorphism between black mulberry genotypes was very low. While no polymorphism was observed in the great majority of 15 genotypes of black mulberry genotypes, the lowest similarity level in this species is 94% in dendrogram. The lowest level of similarity within the other species were 96% in the weeping mulberry, 80% in the white and wild white mulberry and 75% in the everbearing mulberry genotypes.

The wild and everbearing white mulberry genotypes were most likely grown from seed. Therefore, these mulberry genotypes showed the least similarities and the greatest polymorphism was obtained from these genotypes. Although 100% similarities were not obtained within the group, polymorphism was low in weeping mulberry genotypes. Significant differences were determined within the group in white mulberry genotypes, and 100% similarities were not observed. The majority of black mulberry genotypes were similar to each other, most of which can be caused by vegetative propagation from

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a single source by grafting, cutting or dipping. As a matter of fact, previous studies indicate that seeds of black mulberry genotypes hardly germinate without any pre-treatment (Güneş and Çekiç, 2004). In addition, this example is true because the sample area is narrow and there is only one or two black mulberry trees in producer gardens. The results of this study will then shed light on the work to be done for vegetative propagation in this area. Also, the principle component analysis generally put the genotypes into different clusters as parallel to morphological differences of the mulberry species (*Fig. 4*).

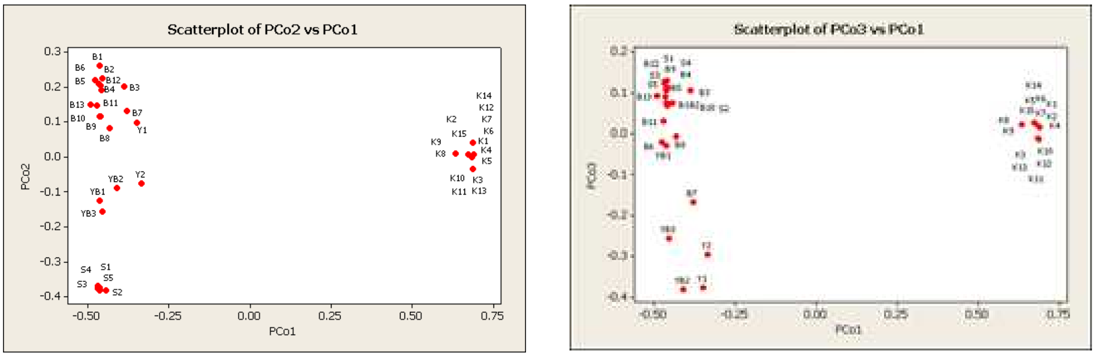

Figure 4. *The principle component analysis of mulberry genotypes according to 15 ISSR primers*

Conclusion

The results of the study mean that molecular tools are more reliable than the phenotypic observations as the well-known fact. Screening of the 38 genotypes of different mulberry species revealed that banding profiles obtained with fifteen ISSR primers were enough to distinguish the diversity between species or within the species. The results indicated that the ISSR technique is effective to develop genotype-specific banding patterns valuable for genotype identification. Since ISSR-PCR technique does not require previous DNA sequence information and uses very small quantity of DNA, it is considered as one of the most widely used techniques for genotype identification and genetic diversity studies. These results mean that molecular tools are more reliable than the phenotypic observations for evaluating variations and monitoring genetic stability.

The wide variation in genetic distance among the different mulberry species revealed by ISSR techniques reflected a high level of polymorphism at the DNA level. The genetic similarity of different mulberry species is low as indicated ISSR analyses. The variability in black mulberry genotypes within the species has been characterized as quite low. We can conclude that the saplings of black mulberry were mostly obtained by budding or cutting from local sources. There are some flows in other mulberry species from outside the province as we can see wide variation within the species.

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MIGRATION AND RETENTION OF PHENOL IN THREE NATURAL SOILS AND EFFECT OF SOLVENT

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Abstract. Soil contaminated with phenol poses a serious threat to the groundwater and soil organisms. In this paper, column transport experiments were used to investigate the retention and the mobility of phenol in three types of natural soils from northern Algeria. The effect of methanol, as an organic solvent, on migration of phenol through soil columns was also investigated. Transport parameters such as the retardation factor and the partition coefficient were calculated using "Laboratory Flow-Through". The use of "Laboratory Flow-Through" method showed that phenol was more retained in Corso: a soil with higher organic matter content (7.22%) than Chorfa and Maâtkas (2.72 and 1.87%, respectively) and the retention increased with soil depth. The retardation of the transport of phenol was reduced in the presence of methanol which increased remarkably the mobility of phenol and its retention was much reduced especially at a depth of 5 cm by 25.55% in Corso, 19.05% in Chorfa and 9.18% in Maâtkas.
Keywords: *Laboratory Flow-Through (LFT) method, methanol, mobility, soil pollution*

Introduction

Phenolic compounds are persistent organic pollutants which have been widely used in chemical plants, metallurgical plants and petroleum refineries (Comninellis and Pulgarin,1991; Li et al., 2005; Zhang et al., 2011), with a great variety of functions, in the production of many and different products: plastics, explosives, medicines, paints, detergents, pesticides, anti-oxidants, among others; in fact, phenol is considered among the most serious environmental contaminant which can affect air, groundwater, surface water and soil. It is very harmful even at low concentrations (Din et al., 2009; Pal et al., 2011), since it is toxic, carcinogenic, teratogenic and mutagenic to humans (Zhang et al., 2011).

Phenol is a colourless to light pink crystalline solid liquefies upon contact with water. It is moderately volatile at room temperature and has an acrid odour (Earl et al., 2003). Generally phenol is soluble in most organic solvents and it has a high solubility within water (from 67 g L^{-1} at 16 °C to 84 g L^{-1} at 20 °C) (Earl et al., 2003). Phenol in the aqueous phase is subject to chemical oxidation and adsorption (Sivasubramanian et al., 2012) and it is readily biodegraded under aerobic conditions in all environmental media, including soil and sediment; however, phenol degradation is slower under anaerobic conditions than under aerobic conditions and acclimation times are longer (Earl et al., 2003). A review of biodegradation studies in groundwater indicates that phenol is degraded under methanogenic, sulphate-reducing, iron-reducing and nitrate reducing conditions, with a half-life range of 22 to 533 days (Earl et al., 2003). As a result and based on its low adsorptivity to soil, phenol trickles down through different strata and migrates along with groundwater flow due to tortuosity and favourable

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hydraulic conductivity of soil (Pal et al., 2014); however, information on levels of phenol in soil is limited (Earl et al., 2003).

Natural soil means soil from the ground (without further modification in the composition), which is distinguished from commercial potting soil. It is composed of mineral component and organic material or humus. Scientists usually test disturbed natural soil samples for soil texture and type and contaminant analysis, among other evaluations; it is easier to collect.

Phenol migration through natural soil has already been experimentally studied by researchers using laboratory batch adsorption test and vertical soil column tests. Smith et al. (1999) showed that phenol was relatively mobile in loamy sand and organic carbon content was found to be the most important soil component controlling retention potential of soils followed by clay mineral content (Khan and Anjaneyulu, 2005; Pal et al., 2014). Different studies showed that addition of other compounds may have an effect on phenol mobility. It is anticipated that the addition of Heavy metals may substantially alter pollutants mobility (Smith et al., 1999); however, compounds such as organic solvents, which were widely used to extract organic contaminants from contaminated soils (Osman and Saim, 2013), may have the opposite effect by increasing the mobility of phenol in soil. Nevertheless, experimental studies of the effect of methanol on the phenol vertical migration through soil media are lacking.

This paper aims to study retention and migration of phenol in three types of natural soils from northern Algeria. For this study, column experiments were used. “Laboratory Flow-Through” (LFT) method (United States, 1999) was used to determine the important parameters governing phenol migration. The effect on phenol mobility of the use of methanol, as an organic solvent, was also established.

Materials and methods

Sampling and soil characterization

Three types of soil were taken from three different regions of northern Algeria, namely, Chorfa (CH) situated in the province of Bouira (SE Algiers), Corso (CO) situated in the province of Boumerdès (E Algiers) and Maâtkas (MA) situated in the province of Tizi Ouzou (E Algiers). These three regions are representative of northern Algeria, as 90% of the population living in northern regions which are most likely to be polluted by phenol used in industrial and agricultural activities especially pesticides. Fifteen (15) soil samples were collected, few hours before the characterization, from multiples spots of each site, by using an Edelman hand auger and then mixed. Only the top 0-20 cm soil layer was sampled. In the laboratory, soil was air dried at ambient temperature, pre-treated by removing any large pieces of debris (e.g. Gravel, plant residues, etc.), gently crushed to break aggregates, homogenized and then screened through a 2 mm stainless steel sieve prior to analysis. These analyzes do not lead to any changes in the composition of the soil sample; therefore, it is still natural. The particle size distribution of soil was measured by laser diffraction (Malvern Mastersizer 2000 instrument). Soil textures were determined according to USDA textural classes (United States, 1993). Organic matter content (OM) was measured by calcination at 550 °C. Organic carbon content (OC) was estimated by dividing the organic matter content by assumed constant conversion factor 1.724; in addition porosity and bulk density (ρ) were also determined.

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Chemicals

Phenol, C_6H_6O was obtained in crystal form (99% purity) from Merck Co. (Darmstadt, Germany). Its solubility in water is 76.04 g L^{-1}. Two stock solutions of phenol were prepared by dissolving 70 mg of phenol in 1000 ml of distilled water for the first one and 70 mg of phenol in 1000 ml of a mixture of methanol/distilled water (50:50, v/v) for the second to examine the effect of methanol, as an organic solvent, on transport of phenol. 100 mg of Mercury (II) Chloride ($HgCl_2$) was added to each stock solution as biocide to avoid phenol degradation. As phenol degrades when in contact with air and sunlight, care was taken to keep the stock solutions away from both. $NO3^-$ [KNO3] is used as inorganic non-reactive tracer, prior to each non-conservative phenol transport experiment to test columns performance and to determine retardation factor (R) of phenol within soil columns. Nitrate is widely used as tracer to study solute mobility and transport process (Braun et al., 2015; Zhen et al., 2015). A solution of KNO3 (100 mg L^{-1}) was prepared using distilled water.

Column leaching experiments

Column leaching experiments were carried out in eighteen vertical propylene columns (1.3 cm inner diameter × 6.5 cm length), containing CH-soil for six of them, CO-soil for six others and MA-soil for the last six ones. Nine of these columns were used to study the transport of phenol dissolved in distilled water and the last nine ones were used to study the transport of phenol dissolved in a mixture of methanol/distilled water (50:50, v/v). A 2 mm thick polystyrene filter was inserted in the base of each column. This filter also served as support for the soil at the bottom of the column. Negligible adsorption of phenol onto the column material was observed in phenol tracer test of the empty system. The columns were packed with soil in layers and were shaken to insure uniform mixing between layers. The soil was approximately 1.7, 3.3 and 5 cm thick for each type of soil (*Fig. 1*).

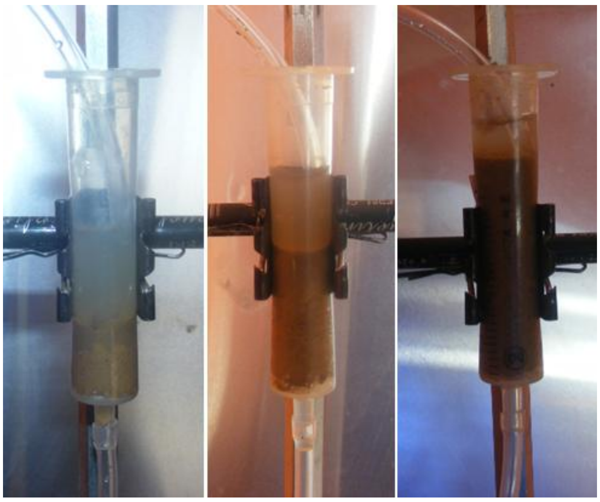

Figure 1. *Soil columns: 1.7 cm thick (left), 3.3 cm thick (middle) and 5 cm thick (right)*

The tracer solution containing $NO3^-$[KNO3] (100 mg L^{-1}) was applied on the top of each first nine soil columns at a constant pressure head under steady state flow conditions, allowing the influents to flow through the soil under positive pressure head

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(Rodriguez-Liebana et al., 2014). The transport of phenol dissolved in distilled water (70 mg L^{-1}) was performed in the same way as the transport of tracer and in the same soil columns. Transport of phenol dissolved in a mixture of methanol/distilled water (50:50, v/v) (70 mg L^{-1}) was performed in the last nine soil columns in the same way as the transport of the two first solutions to investigate the effect of methanol on phenol transport. Leachate samples were collected and analyzed for $NO3^-$ and phenol concentrations by UV-VIS spectrophotometer at wavelength of 220 nm and 510 nm, respectively. The pore volume (PV) or V_0 of the packed columns was estimated as follows: V_0 = column total porosity× column volume. The breakthrough curves (BTCs) of $NO3^-$ and phenol from the packed columns were generated by plotting the relative concentration (concentration of effluent/ concentration of influent: C/C_0) in the leachate versus the relative pore volume (V/V_0) (*Fig. 2*).

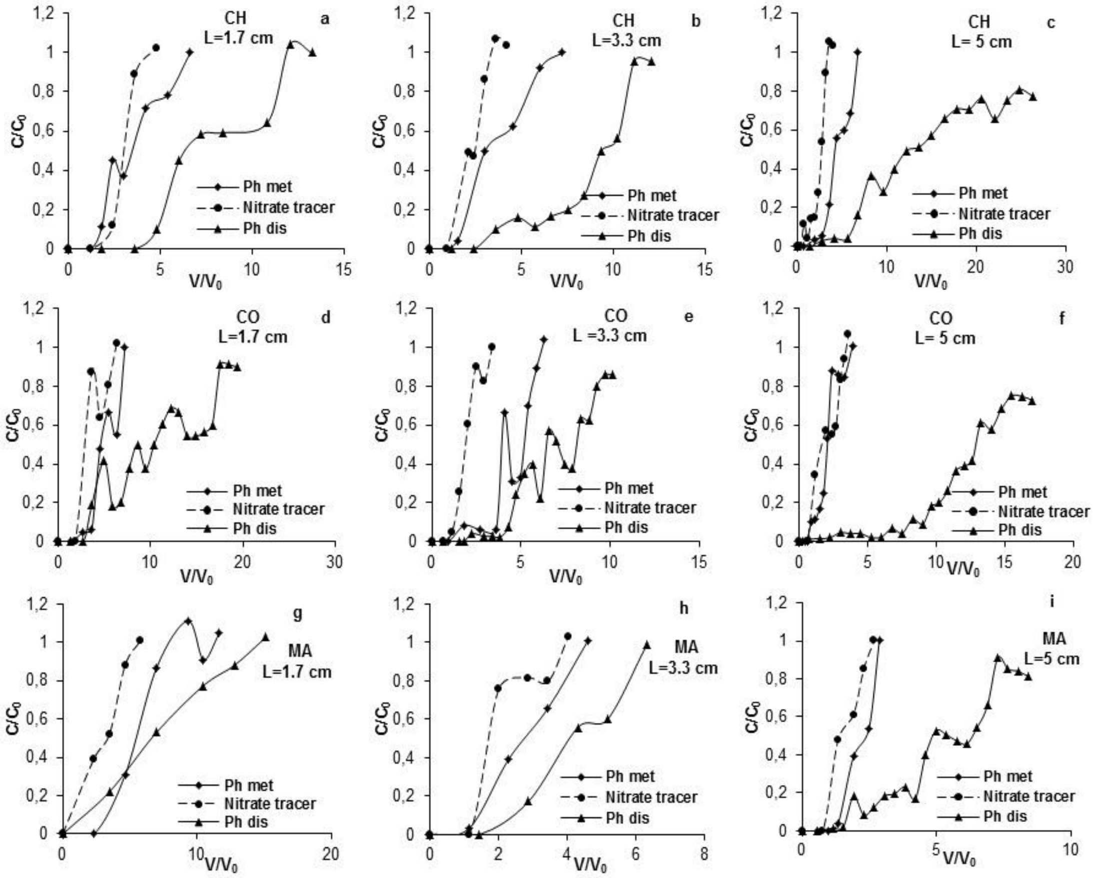

Figure 2. *Measured breakthrough curves of phenol and tracer for various soil columns at different depths: CH at L = 1.7 cm (a); CH at L = 3.3 cm (b); CH at L = 5 cm (c); CO at L = 1.7 cm (d); CO at L = 3.3 cm (e); CO at L = 5 cm (f); MA at L = 1.7 cm (g); MA at L = 3.3 cm (h); MA at L = 5 cm (i). Ph_{dis} and Ph_{met} refer to phenol dissolved in distilled water and phenol dissolved in a mixture of methanol/ distilled water (50:50, v/v), respectively*

Mathematical modelling

The solute transport model, which is considered the dominant mechanism of pollutant transport in porous media, was used in our study. This model is based on the one-dimensional convection-dispersion Richards equation (*Eq. 1*):

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$$R\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} - v\frac{\partial C}{\partial x} \quad \text{(Eq.1)}$$

C is the solute concentration in the aqueous phase [ML^{-3}], t is the time [T], D is the hydrodynamic dispersion coefficient [L^2T^{-1}], X is the spatial coordinate [L], and v is the average pore water velocity [$L\ T^{-1}$].

The retardation factor (R) can be defined as (*Eq. 2*):

$$R = 1 + \frac{\rho}{\theta} K_d \quad \text{(Eq.2)}$$

where θ is the volumetric water content [$L^3\ L^{-3}$], ρ is the bulk density of soil [ML^{-3}] and k_d is the partition coefficient [L^3M^{-1}] describing the partitioning of aqueous phase constituents to a solid phase.

For a solute transport without adsorption: $k_d = 0$ then R = 1. R can be calculated from the breakthrough curves using “Laboratory Flow-Through” (LFT) method (*Eq. 3*):

$$R = \frac{V_2}{V_1} \quad \text{(Eq.3)}$$

where V_2 is the volume of the eluted phenol solution when C/C_0 (phenol) = 0.5 and V_1 is the volume of the eluted tracer solution when C/C_0 (tracer) = 0.5 (United States, 1999); therefore, k_d is determined using *Equation 2*.

Results

Soils properties

The measured soils properties are summarized in *Table 1*. Based on texture, CH is loamy sand soil and CO and MA are sandy soils.

Table 1. *Properties of the natural soils (CH, CO and MA) used in study*

Soil	Sand (%)	Silt (%)	Clay (%)	Texture	OM (%)	OC (%)	Porosity (%)	ρ (g cm^{-3})
CH	73.31	24.69	1.98	Loamy sand	1.87	1.08	37.64	0.97
CO	90.49	8.88	0.62	Sand	7.22	4.18	50.21	0.91
MA	91.15	8.84	0.008	Sand	2.72	1.57	39.39	1.03

CH, CO and MA, refer to soils collected from Chorfa, Corso and Maâtkas, respectively; OM, OC and ρ refer to organic matter content, organic carbon content and bulk density, respectively

Transport of phenol in soil columns using “Laboratory Flow-Through Method”

Phenol and tracer breakthrough curves (BTCs) for the soil columns are presented in *Figure 2* and transport parameters calculated using “Laboratory Flow Through” (LFT) method are listed in *Table 2*. The same parameters of columns are considered for the transport of both phenol dissolved in distilled water and phenol dissolved in a mixture of methanol/distilled water (50:50, v/v). The recovery rates of tracer were approach 100% in almost all soil columns, which can be considered ideal (Gibert et al., 2014).

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Transport of phenol dissolved in distilled water

The transport of phenol dissolved in distilled water was retarded in almost all soil columns with late breakthrough curves (BTCs) especially at a depth of 5 cm and in all CO-soil columns (*Fig. 2*). The transport of phenol in the sandy soil MA was the greatest with recovery rates (RR) ranging from 90.97 to 102.77%; however, the recovery rates (RR) of phenol were poor in the sandy soil CO especially at a depth of 5 cm with 75.07% and in the loamy sand soil CH at a depth of 5 cm with 80.80% (*Table 2*).

Transport of phenol dissolved in a mixture of methanol/distilled water (50:50, v/v)

The (BTCs) showed nearly conservative transport behaviour especially at a depth of 5 cm in CO-soil and in MA-soil, where they were slightly delayed relative to the (BTCs) of the tracer (*Fig. 2*).

Table 2. *Retention and transport parameters of phenol in natural soils calculated using column experiments*

Phenol	Soil	Column number	L (cm)	Transport parameters calculated using LFT method		Recovery rate (RR, %)
				k_d (L kg^{-1})	R [-]	
$Phenol_{dis}$	CH	1	1.7	0.38	2	99.90
		2	3.3	1.3	4.42	95.64
		3	5	1.46	4.85	80.80
	CO	4	1.7	1.33	3.43	91.25
		5	3.3	1.25	3.29	85.84
		6	5	3.63	7.62	75.07
	MA	7	1.7	0.37	2	102.77
		8	3.3	0.46	2.23	98.94
		9	5	0.88	3.33	90.97
$Phenol_{met}$	CH	1	1.7	0.08	1.23	99.90
		2	3.3	0.16	1.42	99.92
		3	5	0.21	1.57	99.85
	CO	4	1.7	0.31	1.57	100.01
		5	3.3	0.60	2.11	104.05
		6	5	0.06	1.12	100.62
	MA	7	1.7	0.24	1.66	104.64
		8	3.3	0.24	1.66	100.74
		9	5	0.24	1.66	100.15

Subscripts $_{dis}$ and $_{met}$ refer to phenol dissolved in distilled water and phenol dissolved in a mixture of methanol/distilled water (50:50, v/v), respectively. CH, CO and MA, refer to soils collected from Chorfa, Corso and Maâtkas, respectively. L, k_d, R and LFT refer to soil depth, partition coefficient, retardation factor and 'laboratory flow-through' method, respectively

The recovery rate increased to reach approximately 100% in almost all soil columns (*Table 2*) and the highest reductions in the retention of phenol were observed at a depth of 5 cm and reached 19.05, 25.55 and 9.18% in CH, CO and MA, respectively (*Fig. 3*).

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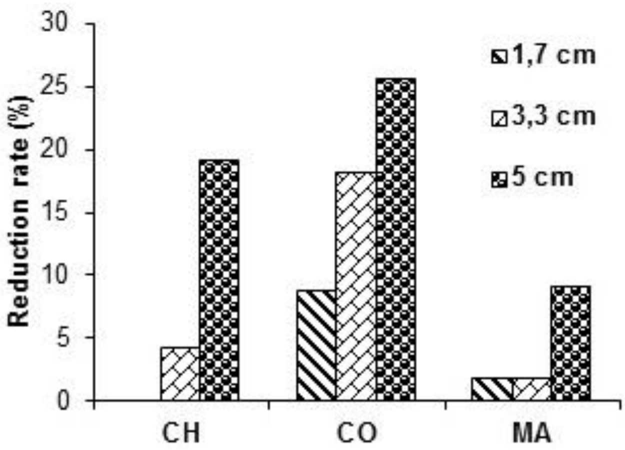
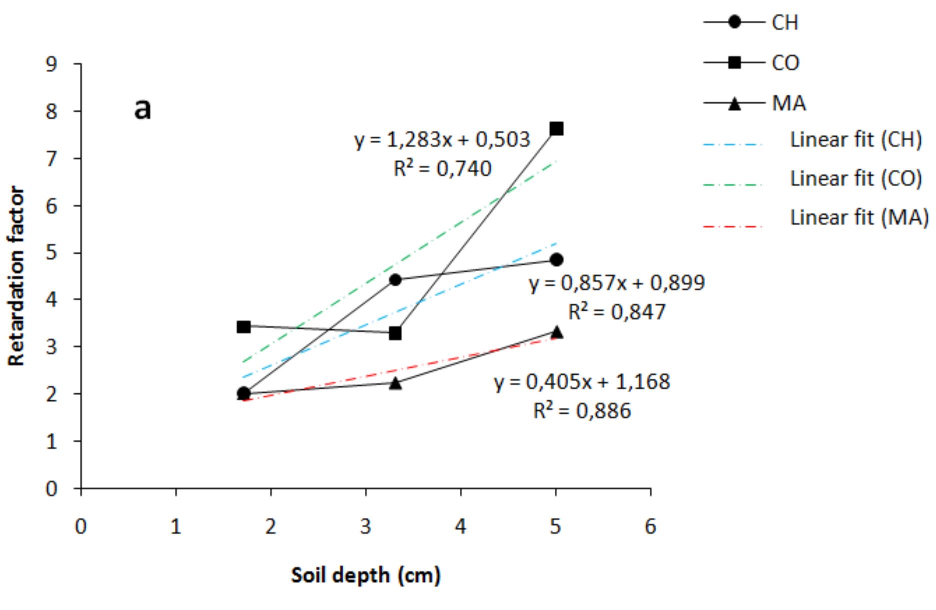


Figure 3. *The reduction rate of the retention of phenol in various soil columns at different depths. Reduction rate is the difference between recovery rate of phenol dissolved in distilled water and recovery rate of phenol dissolved in a mixture of methanol/ distilled water (50:50, v/v)*

Variation of the retardation factor with soil depth

As shown in *Figure 4,* the changes in the retardation factor values (R) measured using (LFT) method were found to be positively correlated with soil depth, for transport of phenol dissolved in distilled water (a), and followed the linear equations $R = 0.857\ L + 0.899$ ($r^2 = 0.847$), $R = 1.283\ L + 0.503$ ($r^2 = 0.740$) and $R = 0.405\ L + 1.168$ ($r^2 = 0.886$) for CH, CO and MA, respectively, which suggested that the retardation of the transport of phenol increased with soil depth.

However, for transport of phenol dissolved in in a mixture of methanol/distilled water (50:50, v/v) (b), there is no significant correlations between retardation factor values (R) and soil depth, except for CH, where the retardation factor value (R) increased with soil depth and followed the linear equation $R = 0.102\ L + 1.063$ ($r^2 = 0.992$). Therefore the retardation of the migration of phenol through CH increased with soil depth.



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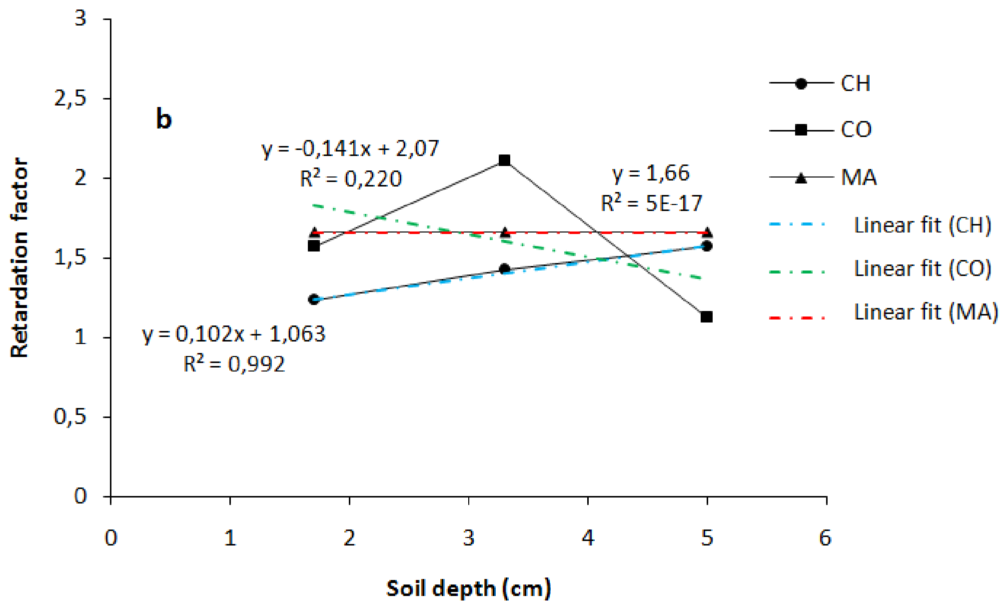

Figure 4. *Relationship of the retardation factor (R) measured using (LFT) method for phenol dissolved in distilled water (a) and phenol dissolved in a mixture of methanol/ distilled water (50:50, v/v) (b) with soil depth*

Discussion

Effect of soil composition on retention and migration of phenol

For transport of phenol dissolved in distilled water, the calculated values of k_d (*Table 2*) denote that retention of phenol was higher in CO-soil (1.33, 1.25 and 3.63 L kg^{-1} at a depth of 1.7, 3.3 and 5 cm, respectively) than in MA-soil (0.37, 0.46 and 0.88 L kg^{-1} at a depth of 1.7, 3.3 and 5 cm, respectively), which were both sandy soils, and it was even higher in CO-soil (1.33, and 3.63 L kg^{-1} at a depth of 1.7 and 5 cm, respectively) than in CH-soil (0.38 and 1.46 L kg^{-1} at a depth of 1.7 and 5 cm, respectively): soil which has the greatest clay content (*Table 1*), except at a depth of 3.3 cm (1.3 L kg^{-1} for CH and 1.25 L kg^{-1} for CO); it is well known that natural clays have good capacity for the adsorption of organic molecules (Djebbar et al., 2012). This fact is supported by high organic matter content (OM) of CO-soil (7.22%) compared to the two other soils (1.87% for CH and 2.72% for MA; *Table 1*); it is well known that, once in soil, organic pollutants bound to organic matter (OM) have reduced mobility due to their low solubility (Valentín et al., 2013). In spite of the high solubility of phenol compared to the other organic pollutants which are less hydrophilic, OM may be of great importance for adsorption of polar organic compounds like phenol (Delle Site, 2001). Based on retardation factor values (R), the soils can be presented in the following orders of retardation of phenol migration: CO> CH > MA at a depth of 5 cm, CH > CO > MA at a depth of 3.3 cm and CO > CH = MA at a depth of 1.7 cm (*Table 2*).

Effect of methanol on retention and migration of phenol in natural soils

The retention of phenol was very low with values of k_d ranging from 0.06 to 0.6 L kg^{-1}, which were much lower than those obtained from transport of phenol dissolved in distilled water. Therefore, the retardation factor values (R) were

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remarkably lower than those observed from transport of phenol dissolved in distilled water (*Table 2*). It is well known that organic solvents such as methanol are often used to extract phenols from environmental samples (Afghan and Chau, 1989) and types of environmental matrices such as soils (Mahugo Santana et al., 2009); in addition, methanol, as one of the most polar organic solvent, was used by Osman and Saim (2013) to extract phenol, as one of the most polar contaminant, from soil. As a conclusion, our results confirm the opposite effect of methanol on the retention of phenol in our soils. In our case of study, this caracteristic of methonol accelerated phenol vertical migration process through soil.

Conclusion

LFT method based on the one-dimensional convection-dispersion equation (*Eq. 1*) was used to investigate migration and retention of phenol in three types of natural soils. Our results confirm that organic matter can decrease the risk of groundwater contamination (Rodriguez-Liebana et al., 2014). The use of LFT method indicates that phenol was more retained in sandy soil “CO” rich in organic matter than in sandy soil “MA” and in loamy sand soil “CH” both poor in organic matter. The mobility of phenol decreased with increasing soil depth. The effect of methanol, as an organic solvent, on transport of phenol was also investigated. Results indicate that methanol increased remarkably the mobility of phenol through soil columns and decreased its retention. As a negative effect, methanol can increase the risk of groundwater contamination, but it can also be used for cleaning up soils contaminated with phenol, as a positive effect. These results can be generalized and utilized in practice to estimate migration of phenol at large scales in the northern regions of Algeria to predict the gravity of the risk of contamination of groundwater and soil. Further research can be conducted with undisturbed soil samples. In addition, other factors that may influence phenol migration in natural soil, such as pH, should also be considered for future studies.

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PHYTODIVERSITY OF MIDFIELD BALKS (ENVIRONMENTAL ISLANDS) IN A SELECTED AREA IN NORTH-WEST POLAND

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Abstract. In the years 2007-2009 the differentiation of flora on the area of balks of different length was assessed. The studies of flora were carried out in the area of 7772 ha of Ińsko Lakeland in Poland. For the detailed studies 72 balks were chosen, formed from roadsides and divided into two groups according to the length. Topographic maps in the scale 1:10 000 were used for the assessment of earlier functions of the studied objects. It was shown that in the areas of an arable surface characteristic of average farms as regards their size in the European Union, the size of arable land and the length of balks related with it had an influence on phytodiversity. Despite the fact that the number of phytocoenoses was larger on shorter balks, their specific poverty did not affect biodiversity significantly. The presence of a large number of melliferous species influenced not only phytodiversity of balks, but it also resulted in the improvement of nutritive conditions, among others, for bees. A larger number of plant species on long balks, including also melliferous and therapeutic species, proves correct management of the production space in a long time period.
Keywords: *fields, flora, Ińsko Lakeland, phytocoenoses, bees, melliferous and therapeutic plants*

Introduction

In the middle of the 19th century a change of the production space started leading to its rationalization and intensification (introduction of mineral fertilization, soil reclamation, simplified crop rotation, land integration). These changes resulted in the reduction of species diversity in the cultural landscape (Kędziora et al., 2012; Pe'er et al., 2014; Horta et al., 2015). Cultivation of old species of wheat (einkorn wheat, emmer wheat, spelt, Polish wheat and club wheat), wild rye, bristle oat, millet, buckwheat and multispecies crops: flax, hemp, tobacco and hop was discontinued in favour of monocultures: wheat, maize, rye. There was a change in the density of boundary nature objects such as balks and midfield roads and other forms of environmental islands. The term "environmental islands" was coined in 1967 by McArthur and Wilson. It describes surface objects, such as: groups of trees, water ponds, water-free pits and grasslands. Polish researchers have extended the scope of this definition to cover the linear forms as well: drainage ditches, roadsides and farmbounds (Loster, 1991; Ratyńska, 2002; Karg, 2003, 2005; Symonides, 2010). Balks, i.e. narrow, uncultivated strips of grass

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vegetation participate, due to their location, in maintaining biological diversity of agricultural landscape, constituting valuable habitats for plants (Štefanová and Šálek, 2014).

In order to maintain biodiversity in monoculture cultivation it is important not to apply chemical plant protective agents on the fringes of the fields. Dobrzański (2009) shows that on large plantations the width of individual fields should not exceed 100 m, and that of balks 1 m, which inclines towards maintaining areas of increased fragmentation. A positive aspect of land consolidation is reduction of the cost of the performed cultivation and limitation of potential neighborhood conflicts. However, land fragmentation makes it possible to lower the risk related to spatial variability of meteorological conditions and natural catastrophes (Penov, 2004; Di Falco et al., 2010). According to Kazlauskaite-Jadzevice et al. (2016), for the purpose of preserving biodiversity, the traditional Lithuanian landscape should be maintained and even further developed, particularly on highly undulating areas (Feiza et al., 2008; Kinderiene and Karcauskiene, 2016).

The quality of soils in the European Union is strongly differentiated, from quite fertile brown soils in the west to weak podsol soils in the central part (Directive, 2004**).** In the countries of the "old" EU, the process of food production is strongly mechanized and is of an intensive character. For example, in Great Britain 95% of agricultural acreage belongs to the farms of the surface exceeding 20 ha. Larger fragmentation is observed in the south of Europe, e.g. in Greece, Portugal and Italy. The differentiation of the surface of plots depends on the size of a farm, the degree to which the field works were mechanized (Noga, 2005). An average size of arable land in the EU changed depending on the number of membership countries (for example the EU -15: 23 ha, and currently the EU - 27: 13.8 ha).

Taking into consideration the diversity of economic and social conditions in the European Union, the arable land in the North-West Poland was taken for the study. In the area set for the study, the most dominant were brown soils with farms of an average size of 21 ha. In the EU agricultural and environmental programs according to package 9 (of the buffer zone), the emphasis was laid on the protection of land against pollution of agricultural origin. Such function is played, among other things, by midfield balks which also results in maintaining biodiversity. Considering these regularities it was assumed that fragmentation of arable land will affect the diversity of balks. In view of the above, the aim of the study was to assess the differentiation of flora in the area of balks of different length. The usability of flora as bee nutrients was also studied and the presence of therapeutic species was determined.

Material and methods

Study object

In the years 2007-2009 the studies of flora were carried out in the area of 7772 ha of Ińsko Lakeland in Poland. This mesoregion is characterised by a diversified terrain relief with moraine hills of steep slopes and of considerable relative heights. Wet dells occur there as well as numerous lakes and rivers. This mosaic of diversified habitats is additionally enriched by an irregular course of boundaries between fields and forests (Borzyszkowski et al., 2016). The arable land in the studied area constituted above 60% of the surface and is dominated mainly by soils of complex 4 and 5, i.e. by very good and good rye soils, determined as leached brown and acid ones. In the structure of the

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surface layer of soils, light loamy and heavy loamy sand prevailed (Gamrat, 2012). An average surface of arable land in this area amounted to 21 ha, which reflects approximate conditions in the European Union.

Analysis of flora

For the detailed studies 72 balks were chosen, formed from roadsides and divided into two groups (36 objects in each group) according to the length: I - up to 500 m, II - >500 m (*Fig. 1*). The width of the balks varied from 50 to 70 cm. Topographic maps in the scale 1:10 000 were used for the assessment of earlier functions of the studied objects.

According to the description prepared by Dzwonko (2007), 72 phytosociological releves were taken and used for the identification of 11 vegetation communities determined in the synthetic table which refers to shorter and longer balks. The surface of releves varied from 10 (0.5 m × 20 m) to 14 m^2 (0.7 m × 20 m). The species composition of the plans was also analysed with reference to their usability as bee nutrients (along with defining their melliferous potential and length of flowering), additionally the occurrence of therapeutic species was also assessed (including ornamental, cosmetic and edible ones).

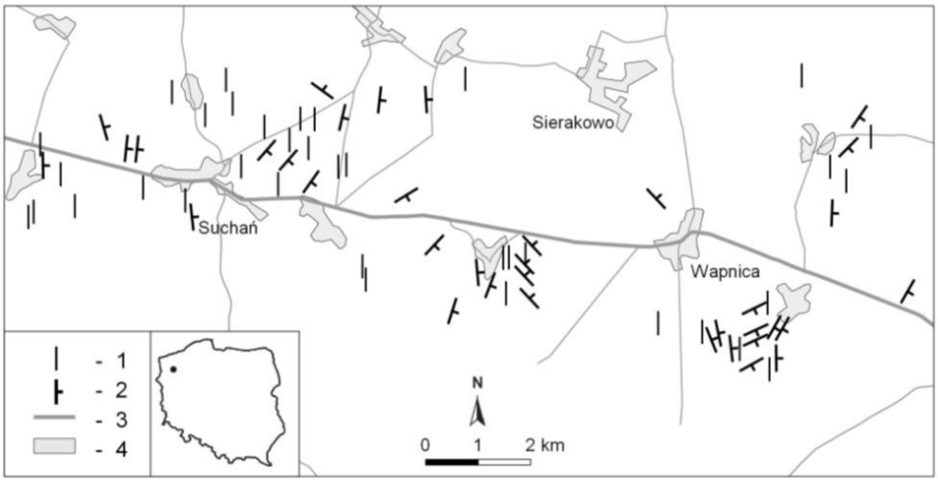

Figure 1. *A schematic map showing studied balks on the Ińsko Lakeland. (1 - short balks, 2 - long balks, 3 - roads, 4 - buildings)*

Analysis of melliferous plants

The species of plants found at the balks were classified according to Lipiński (2010) and Pogorzelec (2018) as a food base for bees, i.e. giving their values of honey yield [kg/ha or kg/tree or bush] and the time of flowering. Floristic observations were made during floristic studies. The frequency of bees on melliferous plants was determined: 3 - frequent, 2 - weak, 1 - sporadic or presence of other pollinators, x - no insects were observed (*Table 3*).

Statistical analysis

The results concerning the number of plant species occurring on the balks were developed on the basis of the univariate analysis of variance. Additionally, such factors

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as the complex, type of soil and surface layer were tested. The significance of differences was evaluated using the Tuckey's test at the significance level α = 0.05. For the isolated groups of balks the linear relationship was determined between the number of all the species of plants and the number of plant species according to their sociological belonging. All statistical analyses were performed with Statistica 12 PL software (Statsoft).

Results

In the studied area 102 species of plants were observed, of which balks from group II (of the length of the object amounting to 916 m) were characterised by higher phytodiversity (89 species). Shorter balks showed a 1.5 times decreased number of plant species (59).

Out of the species isolated according to their sociological belonging, ruderal species dominated (53 species). There was a smaller number of meadow species (39 species). *Triticum aestivum* L. was shown as an agricultural species. Trees and shrubs occurred rarely, there were individual specimens*: Alnus glutinosa* Gaertn., *Betula pendula* Roth., *Crataegus monogyna* Jacq., *Prunus domestica* L., *P. padus* L., *Rubus caesius* L., *R. idaeus* L., *Salix alba* L., except for *Sambucus nigra* L. which formed a vegetation patch in the area of one object.

Large diversity of the number of species at 72 isolated research objects resulted in the observation of statistically significant differences between the average number of species on the balks of group I (8) and group II (11). Such diversity was not recorded as regards agricultural and ruderal trees and shrubs. In the case of meadow species - their larger number occurred on the long balks (*Fig. 2*).

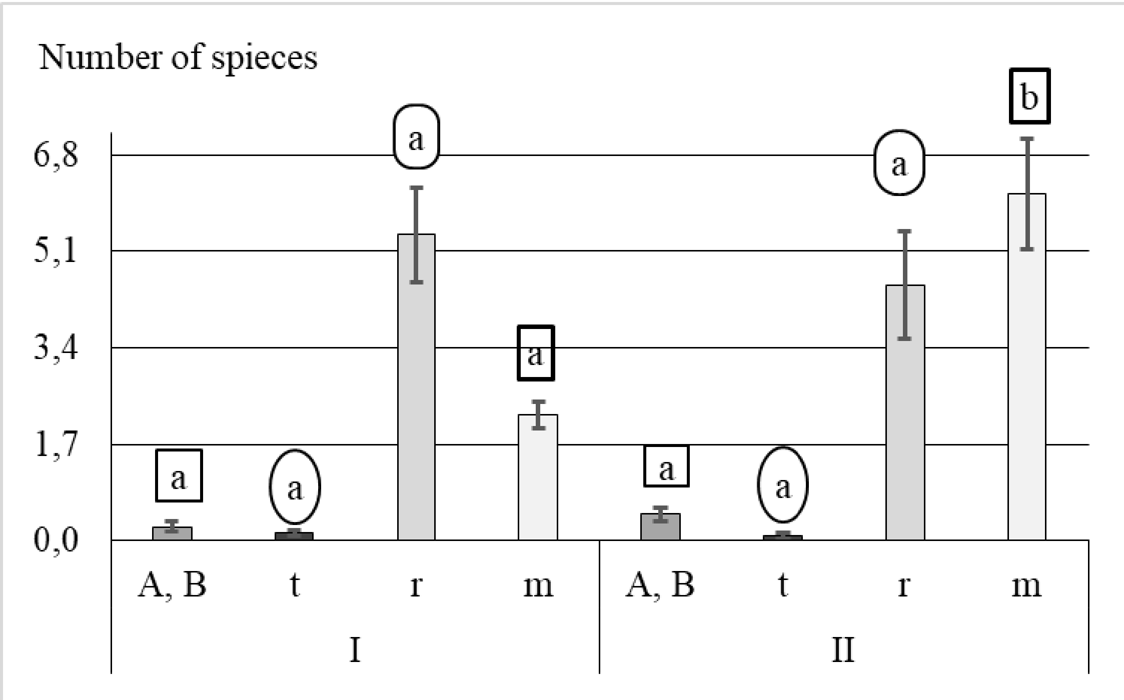

***Figure 2.** The number of plants on the balks. (I - group of balks (short), II - group of balks (long), A, B - trees, shrubs, t - tillage species, r - ruderal, m - meadow)*

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Arable land of good agricultural quality constitutes about 2/3 of the studied area. However, considerable diversity of the terrain both in the period when PGRs (State Agricultural Farms) functioned to the year 1989 and at present were not conducive to a large consolidation of arable land. This fact had an influence on the diversity of this landscape.

The largest number of ruderal species according to sociological groups (*Table 1*) was characteristic of the very weak rye-lupine complex (complex 7).

Table 1. *The mean number of plant species according to their nationality sociological*

Group	Soil complexes				Soil type		Mechanical composition of the soil	
	2	4	5	7	lb	ab	lls	lss
G	11.0[a]	8.2[a]	9.8[a]	21.0[a]	9.0[a]	11.0[a]	11.0[a]	8.0[a]
A, B	0.3[a]	0.3[a]	0.5[a]	1.0[a]	0.3[a]	0.6[a]	0.5[a]	0.2[a]
t	0.1[a]	0.1[a]	0.2[a]	0.0[a]	0.1[a]	0.3[b]	0.1[a]	0.1[a]
r	4.8[a]	5.0[a]	4.2[a]	15.5[b]	4.7[a]	5.7[a]	5.0[a]	5.0[a]
m	5.8[a]	2.9[a]	5.0[a]	4.5[a]	4.1[a]	4.4[a]	5.2[a]	3.0[b]

Group: G - General, A, B - trees, shrubs, t - tillage species, r - ruderal, m – meadow
Soil type: lb - leached brown soil, ab - acid brown soil
Mechanical composition of the soil: lls - light loamy sand, lss - loamy sand strong

In the studied area leached brown soils dominated (74%) in the surface layer with light loamy sands (54%). The diversity of the type of soils and their mechanical composition in the surface layer did not influence the number of species: trees and shrubs and green species from ruderal habitats. The type of soil influenced the number of agricultural species and the mechanical composition of soils had an influence on the number of meadow habitats.

In both groups of balks a statistically significant correlation was observed between the general number of plant species and the number of those from ruderal habitats (*Figs. 3* and *4*). A stronger relation occurred for the area of balks from group I (0.96) than from II (0.70).

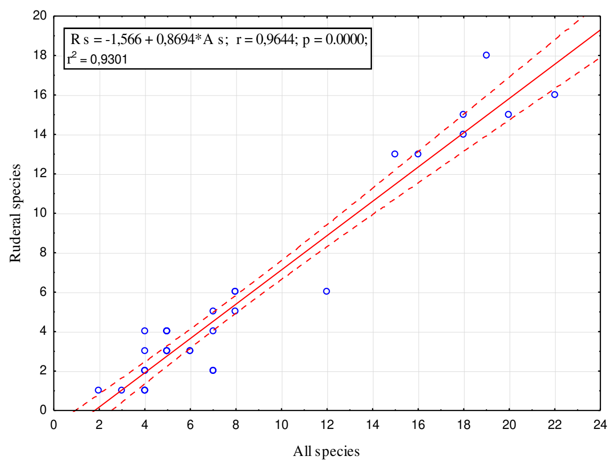

Figure 3. *Relationship between number of all species and ruderal species (balks I)*

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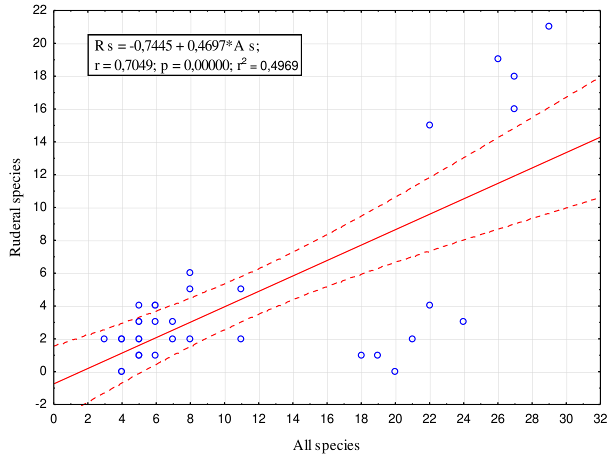

Figure 4. *Relationship between number of all species and ruderal species (balks II)*

In the case of longer balks (group II) additionally a significant linear dependence was observed between the number of plant species and the number of those from meadow (*Fig. 5*). In regression, the r^2 coefficient of determination is a statistical measure of how well the regression line approximates the real data points. It should be noted that in the case of longer baulks only slightly below 50% was explained by the positive correlation between the number of all species and ruderal species or between the number of all species and meadow species (coefficient of determination was from 50 to 46%).

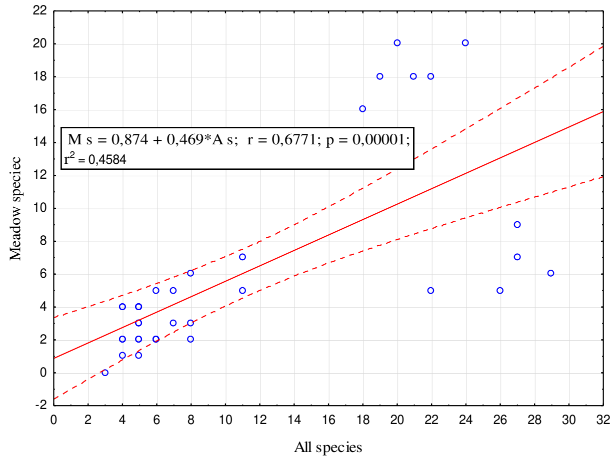

Figure 5. *Relationship between number of all species and meadow species (balks II)*

In the area of all the objects analysed from the phytosociological angle, 11 communities were found, including 10 in the area of shorter balks.

Out of phytocoenoses (marked with a grey colour) the most frequent were phytocoenoses of the grasses: community *Lolium perenne* (25 objects) and association *Agropyretum repentis* (18 objects). Other gramineous phytocoenoses were not so numerous: community with *Dactylis glomerata* (7 objects), with *Apera spicae-venti* (6 objects) and with *Agrostis capillaris* and *Arrhenatheretum elatioris* (4 objects – *Table 2*).

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Table 2. *Characteristics of communities studied balks*

Sort of balks	Long balks		Short balks	
Constancy S-Coefficient D for 36 phytosociological releve's	**S**	**D**	**S**	**D**
	1		2	
I. ChCl. *Molinio-Arrhenatheretea**, ChO. *Arrhenatheretalia elatioris*				
Lolium perenne D	II	3244	II	2701
Dactylis glomerata	I	604	I	2604
Arrhenatherum elatius	I	1000	I	1944
Lotus corniculatus	I	4	I	660
**Lathyrus pratensis*	I	70	I	590
Achillea millefolium	I	208	I	1611
Pastinaca sativa	II	49	I	1750
Taraxacum officinale	I	132	I	778
Heracleum sphondylium	.	.	I	340
Trifolium repens	I	14	I	104
ChO. *Plantaginetalia majoris*				
Agrostis capillaris D	I	257	.	.
Plantago major	I	292	I	243
Poa annua	I	14	I	243
ChO. *Molinietalia caeruleae*				
Cirsium oleraceum	II	83	I	486
Deschampsia cespitosa	I	340	.	.
II. ChCl. *Agropyretea intermedio-repentis*				
Elymus repens	I	1903	II	3604
Equisetum arvense	I	69	I	486
Convolvulus arvensis	.	.	I	1507
III. ChCl. *Stellarietea mediae*				
Apera spica-venti	I	868	I	972
Chenopodium album	I	222	I	1056
Capsella bursa-pastoris D	I	69	I	1264
Lactuca serriola D	I	69	I	1507
Linaria vulgaris	I	69	I	1507
Tripleurospermum inodorum	I	104	I	1076
Erigeron canadensis D	I	63	I	972
Papaver rhoeas	I	14	I	729
Polygonum aviculare	I	111	I	292
Chamomilla recutita	I	70	I	243
Rumex crispus D	.	.	I	535
IV. ChCl. *Artemisietea vulgaris*, SCl. *Artemisienea**				
Artemisia vulgaris	I	292	II	3056
Urtica dioica	I	618	I	1944
Rumex obtusifolius	I	14	I	1507
Cirsium arvense	I	83	I	4861
**Oenothera biennis*	I	69	I	1701
**Picris hieracioides* D	.	.	I	1750
**Verbascum thapsus* D	I	69	I	1750

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**Galeopsis pubescens*	.	.	I	1507
**Melilotus officinalis*	I	69	I	1458
**Medicago lupulina* D	.	.	I	1264
**Ballota nigra*	I	14	I	1215
**Malva sylvestris*	I	42	I	722
**Cirsium vulgare*	I	14	I	486
**Berteroa incana*	I	14	I	292
**Arctium lappa*	I	69	I	2431
**Lamium album*	I	14	I	1458
SCl. *Galio-Urticenea*				
Fallopia dumetorum	I	69	I	1750
Others				
Sambucus nigra b	I	97	I	340
Prunus domestica b	.	.	I	347
Salix alba a	I	14	I	292
Alnus glutinosa b	I	14	I	243
Triticum aestivum	I	525	I	632

Sporadic species - I: *Alchemilla pastoralis*, *Agrostis stolonifera*, *Bromus hordeaceus*, *Matricaria discoidea*, *Crepis biennis*, *Cynosurus cristatus*, *Daucus carota*, *Festuca rubra*, *Juncus articulatus*, *J. effusus*, *J. inflexus J. tenuis*, *Lysimachia nummularia*, *Plantago lanceolata*, *Phleum pratense*, *Potentilla anserina*, *Rumex acetosa*, *Vicia cracca* (1), *Lysimachia vulgaris* (1, 2); II: *Falcaria vulgaris* (1), *Tussilago farfara*, *Bunias orientalis* (2); III: *Anagalis arvensis*, *Centaurea cyanus*, *Consolida regalis*, *Lamium purpureum*, *Fallopia convolvulus* (1); IV: *Anthriscus sylvestris*, *Silene latifolia*, *Solidago canadensis* (1, 2), *Aegopodium podagraria*, *Cichorium intybus*, *Rubus caesius* b, *Tanacetum vulgare* (1), *Artemisia absinthium* (2); Others: *Anthoxanthum odoratum*, *Betula pendula* a, *Calamagrostis epigejos*, *Centaurea scabiosa*, *Crataegus monogyna* b, *Galium verum*, *Jasione montana*, *Prunus padus* b, *Rubus idaeus* b, *Rumex acetosella*, *Sedum acre*, *Thymus serpyllum*, *Trifolium arvense*, *T. campestre* (1), *Gnaphalium sylvaticum* (2)

Cl. - class, O. - order; constancy, Ch. - characteristic species, I-V - order number, S: I - proportional participation of species < 20%, II - 40-20%, III - 60-40%, IV - 80-60%, V - 100-80%; D - the coefficients of cover (from 1 to 8 750) were estimated for 36 phytosociological releve's; . not present

The indicated species belonged to 10 phytosociological classes, 2 subclasses, 17 orders. The most numerous were the species of meadow and ruderal habitats (respectively *Molinio-Arrhenatheretea* class - 33 and *Artemisietea vulgaris* - 25 species) and segetal ones (*Stellarietea mediae* class - 16 species). The species of ruderal habitats that formed numerous patches were, among others: *Artemisia vulgaris* L. (D-3056), *Urtica dioica* L. (D-1944), *Picris hieracioides* L., *Verbascum thapsus* L., *Fallopia dumetorum* (D-1750), *Galeopsis pubescens* Willd., *Rumex obtusifolius* L. (D-1507). As to the meadow species, the following were presenrt in large numbers: *Dactylis glomerata* L. (D-2604), *Lolium perenne* L. (D-3244, 2701), *Arrhenatherum elatius* (L.) P. Beauv. ex J. & C. Presl (D-1000, 1944), *Achillea millefolium* L. (D-1611). At present, numerous species from segetal habitats were, among others: *Lactuca serriola* L., *Linaria vulgaris* Mill. (D-1507), *Capsella bursa-pastoris* (L.) Medik. (D-1264), *Tripleurospermum inodorum* (L.) Sch. Bip. (D-1076). Less numerous, as far as species are concerned, however often present, were: *Agropyretea intermedio-repentis* class, *Koelerio glaucae-Corynephoretea canescentis* and *Epilobietea angustifolii* in a form of the following species: *Elymus repens* (L.) Gould (D-1903, 3604) and *Convolvulus*

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arvensis L. (D-1507). Species belonging to other phytosociological classes (*Festuco-Brometea*, *Rhamno-Prunetea*, *Salicetea purpureae*, *Trifolio-Geranietea sanguinei*) occurred in small numbers (from 1 to 3 species).

Herbal plants were classified according to the way of their use as: food base for bees (67), therapeutic (64 species), ornamental (22 species), cosmetic (15 species), and seasoning herbs (22 species). Despite the domination of grass communities, there were also numerous dicotyledonous species with a melliferous function (*Table 3*).

Table 3. *Frequency of bees on melliferous plants found in the examined areas together with the value of honey yield [kg/ha or kg/tree or bush] and the time of flowering beginning*

Frequency of bees/name of the plant species/values of honey yield
Melliferous plants flowering in spring (from March)
[3] *Lamium album* (368)*, [3] *Taraxacum officinale* (200), [3] *Salix alba* a, [3] *Rubus idaeus* b (po 150), [3] *Lamium maculatum* (140), [3] *Prunus domestica* b (43), [3] *Lamium purpureum* (38), [2] *Berteroa incana*, [2] *Capsella bursa-pastoris*, [1] *Alnus glutinosa* a, b, [1] *Betula pendula* a, [1] *Sambucus nigra* b, [x] *Prunus padus* b (25), [x] *Crataegus monogyna* b (15), [x] *Tussilago farfara*, [x] *Linaria vulgaris*, [x] *Rumex acetosella*
Melliferous plants flowering in summer (from June)
[3] *Arctium major* (200), [3] *Melilotus officinalis* (200), [3] *Jasione montana* (160), [3] *Malva sylvestris* (160), [3] *Thymus serpyllum* (150), [3] *Trifolium repens* (90), [3] *Lotus corniculatus* (40), [3] *Cirsium arvense*, [3] *C. vulgare*, [3] *Chenopodium album*, [3] *Convolvulus arvensis*, [3] *Heracleum sphondylium*, [2] *Oenothera biennis*, [2] *Rumex crispus*, [1] *Medicago lupulina* (200), [1] *Cheopodium album*, [1] *Plantago lanceolata*, [1] *Rumex obtusifolius*, [1] *Urtica dioica*, [1] *Lathyrus pratensis* (0,8), [1] *Matricaria discoidea*, [1] *Fallopia convolvulus*, [1] *F. dumetorum*, [1] *Erigeron canadensis*, [1] *Galeopsis pubescens*, [1] *Papaver rhoeas*, [1] *Silene latifolia*, [x] *Centaurea scabiosa* (500), [x] *C. cyanus* (350), [x] *Ballota nigra* (300), [x] *Rubus caesius* b (26), [x] *Aegopodium podagraria*, [x] *Alchemilla pastoralis*, [x] *Anthriscus sylvestris*, [x] *Consolida regalis*, [x] *Crepis biennis*, [x] *Daucus carota*, [x] *Hieracium umbellatum*, [x] *Lysimachia vulgaris*, [x] *Sedum are*
Melliferous plants flowering in autumn (from August)
[3] *Cirsium oleraceum* (500), [3] *Cichorium intybus* (40), [3] *Verbascum thapsus*, [1] *Pastinaca sativa*, [1] *Picris hieracioides*, [x] *Solidago canadensis* (700), [x] *Trifolium arvense* (30), [x] *Artemisia absinthium*, [x] *A. vulgaris*, [x] *Galium verum*

[3] - frequent, [2] - weak, [1] - sporadic or presence of other pollinators, [x] - no insects were observed, * - values of honey yield *yield* [kg/ha or kg/tree or bush]

This species on the fields are a valuable food base for bees, especially when the cultivated species are no longer flowering, i.e. in spring and late summer. The flora of the balks are numerous species on which bees forage for a long time, for example from March to September or October, and are also characterized by high honey yield values. They are the so-called common “weeds” (grasshopperdead-nettles, shepherd’s purse and sorrel). In summer, the main species begin to flower, including those that bloom from June to October (including: *Erigeron canadensis* (L.) Cronquist, *Galeopsis pubescens* Willd., *Hieracium umbellatum* L., *Malva sylvestris* L., *Fallopia convolvulus* (L.) Á. Löve, *Urtica dioica* L.). High value of honey yield of autumnal melliferous plants is a caloric food just before the wintering of bees. The diversity of plant species on the balks, through a different flower structure, can provide a food base for numerous (other than bees) pollinating insects, for which balks are not only a place of feeding but also nesting. The plants weakly covered by honeybees, and many other pollinators are, among others: *Fallopia dumetorum* (L.) Holub, *Lathyrus pratensis* L., *Matricaria discoidea* DC., *Papaver rhoeas* L., *Silene latifolia* Poir.

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Many of therapeutic species show an influence on at least three systems of the human body (for example: digestive system, respiratory system, excretory system, immune system, nervous system and also on dermal layers of the skin). Among others, these are: *Artemisia absinthium* L., *Ballota nigra* L., *Berteroa incana* (L.) DC.), *Centaurea cyanus* L., *C. scabiosa* L., *Chamomilla recutita* (L.) Rauschert, *Ch. suaveolens* (Pursh) Rydb., *Chenopodium album* L., *Cichorium intybus* L., *Cirsium arvense* (L.) Scop., *C. oleraceum* (L.) Scop. and *Equisetum arvense* L. Out of cosmetic species there were, among others: *Aegopodium podagraria* L., *Erigeron canadensis* (L.) Cronquist, *Papaver rhoeas* L., *Potentilla anserina* L., *Urtica dioica* L. Seasoning herb species are, among others: *Achillea millefolium* L., *Artemisia absinthium* L., *A. vulgaris* L., *Capsella bursa-pastoris* (L.) Medik., *Chamomilla recutita* (L.) Rauschert, *Cirsium oleraceum* (L.) Scop., *Crataegus monogyna* Jacq., *Elymus repens* L., *Heracleum sphondylium* L., *Lactuca serriola* L., *Pastinaca sativa* L. Decorative herb species are, among others: *Lysimachia nummularia* L., *Malva sylvestris* L., *Plantago lanceolata* L., *Oenothera biennis* L. and *Solidago canadensis* L.

On the terrain of balks it was shown that 31% of the species belong to the group of very burdensome weeds. These species are resistant to breaking (e.g. they possess long rhizomes or a strong taproot system) and they spread quickly, which is connected with the number of yielded seeds. These are, among others: *Achillea millefolium* L., *Aegopodium podagraria* L., *Anagalis arvensis* L., *Apera spica-venti* (L.) P. Beauv, *Capsella bursa-pastoris* (L.) Medik., *Centaurea cyanus* L., *Chenopodium album* L., *Consolida regalis* S. F. Gray, *Equisetum arvense* L. The presence of dicotyledonous plants, strongly rooted, i.e. 32 species of weeds and 12 species of grasses (such as, for example: *Cynosurus cristatus* L., *Dactylis glomerata* L., *Deschampsia cespitosa* (L.) P. B., *Elymus repens* (L.) Gould, *Lolium perenne* L., *Phleum pratense* L.) conducive to strong sodding of subsoil, was related to the earlier use of the balk as a field track.

Discussion

Diversity of the species occurring in the agricultural landscape is caused by the presence of constructive elements such as areas used agriculturally, grassland and unused areas, to which belong drainage ditches, water canals, hedges and balks, but also biotopes of a high degree of naturalness such as fragments of forest ecosystems or afforestation. In the space-time system of the agricultural landscape it is important that there occur anthropogenic species strongly related with a given form of the object or with historical kind of the use of land (Harrach and Sauer, 2002). The studied area of the Lake District is characterized by a varied landscape structure composed between numerous hills (> 150 m above sea level). Fields are separated by narrow areas of forest ecosystems, large and small lakes with a long shape (Wapnica Lake, Wapnica Południowa, Sierakowskie, At Poplar, Sicko) and water ponds. The richness of so many natural elements of the landscape is conducive to the presence of various forms of environmental islands, and the diversity of sculpture facilitates the existence of numerous midfield balks. Aavik and Lira (2010) determined the main factors of plant species richness and composition in field boundaries. They surveyed the vegetation of field boundaries in organic and non-organic farms, and recorded the field boundary type and width. It was found that field boundary type and landscape structure described most of the variation in species composition of these boundaries, while organic farming had little effect. Boundary type had a specific impact on species diversity ditches enhanced

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the richness of nature-value species and suppressed agrotolerants; woody boundaries hosted only a few agrotolerant species and road verges increased the species richness of both species groups. The richness of nature-value species benefited from wider open boundaries, while narrow boundaries hosted more agrotolerant species.

A large number of fragmented plots significantly influences farmers' unwillingness to implement new technologies and innovations which could improve efficiency of agricultural production. The owners of larger rural areas consent to technological novelties and modern solution much more willingly than the owners of smaller and fragmented plots. However, in Bulgaria, the case of land fragmentation caused abandonment of the use of irrigation systems, which turned out to be unprofitable in such small areas (Penov, 2004). In the 1950s and 1960s, water conditions were regulated in the studied area, with numerous ditches and drainage channels present. The nearest surroundings of the ditches are grasslands, usually of a long shape, separating the individual areas of the fields. The undulating terrain of the research (close distance between the contours within 50-70 m) and the extensive network of drainage ditches, despite the use of intensive mechanical and chemical treatments, enabled the presence of numerous balks. Fragmentation of arable land is more and more frequently regarded as the main cause of difficulties with undertaking agricultural activity, particularly in well developed countries. Therefore, the best economic effect was achieved due to specialization and intensification of plant production. Despite many negative effects of land fragmentation, some positive features can be found in this situation in the agricultural space. It is obvious that fragmentation of the plots increases the costs of production, but lowers its risk (Dudzińska, 2012). The studied area in its north-eastern part adjoins the natural area of Ostoja Ińska (PLB 320008), and in the southern part, on a considerable length it borders with another natural area - the Ina Valley near Recz (PLH320004). Such a neighborhood between two such valuable protected areas, i.e. an area of special protection of birds and of significance for the community, makes it a cover area. Its proper functioning in the landscape is a buffer for negative external factors. At large fragmentation and dispersion of arable fields it is possible to avoid negative effects of weather anomalies such as droughts, floods, fires, hailstorms and other unexpected spatial factors (pest infestation).

As a result of large-area economy and, in considerable areas, monoculture economy significant floristic impoverishment of arable land took place, both in a direct way (field areas - segetal plants) and in an indirect way (areas of marginal habitats - seminatural way), both in a thought over way (application of agrotechnical treatments) and in a thoughtless way (devastating activities accepted). At present, the agricultural landscape undergoes treatment aiming at restoring the previous - natural gene pool (Bauer and Wing, 2010; Paudel et al., 2015). Increased biodiversity is favored by the proximity of other forms of environmental islands. 83% of the studied area was connected to field roads. A few balks neighbored with another object (for example small ponds, grasslands or excavations) or they were located nearby (up to 20-30 m).

Intensification of agricultural production leads to a threat to the habitat and biological diversity. Up to the 1990s, farmers' efforts were directed towards the elimination of weeds (Horta et al., 2015). This study also confirmed Skrajnej on the Kałuszyńska Upland (2010). Through "rapaciousness" of seeds, seedlings and cuttings, as well as increased survival of seedlings in the periods of environmental stress, weeds are treated as nutritive competitors of farm plants. Therefore, some species have been completely eradicated. The share of common and very common species amounted to

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only 8%, although some were characterized by a significant degree of coverage. In the studied area, among the species with a wide range of occurrences the following were recorded: *Equisetum arvense* L., *Elymus repens* (L.) Gould, *Convolvulus arvensis* L., *Capsella bursa-pastoris* (L.) Medik. and *Stellaria media* (L.) Vill.). However, among these cosmopolitan species, only the couch-grass occupied larger areas.

At present, larger and larger significance is attached to diversity. Therefore, the approach to the problem of weed control changes. From the point of view of the amount of crop yields, complete elimination of weeds is not necessary in all conditions. If weed infestation is reduced to the level not threatening farm plants and economic factors are taken into consideration, the effect of diversity protection can be achieved (Gerowitt et al., 2003). In the studied area among the species threatened by extinction to various degrees only one species was observed - *Consolida regalis* S. F. Gray - as sporadic in association with *Agropyretum repentis*, and out of plants constituting the place of living and reproduction of animals, there were, among others: *Chenopodium album* L., *Cirsium arvense* (L.) Scop., *Stellaria media* (L.) Vill., *Galium aparine* L., *Polygonum aviculare* L., *Rumex obtusifolius* L., *Sonchus arvensis* L., *Tripleurospermum inodorum* (L.) Sch. Bip., *Urtica dioica* L. Van Acker (2009) noticed that many plant species can survive in the soil even up to a few years. If weeds did not cause loss to crop yields, they could be treated as “a good and indispensable neighbor” of the cultivated plants. Weed control and creation of the conditions limiting their reproduction and formation of seeds is conducive to farm plants. However, a negative influence of the liquidation of weeds on the functioning of the ecosystem through the disturbance of soil processes and nutritive dependences between plants, microflora and fauna cannot be avoided. However, in the studied area of the balks, the so-called weeds form communites with *Apera spica-venti* and *Chenopodium album*.

Areas classified as wasteland, including midfield balks, are a valuable habitat as a food base for bees. Mačukanović-Jocić and Jarić (2016) in their research on the role of phytocoenoses in maintaining the diversity of melliferous species, showed their greatest share (over 80%) in the *Consolido-Polygonetum avicularis*. In earlier studies Jarić et al. (2013) as phytocoenoses with the highest share of melliferous species they pointed to *Petasitetum hybridi* (70%). Among the species found in the analyzed areas, species constituting the core of the phytocenosis *Consolido-Polygonetum avicularis*, i.e. larkspur and knotgrass, have been demonstrated. However, *Consolida regalis* S. F. Gray appeared as sporadic species, and *Polygonum aviculare* L. occupied slightly larger areas, mainly in contact with the roadside. Among the listed communities, the most melliferous species (64%) were demonstrated in one of the *Lolium perenne* community.

The studies by Denisow and Wrzesień (2007) on the vegetation of balks in the areas of communes near Lublin showed the presence of 214 plant species, 80% of which constituted bee nutrient taxa. The definite majority of melliferous species was connected with meadow, nitrophilous and ruderal communities. Of the species found in the midfield studied balks 67 plants showed melliferous properties. Bee nutrients of high melliferousness and the coefficients of cover (D) were, among others: *Ballota nigra* L. (values of honey yield 368 kg/ha, D-1215), *Melilotus officinalis* (L.) Pallas (200, 1458), *Medicago lupulina* L. (200, 1264), *Salix alba* L. (150, 292), *Trifolium repens* L. (90, 104). Considering the production of pollen, the most valuable melliferous plants commonly found in most communities were: *Taraxacum officinale* F. H. Wiggers coll. and *Cirsium arvense* (L.) Scop., occupying larger areas and already less numerous: *Cichorium intybus* L., *Daucus carota* L., *Medicago lupulina* L., *Rubus caesius* L. In the

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studied area, grass species predominated on the surface, such as: *Apera spica-venti* (L.) P. Beauv, *Arrhenatherum elatius* (L.) P. Beauv. ex. J. & C. Presl, *Dactylis glomerata* L., *Elymus repens* L., *Lolium perenne* L. These species are valuable for bee-keeping due to the heavily occupied areas. This is confirmed by Ceglińska's (2008) study, which has recorded the presence of pollen grains in all honey samples. Among all taxa pollen grains present in one sample, *Poaceae* pollen has reached the highest frequency (70%). The study by Link (2007) of long and narrow belts of balks in the extensively developed fields (each research area amounted to 4.1 ha) in the vicinity of Bronowice, lists 153 plant species, whereas in the intensively managed areas only 59 species. He emphasized the role of balks in the middle of intensively managed agricultural landscapes. The ecological function of these objects is not to be overestimated, even if they constitute 0.8% of the whole surface. In the analysed research area 45% of the observed flora of the balks showed melliferous properties, and these were species characterised by high melliferousness (over 300 kg/ha). Maintenance of a natural habitat or natural habitat elements in a farm is important for behaviour of bees in functionally differentiated communities of agroecosystems (Forrest et al., 2015). This study examined how the quality of a field boundary habitat and farming system (organic or conventional) affect species richness and abundance of diurnal lepidopterans and bumblebees in boreal agricultural landscapes. The results showed positive effects of field boundary area on lepidopteran diversity, as well as positive effects of nectar flower abundance on lepidopterans and bumblebee abundance. The results suggested that a successful conservation strategy for lepidopterans present in boreal agroecosystems depends on proper management of field boundaries, irrespective of farming regime. The results concord with a general hypothesis that the effects of organic farming might be overpowered by the effects of landscape structure in heterogeneous landscapes (Ekroos et al., 2008).

Apart from bee nutrient properties flora of the balks provides raw materials for the production of pharmaceuticals. It was proved that 75% human population benefits from therapeutic properties of herbs. Particularly precious are those gathered in the areas of high sanitary values and which are subject to the protection of biological diversity and such is the area of balks (Abbas et al., 2015). In the studied terrain 63% of plants possessed therapeutic properties affecting at least three human ailments. They were meadows species: yarrow (with carminative, antispasmodic, anti-haemorrhagic, antiallergic properties), hogweed (shows anti-diarrheal properties, regulates digestion, is used in laryngitis and physical exhaustion) or curly dock (astringent, antidiarrheal, antibacterial, anti-anemic, detoxifying effects). Only a few of them are helpful in treating one condition. Therapeutic species that are helpful in treating many diseases predominate.

The results indicated a positive influence of integrated farming on biodiversity of herbal and bird communities in the conditions of agricultural landscape. Especially noteworthy was the positive importance of integrated meadows as the type of farmland with the highest species diversity, due to the interconnectedness of all components of the ecosystem (Štefanová and Šálek, 2014). The flora of the balks of the studied area is structurally diverse also in terms of heights. Thus, it enables convenient conditions for the creation of ecological niches for animals staying temporarily or permanently in the fields. The highest layer, although only in few boughs, is trees. Mainly it was a white willow with the height (on non-topped specimens) of up to 16 m. Single specimens of shrub were found more often for example (up to 5 m in height) - elder. In local

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depressions of the studied area, herbaceous plants grew up to 1.5 m (mugwort or nettle). The remaining sward was definitely lower, except for inflorescences of some grasses (oat-grass, tufted hair grass) or dicotyledons (common chicory, evening primrose, melilot, mallow).

Halada et al. (2011) identified the habitat types listed in the Habitats Directive Annex I that require low-intensity agricultural management for their existence. They discussed that habitat types for which either doubts remain on their dependence on agricultural management, or the relation to extensive farming practices exists only in part of their area of distribution in Europe or under certain site conditions, respectively. Assessments of the conservation status of habitats of European Importance by 25 EU Member States in 2007 showed that habitats identified in the study as depending on agricultural practices had a worse status than non-agricultural habitats.

Plant communities in the area of balks constitute poor phytocoenoses dominated mainly by grasses (for example *Convolvulo arvensis-Agropyretum repentis*), the participation of which is linked with a high level of fertilization in in the neighbouring fields. In the studied area 14 species of grasses were observed, out of which 6 constituted communities. These were: communities with *Apera spicae-venti* from *Stellarietea mediae* class (crop fields communities), association *Agropyretum repentis* from *Agropyretea intermedio-repentis* class (semi-perilous pioneer communities), association *Arrhenatheretum elatioris* from *Molinio-Arrhenatheretea* class, communities with *Dactylis glomerata*, communities with *Lolium perenne,* communities with *Agrostis capillaris* (semi-natural and anthropogenic turf meadow and pasture communities). Literature data also prove that on edge belts there is a slight number of segetal species from class *Stellarietea mediae* (Denisow and Wrzesień, 2007). However, Gargała and Trąba (2014) conducted studies in the south of Poland (Podkarpacie - Subcarpathia) and showed higher participation of such species (to 50%). In the research area of balks in Ińsko Lakeland (Pojezierze Ińskie) segetal species from class *Stellarietea mediae* constituted 14%. Among them two species: *Apera spica-venti* (L.) P. Beauv and *Chenopodium album* L. dominated in communities.

Balks are treated as linear elements in the strongly changed landscape which particularly refers to the area of arable fields. They are under strong pressure due to their small surface in relation to a very long line of adjacency, which results in poverty of plant species (Ratyńska, 2011). The width of the examined balks in the Ińsko Lakeland ranged from 50 to 70 m. The average length of short/long balks was 246 m and 916 m respectively, at a minimum of 76 m and 522 m respectively, and a maximum of 421 m and 2552 m. No relationship was observed between the increase in the width of the balks and the increase in the number of plants present there. In order to sustain biodiversity and maintain species, propositions difficult to accept can be met, such as undersowing weeds and sowing worse cleaned seeds and leaving some weeds in the field. Such a situation takes place in Western Europe countries of higher intensification of agriculture. In relation to this, the choice of undersown segetal plants becomes significant. An important criterion of the selection of these plants will be high reliability and therapeutic properties. Fast urbanization, habitat destruction, over-grazing and over-harvesting of medicinal plants has resulted in a loss of native species and traditional knowledge among the local communities. Efforts are required to conserve habitat as well as indigenous knowledge of the study area (Abbas et al., 2015).

The clearly significant effect of study by Tuomisto et al. (2012) is somewhat in contrast with the contradictory findings obtained in some areas of Western and Eastern

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Europe. In Western Europe, such studies could probably have led to ambiguous results because the landscape devastation there had reached a much higher level, and thus regeneration of communities of organisms is a long-term matter and requires synergistic interaction of multiple positive factors. The studied area of the lake district is characterized by average soil quality, strongly undulating terrain and locally diversified humidity conditions, which do not induce farmers to significantly increase the anthropogenic pressure associated with the cultivation of these fields.

Losses of biological diversity which result from the land use can lower their ecological role (Hooper et al., 2012). Bee reactions are not equally negative (Winfree et al., 2009) because the mosaic of seminatural and arable habitats which differ as regards vegetation, soil and water conditions compensates transformations in the economic production space, which illustrates that these habitats are richer as regards biodiversity by 10.5% (Julier and Roulston, 2009). The selection of therapeutic species should take into account their place of origin (the areas under protection) and local soil-weather conditions. Plant undersowing can be regarded as highly beneficial in the case of species particularly threatened by extinction in a given area. For this reason it is possible to sustain the natural state of vegetation and propagation of useful organisms. This is important from the point of view of environmental protection and can be of larger significance if the plants are grown in large areas. Relatively well maintained balks characterised by significant biodiversity in the area of Ińsko Lakeland can constitute a good point of reference to determine a beneficial specific composition on soils representing average soil conditions for cultivation in Europe.

Conclusions

1. It was shown that in the areas of an arable surface characteristic of average farms as regards their size in the European Union, the size of arable land and the length of balks related with it had an influence on phytodiversity. Despite the fact that the number of phytocoenoses was larger on shorter balks, their specific poverty did not affect biodiversity significantly.
2. The presence of a large number of melliferous species influenced not only phytodiversity of balks (102 species), but it resulted in the improvement of nutritive conditions, among others, for bees. Variety of plant structure (trees, shrubs, herbs), considerable flowering length (from March to October) and various flower construction of the so-called field weeds can provide a food base for many pollinators, including honeybees.
3. A larger number of plant species on long balks, including also melliferous (67) and therapeutic species (64), proves correct management of the production space in a long time period.
4. More and more climatic changes are pushing us to undertake further, more detailed studies of the physical factors and flora of the balks that shape the surrounding biodiversity.

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INVESTIGATING THE PHYSIOLOGICAL RESPONSES OF THREE ENDAPHIC STRAINS OF CYANOBACTERIA TO CRUDE OIL CONCENTRATIONS IN LIMITED SALINITY AND IRRADIATION CONDITIONS

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Abstract. The objective of the present study is to investigate the physiological responses of three soil strains of cyanobacteria (collected from areas contaminated with oil in southern Iran and from paddy-fields in north of Iran) to crude oil concentrations in limited salinity (NaCl) and irradiance conditions. Three cyanobacteria species of *Fischerella* sp. ISC107, *Nostoc* sp. ISC101, *Phormidium* sp. ISC108 were assayed. After purification, samples were grown in BG11 and $BG11_0$ media. The results showed that the efficiency of hydrocarbon source utilization of *Phormidium* sp. ISC108 was higher than in other cyanobacteria in crude oil treatment. The highest growth rate was observed at 3% crude oil concentration in *Phormidium* sp. ISC108. Also, the highest levels of allophycocyanin, phycocyanin, phycoerythrin, chlorophyll, carbohydrate, protein and phycobiliprotein were obtained from cyanobacteria *Phormidium* sp. ISC108 had larger phycobilisomes than the other strains (3.99 µg/gdw). The highest ratio of PSII/PSI was observed in *Fischerella* sp. ISC107. The highest levels of photosynthetic indices were assigned to 3% crude oil. In fact, reducing photosynthetic efficiency in higher levels of crude oil could be related to the toxic effect of organic hydrocarbons on algae growth. It can be concluded that the cyanobacteria can be used as a bioremediation for decomposing crude oil especially in the coastal area of the Persian Gulf and the Caspian Sea. The results of this research can be valuable for reducing the contamination which affects the fauna and flora of the marine ecosystems in Iran.
Keywords: *crude oil, cyanobacteria, allophycocyanin, chlorophyll, carbohydrate, phycobiliprotein*

Abbreviations: APC: Allophycocyanin; Chl: Chlorophyll; PE: Phycoerythrin; PC: Phycocyanin; PBP: Phycobiliproteins; PSI, PSII: Photosystem I, II; PBP/Chl: PSII/PSI ratio; Phycobilisome size: PC+PE/APC

Introduction

In tropical and temperate crude oil production sites, cyanobacterial mats often develop on petroleum contaminated zones in surface soils and water environments. Cyanobacterial mats are microbial combinations composed of photosynthetic and non-photosynthetic bacteria and fungi embedded in cyanobacteria mucilage (Shruthi and Rajashekhar, 2014). Factors that influence the uptake, bioconcentration and degradation of many chemical pollutants include extremely limited light, the structure and concentration of the chemical substances, pH, temperature and nutrient availability of the medium, cell size, number and physiological capabilities of microorganisms (Amirlatifi, 2011). Crude oil is a highly toxic mixture of more than 10000 different hydrocarbons. Accidental spills of crude oil in environment cause severe contamination of marine and continental ecosystems. Contamination is an important problem in waters

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due to spill of processed petroleum derivates especially diesel and fuel (Liu et al., 2009). These pollutants can potentially be degraded by a great variety of soil and aquatic microorganisms. Bacteria, filamentous fungi, yeasts, and cyanobacteria are known to be important hydrocarbon degraders (Prince et al., 2010; Das and Chandran, 2011).

In fact, toxicity and transformation of pollutants may change by cyanobacteria or microalgae depending on the species. The strains of microalgae (*Stichococcus* sp., *Chlorella* sp. and *Scenedesmus quadricauda*) and cyanaobacteria (*Nostoc* sp., and *Phormidium* sp.) are capable to grow without any apparent toxic effect in medium containing 1% black oil and bacterial protectants. The bacteria protect the algae from the toxic effect of the oil, while the cyanobacteria provide the bacteria with oxygen (O_2) and other extracellular carbon-rich exudates (Stal, 1995). For example, El-Sheekh et al. (2013) revealed that the growth of *Scenedesmus obliquus* was increased in the presence of high concentrations of crude oil as compared with *Chlorella vulgaris* and the highest growth of *Scenedesmus obliquus* was obtained at 0.5% crude oil, whereas, in *C. vulgaris*, the highest rate of growth was obtained at 2% crude oil. Some metabolic pathways are inhibited or abolished and some others are enhanced or induced when a change in the environment exceeds a certain threshold level. There are certain reports on the responses of cyanobacteria to oil pollution (Abed et al., 2015; Shruthi and Rajashekhar, 2014; Tamkini et al., 2015) regarding the metabolic versatility of cyanobacteria in different strains. For example, Iranian Stigonema strain Fischerella sp. FS 18, showed the best growth at 0.5% salinity (Soltani et al., 2011) but this may have changed and reached to 1% at different alkalinity (Amirlatifi et al., unpublished data) and Irradiance (Soltani et al., 2007).

Despite of this high potential of microalgae, there is not much attention to the physiological activities of these microorganisms under petroleum contamination. Recent works are changing and some preconceived notions concerning the adaptation of microalgae to extreme environments. As might be expected, physiological acclimatization is enough to achieve adaptation under slightly stressful conditions (Costas et al., 2008). It seems logical to believe that these versatile organisms may have powerful acclimation and adaptation mechanisms to help them stay in extreme habitats, such as oil-polluted soils for a long time. Crude oil rapidly inhibits photosynthesis and growth of microalgae, but little is known about the mechanisms that allow the adaptation of microalgae to extreme environments.

In order to enhance the knowledge on the adaptation of microalgae, including Cyanobacteria to extreme petroleum contamination (Carrera-Martinez et al., 2011), investigating the ecophysiological behaviors of native organisms under field and laboratory conditions is of priority. Southern and northern epidaphic and endaphic cyanobacteria are unexplored. Despite the fact that the southern provinces are oil-rich regions and oil is strategic for Iran, and given that cyanobacteria have been found to have significant potential in the biotechnology of medicine, industry, fisheries presently, little is known about the ecophysiology of the epidaphic and endaphic cyanobacteria of the oil and gas fields in the south and north of Iran (Soltani et al., 2006).

In this way, the demonstration of these organisms could be the reason for future exploitation from various aspects, including physiology and ecophysiology. As it was noted, given that oil and agriculture are of particular importance in the Iranian economy such studies are considered strategic and also the problem of survival and growth under

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various stress conditions could be useful for applied dimensions considering the necessity of using soil reformers in the future (Soltani et al., 2006).

Combined review of three concentrations of oil; the intensity of limited light can be considered as innovative work in the present study. The microbial stacks and shells should be able to deal with these three factors simultaneously both in flood conditions (rice cultivation) and under the conditions of the underlying layer of oil, and in combination condition. In most studies performed, one or two factors have taken under consideration (Rajendran et al., 2007). However, due to the lack of data for comparison with other indigenous specimens in the country and elsewhere in the world, it is necessary to use reports than detailed scientific analysis (Shokravi et al., 2002).

The objective of present work was to investigate physiological responses of three soil strains of cyanobacteria (collected from areas contaminated with oil in southern Iran and from paddy-fields in north of Iran) to crude oil concentrations in limited salinity and irradiance conditions. The authors hope that, the present work would be an attempt for better understanding of potential native cyanobacteria for both agricultural biotechnological and oil bioremediation projects.

Materials and methods

Isolation of strains

Fischerella sp. ISC107, *Nostoc* SP. ISC101, *Phormidium* sp. ISC108 were collected from north (paddy-fields of Golestan province, near the Caspian Sea) and south (petroleum contaminated soils near the Persian Gulf) (*Fig. 1*). The collected samples were cultured by ordinary methods (Lowry et al., 1971). Purification and liquid cultures were performed by solid agar and routine procedures with BG11 and $BG11_0$ media (Andersen, 2005). $BG10_0$ was used that included the following ingredients: $MgSO_4{\cdot}7H_2O$, 0.3 mM; $CaCl_2{\cdot}2H_2O$, 0.25 mM; $K_2HPO_4{\cdot}3H_2O$, 0.18 mM; $Na_2MgEDTA$, 0.003 mM, citrate ferric 0.02 mM; citric acid, 0.029 mM; Na_2CO_3 0.188 mM; microelements 1 $ml{\cdot}L^{-1}$. All chemical materials were purchased from Merck Company (Germany) and used without further purification.

Culture conditions

Stock cultures were grown in the $BG11_0$, and BG11 culture media. Each cyanobacterium was pre-cultured in Erlenmeyer flasks at pH~7 and at temperature of 25 °C. Cells were collected from stock cultures in logarithmic phase of growth and were used as inoculate for experiments. Cells from stock culture were inoculated in 300 ml of $BG11_0$ medium in 500 ml Erlenmeyer flasks stoppered with cotton plugs. The following treatments were used: 0 (control), 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% crude oil and limited irradiation (5 $\mu Em^{-2} s^{-1}$) and limited salt (17 mM NaCl) for incubation period of 14 days.

Analytical methods

Growth measurements and pigment composition

Cell growth was checked by evaluation of algal dry weight, every 2 days in duplicate (Kaushik, 1987; Shruthi and Rajashekhar, 2014). Chlorophyll *a* (Chl. *a*) concentration was evaluated performing overnight extractions with methanol. Centrifuged extracts were measured at 665 nm and calculated by the extinction coefficient (Lowry et al.,

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1951). Phycobiliproteins were extracted after osmotic shock and evaluated spectrophotometrically at 750, 652, 615 and 562 nm. Oxygen (O_2) evolution was measured using a Clark-type sensors measuring oxygen partial pressure from Hach Chemical Company (Shruthi and Rajashekhar, 2014). The total carbohydrates were measured using the anthrone-thiourea method (Herbert et al., 2010), and soluble proteins were estimated using bovine serum albumin as the standard (Liu et al., 2009).

Statistical analysis

Statistical differences were examined using the General linear multivariate model test by SPSS software version 16.0.

Figure 1. *The sampling location: a) North of Iran (paddy-fields of Golestan province, near the Caspian Sea) and b) South of Iran (petroleum contaminated soils near the Persian Gulf*

Result and discussion

Cyanobacteria isolated from northern paddy-fields and southern oil fields of Iran include *Fischerella* sp. ISC107, *Nostoc* sp. ISC101, *Phormidium* sp. ISC108. The results of the physiological responses of isolated samples to the increase of crude oil in limited light and limited salinity conditions were 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ and 17 mM, respectively ($p < 0.01$).

Table 1 shows the growth rate (μ), the doubling time (G) in the three samples under oil treatments from zero to 10%. As it is shown, growth rate and doubling time was positive for all three samples. Investigating the doubling time indicated that the highest amount of time to be doubled was observed in the control concentrations for *Phormidium* sp. ISC108, 1 and 6% for *Fischerella* sp. ISC107 and 4% for *Nostoc* sp. ISC101. On the other hand, the growth rate in *Fischerella* sp. ISC107 and *Nostoc* sp. ISC101 showed an increasing trend from zero to 10% of crude oil, while a different process was observed in *Phormidium* sp. ISC108, so that the highest rate of growth increased to 3% concentration, and with increasing concentrations up to 4%, where reduction in growth rates was observed ($p < 0.01$). Sample survival in oil treatments indicates that the sample could not increase the biomass in the treatments and only retained itself from death, and again, increasing trend was observed to 6% concentration, then, a decreasing trend was observed with increasing concentrations up to 10% (*Table 1*) ($p < 0.01$). Kiayi et al. (2013) found that the time of cell division, and hence, the doubling time was decreased with the increase in growth, the results are consistent with the obtained results in this paper (Kaushik, 1987).

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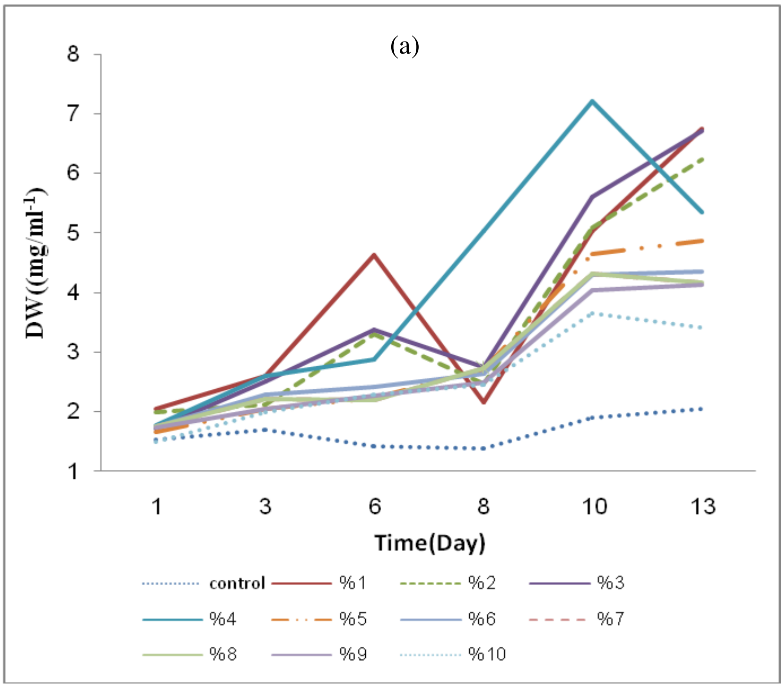
Table 1. *Specific growth rate (μ) and doubling times (G) of three cyanobacteria strains in limited light and limited salinity conditions were 5 μE·m^{-2}·s^{-1} and 17 mM*

SP		Oil percentage (%)										
		0	1	2	3	4	5	6	7	8	9	10
Phormidium SP. ISC108	G	1.73	0.62	0.68	0.66	0.86	1	0.84	0.93	0.99	0.96	1.19
	μ	0.4	1.1	1.02	1.04	0.8	0.69	0.82	0.74	0.7	0.72	0.58
Fischerella SP. ISC107	G	1.31	1.74	0.94	1.12	1.02	1.37	1.74	1.57	1.42	0.9	0.92
	μ	0.52	0.39	0.73	0.61	0.67	0.5	0.39	0.44	0.48	0.76	0.75
Nostoc SP. ISC101	G	1.37	0.82	0.94	0.99	1.18	1.06	1.02	0.79	0.82	0.95	0.66
	μ	0.5	0.83	0.73	0.69	0.58	0.65	0.67	0.86	0.83	0.72	1.03

Figure 2 presents the growth curve of three samples under crude oil treatment. As it is the logarithmic phase of *Phormidium* sp. ISC108 an increasing trend ($p < 0.01$) was observed. This trend was observed in all treatments in this sample. The samples showed a decrease in growth after the ninth day until the thirteenth day ($p < 0.01$). In addition, the absorption rate in this curve was higher than those of the other two samples.

The results indicated the survival of the *Phormidium* sp. ISC108 in all oil treatments. Also an upward trend was observed in the study of the logarithmic phase of *Fischerella* sp. ISC107 and *Nostoc* sp. ISC101 strains. So, in different concentrations of crude oil treatment, they first encountered a decline in growth rate and again showed an increasing trend ($p < 0.01$). Generally, the productivity of the hydrocarbon resource of *Phormidium* sp. ISC108 was higher than those of other strains in crude oil treatment (*Fig. 2a, b, c*). Amirlatifi et al. (2013) reported that biomass amounts was 3.88, 4.35 and 4.60 mg/ml^{-1} on the 12th day of inoculation at control, 1 and 7% concentrations, respectively. This increasing trend was consistent with the results of this study.



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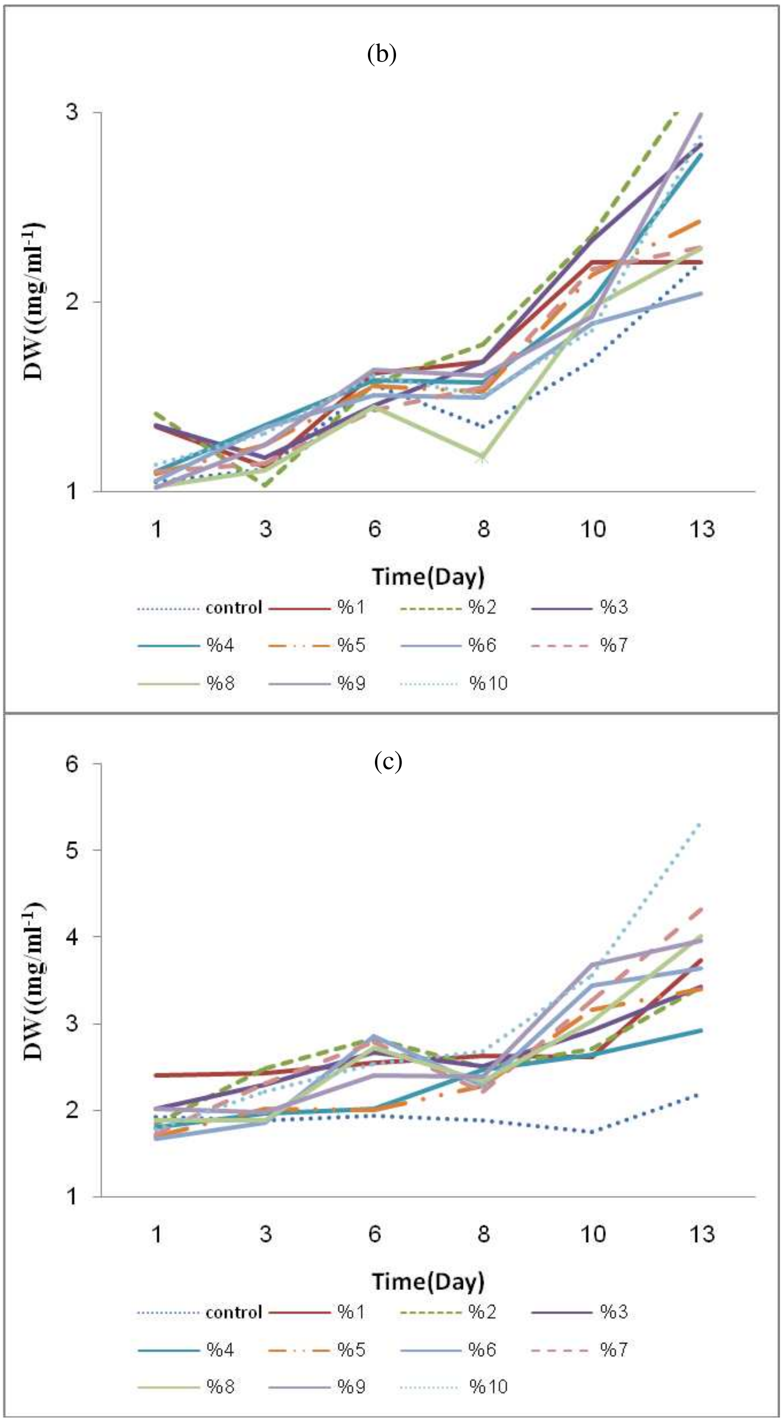

Figure 2. *Dry weight (DW) in different crude oil concentrations. limited light and limited salinity conditions were 5 $\mu E{\cdot}m^{-2}{\cdot}s^{-1}$ and 17 mM. (a) Phormidium SP. ISC108; (b) Fischerella SP. ISC107; (c) Nostoc SP. ISC101*

Figure 2 shows that in cyanobacteria *Phormidium* sp. ISC108; lag phase is not observed throughout the treatments with different oil levels. The progressive phase with a roughly identical gradient was observed in the first days after inoculation, which results from the rapid adhesion of the sample to the environment. On the third day, and

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after the seventh day of inoculation, the growth rate changes and the effect of oil treatments could be observed.

Growth up to 1.04 mg/ml^{-1} is due to the high uptake of this sample (in general) with high concentrations of crude oil. This growth is reduced in two other cyanobacteria (*Fig. 2a, c*). In *Fischerella* sp. ISC107, although a decline was observed in growth rate, generally due to habituation to oil change conditions was more sensible in the first days after inoculation (*Fig. 2b*). The pattern of behavior in this cyanobacterium is due to the absence of a negative growth phase until the third day at a concentration of 3% of the specific crude oil. However, a sample under the influence of time could be adapted to this concentration (*Fig. 2b*). The existence of a long-term delayed phase in salinity of 3% in cyanobacterium *Nostoc* sp. ISC101 is remarkable, which continues even as negative growth until the eighth day.

In addition, in this sample, the lower level was observed in the treatments except at 10% with high growth slope as the same as *Phormidium*, but latent and negative phase is more remarkable, which generally is due to the incapability to habituate in short time (less than 72 h) to high concentrations of oil (*Fig. 2c*). The shorter lag phase and the same trends of growth curves that can be observed in other samples showed that *Phormidium* is not sensitive to higher concentration of oil through the more growth and will not be affected in the short time.

On the contrast, the growth rate in *Table 1* indicates that the amount of produced biomass in such a situation is higher than those of other treatments (*Table 1*). The effect of oil concentration on all three samples is considerable and the amount of biomass production in cyanobacteria is affected by combination of oil concentrations, irradiation and salinity stresses (*Table 1*). The results of mean comparisons using Tukey's test shows that dry weight in different genera under crude oil was classified into three groups and the genus *Phormidium* sp. ISC108 and *Fischerella* sp. ISC107 had the highest and the lowest amount (*Table 2*).

Table 2. *Dry weight (DW) in three strains of cyanobacteria under limited light and limited salinity conditions were 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ and 17 mM. Data shows X ± SE*

SP	**DW (mg/ml^{-1})**
Phormidium SP. ISC108	3.23 ± 1.628^{a}
Fischerella SP. ISC107	$1.67 \pm .543^{c}$
Nostoc SP. ISC101	$2.58 \pm .737^{b}$

The amounts of dry weight in different days also shows a significant difference, so that the values were classified into 5 categories, the highest and the lowest amounts of dry weights were observed on day 13 and the first day, respectively (*Table 3*). On the other hand, the dry weight contents at different concentrations of crude oil were classified into three categories, which showed a significant difference, so that the control concentration had the lowest value and concentrations of 1-5% crude oil shows the highest amount (*Table 4*). Dhull et al. (2014) found that by increasing the concentration of carbon in the medium, the biomass production rate increases in some microalgae species, which is consistent with the results of this study.

Lack of salinity or salinity of 17 mM, could affect the results. The same amount of salinity and the lack of involvement in such surveys is a defect for any process with a biotechnological approach. Gu et al. (2012) shows that the growth and production of

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pigments in *Nannochloropsis oculata* under pond conditions and using *in vitro* photo-bioreactor is affected by salinity. The salinity range was from 15 to 55 g/l^{-1} in the form of five treatments. Reducing salinity from 55 to 15 g/l leads to increasing growth and chlorophyll content. Lack of lag phase in the presence of high concentrations of oil, could be considered. In the studies of Amirlatifi et al. (2011) and Abed et al. (2015), the absence of lag phase has been evaluated from the viewpoint of the outflow of wall-forming compounds and nitrogen metabolism.

***Table 3**. Dry weight (DW) in different days under limited light and limited salinity conditions were 5 µE·m^{-2}·s^{-1} and 17 mM. Data shows X ± SE*

Time(Days)	DW(mg/ml^{-1})
1	1.60±.370^{e}
3	1.84±.511^{d}
7	2.23±.721^{c}
9	2.27±.886^{c}
11	3.30±1.531^{b}
13	3.72±1.382^{a}

***Table 4.** Dry weight(DW) in different crude oil concentrations under limited light and limited salinity conditions were 5 µE·m^{-2}·s^{-1} and 17 mM. Data shows X±SE*

Crude oil(%)	DW (mg/ml^{-1})
0	1.69±.341^{c}
1	2.76±1.42^{a}
2	2.68±1.258^{a}
3	2.74±1.394^{a}
4	2.73±1.769^{a}
5	2.72±1.588^{a}
6	2.40±1.091^{b}
7	2.44±1.01^{b}
8	2.34±.991^{b}
9	2.41±.950^{b}
10	2.42±1.039^{b}

A sample that can be adapted to the new conditions at the first days after inoculation will not have problem with wall-forming compounds and subsequent reproduction for entry into the log phase (Soltani et al., 2011). Under laboratory conditions, cyanobacteria have shown a delayed phase or a negative growth phase depending on the acidity and alkalinity conditions (Amirlatifi, 2011).

Shokravi et al. (2002) reported that the *Fischerella* sp., *Nostoc* sp. and *Oscillatorian* cyanobacteria have shown a lag phase when they enter the natural environment due to their incompatibility with the environment in the first days. It should not be said which factors have contributed to the difference in growth behavior, but this difference has always been existed.

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Compared with the laboratory conditions employed in the present work, as well as studies by Amirlatifi et al. (2011), Pakzad et al. (2011) and Badeli et al. (2012), although neither of the three environments has shown significant differences in terms of growth on the twelfth day, under laboratory conditions, this behavior is not observed at least within the first few days. It was not possible to follow the growth curve in the days following the twelfth day, and it is possible that the cyanobacteria would be retrieved, but it cannot be commented on this due to the lack of investigation on this issue.

Under laboratory conditions, the behavior of samples is different, and the progressive and stagnant phase continues until the 12^{th} day. This indicates the difference between the behaviors of the natural, and, the laboratory environments. In the studies of Chekigar et al. (2012) and Marvizade (2012) on cyanobacterium *Microchaete* sp. it has been shown that cyanobacteria changes trend in carbon dioxide and salinity treatments in the last days and shows a pattern similar to that in the natural conditions in this study.

In this study, such a pattern was related to the change in photosynthetic mechanism (Marker, 1972) and the carbon dioxide condensation mechanism (Chekigar, 2012) under laboratory conditions. It is likely that such a situation exists in the natural environment, as well. Particularly, under the natural environment, there was no carbon dioxide inoculation. However, it is definitely not possible to comment because the condensation-inhibiting compounds were not used in this study.

Table 5 deals with the effect of oil concentrations on the ability of cyanobacteria to habituate with limited light. Regarding the severity of extreme limited light, surveys conducted in Iran have been limited to surveys devoted to paddy-field of Northern Province in Iran.

Table 5. *Photosynthetic pigments amount in different cyanobacteria grown under limited light and limited salinity conditions were 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ and 17 mM. Data shows X ± SE*

SP	APC	PC	PE	Chl	Carbo	Pro	PBP	PC+PE/APC	PBP/Chl
	($\mu g/g^{-1}$ dw)								
Phormidium SP. ISC108	4.79±4.59[a]	10.62±7.14[a]	8.48±2.51[a]	4.26±.23704[a]	633.60±2.38[a]	677.10±3.93[a]	23.89±1.30E1[a]	3.99±1.21[a]	5.60±0.75[c]
Fischerella SP. ISC107	3.24±1.59[b]	4.60±2.04[c]	4.79±1.90[c]	1.03±.09844[c]	281.40±2.12[c]	344.70±1.16[c]	12.63±4.07[c]	1.69±3.32[b]	12.26±1.35[a]
Nostoc SP. ISC101	4.54±1.69[a]	7.38±2.249[b]	6.38±1.56[b]	2.52±.13139[b]	506.00±2.26[b]	462.70±2.22[b]	18.32±4.762[b]	0.96±0.56[b]	7.27±0.57[b]

APC: Allophycocyanin, PE: Phycoerythrin, PC: Phycocyanin, Carbo: Carbohydrate, Pro: Protein,Chl: Chlorophyll, PBP: Phycobiliproteins

The results of the mean comparisons obtained from the physiological traits with Tukey's test shows that PC (Phycocyanin), PE (Phycoerythrin), Chl (Chlorophyll), Carbo (Carbohydrate), Pro (Protein) and PBP (Phycobiliproteins) traits were classified into three groups in different genus, so that the genus *Phormidium* sp. ISC108 had the highest and *Fischerella* sp. ISC107 had the lowest amount. Also, evaluation APC (Allophycocyanin) values showed that the genus *Fischerella* sp. ISC107 showed the lowest amount but other genus show the highest amount.

The study of PBP/Chl and PC+PE/APC values showed that the concentration of 10% crude oil was the highest value (*Table 6*). In fact, the ratio of PBP/Chl shows the relationship between the photosystem II and the photosystem I. It should be noted that these results were precisely the same as that found for the comparison of the mean values of PBP / Chl (PSII/PSI ratio). Also, the results of the measurement of phycobilisome (PC+PE/APC) showed that the *Phormidium* sp. ISC108 strain had the

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highest value (3.99; $P \leq 0.01$). On the other hand, PBP values were classified into three categories, as *Fischerella* sp. ISC107 showed the minimum amount but *Phormidium* sp. ISC108 showed the maximum amount (*Table 5*).

Table 6. *Photosynthetic pigments amount in different crude oil concentrations under limited light and limited salinity conditions were 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ and 17 mM. Data shows X±SE*

Range of crude oil (%)	APC	PC	PE	Chl	Carbo	Pro	PBP	PC+PE/APC	PBP/Chl
	($\mu g/g^{-1}$ dw)								
0	4.62±2.84^{b}	6.76±4.27^{bcd}	6.07±2.30^{cd}	3.38±0.10^{ab}	463.78±27.82^{c}	442.31±18.15^{f}	17.09±1.27E1^{de}	2.77±6.044^{bc}	5.06±10.59^{b}
1	3.93±3.95^{bc}	6.70±4.20^{bcd}	6.43±3.41^{bc}	2.86±0.23^{c}	501.00±21.96^{bc}	528.81±4.10^{bc}	17.97±1.01E1^{d}	6.23±17.15^{b}	6.28±9.78^{b}
2	3.33±1.64^{c}	6.18±3.38^{d}	6.17±2.76^{c}	2.42±0.17^{d}	384.12±.19.37^{cd}	357.62±15.22^{g}	15.25±7.90^{e}	3.33±5.31^{bc}	6.30±4.85^{b}
3	5.94±4.16^{a}	12.94±8.58^{a}	9.61±1.52^{a}	3.58±0.3541^{a}	742.50±30.32^{a}	884.62±52.07^{a}	28.49±9.11^{a}	3.79±0.71^{bc}	7.96±8.99^{b}
4	3.79±2.82^{bc}	6.57±3.75^{cd}	6.13±2.28^{b}	2.03±0.12^{d}	405.22±22.52^{d}	349.55±19.52^{g}	16.38±8.29^{e}	2.62±5.77^{bc}	8.06±5.96^{b}
5	4.20±3.46^{bc}	7.09±4.74^{bcd}	6.82±3.00^{b}	3.36±0.32^{ab}	465.46±26.95^{c}	449.72±20.55^{ef}	18.65±1.05E1^{b}	4.48±13.80^{b}	5.55±1.922^{b}
6	4.71±3.64^{b}	6.90±5.21^{bcd}	6.29±2.54^{bc}	2.83±0.18^{c}	533.27±28.24^{b}	530.52±25.67^{bc}	18.21±1.06E1^{c}	1.22±0.88^{c}	6.43±4.95^{b}
7	4.20±2.63^{bc}	7.63±4.18^{bc}	6.39±2.36^{bc}	3.08±0.24^{c}	538.71±29.13^{b}	552.83±19.36^{b}	18.22±9.56^{bc}	3.34±229.32^{bc}	5.91±5.64^{b}
8	4.19±2.81^{bc}	7.88±6.32^{b}	6.37±2.12^{bc}	2.13±0.09^{d}	428.92±28.82^{de}	480.81±20.84^{de}	18.08±1.12E1^{cd}	2.40±4.01^{bc}	8.49±10.80^{b}
9	3.60±2.25^{c}	7.40±3.78^{bcd}	5.54±0.97^{d}	1.58±0.06^{e}	423.53±23.57^{de}	513.13±23.94^{cd}	16.11±6.61^{e}	3.22±5.37^{bc}	10.19±8.98^{b}
10	3.60±2.05^{c}	6.78±2.75^{bcd}	6.18±1.82^{b}	1.40±0.08^{e}	324.01±15.73^{d}	353.49±19.37^{g}	16.12±6.02^{e}	8.77±25.52^{a}	11.51±15.01^{a}

APC: Allophycocyanin, PE: Phycoerythrin, PC: Phycocyanin, Carbo: Carbohydrate, Pro: Protein, Chl: Chlorophyll, PBP: Phycobiliproteins

Shokravi and Soltani (2011) studied the simultaneous effect of extremely limited light of 2 micromoles quanta per square meter per second and pH was studied on cyanobacteria *Hapalosiphon* sp. FS56. Soltani et al. (2006) studies on *Fischerella* sp. FS 18 showed that this species not only has the ability to survive in this intensity, but also has high photosynthetic and high nitrogen activity. The studies by Amirlatifi et al. (2013) has confirmed the same result for the *Nostoc* sp. FS 76 and *Anabaena* sp. FS 77.

The results of the mean comparison of physiological traits using Tukey's test showed in different concentrations of crude oil that the various amounts of APC in different concentrations of oil (0 to 10%) were classified in 3 groups. Moreover, the lowest amount of APC was observed in 3% crude oil and the highest amount was obtained in 2, 9 and 10% concentration of crude oil. While in PC amounts, the lowest values were observed at 2% crude oil concentration and the highest values were at 3% crude oil, which were classified in four separate categories. The study of PE values also showed that a sample of 9% and 3% oil content had the lowest and the highest value, respectively.

In a study by Hamouda et al. (2016), it was recorded that with the increase in crude oil content, the amount of carotenoides was increased in *Calothrix. kesseleri* and *Anabaena oryzae*, while according to Liu et al. (2009), the amount of carotenoids was decreased in mixotrophic cells than photo-autotrophic cells, which may be due to the toxic effect of organic hydrocarbons on the growth of algae, which is consistent with the results of this study and with the results of Liu et al. (2009). Also, the results of Chl values in various oil concentrations show that concentrations of 9 and 10% were the lowest and the concentrations of 3% were the highest amounts and the data were categorized in five separate categories.

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El-Sheekh et al. (2013) reported that the highest amount of chlorophyll (6.13 µg ml^{-1}) was obtained in *Chlorella vulgaris* fifteen days after inoculation with 1.5% concentration of crude oil, while in the samples examined in the present study, the highest amount (3.58 µg/gdw; $P \leq 0.01$) was obtained at a concentration of 3% crude oil.

The obtained results from Carbo values indicated that the values were classified into 7 categories, so that a concentration of 10% crude oil and a concentration of 3% crude oil had the lowest and the highest values, respectively. In the following, the physiological traits showed that the protein traits were classified into 7 groups, as the concentration of 10% and 3% showed the lowest and the highest values, respectively.

By investigating PBP values, it was concluded that the concentration of 3% crude oil was the highest and the concentrations of 2, 9 and 10% crude oil contained the lowest values, so that the data were categorized into five categories. Amirlatifi et al. (2013) found that the concentration of photosynthetic pigments depends on the concentration of crude oil that by increasing crude oil concentration, phycobiliproteins concentration was reduced. The results of the present study are also consistent with the mentioned study.

It seems that the reason for the reduction of phycobiliproteins in algae under conditions of environmental stress is resulted from the induction of biosynthesis of certain proteins. It can also affect the stability of the protein and its re-rotation by the protein proteolytic rate.

Pittmanet al. (2010) reported that the optical intensity limited to 10 µm quanta per square meter per second has been used on the cyanobacteria *Nostoc* sp. UAM206 and has reduced the growth and photosynthesis activity in comparison with 60 µM Quanta per square meter per second and 300 µM quanta per square meter per second, respectively.

Studies by Marvizade (2012) and Chekigar et al. (2012) have shown that the two species studied in this article retain their survival and have high photosynthetic and nitrogen activity in extreme conditions of 2 µM Em^{-2} s^{-1} in the presence of white light, which is far lower than other tested species such as *Nostocsp* FS 76 and *Anabaena* sp. FS 77 (Amirlatifi, 2011; Badeli et al., 2012).

Table 5 and the results table of photosynthesis (*Table 6*) show that the application of oil concentration not only does not entail tension on the photosynthetic apparatus, but also significantly increased the efficiency of this apparatus ($p < 0.01$). The maximum rate of photosynthesis increases significantly in these conditions ($p < 0.01$). This issue is in full compliance with the increase in sample growth rate under the above conditions.

Measurements of photosynthetic pigments (*Tables 5* and *6*) are due to phycobilisome system. It seems that in addition to the main pigments, the rode sections and the core sections of the phycobilisome is oscillated under the influence of oil concentrations.

It seems that the increase in oil concentration has significant effect on the increase of light absorption power ($p < 0.01$). In addition, salinity not only does not damage the phycobilisome system, but also adds to the length and power of its optical absorption. This is a major biotechnological achievement that could be particularly used in the process of inoculation of algae, for agricultural and for other purposes. Whether non-aeration (although it reduces the activity of the phycobilisome system a little) increases the limited light absorption efficiency and amplifies it through the phycobilisomes is another biotechnological achievement that can be useful in intensive culture through eliminating of carbon dioxide induction (Montagnolli et al., 2015; Prince et al., 2010).

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Significant increase in total sugar and total protein content (*Tables 5* and *6*) is remarkable from two perspectives ($p < 0.01$). Coordination with the potential of photosynthesis and nitrogen activation on the one hand, and the efficiency of the phycobilisome system on the other, is shown to be capable of generating total carbohydrate and protein. In other words, salinity not only does not reduce the level of carbohydrate pool and protein, but also significantly increases it ($p < 0.01$).

Relative absorption studies under in vivo conditions could be considered as evidence of the situation of the pigmentation of the reaction center structures, the light collector complex and phycobilisome antennas (qualitatively).

It seems that the phycocyanin level is interacted with the growth rate observed in both cyanobacteria, as the main and permanent barrier of phycobilisome in both cyanobacteria, under the influence of extreme limited light and salinity, although the absorption peaks in cyanobacteria are moved a little affected by combined stresses.

Soltani et al (2007) stated that cyanobacteria *Fischerella* sp. FS 18 loses its central allophycocyanin under acidity and alkalinity treatments and changes in light intensity.

Shokravi et al. (2011) in their research declared that Cyanobacteria *Hapalosiphon* sp.FS56 loses its allophycocyanin part under combined treatments of limited extreme light, acidity and alkalinity, in extreme alkaline conditions. Both samples contain phycoerythrin parts. Ahmadi Livani et al. (2009) reported that salinity and limited irradiation treatments in cyanobacterium *Hapalosiphon* sp. FS56 have not removed the allophycocyanin.

However, the results of this study indicate that the content of phycobilisome is completely preserved in extreme light stress conditions (2 micromoles quanta per square meter/second) and salinity in both cyanobacteria. In the studies of Tamkini et al. (2015), it has been shown for the first time that short-term irradiation and carbon dioxide treatments could lead to the displacement of pigments. This finding was also used in this study. It appears that the physiological fluidity of the sample in short periods of time (in shock) at very low light intensities can be as high as 2 $\mu M\ Em^{-2}\ s^{-1}$. The results of Tamkini et al. (2015) showed that optical shock and carbon dioxide could lead to extreme reactions.

In addition to this, the combination of crude oil, light and carbon dioxide shocks can change the color of the pigment and, of course, the size of the phycobilisome, even in the five to ten minute period. This is also compatible in other light intensities.It is appeared that light and carbon dioxide, combined with salinity affect the state of light-absorbing pigments. Interestingly, in this case, there are microsporine-like amino acids (MAAs) that have been considered as anti-ultraviolet treads. It was found in the studies by Badeli et al. (2012) and Amirlatifi et al. (2011) that the species of *Nostoc* sp. FS 101 and *Anaphana* contain more. This significant photosynthesis continues to 7% oil values (ANOVA, $p < 0.05$).

About *Microcheate* sp. FS13*,* photosynthesis is similar but its value is less (approximately 44 micromoles oxygen per dry weight unit), which is equivalent to the ones have been obtained from *Nostoc* sp. FS 101 and *Annapana* by Amirlatifi et al. (2013) and Badeli et al. (2012).

Absorption spectra in vivo in the first days after inoculation and comparison with the days after inoculation in different concentrations indicate the effect of a time factor on the behavior of the phycobilisome system of the sample in different oil conditions at different times. In the first hours after inoculation, both samples have high-density

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spectra, in which phycobilisome system has its pigments fully, especially phycocyanin that is detectable. But then, the sample behavior changes under the influence of salinity.

The change in the behavior of the phycobilisome system is natural and is due to the metabolic fluidity of the sample, but the important issue is the time problem. In the short and medium term studies, the problem of the effect of short time (shock) on the process of changing the phycobilisome system has been evaluated.

Conclusion

The marine environment is highly susceptible to pollution by petroleum, and so, it is important to understand how the microorganisms degrade hydrocarbons. In this research, the effects of crude oil under limited light and limited salinity on physiological characterization of the cyanobacteria *Fischerella* sp. ISC107, *Nostoc* SP. ISC101, *Phormidium* sp. ISC108 were investigated. This study showed that growth rate and doubling time was positive for all three samples, but *Phormidium* sp. ISC108 had the highest rate of growth in elevated oil concentration. Photosynthetic pigments amount in different cyanobacteria grown under the limited light and limited salinity conditions showed that *Phormidium* sp. ISC108 had the highest amount of APC, PC, PE, Chl, Carbo and Protein. In this research salinity significantly increased the levels of carbohydrate pool and protein and photosynthetic pigments amount ($p < 0.01$). Limited light eliminated induction of carbon dioxide concentration systems. Increasing oil concentrations up to 3% are significantly enhanced photosynthetic pigments amount, carbohydrate and under limited light and limited salinity but it decreased towards high concentrations ($p < 0.01$). It can be concluded that the cyanobacteria can be used as a bioremediation for decomposing crude oil especially in the coastal area of the Persian Gulf and the Caspian Sea. The results of this research can be valuable for reducing the contamination which affects the fauna and flora of the marine ecosystems in our country.

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CYCLANILIDE TREATMENTS INCREASE LATERAL BRANCHING OF APPLE AND PEAR NURSERY TREES

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Abstract. This study was carried out to investigate the effects of different cyclanilide (Cyc) and Perlan (Pr) doses on formation of lateral branches on apple [Golden Delicious (GD) and Starking Delicious (SD)] and pear (Deveci and Williams) nursery trees grafted on seedling rootstocks. Except for 5 mg L^{-1} Cyc in Deveci pears, all Cyc concentrations applied in this study increased significantly the number of lateral branches on both apple and pear trees in nursery. In GD apple, while control trees had 2.5 lateral branches, the trees treated 100 mg L^{-1} Cyc had 12.7 lateral branches per trees. Except for 250 mg L^{-1} in GD apple, all other Pr treatments did not cause a significant increase in the number of lateral branches on apple and pear trees. Crotch angles of SD apples decreased significantly with 750 mg L^{-1} Pr treatments. The 250 and 500 mg L^{-1} Pr treatments significantly reduced number of lateral branches of Williams pears. It was concluded based on present findings that entire Cyc doses could be used as an efficient tool to increase the number of lateral branches in apples and pears; high Cyc doses could also be used to reduce the first branch height in both apple cultivars and Deveci pears.
Keywords: *auxin, benzyladenine, bio-regulator, feather, perlan*

Introduction

For a profitable fruit growing, there are many factors to consider when establishing an orchard. These factors include rootstock, cultivar, tree density, training system, pruning methods and support systems. The primary target of the systems developed for the orchards is to maintain high yield levels through early and late yields and to get exceptional fruit quality (Robinson, 2003). The basic factor for early production is to get fruits from the initial years. Therefore, branched trees are used while establishing orchards (Coşkun, 2011). Number of lateral branches, crotch angle and the first branch height are significant factors in having early and high yields (Barritt, 1992; Hrotko et al., 1996; Kviklys, 2006; Koyuncu and Yıldırım, 2008; Magyar et al., 2008).

The formation of lateral branches can be controlled through external applications of growth regulators effective in apical dormancy. Cytokinins are known to inhibit apical. Previous researches indicated that benzyladenine (BA), a synthetic cytokinin, stimulated lateral branch formation (Faust, 1989; Atay and Koyuncu, 2016; Ipek et al., 2017). Perlan (Pr) or Promolin ($BA+GA_{4+7}$) have an active ingredient of cytokinins and gibberellin and these commercial products were also reported to stimulate lateral branch formation in different fruit species (Cody et al., 1985; Elfving, 1985; Yıldırım et al., 2010). Another approach to stimulate lateral branch formation is to inhibit transport of auxins which stimulate apical dormancy. It was reported in previous studies that 2,3,5-triiodobenzoic acid and n-propyl-3-t-butylphenoxyacetate and similar chemicals inhibited polar auxin transport and increased the number of lateral branches (Jacyna and Dodds, 2001). Cyclanilide (Cyc) has emerged as a bio-regulator inhibiting auxin transport and it was

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reported in previous studied that Cyc stimulated shooting of lateral buds in different fruit species (Tamas, 1995; Mickelbart, 2011). On the other hand, effects of such chemicals usually vary based on species and cultivars (Wustenberghs and Keulemans, 1999). Previous studies were mostly carried out over the trees grafted on clonal dwarfing rootstocks. On the other hand, in Turkey, nursery trees grafted on seedling rootstocks are still used at considerable rates in apples and pears. These trees generally yield late because of strong vegetative growth. Therefore, in nursery trees grafted on strong rootstocks, breaking apical dormancy and stimulating lateral-branch formation are even more important. This study was conducted to investigate the effects of Cyc and Pr treatments at different doses on lateral branch formation in Golden Delicious and Starking Delicious apple cultivars and Williams and Deveci pear cultivars grafted on seedling rootstocks.

Materials and methods

Plant material

This study was conducted in a commercial nursery orchard in Tokat (Turkey) located between 40° 18’ north latitude and 36° 13’ east longitude in 2015. Experimental site has a loamy soil texture with moderate lime, low organic matter, sufficient P_2O_5 and K_2O levels. Annual average temperature (based on 55-years data) is 12.6 °C, average relative humidity is 62.8% and average precipitation is 432.1 mm (Tokat Meteoroloji Station). In the study years average temperature, average relative humidity and average precipitation are measured as in 13.1 °C, 62.7% and 318.1 mm, respectively. Fertilization was performed in all experimental plots in accordance with the results of soil analyses. Irrigations were performed with drip irrigation. Golden Delicious (GD) and Starking Delicious (SD) apple cultivars were grafted on apple seedling rootstocks *(Malus domestica* L), Williams and Deveci pear cultivars were grafted on pear seedling rootstocks *(Pyrus communis* L.*)* with T-budding.

Treatment

Concentration

Apple nursery trees were subjected to 250, 500, 750 and 1000 mg L^{-1} Perlan (18.8% 6-benzyladenine (BA) and 18.5% GA4+7) and 25, 50, 75 and 100 mg L^{-1} Cyclanilide (Cyc) treatments; pear trees were subjected to 250 and 500 mg L^{-1} Perlan and 5, 10, 15 and 20 mg L^{-1} Cyclanilide treatments. All experimental solutions were applied with 0.1% surfactant. Control trees were subjected to only distilled water treatments with surfactant.

Experimental design

Nursery trees were planted with 80 cm plant spacing and 15 cm row spacing. Standard cultural practices were performed regularly. All treatments were performed in the first week of July when the trees reached to a height of 70-80 cm from the soil surface. Treatments were sprayed about 15-20 cm above the canopy.

Investigated parameters

At the end of growth season, tree trunk diameter, tree height, lateral branch length, height of the first branch, crotch angle, number of lateral branches were determined.

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Trunk diameter was measured with a caliper from 5 cm above the graft union. Tree height was measured as the height from the graft union to tip of the tree. Lateral branches longer than 10 cm were counted in each tree to get number of lateral branches per tree. Their lengths were measured to get average lateral branch length. The crotch angle was measured with a protractor. The height of first branch was taken as the distance from the bud union to the first induced feather longer than 10 cm.

Statistical analysis

Experiments were conducted in randomized block design with 3 replications with 10 trees in each replicate. The normality of the data was confirmed by the Kolmogorov-Smirnov test and the homogeneity of variances by the Levene's test. Except for number of lateral branches, resultant data were subjected to variance analysis. Means were separated by Duncan's multiple range test. Since the number of lateral branches did not have a normal distribution, they were analyzed with Friedman test.

Results

As compared to control treatment, neither Cyc nor Pr treatment resulted in significant changes in trunk diameters of either apple cultivars (*Table 1*). A similar response was observed in tree heights. Relative to control treatment, decreases in tree height were observed only with 100 mg L^{-1} Cyc and 750 mg L^{-1} Pr treatments of SD cultivar. Experimental treatments did not result in significant differences in lateral branch lengths of GD. Although some treatments created differences in lateral branch lengths of SD cultivar, both Cyc and Pr treatments did not result in significant changes in lateral branch lengths as compared to control treatments.

Table 1. *Effects of cyclanilide and perlan treatments on trunk diameter, sapling height, lateral branch length of Golden Delicious and Starking Delicious apple cultivars*

Treatments (mg L^{-1})	Trunk diameter (mm)	Tree height (cm)	Branch length (cm)
	Golden Delicious		
Control	14.1 ab*	171.5 a	41.9 a
25 Cyc	13.6 ab	159.7 a	30.8 a
50 Cyc	15.62 a	171.6 a	33.5 a
75 Cyc	14.65 ab	154.8 a	31.6 a
100 Cyc	14.8 ab	157.5 a	29.9 a
250 Pr	16.0 a	174.3 a	33.8 a
500 Pr	16.3 a	180.6 a	30.5 a
750 Pr	14.6 ab	160.3 a	39.1 a
1000 Pr	12.0 b	140.9 a	38.6 a
	Starking Delicious		
Control	16.3 ab	211.7 a	50.6 ab
25 Cyc	16.9 a	198.1 ab	49.9 ab
50 Cyc	15.0 ab	179.1 ab	40.5 ab
75 Cyc	15.1 ab	175.8 ab	36.3 ab
100 Cyc	13.1 ab	160.0 b	32.6 b
250 Pr	13.5 ab	173.9 ab	56.3 a
500 Pr	15.2 ab	191.3 ab	38.1 ab
750 Pr	12.6 b	150.6 b	29.3 b
1000 Pr	14.5 ab	170.2 ab	30.3 b

*Means in the same columns with the same letter do not differ according to Duncan's test at $P < 0.05$

Low Cyc doses did not have significant effects on the first lateral branch height. However, higher doses (75 and 100 mg L^{-1}) result in significant decreases in both cultivars (*Table 2*). All Pr doses did not cause a significant change in the first lateral branch height of GD apples. In the SD apple tree, while 1000 mg L^{-1} Pr treatment decreased the first lateral branch height, effects of the other doses were not significant when compared to control treatment. Crotch angles of SD cultivar significantly decreased only with 750 mg L^{-1} Pr treatment. Effects of the other treatments were not found to be significant.

Table 2. *Effects of cyclanilide and perlan treatments on the first branch height and crotch angle of Golden Delicious and Starking Delicious apple cultivars*

Treatments (mg L^{-1})	Height of the first branch (cm)	Crotch angle (°)
	Golden Delicious	
Control	71.2 a*	56.2 ab
25 Cyc	55.0 abc	58.4 ab
50 Cyc	39.9 abc	59.2 ab
75 Cyc	33.9 bc	61.6 a
100 Cyc	20.7 c	62.2 a
250 Pr	69.2 ab	55.1 ab
500 Pr	72.0 a	55.4 ab
750 Pr	63.1 ab	57.6 ab
1000 Pr	61.1 ab	51.1 b
	Starking Delicious	
Control	102.0 ab	77.5 a
25 Cyc	64.6 bcd	73.5 a
50 Cyc	68.3 bcd	71.0 a
75 Cyc	42.9 d	67.9 a
100 Cyc	31.2 d	65.6 a
250 Pr	111.3 a	67.5 a
500 Pr	98.2 ab	70.6 a
750 Pr	85.8 abc	44.4 b
1000 Pr	53.5 cd	66.0 a

*Means in the same columns with the same letter do not differ according to Duncan's test at $P < 0.05$

Entire Cyc treatments remarkably increased the number of lateral branches in both apple cultivars. While the number of lateral branches was 2.5 in control treatment of GD cultivar, the value increased to 12.7 with 100 mg L^{-1} Cyc treatment. In GD cultivar, significant differences were observed in number of lateral branches the among the Cyc application concentrations. Number of lateral branches in 50, 75 and 100 mg L^{-1} Cyc treatments was remarkably higher than both the control and 25 mg L^{-1} Cyc treatment. Similarly, Cyc treatments increased number of lateral branches also in SD cultivar, but the differences between Cyc doses were not found to be significant. On the other hand, effects of Pr doses on number of lateral branches varied based on the cultivar and treatment dose. Relative to control treatment, only 250 mg L^{-1} Pr treatment significantly increased number of lateral branches in GD cultivar (*Fig. 1*).

No treatment was significantly different from each other and control with respect to trunk diameters and tree heights in Deveci and Williams pear cultivars. The differences in lateral branch lengths of the treatments were not found to be significant in Williams cultivar. In Deveci cultivar, 250 mg L^{-1} Pr-treated trees had significantly higher tree

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height than 15 and 20 mg L^{-1} Cyc-treated trees, but no treatment caused a significant change relatively to control (*Table 3*).

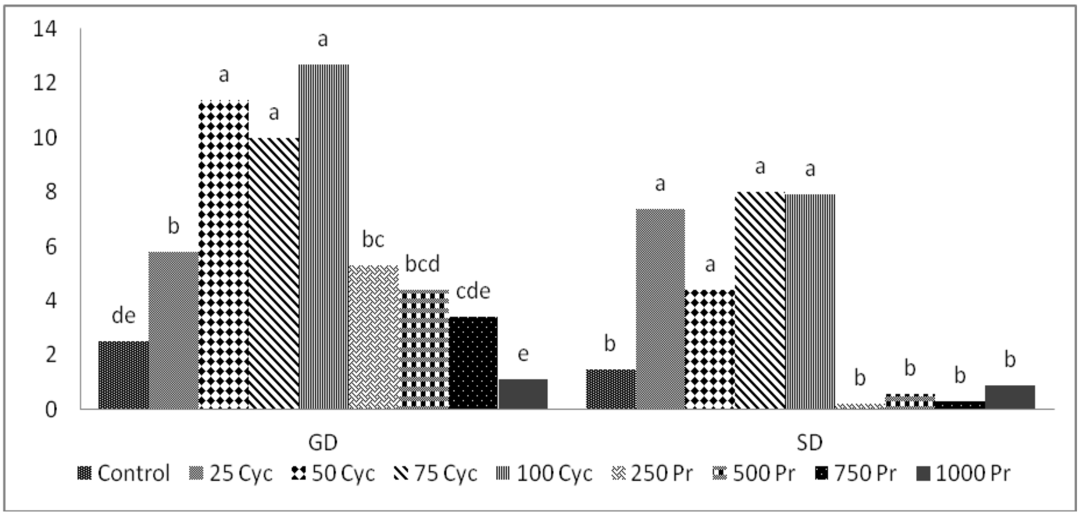

Figure 1. *Effects of cyclanilide and perlan treatments on the number of lateral branches of Golden Delicious and Starking Delicious apple cultivars*

Table 3. *Effects of cyclanilide and perlan treatments on trunk diameter, sapling height and lateral branch length of Deveci and Williams pear cultivars*

Treatments (mg L^{-1})	**Trunk diameter (mm)**	**Tree height (cm)**	**Branch length (cm)**
		Deveci	
Control	12.84 a*	153.27 a	25.36 ab
5 Cyc	10.78 a	115.60 a	25.51 ab
10 Cyc	11.12 a	114.33 a	21.25 ab
15 Cyc	12.87 a	123.47 a	19.04 b
20 Cyc	11.49 a	115.07 a	18.31 b
250 Pr	11.31 a	123.93 a	29.69 a
500 Pr	12.03 a	140.20 a	25.57 ab
		Williams	
Control	12.16 a	126.80 a	12.50 a
5 Cyc	13.46 a	123.60 a	22.20 a
10 Cyc	13.90 a	114.47 a	18.86 a
15 Cyc	11.88 a	106.80 a	17.91 a
20 Cyc	11.57 a	101.07 a	16.54 a
250 Pr	11.07 a	113.27 a	14.00 a
500 Pr	11.65 a	123.53 a	-

*Means in the same columns with the same letter do not differ according to Duncan's test at $P < 0.05$

The first branch height and crotch angle of Williams cultivar under Cyc and Pr treatments were similar to the control treatments. In Deveci cultivar, on the other hand, Pr treatments yielded similar values with the control saplings, but Cyc treatments (except for 5 mg L^{-1} Cyc) significantly reduced the first branch heights. Although the crotch angles of 15 mg L^{-1} Cyc-treated trees were significantly higher than the crotch angles of 5 mg L^{-1} Cyc-treated trees, both treatment did not differ from control treatment respect to crotch angle (*Table 4*).

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Table 4. *Effects of cyclanilide and perlan treatments on the first branch height and crotch angle of Deveci and Williams pear cultivars*

Treatments (mg L^{-1})	**Height of the first branch (cm)**	**Crotch angle (°)**
	Deveci	
Control	127.00 a*	35.93 ab
5 Cyc	93.33 ab	34.07 b
10 Cyc	72.35 b	45.67 ab
15 Cyc	71.05 b	49.42 a
20 Cyc	64.43 b	43.61 ab
250 Pr	97.93 ab	38.31 ab
500 Pr	113.53 a	43.00 ab
	Williams	
Control	47.80 a	41.17 a
5 Cyc	69.75 a	51.46 a
10 Cyc	48.97 a	50.97 a
15 Cyc	54.01 a	47.72 a
20 Cyc	42.07 a	44.19 a
250 Pr	59.00 a	40.00 a
500 Pr	-	-

*Means in the same columns with the same letter do not differ according to Duncan's test at $P < 0.05$

The experimental treatments had significant effects on number of lateral branches in both pear cultivars. Except for 5 mg L^{-1} Cyc treatment, other Cyc treatments significantly increased number of lateral branches in Deveci cultivar. In this pear cultivar, while the number of lateral branches per tree was 0.93 in control treatment, the value increased to 5.68 with 15 mg L^{-1} Cyc treatment. Number of lateral branches in Pr-treated trees of Deveci cultivar was similar with the control treatment. In Williams cultivar, all Cyc treatments significantly increased number of lateral branches. However, both Pr doses significantly reduced the number of lateral branches. As compared to 5 mg L^{-1} Cyc treatment, 10 and 15 mg L^{-1} Cyc treatments were significantly more effective on increasing number of lateral branches (*Fig. 2*).

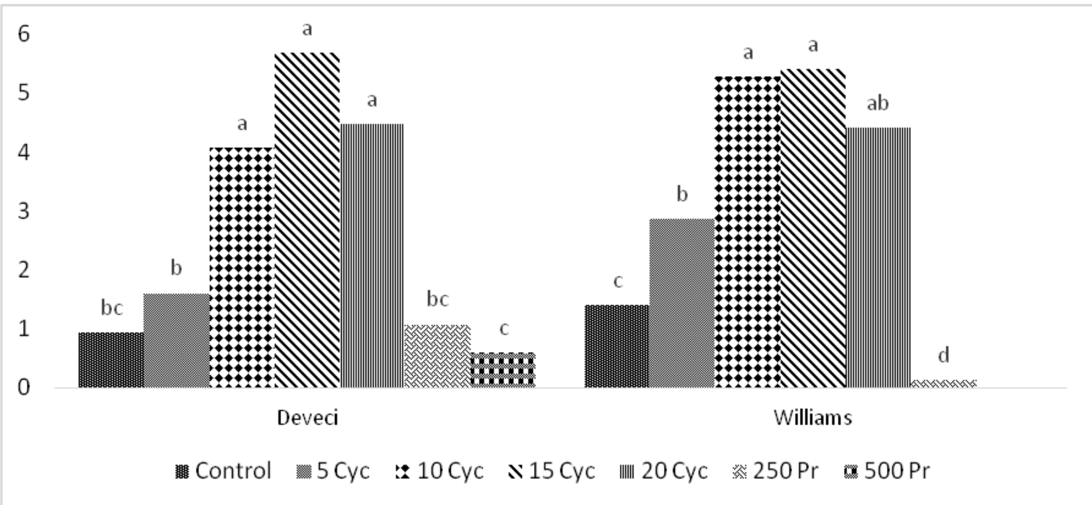

Figure 2. *Effects of cyclanilide and perlan treatments on the number of lateral branches of Deveci and Williams pear cultivars*

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Discussion

Cropping is usually delayed in orchards established with trees with less number of lateral branches (Elfving, 2010). Therefore, some plant growth regulators are used to reduce the effects of apical dormancy and thus to stimulate lateral branch development. It was reported in previous studies that benzyladenine (BA) applied either alone or together with gibberellic acid (GA_{4+7}) increased number of lateral branches in apple and pear saplings (Neri et al., 2004; Koyuncu and Yıldırım, 2008; Yıldırım et al., 2010). In present study, the effects of Pr treatments varied based on species and cultivars. More efficient and stable outcomes were achieved from Cyc treatments. Even remarkable at higher doses, all Cyc treatments distinctively increased number of lateral branches both in apples and pears. A similar case was reported by Elfving and Visser (2006a) for cherry saplings. Previous researchers also indicated that Cyc treatments yielded better outcomes for lateral branch development than Promalin ($BA+GA_{4+7}$). Researchers also indicated that the effects of Promalin varied based on species, but Cyc had more stable outcomes for different species (Elfving and Visser, 2006a, b; Ipek et al., 2017; Lordan et al., 2017).

Previous researchers indicated that the effects of both Pr and Cyc on trunk diameter and tree height varied depending on species, cultivars, rootstocks and treatment doses (Gastol and Poniedzialek, 2003; Rossi et al., 2004; Elfving and Visser, 2006a, b; Kalyoncu et al., 2011; Sazo and Robinson, 2011; Moghadam and Zamanipour, 2013; Lordan et al., 2017). However, none of these studies mentioned about a negative impact of those treatments on nursery tree quality parameters. In present study, both Pr and Cyc treatments generally did not have any negative effects on trunk diameter and tree height of apple (GD and SD) and pear (Deveci and Williams) cultivars.

Some researchers reported that BA and Promalin treatments increase lateral branch lengths of apple saplings (Greene et al., 1990; Kalyoncu et al., 2011). Gastol and Poniedzialek (2003) carried out a study with 3 different apple cultivars and indicated that effects of Promalin treatments on lateral branch lengths varied with the years and climate conditions. Elfving and Visser (2005) reported that different Cyc doses did not result in significant changes in lateral branch lengths of Cameo apples grafted on M26 rootstocks, but significantly reduced lateral branch lengths of Fuji apples. Despite the differences in lateral branch lengths of treatments in Deveci pear and Starking Delicious apple cultivar, none of the treatments resulted in significant changes in lateral branch lengths of apples and pears as compared to the control treatments. Such findings revealed that response of nursery trees to Pr and Cyc treatments with regard to lateral branch length might vary based on the rootstocks and cultivars.

Although being changed based on intended market, the first branch height is a significant criterion for all markets. Contradictory outcomes were reported in previous studies about the effects of Pr and Cyc treatments on the first branch heights (Elfving and Visser, 2005, 2006b; Yıldırım et al., 2010; Sazo and Robinson, 2011). Except for Williams cultivar, Cyc treatments decreased the first branch height of the nursery trees, on the other hand Pr treatments generally did not have significant effects on the first branch heights. In previous studies, positive effects of Promalin and Cyc-like growth regulators on crotch angles were reported (Gürz, 2005; Yıldırım et al., 2010). However in this study, no results were obtained to support that both Pr and Cyc could have a beneficial effect on crotch angles of nursery trees of apple and pear cultivars.

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Conclusion

Although there are several studies indicating that external application of growth regulators effective in apical dormancy may stimulate lateral branch formation in fruit trees, effects of such growth regulators usually vary based on the rootstock and cultivars. Present findings revealed that all Cyc doses could be used to increase number of lateral branches in apple (Golden Delicious and Starking Delicious) and pear (Deveci and Williams) cultivars grafted on seedling rootstocks; Cyc doses could be used as an efficient tool to reduce the first branch height in two apple cultivars and Deveci pear cultivar. Also, these growth regulators stimulated shooting of lateral buds through inhibiting apical dormancy and did not have any negative impacts on tree heights.

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FIRST RECORD OF *HYLASTES OPACUS* ERICHSON AND *CRYPTURGUS HISPIDULUS* THOMSON, C. G. (COLEOPTERA; CURCULIONIDAE; SCOLYTINAE) FOR THE TURKISH FAUNA

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Abstract. Scolytinae subfamily (Coleoptera: Curculionidae) is represented by 135 species in Turkey including 8 species of the genus *Hylastes* Ericson and 7 species of the genus *Crypturgus* Ericson. A total of 79 adult individuals of *Hylastes opacus* Erichson and 6 adult individuals of *Crypturgus hispidulus* Thomson, C. G. were obtained from log and pheromone traps in pine stands at twelve localities during 2014. *H. opacus* and *C. hispidulus* were recorded for the first time in Turkey. *H. opacus* were obtained from trap logs of *Pinus brutia* and *Pinus nigra* and also pheromone traps in *P. brutia* stands. Adult individuals of *C. hispidulus* were observed in association with *Orthotomicus erosus* and *Pityogenes pennidens* on *P. brutia* and *P. nigra* log traps.
Keywords: *Hylastes opacus, European bark beetle, Crypturgus hispidulus, Pinus brutia, Pinus nigra, Marmara, Turkey*

Introduction

The Scolytinae subfamily (Coleoptera: Curculionidae) is one of the largest groups of Coleoptera and it is represented by 135 species in Turkey (Wood and Bright, 1992a, b; Knížek, 1998; Selmi, 1998; Sarıkaya and Avcı, 2011; Sarıkaya 2013; Sarıkaya and Knížek, 2013; Cognato, 2015; Lieutier et al., 2016). The majority of species feed on dead or dying tissues and are not normally considered to be of an economic importance. However, such species can become economically important if their galleries create holes in timber used for furniture or veneer, or if they transport pathogenic fungi to living trees during the feeding period by young adults to mature the gonads. The relatively small number of species that attack living trees, saplings or seedlings, or the seeds of commercial crops are sometimes of major economic importance, causing damage estimated in millions of dollars (Knížek and Beaver, 2007).

Species of the genus *Hylastes* Ericson primarily colonize subterranean stem parts of various conifers, predominantly, *Pinus*, *Picea* and *Abies* genera. The genus *Hylastes* has 21 species in conifers throughout the Holarctic region (Webber and Gibbs, 1989; Bright, 2014). It is presented with eight species in Turkey; *H. angustatus* (Herbst,), *H. ater* (Paykull), *H. attenuatus* Erichson, *H. batnensis anatolicus* Knížek & Pfeffer, H. *brunneus* Erichson, *H. cunicularius* Erichson, *H. linearis* Erichson and *H. substriatus* Strohmeyer (Selmi, 1987; Wood and Bright, 1992a; Lieutier et al., 2016; Alonso-

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Zarazaga et al., 2017). Their adults feed on the tender bark near the root collars of seedlings and transplants, often girdling them, the species is frequently considered a noxious pest of nurseries and pine plantations, not only killing small plants but exposing older trees to infestation by wound parasites such as *Fomes* (Basidiomycetes, Polyporales: Polyporaceae) (Hoebeke, 1994). *Hylastes* species are also the vector of black stain root diseases and some of them have been reported as serious pests in forestry (Webber and Gibbs, 1989).

The genus *Crypturgus* Erichson breed in conifers throughout the Holarctic region and fifteen species are currently recognized (Wood and Bright, 1992a; Jordal and Knížek, 2007). It is represented with 7 species in Turkey; *C. cinereus* (Herbst), *C. cylindricollis* Eggers, *C. dubius* Eichhoff, *C. mediterraneus* Eichhoff, *C. numidicus* Ferrari *C. parallelocollis* Eichhoff and *C. pusillus* (Gyllenhal) (Wood and Bright, 1992a; Selmi, 1998; Lieutier et al., 2016; Alonso-Zarazaga et al., 2017). Species of the genus *Crypturgus* have very small body size (0.9–1.5 mm) and inhabit bark of the trees. The species are very abundant throughout the whole Mediterranean basin, but largely secondary, infesting only trees heavily stressed or already infested by more aggressive species. They have no economic relevance; high population density of this species may interfere with the development of associated more aggressive species (Lieutier et al., 2016).

In the current study *Hylastes opacus* Erichson and *Crypturgus hispidulus* Thomson, C. G. were reported for the first time to Turkish fauna.

Material and methods

Specimens of *H. opacus* and *C. hispidulus* were collected from trap logs and pheromone traps in stands of *Pinus brutia* Ten. and *P. nigra* J. F. Arnold in Marmara Region, Turkey (*Fig. 1*).

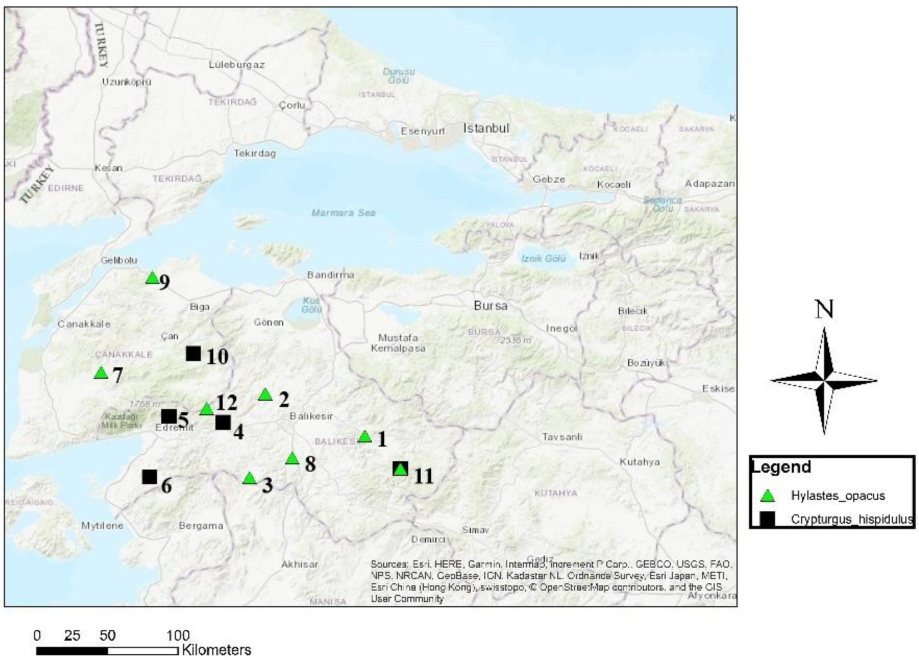

***Figure 1.** Collection sites*

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Pheromone traps baited with the pheromone of *Orthotomicus erosus* Wollaston and trap logs were set up in February 2014 in 12 sites. Coordinates of the collection sites and trap types are presented in *Table 1*. Traps were checked for the presence of beetles at regular intervals. Insects were collected and identified on the basis of characteristic morphological features of the elytra and elytral declivity by using a LEICA S8APO stereomicroscope. Taxonomic keys in (Grüne, 1979; Wood, 1982; Hoebeke, 1994; Jordal and Knížek, 2007) were used to determine the specimens. Color photographs of specimens were prepared by using the stereomicroscope with a LEICA DFC295 digital video camera. Voucher specimens of *H. opacus* and *C. hispidulus* were prepared appropriate methods and preserved at Istanbul University-Cerrahpaşa, Faculty of Forestry, Department of Forest Entomology and Protection.

Table 1. *Coordinates of the collection sites and trap types*

Locality (N)	Geographical positions	Altitude (m)	Trap type	Host plant
1	39°34'46.6"N; 28°21'42.1"E	508	Log trap	*P. brutia*
2	39°46'52.1"N; 27°43'33.5"E	448	Log trap	*P. brutia*
3	39°22'07.0"N; 27°37'34.9"E	275	Log trap	*P. brutia*
4	39°38'26.2"N; 27°27'25.7"E	310	Log trap	*P. brutia*
5	39°40'15.3"N; 27°06'43.5"E	372	Log trap	*P. brutia*
6	39°22'19.5"N; 26°59'19.8"E	311	Log trap	*P. brutia*
7	39°53'11.2"N; 26°40'44.9"E	287	Log trap	*P. brutia*
8	39° 27' 58"N; 27° 53' 57"E	225	Pheromone trap	*P. brutia*
9	40°20'59.8"N; 27°00'24.3"E	133	Log trap	*P. brutia*
10	39°58'41.6"N; 27°16'6.2"E	93	Log trap	*P. brutia*
11	39°24'47.9"N; 28°35'18.6"E	1164	Log trap	*P. nigra*
12	39°42'34.9"N; 27°21'09.3"E	730	Log trap	*P. nigra*

Result and discussion

A total of 79 adult individuals of *H. opacus* and 6 adult individuals of *C. hispidulus* were collected during 2014 from eight localities in Turkey at various altitudes between 93 and 1164 m (*Table 1*). The number, locality and date of captured *H. opacus* and *C. hispidulus* are given in (*Table 2*).

H. opacus can be distinguished from other Turkish species of the genus *Hylastes* by frons without longitudinal ridge (Grüne, 1979; Freude et al., 1981). Its adults were 2.5-3 mm long and were recognized by the following combination of characters: frons and vertex closely and coarsely punctured; pronotum as long as wide and constricted anteriorly; interstriae flat, wider than striae; elytral declivity with filiform setae; and body black with antennae and legs reddish brown and elytra dull (*Fig. 2*).

The distribution area of *H. opacus* includes; Austria, Belgium, Bulgaria, China, Denmark, England, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italia, Japan, Korea, Latvia, Lithuania, Macedonia, Norway, Poland, Russia, Slovakia, Slovenia, Sweden, Switzerland, North America and Canada (Wood and Bright, 1992a; GBIF, 2017; Fauna Europaea, 2018).

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Table 2. *Locality, capturing date and individual numbers of H. opacus and C. hispidulus*

	N	Capturing date	Individuals
Hylastes opacus	1	12 May	14
	2	13 May	1
	3	13 May	24
	7	15 May	4
	8	26 May	17
	9	14 May	1
	11	30 June	11
	12	26 July	7
	Total		**79**
Crypturgus hispidulus	4	1 July	1
	5	27 August	1
	6	13 May	1
	10	15 May	1
	11	30 June	2
	Total		**6**

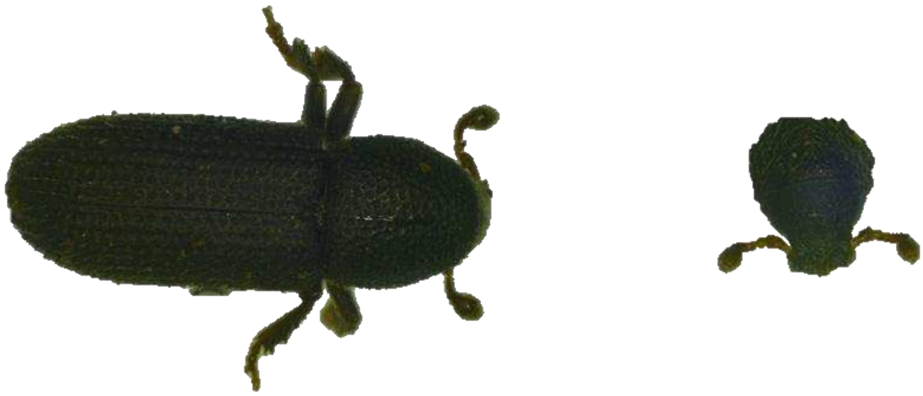

Figure 2. *Hylastes opacus Erichson*

We obtained only six adult of *C. hispidulus* individuals from log traps in Balıkesir and Çanakkale province in 2014. *C. hispidulus* can be differentiated from other Turkish species of the genus *Crypturgus* (except *C. pusillus, C. cylindricollis* and *C. parallelocollis*) by shiny elytra and with round punctures of striae and elytral declivity with individually placed setae. It can be distinguished from *C. pusillus* by fine pronotum with sparsely punctured and elytral discs and sides with erect and long setae. It differs from *C. cylindricollis* and *C. parallelocollis* in terms of sizes (Grüne, 1979; Freude et al., 1981; Lompe, 2002; Jordal and Knížek, 2007). Its adults are 1.2-1.3 mm long and have the following features: interstriae with rows of white to light brown, short, thick and filiform setae; body black (*Fig. 3*).

The distribution area of *C. hispidulus* includes; Austria, Belarus, Bulgaria, Central and North European Russia, Czech Republic, Denmark, England, Estonia, Finland,

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Germany, Hungary, Italia, Latvia, Lithuania, Norway, Poland, Slovakia, Slovenia, Sweden, Switzerland, and Ukraine (GBIF, 2017; Fauna Europaea, 2018).

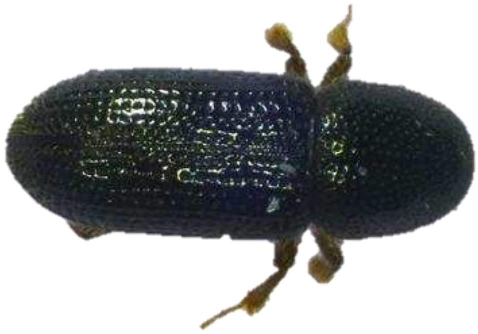

***Figure 3.** Crypturgus hispidulus Thomson, C. G.*

There were records from Greece and Bulgaria of *H. opacus*. Likewise, *C. hispidulus* was reported from Bulgaria (Doychev and Ovcharov, 2006). Although both of them were recorded in the neighbouring countries, *Hylastes opacus* and *Crypturgus hispidulus* were recorded for the first time in Turkey. As mentioned above, the Scolytinae subfamily is represented by 135 species in Turkey. Five of those species are known only in the country. We have contributed 2 species to Scolytinae of Turkey by current article.

H. opacus breeds in the bark of stumps or at the bases of unhealthy *Pinus* spp., chiefly Scotch pine (*P. sylvestris*). It occasionally infests the bark of other conifers for example *Picea* spp. and *Larix* spp. (Freude et al., 1981; Wood and Bright, 1992a; Hoebeke, 1994). We obtained *H. opacus* from trap logs of *P. brutia* and *P. nigra* and also pheromone traps in *P. brutia* stands. *C. hispidulus* lives under the bark of *Picea, Pinus* and less often on larch species. It also occurs in the galleria of other larger bark beetles for example; *Xylechinus pilosus*, *Hylurgops palliatus*, *Polygraphus poligraphus*, *P. subopacus*, *Tomicus minor*, *Pityogenes chalcographus* etc. (Freude et al., 1981). We observed that their adult individuals were associated with *Orthotomicus erosus* and *Pityogenes pennidens* on *P. brutia* and *P. nigra* log traps. Our findings are consistent with previous studies regarding the presence of adult individuals under the bark of pine species (*P. brutia* and *P. nigra*), and the association of *C. hispidulus* with other Scolytine species.

Conclusion

Bark beetles are important disturbance agents in forestry. They can colonize weak or recently killed trees, however a few species are capable of killing healthy trees. One of the most important characteristics of bark beetles is their association with fungi. In this study, where we present two new species for Turkish fauna, our main goal was to examine the association of bark beetles-fungi. Due to the experimental design we are not able to give more detailed information for these two new species. Additional work, including their life cycle should be conducted to determine their impact on forest ecosystem.

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MORPHOLOGICAL HAIR IDENTIFICATION KEY OF COMMON MAMMALS IN TURKEY

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Abstract. The aim of this study was to research the morphology and to make ready a hair identification key for common mammals in Turkey and also to make up a comprehensive method for the preparation of hair identification key for Turkey's mammals. Despite the wide application of this technique in the determination of diet composition in large carnivores, it has not been used in Turkey up to this point, thus this paper is the first study and an appropriate guide for using this technique in ecological studies and management of many species and populations in Turkey. In this research, hair structures of eighteen species representing four mammalian orders including Artiodactyla such as wild goat, chamois, red deer; Carnivora such as leopard, eurasian lynx, gray wolf, brown bear; Lagomorpha and Rodentia were investigated. The studied large carnivore species constitute the main predator species and the others are important prey sources. The microscopic structure of the hair was studied using the hair medulla and cuticle scale patterns. Afterwards, the structural features of hair for each species were comparatively used in order to prepare a descriptive and photographic hair identification key.
Keywords: *microscopic structure, photographic key, medulla, cuticle, wildlife*

Introduction

Hair identification of mammalian species has efficient practices in wildlife, biology, ecology and forensic medicine in particular the study of food habits of carnivores using investigation of prey hair in scats is widely utilized. The purpose of morphological identification of animal hairs is to classify the animal species from an unknown hair pattern to a special taxon based on well-defined, genetically based features (Tridicio et al., 2014). Knowledge of diet of carnivores is an important and essential factor for management and conservation of endangered species (Klare et al., 2011). Several techniques have been suggested to study species diet (Karanth and Sunquist, 1995; Ciucci et al., 1996). Despite these methods, scat analysis has been extensively used because this method is a non–harmful way and scat collections can be easily done throughout the year (Boitani and Powell, 2012). Various hair identification key atlases have been published on European mammal hairs (Day, 1966; Dziurdzik, 1978; Keller, 1978; Faliu et al., 1980; Debrot et al., 1982; Teerink, 1991; Meyer et al., 2002; Cornally and Lawton, 2016). Dietary habits of species are critical for the study of sustainable wildlife conservation and management (Treves and Karanth, 2003). Knowing the answer of question "Who eats what?" is essential for recognizing predator-prey relationships and a healthy ecosystem (Symondson, 2002; Sheppard and Harwood, 2005). Diet is basic principle in animal ecology but is specifically critical in the study of large carnivore species (Gese, 2001; Treves and Karanth, 2003). Scat analysis method is the most widely used to examine carnivore diet preferences (Leopold and Krausman, 1986; Gamberg and Atkinson, 1988). Knowledge of predators' diet is very important

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for sustainable wildlife conservation and management (Anwar et al., 2012). Scat analysis is based on morphological identification of indigestible prey remains such as bone and hair (Symondson, 2002; Sheppard and Harwood, 2005). Carnivore food habits studies from the prey hairs in scats has been widely accepted and used for describing the diet of mammalian predators, because of non-destructiveness of hairs and easily available scats at all times (De-Marinis and Asprea, 2005). The research of hair has been used in wildlife study in order to gather information such as species distribution, population density, species' dietary habits and translocation studies (Waters and Lawton, 2011).

Guard hairs have the ability to be used for identification, as a result, in this study only guard hairs were used to prepare the identification key. Hair structure is made of three layers: the medulla, the cortex and an outermost layer, the cuticle (Oli, 1993). The cortex is the middle layer of hair. It is composed of longitudinal and shriveled cells, which appears under the light microscope as a homogeneous mass without any details. This layer is of limited value for identification (Teerink, 1991). The medulla is composed shriveled dead cells but unlike the cortex they are clearly visible (Teerink, 1991; Hausman, 1920). Hair scale patterns formed by the cuticle and hair cross-sectional patterns formed by the cortex and medulla are the two main characteristics used in species recognition (Moyo, 2005). In identifying hair species it is necessary to compare the scales and medulla from the same parts of the hairs (Hausman, 1920).

The use of hair identification key could be helpful to distinguish hair structures of many related and unrelated species. Methods of direct comparison by microscopy have also been used but they are all time consuming (Oli, 1993). Photographic reference key has been proved the best and easy method for the comparison of characteristics of hairs recovered from the scats of carnivores (Oli, 1993).

In this study, a photographic hair identification key of common mammals of Turkey was developed. In this paper, hair structures of eighteen mammalian species have been investigated. This study is also important for all wildlife studies as important for Turkey. The aim of this study is to present a descriptive and photographic identification key and to provide a descriptive and simple method for the preparation of hair identification keys for other mammals in future studies.

Materials and methods

The structure of guard hair was examined in eighteen different mammal species found throughout Turkey. We investigated the hair structures of eighteen species representing four mammalian orders and nine families including Bovidae, Cervidae, Suidae, Canidae, Felidae, Mustelidae, Ursidae, Leporidae, and Sciuridae (*Table 1*). We collected hair tufts from taxidermied animals housed in Karadeniz Technical University, Wildlife Museum of Department of Wildlife Ecology and Management. All of the samples were dorsal guard hair from mature adult animals. Guard hairs are important in species identification as they exhibit diagnostically reliable features (Eunok et al., 2014). The hair samples were collected from at least five spots in the dorsolateral body region of each specimen (Wallis, 1993). The hairs samples were taken from the dorsal region of the body. De Marinis and Asprea (2005) noted that hairs from other body regions show similar characteristics to dorsal hair, but are often less distinct making identification more problematic.

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Table 1. *Studied mammal species in this research*

Order	Family	Scientific Name	English Name
Artiodactyla	Bovidae	*Capra aegagrus*	Wild goat
		Gazella subgutturosa	Goitered gazelle
		Rupicapra rupicapra	Chamois
	Cervidae	*Capreolus capreolus*	Roe deer
		Cervus elaphus	Red deer
		Dama dama	Fallow deer
	Suidae	*Sus scrofa*	Wild boar
Carnivora	Canidae	*Canis aureus*	Golden jackal
		Canis lupus	Gray wolf
		Vulpes vulpes	Red fox
	Felidae	*Lynx lynx*	Eurasian lynx
		Panthera pardus	Leopard
	Mustelidae	*Martes foina*	Stone marten
		Meles meles	European badger
		Lutra lutra	European otter
	Ursidae	*Ursus arctos*	Brown bear
Lagomorpha	Leporidae	*Lepus europaeus*	European hare
Rodentia	Sciuridae	*Sciurus anomalus*	Caucasian squirrel

Medulla structure

Dead cell and the gaps between them create several patterns of medulla that are good indicators for identification (Teerink, 1991). If hair samples are not prepared for observation under light microscope, in most cases, medulla seems so dark that its structure can hardly be observed. Accordingly, the hair is placed into the mixture solution of 70% hydrogen peroxide and 30% ammonia for about 20 to 60 minutes so that hair colour is washed and the hair becomes transparent (De Marinis and Agnelli, 1993; Rezaie et al., 2012). Then, samples were placed in the water to be thoroughly washed and then a number of samples were placed on gelatine-treated solution and some were placed on liquid paraffin. Comparison between paraffin and gelatine slide displayed a more precise structure of the medulla (Teerink, 1991). Hair samples impregnated with paraffin and gelatine were placed on slides. Then, these slides were studied under a light microscope with a magnification of 100 and 400.

In this study, we have used the hair classification system that was proposed by De Marinis and Asprea (2005) and Teerink (1991). This classification has been based on cellular composition, cellular structure and pattern, medulla margins and width of cortex (Teerink, 1991) (*Fig. 1*).

Cuticle scales pattern

The outermost layer, the cuticle, is made up of a large number of overlapping transparent scales of keratin. Observation placement of scales pattern is usually impossible or can hardly be seen with a light microscope without special preparation (Teerink, 1991; Rezaie et al., 2012). Therefore, it is essential to make a gelatine impression of the hair or to use an electronic microscope (Homan and Genoways,

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1978). Although the electronic microscope showed more detail of cuticular scale pattern, gelatine impressions were more practical (Perrin and Campbell, 1980). For this reason, the mixture of gelatine solution was placed on a slide, consequently hairs were placed on it. After drying for about 20 to 40 minutes, the gelatine will be solid and the hairs can be removed. As a consequence, the scales of hairs remaining on the gelatine can be seen under the light microscope with a magnification of 100 and 400 (Teerink, 1991; Oli, 1993). In this study, scale patterns of hairs are classified according to longitudinal axis of the hair, the structure of scale margins and the distance between scale margins (Teerink, 1991) (*Fig. 1*).

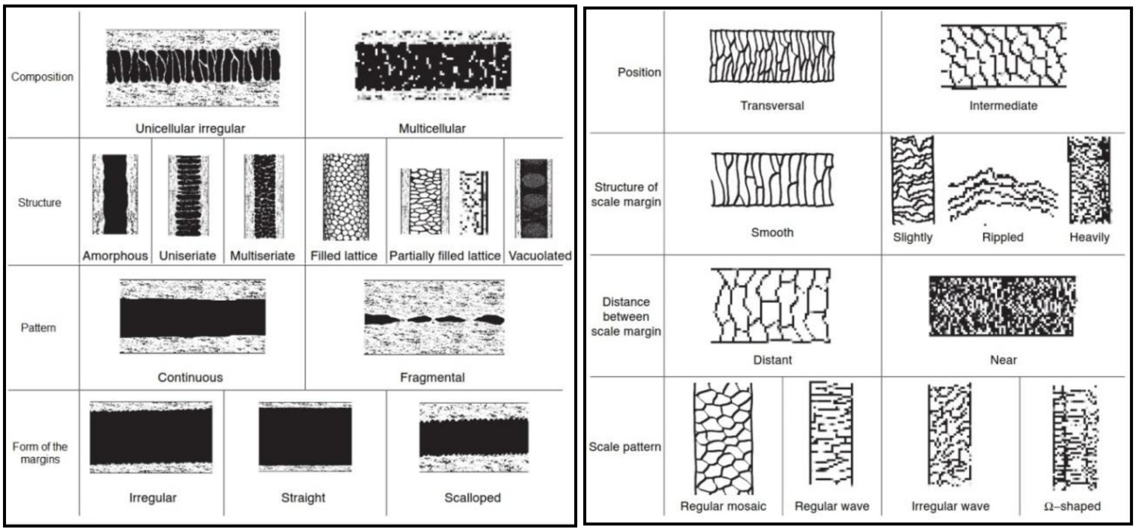

Figure 1. *Medulla (left) and Cuticle (right) classification system used in our hair key to identify common mammals of Turkey (Teerink, 1991)*

Results and discussion

In all species, the medulla structure of the hairs is an important key for the identification of species at the family level and the medulla structure per species is constant throughout the hair length (Homan and Genoways, 1978; Toth, 2002; De Marinis and Asprea, 2005). But at lower levels, such as subfamily and genus, for instance the wild goat and goitered gazelle, the medulla structure does not give the correct results in species identification, due to its high resemblance, so the cuticle pattern was used for identification of both species. The cuticle pattern shows more changes in hair length at family and subfamily levels. The cuticle is a useful tool used to distinguish species among wild ungulates. (De Marinis and Asprea, 2005). Therefore, firstly, in an attempt for microscopic identification, we investigated the medulla structure up to order and family levels and in order to identify at genus and species levels, it is recommended to use the cuticle pattern. As a result, in microscopic morphology the use of medulla structure has priority over the cuticle pattern and the use of the cuticle pattern individually, will cause confusion (De Marinis and Agnelli, 1993).

Keller (1981) separated Cervidae, which have similar medulla structure, from Bovidae on the basis of cross-sections. But hair cross-sectioning is a sophisticated and time consuming process in laboratory. So, we distinguished these taxa by the cuticle of hairs without cross-sectioning. The methods applied in this research can be easily, quickly and economically used in wildlife researches like previous studies (Toth, 2002;

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Chernova, 2003; Eunok et al., 2014). For a wildlife researcher who knows the worked habitat well, these simply obtainable data are very important resources at identifying for species.

Among all studied mammals species, european badger, wild boar and brown bear hairs can be identified without the help of microscopic studies. The hairs of these species can be easily identified by the naked eye based on the general view of the hair, which are hard and thick. However, they have bristly hairs which can be distinguished from those of other species even if the researcher is not highly skilled in hair identification (De Marinis and Asprea, 2005). Medulla and cuticle features of these species are not clearly displayed due to amorphous cellular structure and morphological structures of their hairs. The hair of those animals should be classified by macroscopic view for hair profile and hair general appearance (Eunok et al., 2014).

Medulla features

All investigated Leporidae, Sciuridae family and wild ungulates, except wild boar, showed a multicellular composition. The predator mammals studied, except european badger and brown bear, showed an unicellular composition. European badger and brown bear showed a multicellular composition. The reason of differences medulla composition of these species is their hair structures which have amorphous cellular structure. All studied mammal species' medullary features are listed in *Table 2*.

Table 2. *Medullary features of 18 mammal species from Turkey*

Species	Composition	Structure	Pattern	Form of the margin	Cortex width
Capra aegagrus	Multicellular	Partially filled lattice	Continuous	Scalloped	Narrow
Gazella subgutturosa	Multicellular	Partially filled lattice	Continuous	Scalloped	Narrow
Rupicapra rupicapra	Multicellular	Partially filled lattice	Continuous	Scalloped	Narrow
Capreolus capreolus	Multicellular	Partially filled lattice	Continuous	Scalloped	Narrow
Cervus elaphus	Multicellular	Partially filled lattice	Continuous	Scalloped	Narrow
Dama dama	Multicellular	Partially filled lattice	Continuous	Scalloped	Narrow
Sus scrofa	Multicellular	Amorphous	Continuous	Irregular	Width
Canis aureus	Unicellular	Vacuolated	Continuous	Irregular	Medium
Canis lupus	Unicellular	Vacuolated	Continuous	Irregular	Medium
Vulpes vulpes	Unicellular	Multiseriate	Continuous	Irregular	Medium
Lynx lynx	Unicellular	Uniseriate	Continuous	Scalloped	Medium
Panthera pardus	Unicellular	Uniseriate	Continuous	Scalloped	Medium
Martes foina	Unicellular	Uniseriate	Continuous	Scalloped	Medium
Meles meles	Multicellular	Amorphous	Continuous	Irregular	Medium
Lutra lutra	Unicellular	Uniseriate	Continuous	Scalloped	Medium
Ursus arctos	Multicellular	Amorphous	Continuous	Straight	Width
Lepus europaeus	Multicellular	Multiseriate	Continuous	Scalloped	Narrow
Sciurus anomalus	Unicellular	Uniseriate	Continuous	Scalloped	Medium

Studied Bovidae and Cervidae family species have a partially filled lattice hair structure. Canidae family species except red fox have vacuolated hair structure. Red fox has multiseriate hair structure. Felidae family species have uniseriate hair structure.

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European hare has multiseriate hair structure. Mustelidae family species except european badger have uniseriate hair structure. European badger has amorphous hair structure. Caucasian squirrel has uniseriate hair structure. Faliu et al. (1980) and Keller (1981) found the medullary lattice structure in wild boar. But, we found the medullary amorphous hair structure in wild boar and brown bear. We did not find in brown bear, as previously reported by Debelica and Thies (2009) found the medullary hair structure vacuolated in brown bear. All investigated mammals, showed a continous pattern. Studied Bovidae, Cervidae, Felidae, Leporidae, Mustelidae (except european badger) and Scirudae family species have form of the scalloped hair margin. Canidae family species, european badger and wild boar have form of the irregular hair margin.

Cuticle features

Among the mammals studied, except the leopard, position is transverse. The leopard has the most diverse pattern of cuticle hair so that from the base to the tip of the hair has intermediate structure. All mammals studied, except european badger due to amorphous cellular structure and morphological structures of hair have smooth scale margin. Irregular rippled scale pattern can be observed in all studied species except Bovidae family and wild boar. The pattern of cuticle in the entire hair length of the Bovidae family and wild boar is regularly waved. De Marinis and Asprea (2005) found regularly waved pattern of cuticle in all Cervidae species. But we found them irregularly waved. Eunok et al. (2014) found irregularly waved pattern of cuticle in wild boar. But we found it regularly waved. The pattern of cuticle in european badger is not visible due to morphological structures of hair. All studied mammal species' cuticle features are listed in *Table 3*.

Table 3. *Cuticle features of 18 mammal species from Turkey*

Species	Position	Scale margin	Scale pattern	Distant between scale margin
Capra aegagrus	Transversal	Smooth	Regular wave	Distant
Gazella subgutturosa	Transversal	Smooth	Regular wave	Distant
Rupicapra rupicapra	Transversal	Smooth	Regular wave	Distant
Capreolus capreolus	Transversal	Smooth	Irregular wave	Distant
Cervus elaphus	Transversal	Smooth	Irregular wave	Distant
Dama dama	Transversal	Smooth	Irregular wave	Distant
Sus scrofa	Transversal	Smooth	Regular wave	Distant
Canis aureus	Transversal	Smooth	Irregular wave	Near
Canis lupus	Transversal	Smooth	Irregular wave	Near
Vulpes vulpes	Transversal	Smooth	Irregular wave	Near
Lynx lynx	Transversal	Smooth	Irregular wave	Near
Panthera pardus	Intermediate	Smooth	Irregular wave	Near
Martes foina	Transversal	Smooth	Irregular wave	Near
Meles meles	Transversal	Not visible	Not visible	Near
Lutra lutra	Transversal	Smooth	Irregular wave	Near
Ursus arctos	Transversal	Smooth	Irregular wave	Near
Lepus europaeus	Transversal	Smooth	Irregular wave	Distant
Sciurus anomalus	Transversal	Smooth	Irregular wave	Near

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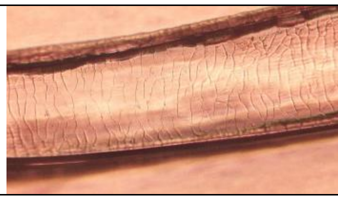
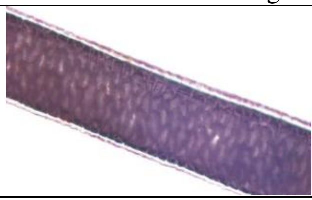
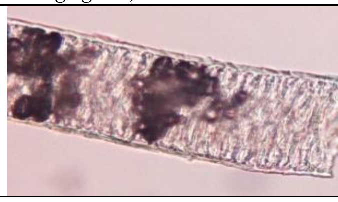
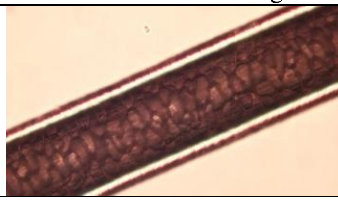
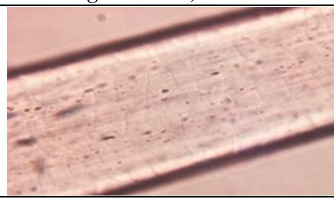
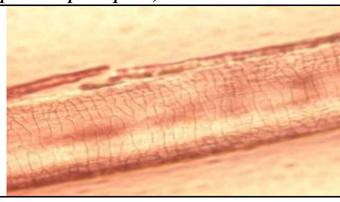
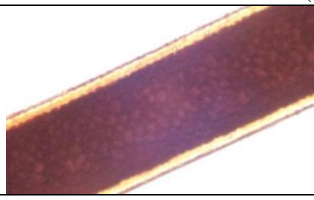
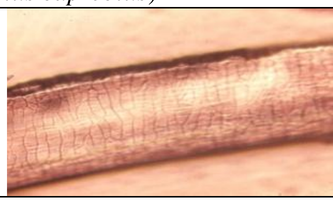
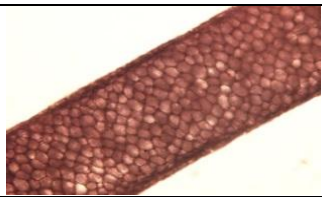
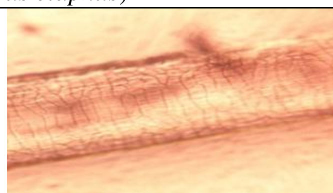
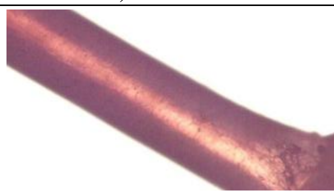
We studied the hair structures of eighteen species representing four mammalian orders and nine families including Bovidae, Cervidae, Suidae, Canidae, Felidae, Mustelidae, Ursidae, Leporidae, and Sciuridae. The identification key was developed by using Turkey's 18 mammal species' hairs medulla and cuticle patterns (*Fig. 2*).

Order	Family	Medulla	Cuticle
Artiodactyla	Bovidae		
		Wild goat (*Capra aegagrus*)	
			
		Goitered gazelle (*Gazella subgutturosa*)	
			
		Chamois (*Rupicapra rupicapra*)	
	Cervidae		
		Roe deer (*Capreolus capreolus*)	
			
		Red deer (*Cervus elaphus*)	
			
		Fallow deer (*Dama dama*)	
	Suidae		
		Wild boar (*Sus scrofa*)	

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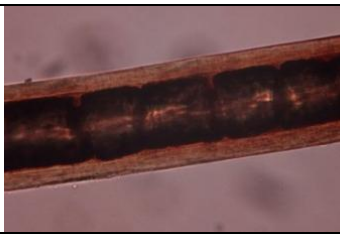
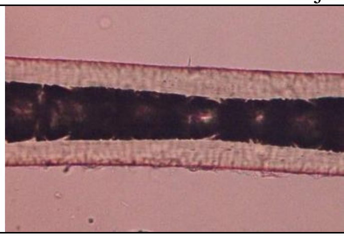
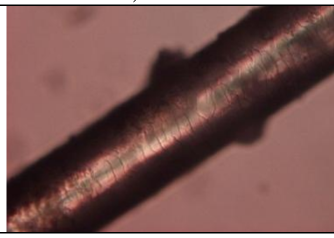
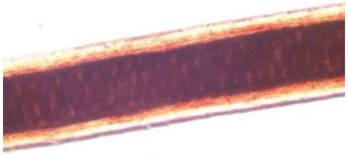
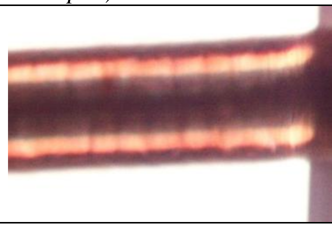
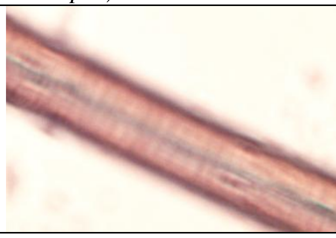
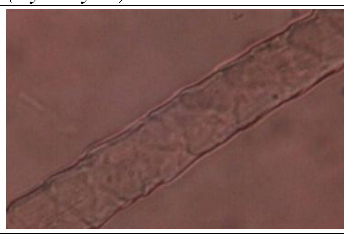
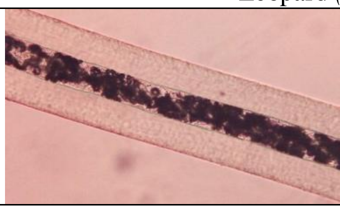
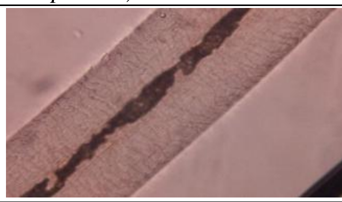
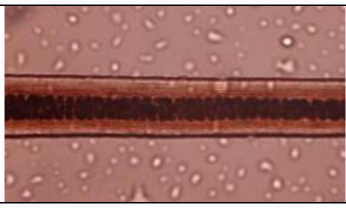
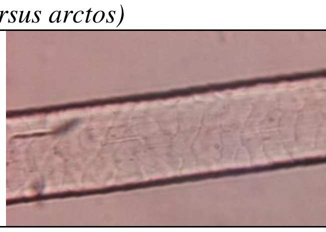
Order	Family		
Carnivora	Canidae		
		Golden jackal *(Canis aureus)*	
			
		Gray wolf *(Canis lupus)*	
			
		Red fox *(Vulpes vulpes)*	
	Felidae		
		Eurasian lynx *(Lynx lynx)*	
			
		Leopard *(Panthera pardus)*	
	Ursidae		
		Brown bear *(Ursus arctos)*	
	Mustalidae		
		Stone marten *(Martes foina)*	

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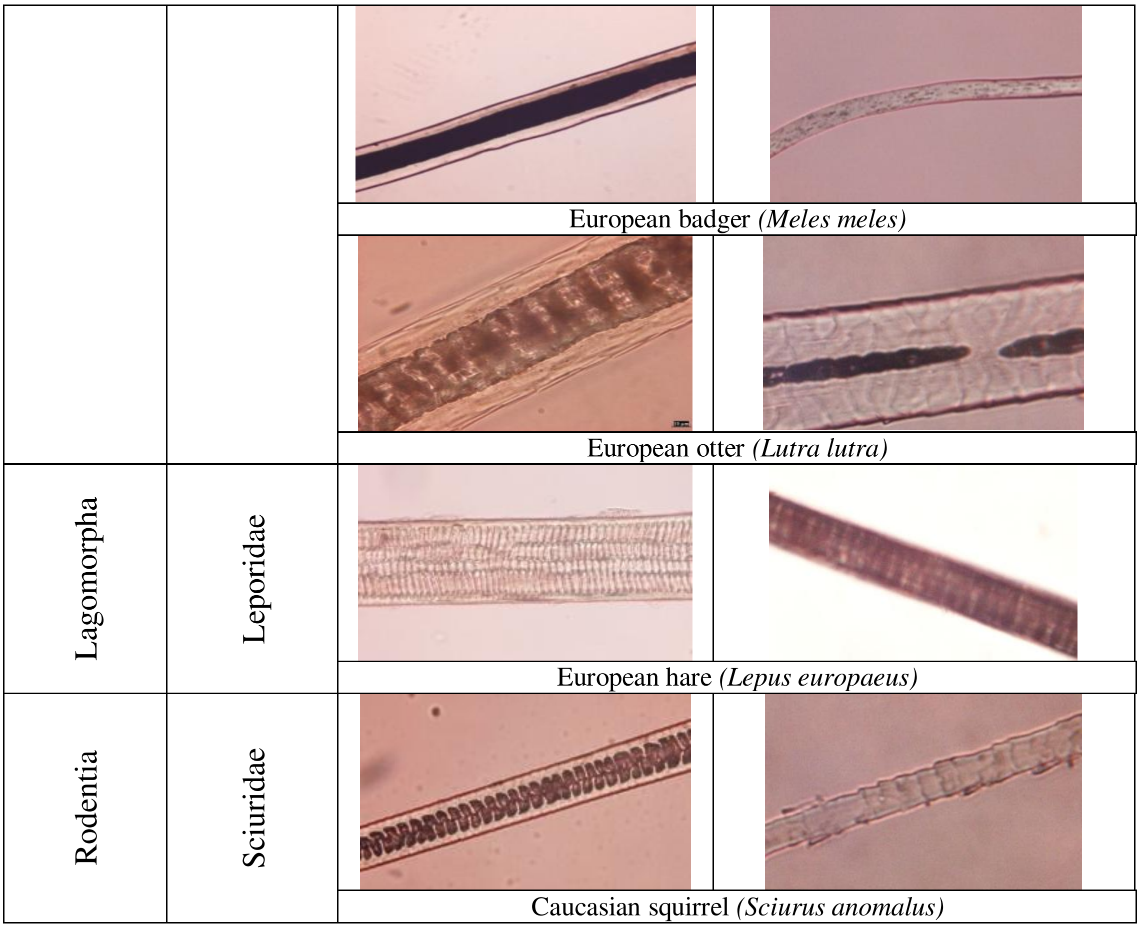

Figure 2. *Medulla (left) and Cuticle (right) patterns used in our hair key to identify common mammal species of Turkey*

Conclusions

No studies have constructed identification keys of mammal hair until this time in Turkey. Mammalian species formed by hair identification key occur in major ecosystems of Turkey such as forests, steppe, grasslands, meadows, alpine, high mountains and rivers and experience a wide range of threats. However, ecology, taxonomy, distribution, population sizes and statutes of those species are uncertain due to limited research. With this study, mammalian species can be easily identified by looking at the hair samples in the scats obtained from field studies, and they will be able to get information about their ecologies.

Our hair key allows hair identification based on morphological structures which are clearly recognizable, considering the hair medulla and cuticle. The present research provided a basic guide for wildlife researchers in ecological studies and population management of prey and predators. The techniques employed in this study are relatively simple and can be easily, quickly and economically applied in routine investigations making it suitable for wildlife studies. It is difficult to determine the species from the hair in the scats with the naked eye except for some species which have hard and thick due to variations considerably hair structures between species. Use of hair identification key will be helped to determine the species.

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In Turkey, wildlife studies on mammalian species are still not enough. Therefore, our hair identification key will be helped to provide basic ecological information for sustainable wildlife conservation and management. The present research provided a basic hair identification key for further research in wildlife studies.

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RICE WEED COMMUNITY COMPOSITION AND RICHNESS IN NORTHERN IRAN: A TEMPERATE RAINY AREA

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Abstract. Knowledge about weed density and composition plays an important role in weed management decision making. Surveys were carried out to study the weed flora composition in rice fields of Guilan Province in Iran. 481 fields from 16 regions were selected for survey. Weed samples were taken with a W-shaped sampling pattern. Weed species were identified and the plant density, percentage frequency, uniformity, and abundance indices were determined. Biodiversity was calculated using Shannon-Weiner (H′) and Simpson (D) diversity indices. A total of 66 species, belonging to 43 genera and 29 families were identified. The highest number of weeds belonged to the two families Cyperaceae and Poaceae with 15 and 8 species, respectively. The most frequent weed species were *Echinochloa crus-galli* (89.8%), and *Paspalum distichum* (79.4%), *Echinochloa oryzoides* (60.3%), *Cyperus difformis* (56.5%), *Ecliptal prostrate* (49.5%), *Cyperus serotinus* (34.7%), *Azolla filiculoides* (34.5%), *Sagittaria trifolia* (31.6%), *Cyperus esculentus* (31.6%) and *Alisma plantago-aquatica* (28.7%). Relative abundance index indicated that the annual weed species were more dominant than the perennial ones. Based on Shannon-Weiner and Simpson's diversity indices Talesh area had the highest weed diversity (H′ = 2.85; D = 0.916) and Rudbar showed the lowest diversity values (H′ = 1.97; D = 0.749).
Keywords: *abundance index, density, dominance, rice fields, sustainable agriculture*

Introduction

Rice is the primary staple food for more than half of the world's population and provides 20% of the total calorie intake of people in the world (Dass et al., 2016). More than half of the world's rice is produced in Asia (Awan et al., 2015). In Iran also rice is the main staple food in most parts of the country. There are almost 600,000 hectares of rice plantation in Iran with a total production of 2,540,000 tons (FAO, 2016). More than 75% of the rice crop is grown in the Provinces of Mazandaran and Guilan, north of Iran. Weeds are considered as the most important challenge to rice production in Iran (Yaghoubi et al., 2010; Tshewang et al., 2016). The mean reduction in rice yield caused by weeds competition is 40-60%, which may reach 94-96% if weeds are not properly controlled (Chauhan et al., 2011). Rice yield reduction resulting from weed competition may vary depending on rice planting methods, type of weed, degree of importance of the weed, agricultural operations, and weather conditions (Jabran and Chauhan, 2015). Information regarding spatial variation of weeds plays an important role in increasing efficiencies of weed management methods. The four main pillars of management suitable for the location of interest are collection of information, its analysis, adoption

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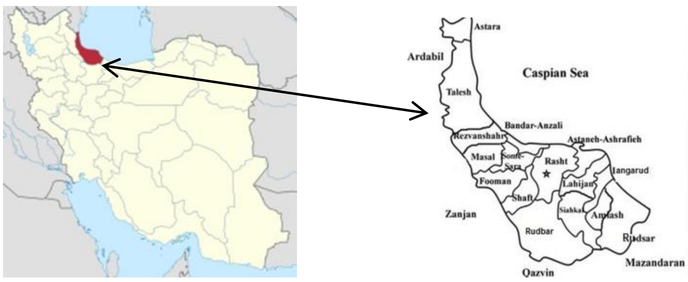
of suitable decisions, and applying management practices based on obtained information (Mohammaddoust Chamanabad, 2011).

Weed community and its distribution in rice fields are determined by the planting method, moisture regime, land preparation, and crop management (Matloob et al., 2015). Weed succession and distribution patterns in rice fields are dynamic in nature, and weed flora composition may be region-dependent (Begum et al., 2008; Uddin et al., 2010). Information concerning presence or absence of weeds, their composition, abundance, importance, and rank depends on weed management strategies and mean rice yield (Begum et al., 2006). Poggio et al. (2004) believe that structures of weed communities and their diversity are determined by environmental and management factors and by interspecific competition between weeds and crop plants and intraspecific competition between plants of the same weed species. Rao et al. (2007) stated that there was a broader spectrum of variation and diversity in rice weeds in direct seeding areas compared to transplanted areas. Weed flora composition in agricultural systems results from seasonal changes, crop rotation, and long-term environmental changes such as soil erosion and climate change. Agricultural operations such as plowing, the crop plant species growth, weed control methods, and fertilizer application changes the natural distribution pattern and availability of resources and, hence, change the structure and composition of plant species (Ahmadvand, 2005; Kraehmer et al., 2016; Lal et al., 2014 Noroz zade et al., 2008). Accurate determination of weed distribution pattern and density at each location is of great importance. Therefore, before any action is taken, the necessary maps of weed distribution pattern and density must be prepared (Lass et al., 1993).

However, detailed information on the presence, composition, abundance, importance and ranking of weed species especially in Guilan province with around 230000 hectare rice fields is rare. Understanding the sociological structure of weeds in rice fields is a pre- requisite for its effective management. Weed management can be an effective step in increasing production and hence in preserving the actual potential yields of crop plants thus increasing production. The objective of this study is to investigate rice weed community richness and composition in the Guilan province, Iran during 2014 and 2016 and their relationships with management.

Materials and methods

The study was conducted in Guilan province, Iran, which is situated in the north of Iran, South of the Caspian Sea and occupies about 14,044 km by area. Guilan Province is located between 36° 34' to 38° 27' latitudes and 48° 53' to 50° 34' longitudes *(Fig. 1)*.



Iran

Guilan

Figure 1. *Geographic map of Guilan province*

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Guilan province has the best type of weather and soil with moderate and humid climate typically known as the moderate Caspian climate. The annual average rainfall is 1356 mm, the annual average temperature is 16.1 °C. During the springs and summers of 2014, 2015 and 2016, when the current study was being done, temperature ranged from 16 to 29.4 °C, monthly average rainfall recorded 213 to 853 mm, average sunshine hours 3320, and relative humidity averaged 45-87%.

The chemical and physical attributes of the soil at the study sites are given in (*Table 1*).

Table 1. *Physico-chemical properties of soil in regions of Gilan province*

Region	Soil properties						
	Clay (%)	PH	EC (ds/m^2)	Phosphorus (mg/kg)	Nitrogen (%)	Potassium (mg/kg)	OC%
Astara	18	6.2	0.55	24.5	0.213	100	2.1
Astaneh-ye Ashrafiyeh	23	7	1	24.4	2.1	245	2.4
Amlash	23	5.6	0.91	39.1	0.16	270	1.85
Anzali	32	6.3	0.63	12.8	0.235	105	2.3
Talesh	21	6.6	0.6	12.2	0.221	106	2.4
Rasht	33	6.8	1.56	11.6	0.161	165	2
Rezvanshahr	24	6.8	0.68	14.1	0.252	100	2.6
Rudbar	30	7.2	0.89	7.9	0.071	190	1
Rudsar	26	6.1	0.9	26	0.167	141	2.4
Siahkal	35	6.9	0.86	7	0.3	175	1.4
Shaft	18	6.2	0.67	6.3	0.168	108	2.1
Sowme'eh Sara	23	6.4	1	18.6	0.222	106	2.3
Foman	26	6.1	0.85	21.7	0.176	116	2.1
Lahijan	29	6.5	1.16	15.5	0.204	178	2.35
Langarud	26	6	1.2	32.3	0.166	178	2.2
Masal	28	7	0.9	16.1	0.234	110	2

Based on the area under rice cultivation in each site of this province, 481 fields were selected from 5 days after transplantation to the end of panicle formation. Weeds in rice fields include annuals and perennials. In each growing season, seeds were sown in the seedling nursery and 21- day-old seedlings were transplanted with 2-3 seedlings per hill. Taking the area of each field into consideration, 0.25 m^2 quadrats were done to take samples using a W-shaped sampling pattern. The weeds in each quadrat were counted and their genera and species were identified. The frequency, field uniformity over all fields, density of the weeds in each field, the mean weed density of the visited fields, and relative abundance of the various species in each County were calculated using the following equations (Thomas, 1985):

Frequency (F) indicates the ratio of the number of fields having a specific weed species to the total number of the fields, and is expressed in terms of percentage. Frequency is concerned with the presence or absence of a species in a quadrat, a field, or a region, and does not refer to the number or quantity of the species (*Eq. 1*):

$$F_k = \sum (Y_i/n) \times 100 \qquad \text{(Eq.1)}$$

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In above equation, F_k represents the frequency of the species, Yi the presence or absence of the species, and "n" the number of visited fields.

Uniformity (U) expresses the percentage infestation of the field by the species of interest and shows the surface area occupied by the weed (*Eq. 2*):

$$U_k = (\Sigma\Sigma X_{ij}/m_i) \quad \text{(Eq.2)}$$

In *Equation 2,* U_k is the uniformity of the field for the species k, X_{ij} the presence or absence of species k in quadrat "j" and in field "i".

Density refers to the counted individuals of each species m^2 in the field of interest (*Eq. 3*):

$$D_{ki} = \left(\frac{\Sigma Z_j}{m_i}\right) \times 4 \quad \text{(Eq.3)}$$

In *Equation 3,* Dki stands for the density of species k in field i and Z_j the number of plants in quadrat j.

Mean density indicates the average number of plants belonging to each species m^2 of the visited fields (*Eq. 4*):

$$MD_{ki} = \left(\frac{\Sigma D_{ki}}{n}\right) \quad \text{(Eq.4)}$$

In the above relation, D_{ki} represents density in each field and "n" the total number of visited fields.

The coverage value indicates the coverage of plants m^{-2} for floating and submerged species averaged over fields sampled (*Eq. 5*):

$$MC_{ki} = \left(\frac{\Sigma C_{ki}}{n}\right) \quad \text{(Eq.5)}$$

In *Equation 5,* C_{ki} represents the coverage of plants m^{-2} for floating and submerged species and "n" the total number of visited fields.

Relative abundance (RA) was used to rank the weed species in the survey and it was assumed that the frequency, field uniformity, and mean field density measures were of equal importance in describing the relative importance of a weed species (*Eq. 9*). This value has no units but the value for one species in comparison to another indicates the relative abundance of the species (Hakim et al., 2013). The relative frequency (RF), relative field uniformity (RFU), and relative mean field density (RMFD) were calculated (*Eqs. 6-8*) by dividing the parameter by the sum of the values for the parameter for all species and multiplying by 100 (Nagaraju et al., 2014).

$$RFk = \frac{\text{Frequency value of species}}{\text{Sum of frequency values for all species}} \times 100 \quad \text{(Eq.6)}$$

$$RFUk = \frac{\text{Field uniformity value of species k}}{\text{Sum of field uniformity values for all species}} \times 100 \quad \text{(Eq.7)}$$

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$$\text{RMFDk}=\frac{\text{Mean field density value of species k}}{\text{Sum of mean field density values for all species}}\times 100 \qquad \text{(Eq.8)}$$

$$\text{RA}_k = \text{RFk} + \text{RFUk} + \text{RMFDk} \qquad \text{(Eq.9)}$$

One of the common methods for studying diversity of plant communities in the ecology of weeds and species uniformity and richness in agricultural ecosystems is to using the Simpson index and the Shannon-Wiener index. Species richness (S) was measured by the mean number of species per treatment (Magurran, 1988). The Simpson index (D) gives more importance to the common species, but the Shannon-Wiener index (H') puts greater importance on rare species and is calculated by *Equations 10* and *11* (Poggio et al., 2004).

$$H' = -\sum_{i=1}^{s}\left(Pi\ Ln(Pi)\right) \qquad \text{(Eq.10)}$$

$$D = 1 - \sum_{i=1}^{s}(Pi)^2 \qquad \text{(Eq.11)}$$

In these equations, Pi represents the relative abundance of a specific species (the i^{th} species) that is calculated as –Pi = ni/N, and Ln is the natural logarithm. In the values obtained for the Shannon-Wiener index, the larger values indicate greater diversity in the weed communities (of the related County). After calculating the Shannon-Wiener index for every County, the Pielou index (E) has been used to determine the uniformity of the community (*Eq. 12*) (Booth et al., 2003).

$$E = \frac{H'}{ln\ S} \qquad \text{(Eq.12)}$$

H' represents the Shannon-Wiener index and S the number of weed species observed in each community (County), and logarithm of S is used in this relation (Magurran et al., 1988). As for the uniformity of the weed community in each County, the closer the obtained value gets to zero the more non-uniform the community is (one weed species is dominant in the community). However, the closer the obtained values are to 1, the more uniform the community will be (we witness the highest species diversity and lack of dominance of a specific weed species) (Mesdaghi et al., 2005; Tang et al., 2014).

After collecting the data and making the calculations required for determining the population indices, this information created the main layer in the project, and was then designed in the format of a databank.

Results

In the present research, a total of 66 species, belonging to 43 genera and 29 families were collected from the 16 representative sites (*Tables 2* and *3*). Thirty- four of the identified species were annuals and 22 perennial species. Moreover, 11 species were

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grass weeds, 17 sedges, 30 broad-leaved species, 4 ferns, 2 vascular gymnosperms, and 2 algae (*Tables 2* and *3*). With respect to life cycle, annual weeds with 34 species were the most diverse. Cyperaceae were the largest family representing with 15 species, followed by Poaceae with 7 species, Lythraceae with 5, Polygonaceae with 4, Asteraceae and Salviniaceae with 3 species and others families with 2 or 1 species (*Tables 2* and *3*). Weeds have non-uniformly distributed in various families including Cyperaceae, Poaceae, Lythraceae, Polygonaceae, Asteraceae, and Salviniaceae that accounted for 36 species (54.5%).

Table 2. *Frequency, uniformity, mean field density, abundance indices and relative frequency, relative uniformity, relative density and relative abundance of weed species in rice fields*

Item	Scientific name	Family name	Frequency	Uniformity	Density (plant m^{-2})	Relative frequency	Relative uniformity	Relative density	Relative abundance
1	*Sagittaria trifolia* L.	Alismataceae	31.60	9.08	0.527	3.96	3.32	0.509	7.79
2	*Alisma plantago-aquatica* L.	Alismataceae	28.69	8.01	0.557	3.6	2.93	0.538	7.07
3	*Alternanthera sessilis* L.	Amaranthaceae	10.19	2.99	0.530	1.28	1.09	0.512	2.88
4	*Eclipta prostrate* L.	Asteraceae	49.48	14.49	2.275	6.21	5.3	2.196	13.7
5	*Bidens tripartita*	Asteraceae	15.38	3.24	0.170	1.93	1.18	0.164	3.28
6	*Xanthium strumarium*	Asteraceae	10.60	2.88	0.268	1.33	1.05	0.259	2.64
7	*Butomus umbellatus*	Butomaceae	6.24	1.82	0.172	0.78	0.67	0.166	1.62
8	*Cyperus difformis*	Cyperaceae	56.55	22.79	9.459	7.09	8.33	9.13	24.56
9	*Cyperus serotinus*	Cyperaceae	34.72	9.62	1.192	4.36	3.51	1.151	9.02
10	*Cyperus esculentus*	Cyperaceae	31.60	11.86	5.127	3.96	4.34	4.949	13.25
11	*Scirpus maritimus*	Cyperaceae	9.77	5.41	1.915	1.23	1.98	1.848	5.05
12	*Cyperus fuscus*	Cyperaceae	9.15	2.56	0.375	1.14	0.94	0.362	2.44
13	*Cyperus rotundus*	Cyperaceae	3.53	0.71	0.067	0.44	0.26	0.065	0.77
14	*Cyperus longus*	Cyperaceae	3.33	0.61	0.038	0.42	0.22	0.037	0.68
15	*Scirpus mucronatus*	Cyperaceae	3.12	0.75	0.037	0.39	0.27	0.036	0.7
16	*Cyperus strigosus*	Cyperaceae	2.49	0.53	0.043	0.31	0.19	0.042	0.55
17	*Pycreus flavescense*	Cyperaceae	1.46	0.28	0.028	0.18	0.1	0.027	0.31
18	*Cyperus odoratus*	Cyperaceae	1.04	0.36	0.024	0.13	0.13	0.023	0.28
19	*Pycreus lanceolatus*	Cyperaceae	0.83	0.25	0.014	0.1	0.09	0.014	0.21
20	*Fimbristylis miliacea*	Cyperaceae	0.62	0.14	0.009	0.08	0.05	0.009	0.14
21	*Cyperus glomeratus*	Cyperaceae	0.42	0.07	0.003	0.05	0.03	0.003	0.08
22	*Cyperus iria*	Cyperaceae	0.42	0.11	0.028	0.05	0.04	0.027	0.12
23	*Bergia capensis*	Elatinaceae	1.66	0.68	0.204	0.21	0.25	0.197	0.65
24	*Equisetum palustre*	Equistmaceae	2.29	0.57	0.060	0.29	0.21	0.058	0.55
25	*Equisetum arvense*	Equistmaceae	0.21	0.04	0.001	0.03	0.02	0.001	0.04
26	*Echinochloa crus-galli*	Poaceae	89.81	34.79	7.452	11.27	12.72	7.193	31.18
27	*Paspalum distichum*	Poaceae	79.42	30.24	8.556	9.96	11.06	8.259	29.28
28	*Echinochloa oryzoides*	Poaceae	60.29	13.57	0.779	7.56	4.96	0.752	13.28
29	*Echinochloa colona*	Poaceae	6.65	1.57	0.083	0.83	0.57	0.08	1.49
30	*Eleocharis palustris*	Poaceae	0.21	0.04	0.001	0.03	0.01	0.001	0.042
31	*Coix lacrima-jobi*	Poaceae	0.21	0.04	0.001	0.03	0.01	0.001	0.042
32	*Digitaria sanguinalis*	Poaceae	0.21	0.04	0.001	0.03	0.01	0.001	0.042
33	*Schoenoplectus juncoides*	Juncaceae	11.02	3.13	0.363	1.38	1.14	0.35	2.88

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34	*Mentha aquatic*	Labiatae	0.83	0.14	0.009	0.1	0.05	0.009	0.16
35	*Ammannia multiflora*	Lythraceae	16.84	5.31	0.688	2.11	1.94	0.664	4.72
36	*Ammannia baccifera*	Lythraceae	10.60	3.10	0.436	1.33	1.13	0.421	2.88
37	*Rotala indica*	Lythraceae	4.16	1.39	0.204	0.52	0.51	0.197	1.23
38	*Ammannia senegalensis*	Lythraceae	3.95	0.96	0.113	0.49	0.35	0.11	0.96
39	*Ammannia gracilis*	Lythraceae	0.83	0.21	0.019	0.1	0.07	0.018	0.19
40	*Ludwigia epilobioides*	Onagraceae	8.52	3.06	0.423	1.07	1.12	0.41	2.6
41	*Polygonum persicaria*	Polygonaceae	15.38	3.63	0.225	1.93	1.33	0.217	3.47
42	*Polygonum hydropiper*	Polygonaceae	6.44	1.57	0.091	0.81	0.57	0.088	1.47
43	*Polygonum hydropiperoides*	Polygonaceae	1.04	0.36	0.019	0.13	0.13	0.018	0.28
44	*Rumex crispus*	Polygonaceae	0.42	0.07	0.003	0.05	0.02	0.003	0.08
45	*Monochoria vaginalis*	Potederiaceae	16.63	8.19	2.969	2.09	2.99	2.866	7.95
46	*Samolus valerandi*	Primulaceae	0.21	0.07	0.003	0.02	0.03	0.003	0.05
47	*Galium aparine*	Rubiaceae	0.21	0.04	0.003	0.02	0.01	0.003	0.044
48	*Veronica anagalis-aquatica*	Scrophulariaceae	2.08	0.43	0.019	0.26	0.16	0.018	0.44
49	*Typha minima*	Typhaceae	1.46	0.25	0.010	0.18	0.09	0.01	0.28
50	*Berula angustifolia*	Umbelliferae	0.83	0.21	0.017	0.1	0.08	0.016	0.197

Table 3. *Frequency, uniformity, relative frequency, relative uniformity and relative weed cover related to the various weed species floating and submerged in rice fields of Guilan Province*

Item	Scientific name	Family name	Frequency	Uniformity	Relative frequency	Relative uniformity	Coverage % (m^2)
1	*Chara vulgaris*	Characeae	0.42	0.21	0.053	0.077	0.023
2	*Nasturtium officinale*	Cruciferae	10.40	2.46	1.305	0.91	0.229
3	*Algue blue-green*	Cyanophyceae	17.26	7.51	2.166	2.746	6.936
4	*Lemna minor*	Lemnaceae	28.27	12.50	3.547	4.571	6.030
5	*Najas marina*	Hydrocharitaceae	12.06	5.59	1.513	2.044	4.798
6	*Najas minor*	Hydrocharitaceae	2.70	1.18	0.339	0.431	0.645
7	*Marsilea quadrifolia*	Marsileaceae	6.24	1.75	0.783	0.64	0.242
8	*Ludwigia palustris*	Onagraceae	2.91	0.78	0.365	0.285	0.125
9	*Potamogeton crispus*	Potamogetonaceae	0.21	0.07	0.026	0.026	0.007
10	*Potamogeton nodosus Poir.*	Potamogetonaceae	19.33	6.87	2.426	2.512	1.383
11	*Ranunculus aquatilis*	Rananculaceae	3.12	0.89	0.392	0.325	0.373
12	*Riccia glauca*	Ricciaceae	2.49	0.93	0.312	0.34	0.147
13	*Azolla filiculoides*	Salviniaceae	34.51	18.69	4.331	6.834	24.316
14	*Azolla pinnata*	Salviniaceae	1.66	1.10	0.208	0.402	1.876
15	*Salvinia natans*	Salviniaceae	0.83	0.36	0.104	0.132	0.016
16	*Hydrocotyle ranunculoides*	Umbelliferae	0.83	0.32	0.104	0.117	0.046

Species with higher abundances had greater uniformity. Among the grass weeds, the most frequent weed species is *E. crus-galli* (89.8%) (*Table 2*), followed by *P. distichum, E. oryzoides* with frequency higher than 50%. *Echinochloa spp* are of the weeds that appear immediately after rice sowing or transplanting (Yaghoubi and

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Farahpour, 2013). And may compete heavily with the rice for nutrients, space, light and water. The most frequent observed species among the sedges were *C. difformis* with 56.5%, *C. serotinus* and *C. esculentus* with frequency of 34.5 and 31.6%, respectively. Among broad-leaved weeds *E. prostrata*, *S. trifolia*, and *A. plantago-aquatica* respectively with 49.5, 31.6, and 28.7%, frequency, were the most abundant weeds (*Table 2*). Among the floating and submerged species, *A. filiculoides*, *L. minor*, and *P. nodosus* with frequently of 34.1, 28.3, and 19.33%, respectively, were the most frequent observed species (*Table 3*).

C. difformis and *P. distichum* were the most abundant species with 9 and 8 plants m^{-2}, respectively. *E. crus-galli* and *C. esculentus* with a density of 5 plants m^{-2}. *E. prostrate* and *Monochoria vaginalis* were the other weed species with more than 2 plant m^{-2}. While other weed species varied from 0.003 to 1.91 plants m^{-2}. Weeds density was very variable for different species (*Table 2*) and it is an important factor for adoption any decision for weed control. Uniformity refers to quantitative measurement of weed distribution in fields and relative abundance values quantify the predominance of a given weed species in a environment by calculating the frequency, field uniformity, and density of a particular weed species relative to all over species observed. *E. crus-gall, P. distichum, C. difformis, A. filiculoides, E. prostrata, E. oryzoides, L. minor, C. esculentus, C. serotinus, S. trifolia, M. vaginalis*, respectively, had the highest uniformity in rice fields of Guilan (*Tables 2* and *3*). Among the grass weeds, uniformity and relative abundance for *E. crus-galli* were 34.7% and 31.18, *C. difformis* with 22.8% and 24.56, respectively, had the highest uniformity and relative abundance among the sedges (*Table 2*), and *A. filiculoides* was present with the highest uniformity and relative plant coverage of 18.5 and 24.3%, respectively, among the floating and submerged weeds (*Table 3*). The most important weeds with high RA value in rice fields were *E. crus-galli* (31.18), *P. distichum* (29.28), *C. difformis* (25.56), *E. prostrate* (13.7), *E. oryzoides* (13.28), and, *C. esculentus* (13.25) (*Fig. 2*).

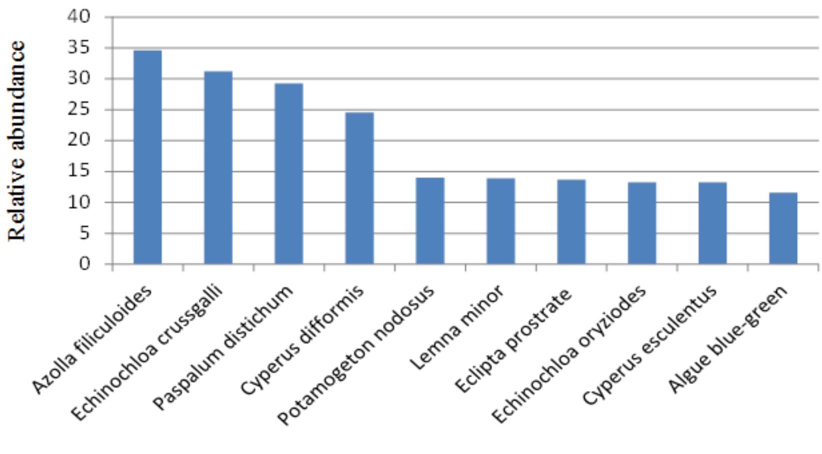

Figure 2. *Top 10 important weed species in rice fields of Guilan Province, Iran*

Rudsar and Langarud regions were the most dominant site registering a total of 47 weed species followed by Rasht and Lahijan (46 sp.), Astaneh-ye Ashrafiyeh (42 sp.), Amlash (37 sp.), Talesh (36 sp.), Foman and Siahkal (34 sp.), Rudbar (32 sp.), Rezvanshahr and Sowme'eh Sara (30 sp.), Masal (28 sp.), Astara (27 sp.) and Shaft and Anzali (25 sp.). The Simpson and the Shannon-Wiener distribution indices had the

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highest values in Talesh and the lowest in Rudbar regions (*Table 4*). It shows that weed diversity was the highest and the lowest in these regions. The Shannon-Wiener index gives greater importance to rare species, and the smaller the value of this index for the relative abundance of a species the rarer the species will be *E. arvense*, *G. aparine*, *S. valerandi*, and *Ch. vulgaris* were among the most rarely found species.

Table 4. *Quantitative indices of weed distribution in various regions of Guilan Province*

Item	Area	Shannon-Wiener index	Simpson index	Pielou index	Species richness
1	Talesh	2.850	0.916	0.795	36
2	Rezvanshahr	2.740	0.909	0.806	30
3	Foman	2.590	0.896	0.734	34
4	Rudsar	2.640	0.892	0.686	47
5	Siahkal	2.530	0.885	0.717	34
6	Langarud	2.450	0.877	0.640	47
7	Astaneh-ye Ashrafiyeh	2.580	0.874	0.690	42
8	Amlash	2.360	0.865	0.654	37
9	Astara	2.230	0.863	0.677	27
10	Rasht	2.420	0.858	0.632	46
11	Lahijan	2.420	0.843	0.632	46
12	Sowme'eh Sara	2.340	0.834	0.688	30
13	Masal	2.645	0.904	0.792	28
14	Shaft	2.050	0.815	0.637	25
15	Anzali	1.990	0.787	0.618	25
16	Rudbar	1.970	0.749	0.568	32

In the present research, *Echinochloa spp* was the most common weed in rice fields of Guilan province, and Langarud and Rudsar Counties each with 47 species and Anzali and Shaft with 25 species (with 71.2 and 37.9%, respectively) had the highest and lowest species diversities. Rezvanshahr city was 0.806 and Rudbar city with 0.568 highest and lowest levels of Pielou index (*Table 4*). As for the Simpson index, any species that is more dominant has a greater relative abundance. This index gives greater importance to the more common species.

Discussion

During the last century, more than 1,800 species were identified in rice fields (Kamoshita et al., 2014). Tshewang et al. (2016) reported weeds in fields in Bhutan included 27 species (13 annual and 14 perennial species) belonging to 10 families. In a study conducted in rice fields of Egypt, 71 weed species were identified, and it was reported that the largest percentages belonged to the Poaceae, Asteraceae and Cyperaceae families with 28, 9, and 7%, respectively. It founded that various factors including the environment, soil, and biological factors such as soil structure, pH, nutrients, and moisture, type of crop, and history of grown crops influenced weed diversity (Hakim et al., 2010; Turki et al., 2002). *E. colona* constituted about 60% of the weed population 60 days after transplantation. In the research by Matloob et al. (2015),

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D. aegypticum, E. colona, and *E. crus-galli* were more common than other grass weeds. In our study, the most common grass weeds were *E. crus-galli, P. distichum* and *E. oryzoides. C. serotinus*, which has also been reported from some Asian countries and from Italy, is apparently confined to transplanted rice. Yakup (2007) reported 70 weed species from rice fields in western Java, with the broad-leaved, Cyperaceae, and grass weeds having the largest number of species. Dominance and relative population of broad-leaved weeds increased by about 62% more than grass weeds and sedges 30 days after rice was transplanted (Singh et al., 2008). In Malaysia, grass weeds formed more than 80% of weed communities in direct seeding of rice. Broad-leaved weeds are more dominant than grass weeds and sedges in direct rice-sowing (Matloob et al., 2015). Scirpus species play an important role among weeds in rice fields (Schaedler et al., 2015), and Begum et al. (2006) reported that *F. miliacea* and *Scirpus grossus* were the most common weeds among the sedges emerged by the change from transplanting to direct rice-sowing in some regions of Malaysia. Singh et al. (2008) observed that most common weeds in rice fields were of annual types that were able to survive even under unfavorable conditions and complete their life cycle from seed to seed during one growing season. All weed species are not identically present in crop plants. *Echinochloa spp* and weedy rice are the most common weeds in rice fields of the world (Kraehmer et al., 2016). In the present study, *E. crus-galli* and *E. oryzoides* were the most dominant weeds. It seems that weedy rice cannot survive in transplanted rice mainly because of handweeding. Kraehmer et al (2016) stated that *C. rotundus, C. iria, and C. difformis* were the most frequent observed Cyperus species in rice fields. In another report submitted by Kraehmer et al. (2016), it was stated that the genera Alisma, Heteranthera, Monochoria, and Sagittaria constituted the most frequent found broad-leaved weeds in rice fields. In our study, the weeds of *E. crus-galli, P. distichum, E. oryzoides, C. difformis, A. filiculoides, A. plantago-aquatica, E. prostrate, C. esculentus, C. serotinus, Xanthium strumarium, Lemna minor* were dominant species. Nithya and Ramamoorthy (2015) identified 23 weed families in rice fields among which nut-grass (*C. rotundus*) was the most dominant because of its biological and physiological characteristics. Despite presence many sedges in our study, *C. rotundus* is not listed here probably because of not being able to survive in flooded rice. Rabbani et al. (2011) collected 20 weed species from five locations in the Punjab region of India and noticed that *C. difformis, E. colona, Euphyllia glabrescens, Cynodon dactylon*, and *P. paspaloides* had the highest abundances in at least 60% of the fields. The following weeds are economically important in India: *E. crus-galli*, *E. colon*, *Cyperus spp*., *Alternanthera spp*., *C. rotundus, Commelina benghalensis, Caesulia axillaris, Ammania spp., Dnebra spp., E. prostrata, F. miliacea,* and *Dactyloctenium aegypticum* (Rao et al., 2015). Begum et al. (2006) reported that *Oryza sativa* L. (weedy rice), *E. crus-galli*, *Leptochloa chinensis*, *L. hyssopifolia*, and *F. miliacea* were the most frequent found species with abundances higher than 30%. *Wolffia globosa* was the most abundant weed in rice fields of North Costal Andhra Pradesh (Nagaraju et al., 2014). Wicks et al. (2003) reported that mean densities of weeds were less than 9 plants m^{-2}, but some species had a higher mean density than the average. Uddin et al. (2009) stated that densities of most species were higher compared to the total weed density. Uddin et al. (2009) stated that *C. compressus* and *C. aromaticus* with 16.7 and 43.6%, respectively, had the highest uniformity. Most of the weeds that had the highest frequencies, uniformity, and mean density in the field are hard to control (Hakim et al., 2013). This is confirmed by the problematic weeds in terms of relative abundance in the rice agro-

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ecosystems. Kandibane et al. (2007) identified 17 weed species in rice fields with *E. colona*, *C. rotundus*, *C. iria*, *C. difformis*, *Panicum repens*, and *Brachiaria mutica* the more dominant ones. Considering the dominance of several weed species in rice fields of Guilan Province and the low values of the index, we can consider unsuitable management of weeds and consecutive rice growing one of the reasons why these species have become dominant. Rezvanshahr and Rudbar had the lowest values of the Pielou index (E = 0.806 and E = 0.568, respectively) (*Table 4*). In Kashmir, the Bandipora and Anantang districts had the highest and the lowest values of the Shannon-Wiener index (H' = 3.755 and H' = 3.271), respectively (Bahaar and Baht, 2012). A study in the greenhouse conducted, floristic diversity was greater *ex situ* (H'= 2.66) than *in situ* (H'=2.53). The high number of weed species found *ex situ* contributed to the great floristic diversity in this area (Mesquita et al., 2013).

The Simpson index had the values of 7.82, 6.82, 6.56, and 6.12 in Kiliyanur, Thailapuram, Thensiruvallur, Aadhanapattu, and Konthamur, respectively, whereas the highest value of the Shannon index (2.46) was that of Kiliyanur. Of the 56 identified species belonging to 23 plant families, 37 species were present in Kiliyanur, 45 in Thensiruvallur, 30 in Thailapuram, 28 in Konthamur, and 32 in Aadhanapattu. Nithya and Ramamoorthy (2015) stated that the Simpson index allowed better comparison between various areas compared to the Shannon-Wiener index. In the present study, *C. difformis*, *P. distichum, E. crus-galli, A. filiculoides* and *C. esculentus* had the highest values of the Simpson index among the 66 weed species identified in the rice fields.

Conclusion

Weed flora composition in rice fields of 16 counties in Guilan Province includes 66 species belonging to 29 families. Eleven species, three grass (narrowleaf) species (*E. crus-galli*, *P. distichum*, and *E. oryzoides*), three sedge species (*C. difformis*, *C. serotinus*, and *C. esculentus*), four broad-leaved species (*E. prostrata*, *S. trifolia*, *A. plantago-aquatica*, and *P. nodosus*), and one fern species (*A. filiculoides*) were the most widespread and of the highest abundances. Depending on weed management, weed growth in transplanted rice begins around 5 days after pulling or transplanting. Perennial weeds such as *P. distichum, P. nodosus, A. filiculoides,* and *B. umbellatus* new species with high density and tolerance to flooding the main tool for weed control in transplanting rice cause numerous problems like using more herbicides and repeating the handweeding in rice fields Every year, due to lack of awareness of the identification and effective factors on weed control of farms, their population is increasing. Therefore, the study of the role of management in the restructuring of communities and species diversity of weeds can be useful in developing strategies and management of weeds.

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SPATIAL-TEMPORAL ANALYSIS OF PATTERN CHANGES AND PREDICTION IN PENANG ISLAND, MALAYSIA USING LULC AND CA-MARKOV MODEL

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Abstract. Penang Island has witnessed rapid urban development and economic growth in recent years. With the ambitious vision to become a developed country by 2020, planners and policy-makers need to know the most likely direction of future urban development without compromising on the forest landscape. In this study, remotely sensed data combined with cellular automata models were used to predict the land use land cover change in Penang Island, Malaysia. Cellular Automata (CA)-Markov models were applied in 1990 and 2006 to predict the land cover in 2016. Land use land cover maps for the study area were derived from 1990, 2006, and 2016 Landsat Images (MSS, TM, and OLI). The accuracy of models is above 80%. A Markov model was applied in 2006 and 2016 to predict the land cover in 2042. The results indicate that the built-up area had expanded more in the east towards the north and south, and also towards the centre of the island and moving upwards to the north. The models suggest limiting urban development in the centre of the island to protect forest landscapes. This study serves as guidelines for other studies which attempt to project land use land cover change in forest landscape while experiencing similar land use changes.
Keywords: *Penang Island, CA-Markov, land use land cover change, accuracy, future land use*

Introduction

Environmentalists and landscape planners who are knowledgeable about land use and land cover (LULC) change are concerned that LULC can affect the global environment (Guan et al., 2011). Majority of the causes leading to biodiversity loss is due to changes in land use which are associated with habitat loss and fragmentation (Sala et al., 2000). Deforestation, urbanisation, agriculture intensification, as well as overgrazing and subsequent land degradation are all classified as land use changes in anthropogenic origin. The existence of nature, viz: slope and elevation are also involved in the change (Lambin, 1997), thus making the dynamics of land use a complex process. This complication which caused the LULC dynamics to change originates from economics, politics, culture and society as well as other legal aspects (Lambin, 1997). However, the main causes of land and forest degradation are severe changes made to urbanisation and agriculture. Such decline in natural resources which affects the environment quality through air pollution (Wu et al., 2012), water pollution (Hua, 2017), and can influence the climate are man-made changes (Angelsen and Kaimowitz, 1999). Therefore, having a good knowledge of land use land cover will be useful in providing information when decision making for using and managing the land use resources (Lu et al., 2004).

A sudden growth in distant senses data in temporal, spatial and spectral resolutions appears to be beneficial as it provides essential tools for identifying changes on the Earth's surface at different scales (Wu et al., 2006; Rogan and Chen, 2004). Several suitable approaches that have been used to model the land use land cover changes by

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using remotely senses data are; statistical models (regression and structure equation model), mathematical models (linear and static), systems model (stock and flow), agent based models cellular models (Cellular Automata (CA) and Markov Chains), and evolutionary models (neural networks) (Parker et al., 2003; Agarwal et al., 2002). Roger and Chen (2004) cited that digital change detection is defined as the process of determining and describing the changes in land use characteristic based on co-registered multi-temporal remote sensing data. These techniques used for assessing change are numerous including both statistical and rule-based methods (Coppin et al., 2004; Rogen and Chen, 2004; Lu et al., 2004).

Modeling LULC change

To detect changes that have happened or are about to take place, LULC change models are used (Veldkamp and Lambin, 2001). These change models involve analysing historical land use data where the past land transformation and transition are evaluated. The transition trend which has been identified is amalgamated with environmental variables to provide an estimate of future land use (Eastman, 2009; Pijanowski et al., 2002). Having an understanding of the factors of change (i.e population growth, soil type, distance to road or other facilities), the models have the ability to provide a probabilistic prediction of where the changes may happen (Overmars et al., 2003). LULC model which was designed to evaluate the cumulative impact of land use change and develop future activities (Veldkamp and Lambin, 2001) which are essential in helping and supporting decision making of land use planning (Guan et al., 2011). In particular, LULC change have been forecasted to be important for understanding and highlighting of potential modifications and alterations that might happen over landscapes in the future. LULC changes have been applied in different situations viz; rural development and urban growth (Kityuttachai et al., 2013), selecting conservation priority areas and setting alternative conservation measures (Adhikari and Southworth, 2012) and simulating rangeland dynamics under different climate change scenarios (Halmy et al., 2015; Freier et al., 2011).

To understand better the dynamics of land use change at different angles, the Markov chain analysis has been extensively used (Baker, 1989; Muller and Middleton, 1994). This chain analysis works on the probability of a system being in a certain state at a certain time can be determined, if the state at an earlier time is known (Bell and Hinojosa, 1977). To put it simply, this method develops a transition probability matrix of land use change between two different dates by providing an estimation of probability that each pixel of certain LULC class will be transformed to another or remains in the class (Eastman, 2009). This method has an added advantage in modeling land use change especially on a large scale (Weng, 2002). Although different from logistic regression, Markov chain analysis cannot assume statistical independence data (Overmars et al., 2003) but is suited for spatial dependent land use data but has the ability to forecast all multidirectional land use changes among all classes land use available (Pontius and Malanson, 2005). One problem that can arise when using Markov chain models is that this method is more suitable for short term projections (Sinha and Kimar, 2013) and not spatially explicit especially not providing the spatial distribution of the changes (Sklar and Costanza, 1991). However, this problem can be overcome by integrating with other different dynamic empirical models e.g. cellular automata models (Guan et al., 2011; Weng, 2002). The CA- Markov is regarded as a spatial transition model as it contains the stochastic aspatial Markov techniques with the

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stochastic spatial cellular automata method (Eastman, 2009) which is capable of predicting the two-way transitions among the available LULC classes (Pontius and Malanson, 2005). Therefore, the main purpose of this study is to demonstrate the ability of CA-Markov models to predict the LULC changes for year 2042 with special attention on the ecologically impaired forest land in Penang Island, Malaysia.

Materials and methods

Study area

Penang Island is located in the northern part of Malaysia which lies within a latitude of 5°12'N to 5°30'N and longitude of 100°09'E to 100°26'E (*Fig. 1*). The total area of Penang Island is approximately 295 km^2, and is also the most populated island in the country with an estimated population of 720 000 (Tan et al., 2011, 2010). George Town is the main capital city which is located in the east region of Penang Island. Generally, Penang Island enjoys an equatorial climate with hot and humid conditions throughout the entire year. In other words, the average mean of daily temperature is about 27 °C, with a maximum and minimum average mean of daily temperature at 31.4 °C and 23.5 °C respectively. Specifically, the average annual temperature varies between 27 to 30 °C (Tan et al., 2011, 2010). Meanwhile, the average humidity is between 70 to 90%, and having the daily mean humidity between 60.9 and 96.8% (Tan et al., 2011, 2010). The average annual rainfall is 267 cm, and the annual total can reach up to a maximum of 624 cm (Ahmad et al., 2006). When the monsoon winds arrive, the population of Penang Island experienced sunshine during the day and rainfall in the evenings.

Figure 1. *Study area of Penang Island*

Since George Town constitutes a unique architectural and cultural townscape in the country, the island received recognition from UNESCO World Heritage Site as one of the tourism centres in Malaysia. This recognition will help to increase the number of visitor arrivals to Penang Island. Nevertheless, an increase in population will increase in the built up area and decline in forest land. Generally, the total area of forest land is approximately 154 km^2 with a population density of 132 persons per km^2. Forest land (41%) was the predominant land use in Penang Island, followed by urban area (18%), agriculture area (8%) and water bodies (33%) in 1990. Urbanisation and agricultural practices indirectly had a negative impact on the fate of ecology and biodiversity of the forestry land. The main reasons for choosing Penang Island for LULC change prediction are the rapid population growth, availability of data sets with zero cost, and

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ecological of forest land impaired (Tan et al., 2011, 2010). According to Tan et al. (2011, 2010), the growing population rate between the year 2000-2010 was higher than the years from 1990-2000 and will continue to increase in the coming future as well as the infrastructure of development. Therefore, identifying the potential areas that are likely to be converted into other land use classes would benefit the decision makers and landscape planners as references and guidelines for the policies to be designed in a better way for sustaining the ecology integrity of forest land.

Data sources

Three LULC vector data sets for Penang Island for 1990, 2006 and 2016 (*Table 1*) were taken from USGS Earth Explorer. But the land use was grouped into four classes, namely built-up area, agriculture area, forest land, and water bodies while the road vector data was used to generate the land change suitability maps. Generally, images for classification were chosen between the months of February to April because of clear sky or cloud free during this period. All the dataset was rectified to Universal Transverse Mercator (UTM) coordinate system, Malaysian Cassini State Plane zone 03, and WGS 1984 Datum and clipped to the island boundaries. For this particular study, different software packages were applied as each one has its strength in certain operations needed for analysis. ENVI v4.7 was used for the classification of images and accuracy assessment; IDRISI Selva v.17 was used for CA-Markov modeling (Eastman, 2009); and ArcGIS v10.1 was used to produce suitable maps and final map. The steps of the research study are shown in *Figure 2*.

Table 1. *Specific of remotely sensed data used for the study*

Sensors	Month/day	Year	Spatial resolution (m)	Path/row	Band combination
Landsat MSS	03/04	1990	60	128/56	1,2,3
Landsat TM	02/21	2006	30	128/56	1,2,3
Landsat OLI	03/20	2016	15	128/56	2,3,4

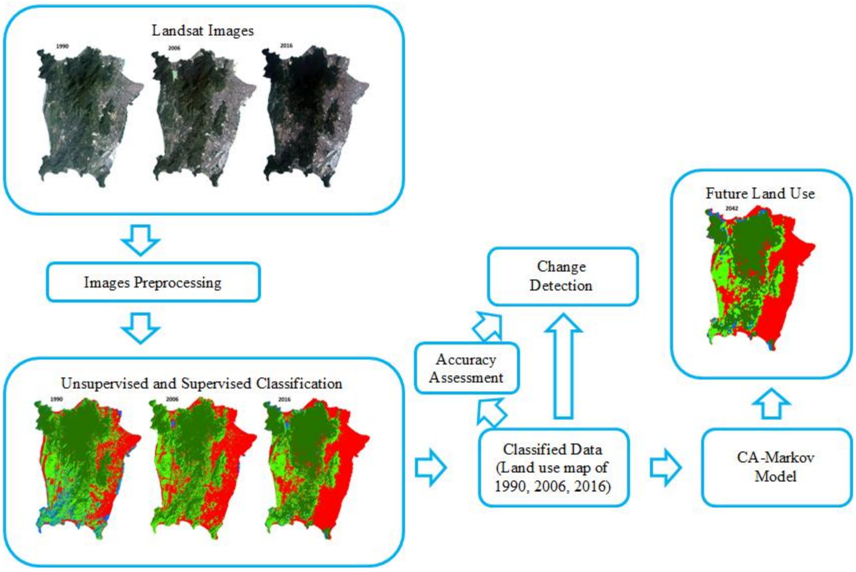

Figure 2. *Demonstration of research methodology as a flowchart*

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Data preprocessing, LULC classification and change detection

To set up a direct link between data and the biophysical phenomena it represents, it is essential to preprocess the satellite images (Parsa et al., 2016). Pre-processing can be achieved by using ArcGIS version 10.1 for geo-referencing, mosaicking and sub setting of the image for the Area of Interest (AOI). The Landsat OLI has experienced spatial sharpening using the panchromatic bands which caused a 15 m resolution. The Landsat MSS and TM images for 1990 and 2006 were originally in 30 m resolution. However, further image processing analysis was carried out using ENVI 4.7. The image appeared in a composite of natural colours using a combination of 3,2,1 for Landsat MSS and TM, and 4,3,2 for Landsat 8. The maximum likelihood supervised and unsupervised classification was performed using several selected regions, and Regions of Interest (ROI) were based on delineated classes of agriculture area, forest land, built up area and water bodies (*Table 2*).

Table 2. *Description of the LULC classes mapped in the study area*

Class name	Description
Vegetation area	Includes all agricultural lands
Forest land	Includes all forest fields
Built up area	Includes all residential, industrial area, commercial, administration, cemetery and transportation, as well as sewage treatment plant (include individual septic tank)
Water bodies	Includes all water bodies (river, lakes, gravels, stream, canals, and reservoirs)

To carry out the LULC change detection, it is proposed to apply the post classification detection method in the ENVI 4.0, for purpose of comparison, by using two classified images to produce change information on a pixel basis. In simple words, the interpretation between the two images will provide changes “from- to” information. All the data were geo-rectified and resampled to ground resolution of 15*15 m for Landsat OLI and 30*30 m for Landsat MSS and TM which was projected toWGS84 UTM with a RMSE of less than 0.5 pixels. Subsequently, the classified images from two different data sets are compared using a cross-tabulation in determining the qualitative and quantitative aspects of changes for the periods from 1990 to 2016. The extent of change and percentage of change can be expressed in a simple formula (*Eqs. 1* and *2*) as follows:

$$K = F - I \qquad \text{(Eq.1)}$$

$$A = \frac{(F-I)}{I}\ x\ 100 \qquad \text{(Eq.2)}$$

where K is the magnitude of changes, A is percentage of changes, F is first data, and I is reference data (Mahmud and Achide, 2012). This research study uses LULC techniques to decide on the differences and to explain the percentage of land use changes with the period of time. Furthermore, the prediction of LULC changes for 2042 will involve IDRISI Selva which will be further explained in CA-Markov model analysis.

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Accuracy assessment

To find out the quality of information provided from the classification process, an accuracy assessment for 1990, 2006 and 2016 images was carried out to establish the quality of information taken from the classification process. It is important to conduct an accuracy assessment for individual classification before the change detection analysis (Behera et al., 2012). Kappa tests are used to measure the accuracy of classification as the test is able to account for all elements in confusion matrix including diagonal elements (Halmy et al., 2015). Kappa test measures predefined producer rating and user assigned rating and are expressed in *Equation 3:*

$$K = \frac{P(A)-P(E)}{1-P(E)} \quad \text{(Eq.3)}$$

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 is defined as the probability of a pixel on the image actually representing a class on the ground. The producer's accuracy will show 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 mentioned earlier, 4 categories of classes were delineated. Each category have a minimum of 50 points to increase the percentage of accuracy assessment (El-Kawy et al., 2011). Therefore, the accuracies of classification for 1990, 2006 and 2016 are 87.31%, 88.49% and 91.62 with a kappa statistic of 0.86, 0.85, and 0.90 respectively. According to Weng (2010) the lowest level for accuracy assessment in identification of LULC categories in remote sensing should be at least 85%. Then, the data will be imported into IDRISI Selva v.14.0 as an ASCHII text file for further analysis.

Markov model

The Markov model is capable of calculating past conditions and predict on how a particular variable changes over time. This model has been extensively used in ecological modeling (Adhikari and Southworth, 2012; Behera et al., 2012). The uses of the Markov model in LULC change modeling is promising due to its ability to quantify the states of conversion between land use types as well as the rate of conversion among the land use type (Pontius and Malanson, 2005). A homogenous of the Markov model for predicting land use change can be represented mathematically as *Equations 4* and *5* (Sinha and Kumar, 2013);

$$L_{(t+1)} = P_{ij} * L_{(t)} \quad \text{(Eq.4)}$$

and

$$P_{ij} = \begin{bmatrix} P_{11} & P_{12} & \cdots & P_{1m} \\ P_{21} & P_{22} & \cdots & P_{2m} \\ \vdots & \vdots & \vdots & \vdots \\ P_{m1} & P_{m2} & \cdots & P_{mm} \end{bmatrix} \quad \text{(Eq.5)}$$

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where, $L_{(t+1)}$ and $L_{(t)}$ are the land use status at time t + 1 and t respectively. $0 \leq P_{ij} < 1$ and $\sum_{j=1}^{m} P_{ij} = 1 \ (i, j = 1,2,3 \ldots .m)$ is the transition probability matric in a state.

Cellular automata (CA) model

The cellular automata (CA) model, is an area spatially dynamic model, which is frequently used for LULC change studies. In this model, the transition of a cell from one land-cover to another depends on the state of the neighbourhood cells (Verburg et al., 2004). Generally, a cell has a high probability to change to land-cover class ‘A’ than to a land-cover class ‘B’ if the cell is closer proximity to land-cover class ‘A’. Similar to the Markov Model, the CA applies previous state information of a land-cover as well as the state of neighbourhood cells for its transition rules. Since CA models have been extensively used in LULC changes analysis especially in forest cover change analysis (Verburg et al., 2004; Messina and Walsh, 2001), it has the ability to integrate with the Markov model and make it a dynamic spatial model.

CA-Markov model

The CA-Markov model is a combination between cellular automata, Markov chain, multi-criteria, and multi-objective land allocation to predict land cover change over time (Parsa et al., 2016; Behera et al., 2012). The Markov model is not only spatial contiguity but is also the probable spatial transitions occur in a particular area over a time. Markov and CA-Markov modules in IDRISI Selva were used to create transition probability and transition area matrix (Eastman, 2009). Transition probability matrix is formed by cross tabulation of two images of different time and determines the probability of a pixel in a land use class to change into another class during that time (Parsa et al., 2016; Eastman, 2009). Transition area matrix contains the number of pixels that are expected to change to a land use class from another class during a time period (Parsa et al., 2016; Behera et al., 2012; Eastman, 2009). Land use map dated 1990 and 2006 were used to create transition probability matrix in order to project land use map for the year 2016. In creation transition probability matrix, Markov module in IDRISI Selva was used and the proportional error was set to be 15% (Eastman, 2009).

IDRISI Selva uses CA-Markov model that repeatedly produce the land use allocation until areas that are predicted by the Markov model are identified. The number of times it is repeated depends on the number of years a projection is made. Here in this study, the number of repeated process performed was 9 because the land use map of 2006 was taken as a base map for projecting the land use in 2016. A contiguity filter of a kernel size of 5*5 pixels that accounts the neighbourhood pixels was used to create spatially explicit continuous weighting factors so that the pixels that are far from the existing land use class have a lower suitability than the pixels that are near. In this study, the filter used was for analysis:

0	0	1	0	0
0	1	1	1	0
1	1	1	1	1
0	1	1	1	0
0	0	1	0	0

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After a predictive data has been built, the model is then validated to test the accuracy of the model. The process of gauging the accuracy of the model will be illustrated in the validation prediction model section. Then, the techniques of CA-Markov for producing the transition probability matrix and transition area matrix is repeated by using land use map dated 2006 and 2016 to cross tabulate to produce the land use map of the year 2042 (*Table 8*). In other words, the actual land use map of 2016 are used as based map to produce simulated 2042 map of LULC at the study area.

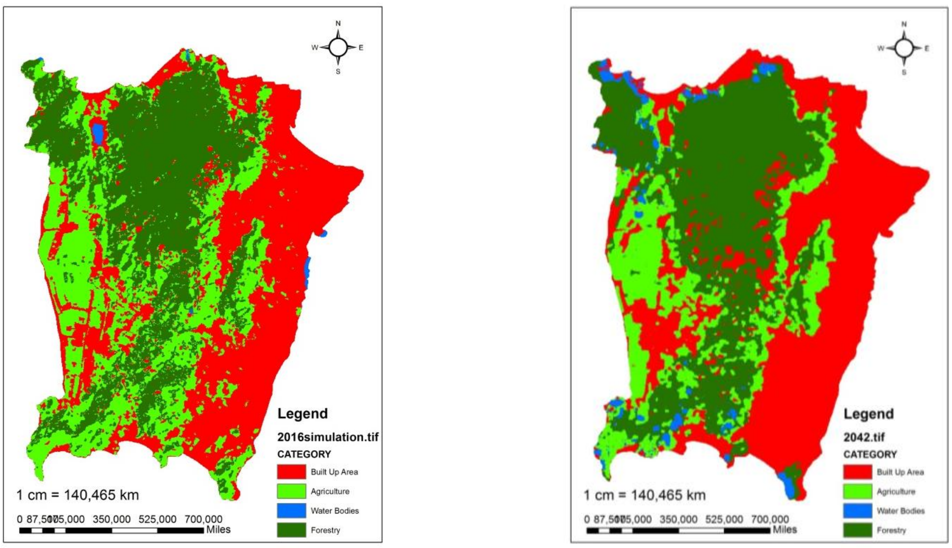
Validation LULC prediction model

For purpose of evaluating and to avoid any miscalculation of the model, it is necessary to investigate between the actual map and simulated map and to compare the output from the model with the actual land use map. The evaluation model is based on the Kappa Index of Agreement (KIA) approach, which is widely used in validation of LULC change predictions (Parsa et al., 2016; Halmy et al., 2015; Behera et al., 2012). Whereas the accuracy assessment process was done using the VALIDATE module in IDRISI Selva. For this study, a simulated land use map of 2016 will be used to compare with the actual land use map of 2016. The results of the comparison between the simulated and actual map for year 2016 can be shown in *Table 3* as well as *Figure 3a* and *b*.

Table 3. *Comparison of actual and projected LULC types in 2016*

Category	2016A (ha)	2016S (ha)
Forest land	110 854	109 350
Built-up area	88 183	83 742
Agriculture area	53 992	62 617
Water bodies	41 971	39 291
Total	295 000	295 000

2016A = 2016 actual map, 2016S = 2016 simulated map



a b

Figure 3. *LULC simulated map of 2016 (**a**) and 2042 (**b**)*

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A Kappa value of 0 illustrates the agreement between actual and reference map (equals chance agreement), the upper and lower limit of kappa is +100 (it occurs when in total agreement) and -1.00 (it happens when agreement is less chance) (Sinha and Kumar, 2013). In this study, the validation indicates K values (Kno = 0.823, Klocation = 0.851; KlocationStrate = 0.851; Kstandard = 0.831) above 0.8 showing satisfactory level of accuracy. According to Pontius and Millones (2011), if the results are greater than 0.8 for each kappa index agreement, then the K statistics are considered accurate. Hence, CA-Markov modeling is suitable for accurate prediction of future LULC's.

Results and discussion

Land use land cover (LULC) changes

According to *Table 4,* the results indicate the forest land, built-up area, agriculture area, and water bodies for 1990, 2006, 2016 and 2042 (*Figs. 3a, b* and *4a, b, c*). In other words, the LULC changes between 1990 to 2006 showed the built-up area and agriculture area have increased by 19 633 ha and 44 302 ha, while the forest land and water bodies have decreased by -7 218 ha and -56 717 ha, respectively (*Table 4; Fig. 4a* and *b*. This situation has occurred because the forest land and water bodies were converted into built-up area and agriculture area. Specifically, forest land and water bodies are likely to transform into agriculture area, before these activities are transformed again into built-up area (*Table 5*).

Table 4. *Area (ha) of LULC type in Penang Island for 1990, 2006, 2016 and 2042*

Category	1990 ha (%)	2006 ha (%)	2016 ha (%)	2042 ha (%)
Forest land	126 330 (43)	119 112 (40)	110 854 (38)	89 568 (30)
Built-up area	53 810 (18)	73 443 (25)	88 183 (30)	97 350 (33)
Agriculture area	25 650 (9)	69 952 (24)	53 992 (18)	61 412 (21)
Water bodies	89 210 (30)	32 493 (11)	41 971 (14)	47 200 (16)
Total	295 000 (100)	295 000 (100)	295 000 (100)	295 000 (100)

ha = hectare; % = percentage

Table 5. *Transition probability of area and matric calculated using land use maps of 1990-2006*

Category		1990–2006			
		Forest land	Built-up area	Agriculture area	Water bodies
Forest land	F	64 894.87	17 500.01	40 326.12	0
	P	52.88	14.26	32.86	0.00
Built-up area	F	4 492.03	50 341.29	8 793.18	0
	P	7.06	79.12	13.82	0.00
Agriculture area	F	7 064.99	14 942.59	25 726.50	66.92
	P	14.78	31.26	53.82	0.14
Water bodies	F	11 732.17	13 594.22	23 074.89	12 450.22
	P	19.28	22.34	37.92	20.46

F = frequency in hectare, P = Percentage

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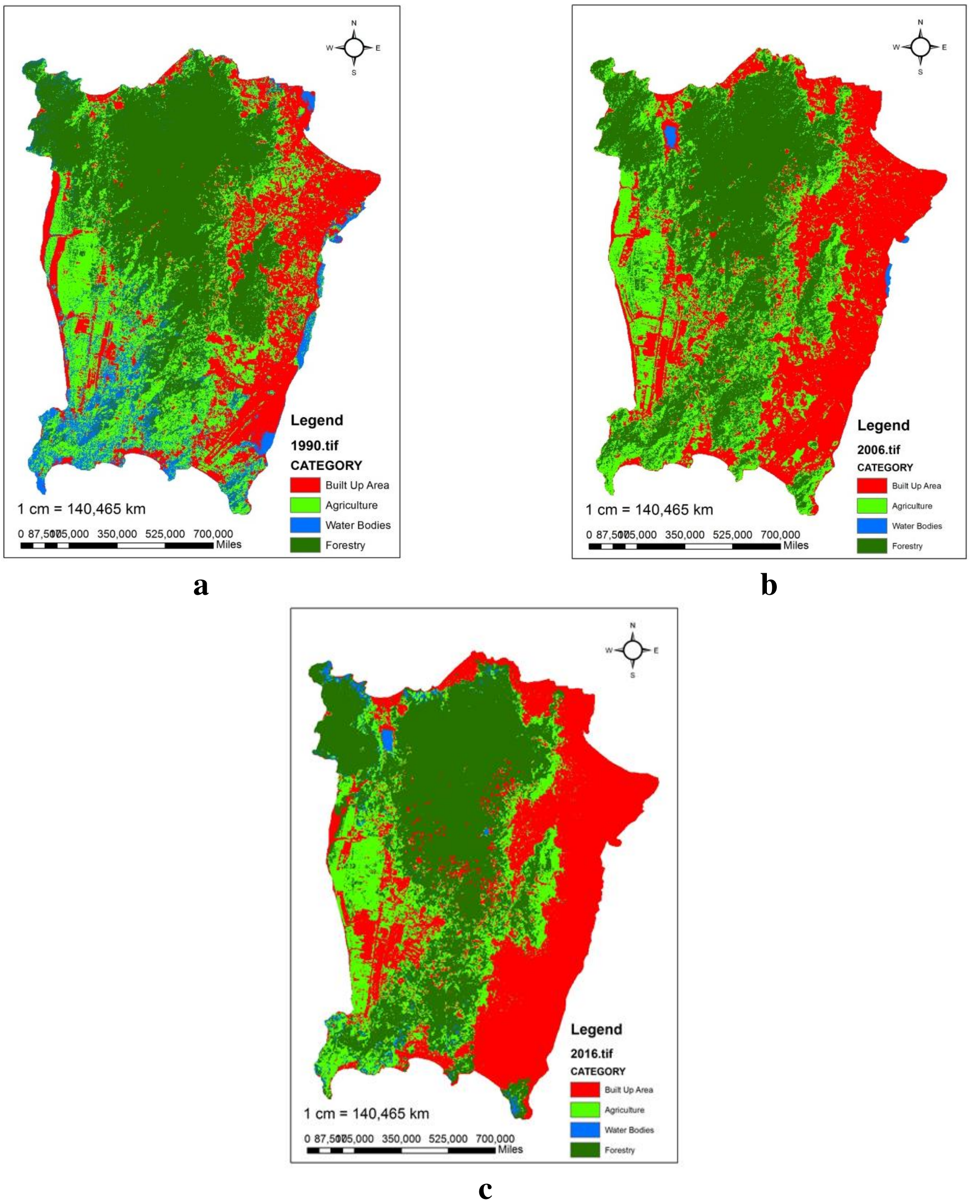

Figure 4. *LULC actual map of 1990 (**a**), 2006 (**b**), 2016 (**c**)*

Sufficient water supply with fertile soil leads to the agriculture activities to be carried out, while abandoned forest land would be in demand for built-up and agriculture activities. In addition, with the country's vision of 2020 to be a developed country, the rapid development becomes prioritised and these encouraged the attraction of local and non-local residents to concentrate on Penang Island. Due to the population growth and rapid urbanisation, the demand for shelter and job opportunities could also increase. Indirectly, this situation will raise the pressure on forest land and water bodies to negatively affect in the quality and quantity, which could bring harm to living species and cause extinction.

Nevertheless, the LULC change for the next 10 years indicated that forest land continued to donate to the coverage area for agriculture activities for 31.22% before its transformation into a built-up area for 29.72% (*Table 6*). Meanwhile, only the built-up

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area will continue to increase especially in the north, south and centre of the island; while others in east, northeast, and southeast show a high cluster of built-up area in Penang (*Fig. 4c*). Besides that, water bodies also showed positive development, where the minority coverage area of forest land and agricultural activities are being converted into water bodies for 6.70% and 10.66%, respectively (*Table 6*). Generally, the recognition of Penang Island as a tourist centre from World Heritage Site in 2008 had raised the economic value of the country, and indirectly increased the tourists' arrival in the island. As a result, the built-up area is detected to have enlarged the area to fulfil the demand for commercial activities such as hotels, restaurants, shopping malls, hypermarkets, etc. As a proof, several residential activities are suspected to concentrate on the surroundings of Penang Hill (which was highlighted as one of the historical heritage attraction as well as for its flora and fauna). Indirectly, this area is exposed to the danger of landslide and rock falls. Simultaneously, although the water especially from the river is not supplied as drinkable sources, but the awareness of the need for quality and quantity of water supply for agriculture activities and aquatic species had been extended and were detected scattered in the north and the south of Penang Island. Hence, these situations will continue to decline from the forest land into built-up area, agriculture activities, and water bodies. Therefore, the natural biodiversity especially living animals on the island will become extinct due to the rampant deforestation for the human activities.

Table 6. *Transition probability of area and matric calculated using land use maps of 2006-2016*

Category		2006–2016			
		Forest land	**Built-up area**	**Agriculture area**	**Water bodies**
Forest land	F	48 683.80	22 697.64	35 897.70	7 703.86
	P	42.34	19.74	31.22	6.70
Built-up area	F	8 016.65	61 143.12	11 346.14	307.09
	P	9.92	75.66	14.04	0.38
Agriculture area	F	17 959.49	18 418.07	18 988.22	6 606.22
	P	28.98	29.72	30.64	10.66
Water bodies	F	320.19	2 092.44	439.34	34 380.03
	P	0.86	5.62	1.18	92.34

F = frequency in hectare, P = percentage

Overall, LULC changes for 26 years from 1990 showing only the built-up area and agriculture area are having an increment steadily for more than 20%; while forest land are having a positive effect for approximately 15% and water bodies are less than 5% (*Table 7*). Increase in built-up area proved that population growth and rapid urbanisation have succeeded in archiving the country's vision of 2020 as a developed country. Indirectly, these results will enhance the demand towards agricultural activities as well as decline in the forest land and water bodies. When the water quantity decreases through the coverage area and evaporation process during droughts season, plus with drastic climate change and water pollution through anthropogenic activities, this resulted to pressuring the supply of water as a whole. So, this situation shows that the water bodies should paid attention to avoid excessive negative impact that may affect the aquatic species and reduce the food supply through agricultural activities.

Simultaneously, forest land provides various benefits in terms of climate regulation, human health, recreation, refuge, fresh water supply, etc. In other words, declining forest land will reduce the possibility of accessible freshwater supply resources. Hence, forest land should be protected from deforestation on a large scale. This is because forest land not only links to the water bodies, but also consists of various species of flora and fauna which exist indigenously or naturally. Therefore, destruction towards forest land and water bodies will bring negative impact and will decline the human quality life.

Table 7. *Transition probability of area and matric calculated using land use maps of 1990-2016*

Category		1990–2016			
		Forest land	**Built-up area**	**Agriculture area**	**Water bodies**
Forest land	F	37 024.42	34 201.93	36 478.90	10 886.75
	P	31.22	28.84	30.76	9.18
Built-up area	F	4 046.80	57 024.39	9 925.31	0
	P	5.70	80.32	13.98	0.00
Agriculture area	F	4 428.09	16 000.08	16 517.75	2 875.08
	P	11.12	40.18	41.48	7.22
Water bodies	F	18 155.45	12 396.60	12 718.51	20 319.94
	P	27.68	18.90	22.44	30.98

F = frequency in hectare, P = percentage

Prediction of future LULC changes

Simulated future LULC change is extended for the next 26 year from 2016, which has been interpreted in *Table 8*.

Table 8. *Transition probability of area and matric calculated using land use maps of 2016-2042*

Category		**2016–2042**			
		Forest land	**Built-up area**	**Agriculture area**	**Water bodies**
Forest land	F	35 795.37	32 849.16	21 204.65	10 361.82
	P	35.72	32.78	21.16	10.34
Built-up area	F	2 170.74	83 063.12	7 532.64	0
	P	2.34	89.54	8.12	0.00
Agriculture area	F	10 317.12	13 986.96	27 662.34	5 735.58
	P	17.88	24.24	47.94	9.94
Water bodies	F	1 979.60	8 694.17	3 174.49	30 737.24
	P	4.44	19.50	7.12	68.94

F = frequency in hectare, P = percentage

The result indicates that the built-up area, agriculture area, water bodies, and forest land are detected to continue to 'grow' in the coverage area of about 18 510 ha (26%), 10 637 ha (12%), 5 365.8 ha (7%), and 4 822 ha (8%), respectively. In other words, the future LULC map only shows the built-up area and water bodies are having an increment in the coverage area, while agricultural activities and forest land are

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suspected to decline. Generally, water provides food and water sources, recreation activities, sustaining the ecosystem, maintaining the humidity of land surface from overheating and the climate changes, etc. The benefits and importance of water resources had increased the awareness of local residents to sustain the quality and quantity from 'disappearance' and be concerned with environmental issues. Nevertheless, LULC map in 2042 resulted to an increase in built-up area showing a sign of warning that the population growth has increased and urbanisation could be achieved in making Penang Island as one of the developed states. Indirectly, the built-up area will increase the demand for water and food sources, shelter, employment, recreations, etc.; as well as creating various numbers of environmental problems such as pollution, the cause of extinction towards living and non-living being. This situation also signalled that 'confiscation' of other class area in fulfillment of the quantity of the population will happen.

As a proof that confiscation could happen, the CA-Markov analysis has shown that forest land and agricultural activities is declining in the coverage area to contribute to the built-up area. To be more specific, future LULC map in 2042 indicated that several areas of forest land in the centre of Penang Island especially surrounding the high hills. This was transformed into built-up area, as well as the north and the west of agriculture area had been converted into water bodies. As explained previously, the forest land plays an important role in providing the sustainability of water resources, food resources, land resources, and air source (which was also referred to as the climatic matter) to provide the comfort continuance of living overall. However, pressured in fulfilling the demand of humans on the 'greedy' attitude had caused the forest land to become a 'victim' of deforestation, which can be shown in *Figure 3b*. There are only several areas that remained as forested land especially the high hills like Batu Feringghi, Bukit Bendera, Bukit Ayer Hitam, and Bukit Relau; including several forested areas at ground land like Teluk Bahang, Pantai Acheh, and Balik Pulau (*Fig. 3b*). Therefore, these areas should be preserved and conserved so that nature can be maintained for its originality and avoid from being damaged or destructed. As a summary, LULC change for 1990 to 2016 shows that the built-up area and agriculture area experienced a gain more than a loss by 29% and 21% (*Fig. 5a*); while forest land and water bodies have a loss more than a gain by -33% and -17% (*Fig. 5a*). Furthermore, the next 26 years of LULC change will indicate that the built-up area, agriculture area, and water bodies are having a gain more than a loss (*Fig. 5b*) by 46%, 2% and 2% (*Fig. 6b*), respectively; while only the forest land are having a loss more than a gain (*Fig. 5b*) by -50% (*Fig. 6b*). Therefore, forest land should be given attention and priority from deforestation on a large scale through various methods of laws, policies, moral and ethical value, religion, and awareness, which are important to prevent it from being constantly taken advantage of by other classes.

Conclusion

This study utilised satellite images (1990, 2006, and 2016) to analyse the land use land cover of Penang Island, Malaysia. Two classified images (1990 and 2006) were used as an input to the CA-Markov model to predict the 2016 land use land cover. The model output is validated with an actual image of 2016. Based on the results, the model is used to predict land use land cover for 2042. The overall results showed a predominant increase in built-up areas from 18% of total land use in 1990 to 77% by

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2042. The increase is found mainly at the expense of forest land, where these areas are reduced from 41% in 1990 to 25% in 2042. Based on the environmental framework of Penang Island in 2042 as planned by City Council of Penang Island, it is noted that Penang Island is planned to expand more to the east towards north and south. Nevertheless, the result of CA-Markov model in this study complied with the 2042 Master Plan, where the built-up areas are expected to encounter an expansion not only from the east to the north and south, but also towards the centre of the island and moving upwards to the north. In this model, it could suggest to limit urban development in the centre of the island to protect forest landscapes. To understand how the changes in the landscapes may influence the distribution of the important species will help guide conservation planning in the area. This study can serve as guidelines for other studies which attempt to project land use land cover change in forest landscape experiencing similar land use changes.

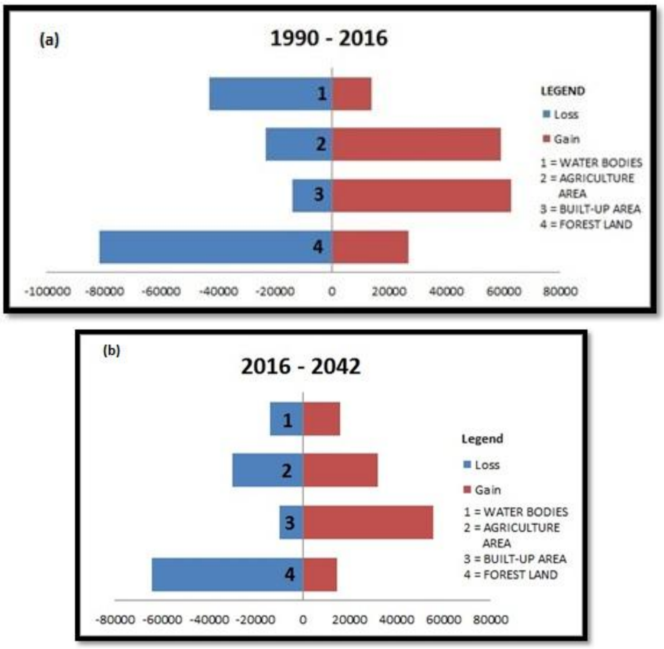

Figure 5. *Loss and gain for 1990-2016 (**a**) and 2016-2042 (**b**)*

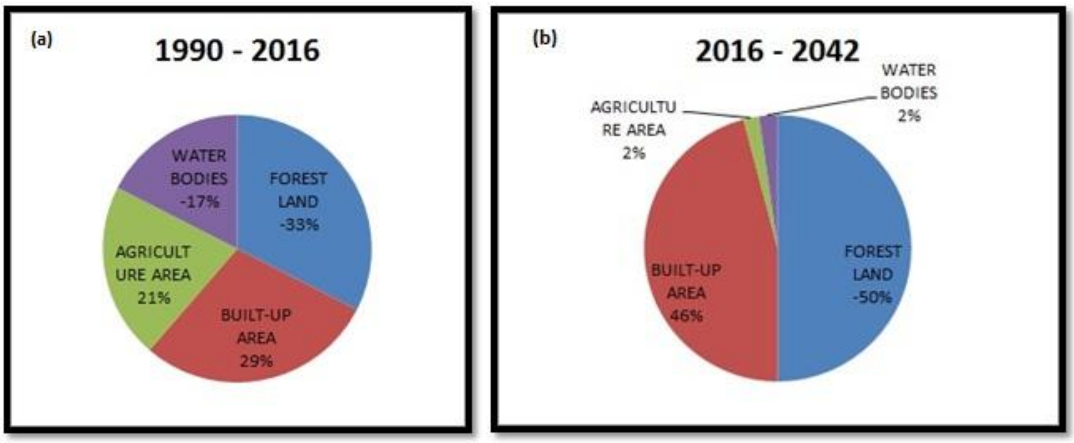

Figure 6. *Net changes for 1990-2016 (**a**) and 2016-2042 (**b**)*

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GOVERNMENT AND RANGE MANAGEMENT IN IRAN (POLICY, LAWS AND PLANS)

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Abstract. Through laws, policies and plans, governments manage natural ecosystems, including rangelands. According to the political and economic approaches, scientific achievements and the ecological and social developments, the mentioned laws and policies must be developed and updated. Investigation of the effects of these measures can play an important role in the improvement of management processes and updating laws and policies. Reduced level and trend of destruction affirms that the government's actions of Iran in rangeland management are not effective enough and need to be reassessed. The present research was carried out aiming at investigating the effectiveness of laws, policies and plans on rangeland management based on a survey of rangeland management specialists. In this research, results and data have been collected based on questionnaire from experts in executive department (Forest, Rangeland and Watershed Organization), Natural resources research specialist and university faculties. Given the facts that a large number of contestants were available and based on the type of this research, the non-probability sampling method (judgment sampling) has been used. Data were analyzed through using SPSS software, descriptive and inferential statics of (X^2) and Kruskal-Wallis test. The total number of specialist rangers in this study was 268 people. The results show that A significant part of the experts participating in this research believe that the nationalization of rangelands has had profound effects on rangeland management systems. In addition, a large number of respondents believe that government policies in relation to rangeland need to be revised and redevelopment plans should also be prepared and implemented in accordance with ecological and social conditions.
Keywords: *laws, land reform, nationalization of rangelands, rangeland survey, grazing permit, rangeland management plan, rangeland management policy, effectiveness, Iran*

Introduction

In the Middle East, livestock breeding and rangeland management flourished thousands of years ago, and rich indigenous knowledge has existed along with deep cultural roots in relation to rangelands in various areas. Based on investigation of historical documents, Javanshir (1999) states that the terms related to the pastoralism are seen in the life of the ancient Persian societies; all of which confirms the importance and development of livestock and range management in the ancient history of this part of the world. This is despite the fact that the science of rangeland management has a relatively short history and only dates back to the early twentieth century. What is currently known as the science of range management first appeared in the US. Holechek et al. (2004) state that in the United States, livestock grazing and

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its impacts on the degradation of pastures were brought into attention back at late 19th century.

Anyway, during the past century, due to population growth, agricultural land development and the expansion of residential areas, the pressure on rangelands has increased dramatically and in many parts of the world the pastures have been degraded and reduced in size. Therefore, many governments have tried to reduce the degradation of pastures and control the grazing process. Through laws, policies and plans, governments manage natural ecosystems and try to improve them during time based on political and economic approaches, scientific achievements, ecological transformations and social changes. Therefore, studying the effectiveness of policies used in rangelands management can play a very important role in guiding governments to improve management processes and update the rules and regulations. Considering the decrease in the area and quality of rangelands, it can be concluded that the government's programs in rangeland management have not been effective enough. So, in order to investigate this issue, some of the laws, policies and plans of rangeland management have been investigated in this research and analyzed based on the experts opinions.

Rangeland areas of Iran

The area and quality of rangelands can be an appropriate indicator in the assessment of the success or failure of management programs and measures. Considering the rangeland areas, Squires et al. (2017) state that more than 85 percent of Iran's surface area is arid and semi-arid, and from the total land area, 84.8 million ha, equivalent to 51.4 percent, consists of pastures. It argued by Farahpour and Marshall (2001) that only 9.3 million ha of pastures (10.3%) are in good conditions and 37.3 million ha are in moderate conditions (41.4%), and the remaining, 43.4 million ha, have poor condition (48.3%). Eskandari et al. (2009) provide the following estimates for the rangeland areas. Their report is important because Eskandari was, at the time, the Director General of the Forests, Rangelands and Watershed Management Organization (FRWO). This rangeland Technical Bureau, which is the main provider of rangelands management and implementer of government policies of Iran, is part of the FRWO, which is also one of the deputies of the Ministry of Agriculture Jihad. Therefore, the above-mentioned report can be considered as a formal report by the government.

1. Niknam (1968) reports the rangeland areas to be 100 million ha.
2. The American FMC Company (1974) reported the rangeland areas to be 90 million ha.
3. The rangeland technical Bureau (FRWO) in 1996, the rangeland area is 90 million ha.
4. Based on digital satellite images, land maps, and vegetations of Iran, FRWO reported the rangeland areas to be 86.1 million ha in 2005 and 84.7 million ha in 2008.

Among the most important causes of the decline in rangeland areas we may refer to increase in population and demand for food, the grazing pressure of livestock, change of use, drought, desertification and government policies. Karimi and Karami Zahkardi (2016) stated that regarding the changes in rangeland areas, despite the importance of rangelands, studies have shown that in the past years, the quality and grading

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rangelands have undergone changes that were due to inappropriate human activities and most of these degradations included rangelands of good and moderate quality. Therefore, these changes can confirm the failure of government policies and programs in the management and conservation of rangelands.

Rules and regulations related to rangeland management

Araghi et al. (1998) state that after the Constitutional Movement, conservation of forests was considered important by legislators. Added was the concern for maintaining the rangelands. In 1960, the issue of rangelands was directly and clearly observed along the forests in the law of forests and rangelands for the first time. The approval of the nationalization of forests and rangelands (in 1963) declared the rangeland as public property belonging to the state. Shamekhi (2009) stated that Islamic jurisprudence is the main legislative basis, and that the Islamic Revolution of 1979 created a new stage in legislation. Also, the Islamic jurisprudence had a great influence on current laws. Motamedi et al. (2007) state that in 1967, after the enactment of "the Law on the Conservation and Use of Forests and Rangelands", and in concordance with the provisions of the Note 1 of the Article 3 of this Law, *"the use of rangelands for which a plan has not been prepared and approved in relation to livestock feeding requires acquiring a grazing permit from the Ministry of Natural Resources and also observance of terms and conditions that are notified by the Ministry*." In fact, according to this law, the rangeland management policy is determined by the government. According to Zohdi (2018), established in recent decades, the rules and regulations related to rangelands and rangeland management in Iran have an impact on rangelands and how they are used, and, on the other hand, they are the basis of the policies governing the management and government organization concerned and have a profound effect on the rangeland management system. Moeenedin (1994) states that since 1963 rangeland have been nationalized and individual ownership have been abandoned to the government and thus management evolves in the rangeland. This was taken on an immediate basis and did take into account the facilities and personnel or the creation of the necessary underlying culture. Ahmadi et al. (2011) state that "the law of the nationalization of forests and rangelands" is a major legal regulation and in which various issues have been referred to such as grazing of livestock in natural resources, requiring the stakeholders to obtain grazing permit from the FRWO during a one –year period, and the issue of excess number of livestock. Due to ambiguities, some legal provisions have an impact on the factors affecting the rangelands negatively and have led to increased degradation. Usefi et al. (2016) asserted that Article 3 of "the Conservation and Use of Forests and Rangelands Act" (adopted in 1967) is the first legal step in rangeland surveys and the issuance of permits, the first grazing conditions for livestock in rangelands were approved and on this basis in 1970 and notified by the government. Moeenedin (1997) states that the rangeland survey that was performed under specific social and political conditions in the 1980s, played a very important role in distorting the balance of livestock and rangelands. Abolhassani et al. (2013) indicated that since the adoption of "the Land Reform Act" and "the Nationalization Act of Natural Resources" in 1964, many reports have been published on intensifying the degradation of rangelands. In order to prevent further degradation of the rangelands, the government set some criteria for the systematic and careful use; however, these

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criteria were not properly implemented and accompanied the sudden increase in the number of livestock at the time.

Rangeland management policies

Baker and Eckerberg (2013) said that in general, a public policy may be defined as "a series of measures approved and followed by the state to solve a problem". In addition, the term "policy" can refer to a proposal or a set of specific measures that the government devotes to addressing a general problem. More precisely, the term "policy" means a formal proposal or operational plan that can be thought of as a centralized and executable tool that requires resource allocation. Zohdi (2018) state that it is not clear whether laws come before policies or they are adopted based on policies. However, what is certain is that they both affect each other. By signing the technical cooperation agreement in the framework of the Point Four Program between Iran and the United States on October 19, 1950, and taking into account the presence of experts from the US and some European countries for these programs, significant events occurred in relation to Iranian rangelands that were the basis of future developments or natural resources, especially the rangelands, of Iran. Since 1953, experts from the Point Four Program Organization have carried out extensive studies of agricultural systems, rangelands and rangeland management in Iran, which resulted in a widespread policy or plan for the Mohammad Reza Shah, called "the Shah and People Revolution", or "the White Revolution". The land reform policies, which include the nationalization of forests and rangelands in 1963, is one of the most important parts of this policy package, had a profound and significant impact on Iran's agricultural and rangeland management systems. Etemadi (1996) says "Despite numerous transient and local laws adopted about forests and rangelands over the past seventy years, the law of forests (adopted in 1943), the law of forests and rangelands (adopted in 1950), Act of Protection and Use of Forests and Rangelands (adopted in 1967) are recognized as milestones among the adopted laws. But the interesting point is that at every stage, a kind of political decision has been the basis for the preparation and adoption of a law. The law of Protection and Use of Forests and Rangelands, approved in 1967, was approved by the Cabinet of Ministers on the basis of a political decision approving the nationalization of forests and rangelands on January 17, 1963. The nationalization of forests and rangelands is the second out of the six principles of the political-economic and social developments of the 1960s. Four months after the final adoption of the Conservation and Exploitation Act, the Ministry of Natural Resources was formed with the merger of forestry, wildlife management, and aquaculture and fishery organizations. Fisher (2015) showed that in the years 1962 to 1966, Mohammad Reza Shah and the Council of Ministers adopted laws aiming at reforming Iran's social, political and economic structure. These reforms are generally known as "Six Point Reforms" or "Shah Revolution". The most important measures in this program were agricultural reforms and land management systems. As a result of these measures, large agricultural lands owned by a small numbers of Khans and landowners were divided among farmers and this was the reason for changing the lifestyle and social patterns of the country. In the following years, other policies were adopted by the government that directly or indirectly affected rangelands. Mashayekhi (1990) states that a major policy that was pursued in Iran was import increase of alfalfa to feed livestock and reduce the pressure on rangelands. Studies have shown that, from 1973 to 1984, alfalfa imports increased about ten times and this has been one of the reasons for the degradation of rangelands, because, with the increase in imports of

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alfalfa, the total supply of feed increased and livestock breeders increased the number of livestock. Then, the large population of livestock, especially in droughts, increased the pressure on rangelands. It was shown by the results that the total area of agricultural land increased by 50%, from 6 to 9 million ha, after 1988. Under these conditions, despite the increase in total agricultural land, the amount of land allocated to forage production has decreased. Also, under the same conditions, the demand for food production in agricultural lands has been increasing, farmers moved their livestock to the rangelands and used extra lands for food production. Therefore, the pressure on the rangelands is increased. Eskandari et al. (2009) stated that, in order to reduce the pressure on the rangelands and increase the production of proteins, the most important policy adopted by the Ministry of Agriculture Jihad was the production of red meat in closed and industrial feedlots and changing the livelihood of rangeland owners. In addition, reforming the cropping pattern and increasing forage production, financial support, government facilities and land allocation have been other policies adopted by this ministry. On the other hand, some of the FRWO's policies for the systematic management of rangelands based on observance of criteria and terms included attraction of capital and knowledge in rangeland management, the provision of rangeland managers with their customary rights in order to reduce the number of small stockbreeders, increase of scientific and technical capacities of rangeland keepers and their role in rangeland management, land use systematization, conversion of low-productivity farms into producers of forage, reduction of population exploiting the rangelands, balancing the number of livestock and rangelands, proper management in droughts, rangeland surveys, identification of common rights of livestock breeders, migration systematization, preparation and implementation of rangeland management plans, precipitation management, collection and production of seeds of quality crops, restoration and improvement of rangelands, insurance plans for rangeland management etc.

Rangeland management plans

Salmasi (1994) declares that the first scientific and technical measure in Iran in terms of rangeland management included the idea of reformation and use of rangelands based on Article 3 of "the Conservation and Use of Forests and Rangelands" (1967) which was the only legal tool for long-term exploitation of rangelands. In these plans, the main purpose was to reform, rehabilitate and properly utilize the rangeland. The first attempt at rangeland plans was made in 1969 with the preparation of extensive rangeland management plans. Since 1977, in the second attempt, experts focused on smaller units of operation, or in other words, the rangeland allotment. FRWO (2009) reported that despite the fact that scientific standards were followed in the establishment of large rangeland management projects, because of the large extent and lack of attention to customary areas and social issues, none of these plans were implemented. These plans were under focus till 1976. Large rangeland management plans were not successful for various reasons. Therefore, small projects were considered by the rangeland Technical Bureau (Government). Since 1976, the preparation and implementation of range plans were made in a new way and began with the emphasis on cooperation between the government and the stakeholders. Several rangeland plans were prepared from 1977 to 1979; however, out of 407 drafted designs, only 87 were approved, and only 30 were implemented, which indicates the failure and un-acceptance of such plans. The rangeland plans faced with a serious ignorance after the Islamic Revolution and they

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were nearly forgotten between 1980 and 1984, but in the years 1982 to 1984, a new attempt was made on large plans. During these years, plans such as forage crops were considered more seriously. In 1984, changes were made in the rangeland exploitation by changing the rangeland survey guidelines and the taking into account the livestock instead of humans (rangeland managers), in such a way that the growth of the stakeholder populations was observed from this year onwards. Since 1987, rangeland projects became part of FRWO's policies and were included as the main administrative program of the government for rangeland management. Also in this year, the duration of rangeland management projects increased from 15 to 30 years in order to increase the share of stakeholders. Composition of rangeland projects was gradually accepted as the main policy of rangeland management and is still the main policy of the state in managing rangelands. Zohdi (2018) state that the early rangeland management plans, prepared based on American schemes, and known as "large rangeland projects", continued until 1976, during which time, 24 projects were established on two million and five hundred thousand hectares and a huge attempt was made to rehabilitate the rangelands and strengthen the vegetation in the framework of these projects. Since 1977, rangeland projects became the main policy of managing rangelands in the framework of rangeland allotments known as "small projects". According to the latest report of FRWO, by August 2017, 14,651 range management projects have been prepared at 34,051,703 ha; of which, 13302 have been approved (31,804,989 ha), 8193 were outsourced and agreements were made with stakeholders as administrators (19,781,615) and 5488 RMP (14,671,245) are under construction. For every specific area, known as the rangeland allotment, these projects are prepared by consultant companies under the supervision of the government and implemented by the stakeholders as the administrators. Rangeland allotment contains a specific area of rangelands that has historically been exploited by an individual or a group of pastoralists, and its boundaries were determined by the surveying boards of the rangelands based on the documents provided by stakeholders and approved by the survey boards.

Materials and methods

The present study was conducted based on evaluation of rangeland management history in Iran and its effective factors. For this purpose, the principles of the subject were investigated by library studies and a review of scientific records and related documents. Based on the results of library studies, a questionnaire was prepared for attaining the expert opinions. Given the large number of rangeland experts were available and taking into account the type of the study, a non-probabilistic and judgment sampling method was used. Data from the survey via questionnaire were analyzed using SPSS software and the descriptive and inferential statistics of Chi square (X2) and Kruskal-Wallis test. The statistical population was divided into three main groups: 1. Rangeland management experts in the administrative area (experts from FRWO and Headquarters of the Natural Resources Departments of the Provinces); 2. Rangeland management experts in the research area; 3. Specialists, faculty members and PhD students in the area of rangeland management. The structure of the questionnaire was based on the results of library studies. This questionnaire is divided into three main sections: questions related to the personal profile of the respondent, questions related to the rules and regulations, and questions related to rangelands. In the rangeland and

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rangeland management section of the questionnaire, items related to government policies and plans are provided. A total of 268 questionnaires were completed and gathered in the present study. To verify the authenticity, validity and reliability of the items, the questionnaire was first distributed among a number of experts for a pilot test, and after revising its defects, the final questionnaire was designed. Finally, the validity of the questionnaire was verified by the Cronbach test.

Results

In order to study the results of this research, the items of the questionnaire were divided into 4 sections based on the assumptions of the research and the responses were analyzed using the statistical methods described above. The results are presented below.

Respondents profiles

From among 268 respondents, 233 were male and 35 were female. The highest number of females belonged to the provincial experts in the administrative area. Among the administrative experts, experts from 28 provinces participated in the study. The average age of retired experts of the rangeland office was 65.9 (oldest group) and the average age of the PhD student group was 34.1 years (youngest group). 71% of respondents aged 27 to 49 years old. Among the 268 respondents, 2 had diploma degree, 32 were undergraduates, 177 were graduates and 57 had doctoral degree (PhD). In terms of academic rank, 177 (66%) were experts, 34 PhD students, 9 research instructor, 24 assistant professors, 16 associate professors, and 8 were faculty members and professors (*Table 1*).

Table 1. *Personal characteristics of respondents*

Education	(%)	Gender	
Diploma	2	Male	233
BSC	32	Female	35
MSC	177		
PHD	57		
Total	268		

Evaluation of the results of the components of the rules and regulations

Thirty one closed, multiple choice items are related to laws and regulations. The results analysis showed that among respondent groups, there is a significant difference in the order of 1% for responses given to the 31 studied variables. This is despite that there was less significant difference between the three main groups of respondents (academics and experts from the research section of FRWO), and that it is at the error level of 5% and confidence level of 95%. In terms of gender, the difference in the responses was significant for only four items at the 5% error level. The results of statistical analysis indicate that the effect of the respondents’ education on the responses was significant at the error level of 5% for the 11 items (*Table 2*).

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Table 2. *Results of evaluating the effectiveness of rangeland management rules and regulations*

Indicator	Percent of responses					Mean	Mode	St.D	CV
	St.Ag (5)	Ag. (4)	Ne.(3)	Dis.(2)	St. Dis.(1)				
The approval of the nationalization of forests and rangelands (1963) had effectiveness	0.4	7.1	26.9	48.5	17.2	2.65	2.0	0.85	32.7
Effectiveness of the Law on the Conservation and Use of Forests and Rangelands (1967) has been positive	0.4	33.6	45.9	17.9	2.2	3.27	3.0	0.73	22.32
The rules are good for managing rangeland	3.0	28.4	19.0	40.7	9.0	2.76	2.0	1.05	38.04
The rules are deterrent	1.5	22.8	45.9	23.5	6.3	2.90	3.0	0.88	30.34
The rules are well implemented	0.4	3.4	38.1	39.6	18.7	2.27	2.00	0.81	35.68
The rules are consistent with ecological conditions, customary relations, and socio-economic issues	0.0	6.3	19.4	63.8	10.5	2.47	2.00	0.68	27.53
Rental ranges to other ranchers are correct and should be regulated	0.7	9.8	23.9	36.9	28.7	2.40	2.00	0.98	40.83
The rangeland survey agenda is good and effectiveness	0.0	10.1	45.5	35.9	8.5	2.92	3.20	0.76	26.03
Grazing in forests is correct	0.0	8.9	37.7	41.8	11.6	2.72	2.00	0.79	29.04
Guild of livestock breeders can be important role in range management	15.7	56.0	17.1	9.0	2.2	3.85	4.00	0.88	22.86
The rules and regulations concerning rangeland need to be reviewed	20.1	60.5	16.9	1.8	0.7	4.12	4.00	0.65	15.78

St.Ag (Strongly Agree), Ag. (Agree), Ne. (Neutral), Dis. (Disagree), St.Dis (Strongly Disagree), St.D (Standard Deviation), CV (Cofficent of Variation), A.S.R (Average Score of Response)

The analysis of responses has shown that a large number of respondents believe the approval of nationalization of forests and rangelands in 1963 did not have a positive effect on rangeland and rangeland management. Also, more than 50% of the responses consider the implementation of the above-mentioned resolution as weak or very weak. Therefore, based on the results, the research hypothesis regarding the positive effectiveness of the nationalization of forests and rangelands is rejected. In connection with the conservation and use law of forests and rangelands (adopted in 1967), the results show that it had a more positive and better effect on rangelands than the nationalization law. Respondents believe that this is a better and more comprehensive law than the nationalization law of forests and rangelands. However, a significant proportion of experts did not provide their opinion in this regard, but at the same time, a large proportion of respondents still do not take the law of protection and use of forests and rangelands as a comprehensive one. The results of this research show that about 50% of respondents believe that approved laws do not meet the requirements of principal management and the others do not consider the rules deterrence appropriate. More than 58% of participants in the research believe that the rules are not being implemented correctly and completely. A significant proportion of respondents (more than 74%) believe that rules related to rangeland management with ecological condition, Conventional relations and social and economic issues have low consistency. Nowadays, many rural councils and some livestock breeders who do not have livestock equal to the number of livestock in Grazing Permit (GP) or Rangeland Management Plan (RMP), Ranges are rented to maintain their privilege and privilege for the purpose of income from this area. In this regard, more than 65% of the experts oppose the lease and regulation of the rangeland.

Reviewed and notified several times so far, the rangeland survey agenda is one of the most important provisions of rangeland management and is the main basis for rangeland surveys. Examination of the results of this agenda declares that a significant number of experts believe that this agenda does not conform to the common user relations and cannot solve the problems of small stakeholders. Also, about 52% of respondents believe that this agenda cannot establish a balance between livestock and rangeland. In contrast, more than 44% of experts believe that the rangeland survey agenda can pave the way for the participation of stakeholders in the determination of the rights to rangeland management. While nearly 61% of respondents take the role of rangeland survey in rangeland management as a positive role, more than 79% of the experts agree with the revision and updating of the guidelines. The results showed that many experts disagree with the issuance or extension of grazing permits in forests. In total, more than 44 percent of respondents consider the effectiveness of the above guidelines very low and more that 45 percent did not have an opinion about this issue. More than 71% of the experts participating in this research consider the role of Guild of livestock breeders, managing pastures and more useful implementation of positive rules. An important point about rules and regulation related to pastures management is that, over 80% of respondents believe that the rules and regulations need to be reviewed and updated, and only 2.5% oppose this claim.

Evaluation of the analysis results of the components related to government policies and programs for rangelands management

In this section of the research, a total of 35 items were used based on the content and objectives, which is grouped and analyzed according to the content and purpose of *Table 3*.

As the analysis of the result of study in *Table 3* shows, more that 57% of respondents believe that plant coverage and the balance of livestock and pasture have been better in the last 3-4 years, in contrast, only 9.5% have opposed this issue. Also, 66.5% of

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participants in the study said that the rate of land use change in the past was less than 2017.

Table 3. *Results of the evaluation of the effectiveness of government policies, plans and programs related to rangeland management*

Indicator	Percent of responses					Mean	Mode	St.D	CV
	St.Ag (5)	Ag. (4)	Ne.(3)	Dis.(2)	St. Dis.(1)				
Rangeland vegetation and equilibrium of livestock and rangeland in the past (30 to 40 years ago) have had better health	7.1	50.0	33.7	7.6	1.9	3.88	4.00	0.71	18.30
Land use change and Grazing pressure has been greater in the past (30-40 years ago)	1.1	7.1	25.3	44.1	22.4	2.34	2.00	0.91	38.9
The great range management plan (RMP) that were planing from 1969 to 1976 were good plans	4.9	14.9	49.6	28.4	2.2	3.08	3.00	0.84	27.27
Management of rural and non-rural rangelands requires a different pattern	24.6	63.1	9.3	3.0	0.0	4.09	4.00	0.67	16.38
Positive Effectiveness of Rangeland Management Plan on Vegetation Improvement, Ranchers Earnings, Livestock Balance and Range and Participation of ranchers	3.4	20.1	43.3	26.1	7.1	3.24	4.00	0.84	25.93
Range management Plan are a good pattern for rangeland management	0.0	5.2	44.4	40.4	10.0	2.8	3.2	0.68	24.3
For manage Rangeland under camel Grazing, needs a special pattern	7.8	48.1	38.0	5.6	0.4	3.65	4.00	0.71	19.45
Range management Plan are a good pattern for Rangeland under camel Grazing	0.0	4.1	51.1	29.5	15.3	2.44	3.00	0.80	32.8
The framework of RMPs should be appropriate to ecological conditions	41.0	50.4	7.1	1.1	0.4	4.31	4.00	0.68	15.78
The government has been successful in managing rangeland	0.0	3.0	36.2	42.5	18.3	2.50	3.50	0.82	32.8

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RMPs need to be updated	16.0	60.8	19.1	3.7	0.4	4.03	4.00	0.68	16.87
The Iranian government's policies for managing rangeland need to be updated	42.2	47.8	7.8	1.9	0.4	4.30	4.00	0.72	16.74
The quality of monitoring of RMPs is good at the county, provincial and national levels	0.0	1.1	23.1	47.4	28.4	2.28	2.00	0.68	29.8
Seed production stations are of great importance in the improvement and rehabilitation of rangelands	14.6	48.1	23.9	11.6	1.9	3.62	4.00	0.93	25.70
Stations of seed production of rangeland plants are well managed	0.0	15.3	16.0	50.0	18.7	2.28	2.00	0.94	41.23
Economic factors play an important role in the participation of livestock breeders in the management of rangelands	46.6	48.1	3.7	1.5	0.0	4.40	4.00	0.64	14.54
Range management Plan in the current situation are not a good policy for managing rangelands	22.0	47.8	14.9	13.4	1.9	3.75	4.00	1.01	26.93

St.Ag (Strongly Agree), Ag. (Agree), Ne. (Neutral), Dis. (Disagree), St.Dis (Strongly Disagree), St.D (Standard Deviation), CV (Coefficient of Variation), A.S.R (Average Score of Response)

More than 50% of the respondents did not provide any opinion about the large RMPs that were produced in Iran between 1969 and 1976, and about 30% of them considered the Plans as good ones. More than 88% of rangeland specialists believed that management of different types of rural and non-rural rangelands need different schemes, while 41% opposed to the fact that current RMP are good models for managing rangelands. Anyhow, 33% agree with this statement and 25% did not give any opinion. In contrast, more than 45% of respondents believe that current RMPs provide good models for nomadic rangeland management, to which only 29% opposed. About 82% opposed to the following statement: “the current framework of rangeland management plans is intact and comprehensive”; which indicates the disapproval of the framework for these projects by the respondents of the study. Results regarding the positive effectiveness of RMPs on improving vegetation, livestock and rangeland balance, income of rangers and their further participation show that more than 33% did not evaluate it positively, and only 23% consider positive impact for RMPs. However, more than 43% did not have any point of view regarding this issue. A large number of respondents believe that rangelands under camel grazing have special conditions and require a specific management model. One of the problems encountered in RMPs is that these are one prescription for all different conditions. Over 91% of the respondents believe that “the framework of RMPs should be adapted to ecological and managerial conditions”. Also, a large number of respondents agree with the utilization of medicinal plants and industrial plants in the rangelands, and believe that this may help the economy of rangeland management. Most respondents (over 90%) said that “*the rangeland management policies of the FRWO have to be improved and upgraded according to the changing situations*”. Also, 67% stated that “*as the rangeland management plans are not suitable for the current rangeland management*”, to which only 14% opposed. The results show that only 6% of respondents believe that there is a good quality for the supervision of natural resource departments of the counties and provincial offices on rangelands and RMPs, and this figure declines to 3.3% for the FRWO. Over 70% of the respondents chose the “weak” and “very weak” scales for the FRWO’s supervision. More than 55% of the experts stated that the legal proceeding of natural resource departments against the offenders is “appropriate to some extent” and 7% find these measures “appropriate”. More than 48% believe that the supervision by the Rangeland Issues Bureau is “weak” or “very weak”. Over 62% of the respondents think that the stations producing the grassland seeds are of high importance, but a large number of the respondents (more than 68%) believe that these stations have not been properly managed in recent years. More than 78% of respondents believe that FRWO had “little” or “very little” success in managing rangelands, and 17.5% said that the FRWO has not been successful at all. So the total proportion of specialists who considered the FRWO is unsuccessful in rangeland management was 96%. In this regard, more than 64% of respondents stated that the policies of FRWO were not effective in managing rangelands. The results of Kruskal Wallis test on rangeland management and policymaking data (*Table 3*) show that at an error level of 5%, different groups of respondents (from experts FRWO, Natural resources research specialist and university faculties) made significant differences in 15 items, gender and educational level in 4 items, scientific rank in 8 items, Education in 6 items show that at an error level of 5%, different.

Discussion

Basically, based on their core strategies, governments define executive policies in each sector and, proceed with the implementation of relevant laws in order to regulate these policies and their enforcement. The management of natural resources and lands in each country is influenced by the large-scale policies and the general approach of governments. In Iran, in the late 1950s and early 1960s, important improvements were made in the agricultural and land management fields, nationalization of forests and rangelands. For the management of renewable natural resources, especially rangelands, as major rulings, the Nationalization of Forests and Rangelands (1963) and the Protection and Use Act (1967) are now five decades old. National rangelands were established and the rangeland management structure and related organizations were formed according to these two laws. The results of this research have shown that, for various reasons, a large number of the respondents believe that “the nationalization of forests and rangelands” in 1963 had no positive effects on the rangeland management system due, primarily, to the fact that its implementation mechanisms were not provided or properly implemented. But, In contrast, a large number of audience were in the belief that “the Law on the Conservation and Use of Forests and Rangelands” in relation to

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rangeland management was better and more complete than “the nationalization of forests and rangelands”. The results showed that, as most respondents indicated, Existing laws and regulations are not concordant with ecological facts, customs, social relations, and social and economic issues. Most of the surveyed statistical population believed that the rules and regulations are weak in implementation. Also, 90% of respondents have emphasized the need to review the relevant laws and regulations. These results are consistent with those of Shamekhi (1992), Salmasi (1994), Azkia (1997), Moeenedin (1994, 1997, 1998), Abdolahpour (2001), Farvar (2005), Eskandari et al. (2009) and Abolhassani et al. (2013). In addition the results indicated that the rangeland survey agenda is not acceptable for the establishment of the balance between the livestock and rangeland and needs to be seriously revised. Moeenedin (1998), Abdolahpour (2001), and Abolhassani et al. (2013) obtained similar results. The above mentioned agenda plays an important role in the determination of the rights of livestock breeders and the establishment of a balance between the livestock and the rangeland; so, it is necessary to be upgraded according to the changes and current conditions. Related to the laws of rangeland management in Turkey, Koc et al. (2014) states that in 1998, the Turkish Parliament approved a rangeland law that empowered the central government to adjust the season of grazing, grazing capacity, to improve the rangelands, and to take other measures to control the use of rangelands. In the present day Turkey, the rangelands of the Republic of Turkey are dedicated to the villages and each village has a right for grazing. Owners of domestic animals in many of these villages still apply the Chergasht (immigration for grazing) method, and in some cases some groups act similar to their predecessors. For the United States, Larson-Praplan (2014) states that one of the first laws approved in relation to rangeland management was the Homestead Act, signed and endorsed by President Abraham Lincoln in 1862. Later, in 1880s, several rules for the management of California rangelands were created. The laws approved in the 1970s have had a great impact on rangeland management. Also, in 1978, the rangeland improvement law was approved and the Clean Water Act was also a part of other related laws that were adopted in this decade. As stated by Holechek (1981), one of the most important events associated with rangeland management was the adoption of the Taylor Grazing Act in 1934, according to which the public land management was assigned to the state-owned grazing service. In response to the pressure of environmental groups, the National Environmental Policy Act was approved by the Congress in 1969. Feller (1998) states that since 1993, significant progress has been made in the field of laws, regulations, standards and guidelines for livestock grazing in public lands of the western US states. Following a two-year attempt, amendments were made to the legislation on grazing and the issuance of permits in 1995. In China, as Peter Ho (2000) indicated, the rangeland law was passed in 1985, through which the rangeland management policy was recognized officially and determined the rights and responsibilities of the government and livestock breeders. According to this law, livestock breeders who were permitted to use grasslands were required to guarantee long-term utilization of the rangelands. In the case of Mongolia, Hannam (2014) states that, according to the law approved in 1992, all lands are state-owned. Mongolia’s environmental law system was supposed to achieve goals of environmental policies based on environmental protection laws in 1995. Mongolia’s environmental law system does not have the ability to effectively manage the major environmental problems in the country. It should be noted, however, that since 2011, the Mongolian government has begun a comprehensive environmental law review. The

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study of the status of rules related to rangelands management in several countries indicates the evolutionary trend in the implementation and enforcement of the above laws in these countries. In recent decades, many countries have been trying to update natural environment laws in line with changes in ecosystems, exploitation communities, climate change, and international achievements derived from important environmental conventions.

Historically, local breeders used grassland cover on the forest floor in the northern forest (Hirkani) and Zagros forests as pasture. Many experts believed that the presence of livestock in forests is one of the most important factors in destroying and preventing natural regeneration of forests, so they emphasized that permit should not be issued for grazing in forests. Therefore, as rangeland management experts, many respondents in this study oppose the issuance and extension of the permit. In addition, due to poor government supervision and the migration of a large part of the villagers to cities, Islamic councils that are responsible for managing rural rangelands in most villages, rangelands that do not have a local livestock breeders are given to other, which is illegal the rangeland survey agenda. But, many experts believe that, given the high frequency of the phenomenon in many parts of the points of the country, it is necessary for the government to organize it. A significant proportion of research respondents opposed leasing of rangelands, while a large number advocated organization of the phenomenon. Currently, permits for medicinal plants, ecotourism, beekeeping, etc. are issued for a variety of individuals who are not generally administrating the rangeland plans. The results have shown that most experts believe that these permits should be issued in the form of a rangeland management plan and only for the administrator. Another important point is that rangeland plans have a single model in all circumstances, while many respondents stated that there should be different plans for in rural and non-rural, ecologically different and camel rangelands. Many experts think that there are problems in rangeland management framework which should be solved. Iran is a country with a diversity of ecological conditions that require different patterns of management. However, the framework for rangelands is the same for all ecological conditions. The results have shown that more than 90% of the respondents of the questionnaire believe that *"the framework of rangeland projects should be designed according to ecological and managerial conditions."* This is a very important issue to consider in policy making and designing management plans. A total of 90% of the rangeland experts believe that *"FRWO policies on rangeland management must be adapted to the conditions."* Also, the results indicate that many experts believe "*Rangeland management plans as rangeland management policy in the present situation, do not address rangeland management"* and a significant number of the audience said in this study that the FRWO was not successful in rangeland management. These are consistent with the results of Alizadeh and Mahdavi (2007), Mirdeylami and Moradi (2017). Regarding RMPs, Amiri Maleki et al. (2009) stated that in general, the implementation of RMPs in the summer rangelands has been very successful, but it has not been successful – and has been deteriorating sometimes – in winter rangelands due to extended implementation time, early grazing, numerous usages, and being positioned in rural areas. In addition many studies have shown that implementation of RMPs is generally better than lack of any plans for the rangelands. In this regard, Dehdari et al. (2014) state that in rangelands with a plan due to the safety of the land owners regarding the ownership of the rangeland and more control of the governmental systems, are less than conflict between the land owners of one rangeland or nearby ranges. Also, the

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implementation of rangeland management plans in rangeland allotments has improved the conditions of the rangelands. Based on the obtained results, Eftekhari et al. (2016) state that there is a significant difference at the error level of 1% between the rangelands for which plans have been implemented and others; the major reason being observance of management principles such as the time of arrival and departure of livestock, the optimal distribution of livestock in the rangeland and the relative realization of livestock and rangeland balance in these rangelands.

Depending on the ecological conditions of rangeland ecosystems and the social characteristics of the stakeholders, different countries apply different policies to reduce the degradation of rangelands, each having different advantages and disadvantages. Hannam (2014) states that due to the ecological hazards that have been caused by the unplanned exploitations, a comprehensive plan has been implemented by the Mongolian government since 2011 to update environmental laws and regulations. Due to the degradation of the Turkish rangelands, being aggravated from 1950s and continued until the 1980s, Koc et al. (2014) believe that excessive grazing has caused a major concern for degradation of rangelands and soil erosion. Therefore, in the year 1980, the Turkish government identified about 14 million ha of severely degraded lands and began to amend and rehabilitate these lands by subsequent laws (such as the 1998 rangeland law). YanBo Li et al. (2014) state that in China three major rangeland management policies have brought about major changes in livestock numbers and rangeland ecosystems: Rangeland Household Contract System (RHCS), Ecological construction projects (ECPs) and Herder Settlement Policy (HSP). The implementation of these policies provided major modifications to rangeland management systems. In the mid-1980s, RHCS began in Chinese major herding areas, and is currently used in the six main provinces of northwestern China. By 2011, this policy covered about 79% of China's exploitable rangelands. Gongbuzeren et al. (2015) state that, over the past 30 years, the Chinese government linked three main policies of the Rangeland Household Contract Policy (RHCP), the Nomad Settlement Policy (NSP), and the RAPs (Rangeland Ecological Construction Projects) to manage the rangelands. These policies have caused great changes in rangeland communities as well as rangeland ecosystems. Many studies have shown that RHCP policy has had a significant negative impact on rangeland management and ecosystems. This is despite that the nomadic system for the exploitation of rangelands evolved over time and has been consistent with the unsustainable environmental conditions of the rangelands. It is also true that NSP policy has had a few positive impacts on livelihood, but most studies have shown major negative impacts of this policy on rangeland ecosystems. Also, studies have shown that RECPS policies have had positive effects on the restoration and regeneration of rangeland ecosystems, but have made negative effects on livelihood of rangeland managers. In general, it can be argued that making changes in any of the components of rangeland ecosystems or the stakeholder communities could lead to changes in other components. Therefore, we have to look for a SES (social-ecological systems) system based on which we can balance social and ecological factors. Government policies in the mentioned countries have had a different impact on rangeland ecosystems and stakeholder communities, but the important point is that ongoing studies are examining the effects of these policies in a continuous manner, that can be very effective in improving and reforming policies and management practices.

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Conclusion

The study of the above-mentioned sources indicates that rangeland management legislation in Turkey, Mongolia and China has not long been a long history, but a few decades ago, but it has evolved and governments are trying to update it. In the United States, the rules for managing rangelands have a longer history and evolved in an evolutionary process with changes in rangeland ecosystems and utilizable communities. The results of this research have shown that there is no specific rule for rangeland management in Iran, and the legal rules of rangeland have been seen in the general framework of the laws of natural resources management. In the past 50 years, despite the many changes in rangeland ecosystems and utilitarian communities, these rules have not been revised and updated. On the other hand, despite the numerous and parallel rules and the poor implementation of the relevant laws, the effectiveness of the rules related to management and conservation of rangelands has greatly decreased. Therefore, due to the wide variation in the level and quality of the rangeland and the changes that have been made in the exploiting communities and the climate change that has been increasing in the Middle East region, it is necessary to complete and update the laws related to the management of rangeland ecosystems. Also, given the international obligations created, these laws must also be in line with these obligations. Another important point in relation to the rules of rangeland and the effectiveness of existing laws is the low and inadequate studies and studies in this area that show the need for comprehensive and in-depth reviews on this issue.

In conjunction with government policies and programs, the results of this study have shown that policies will come at a time when they are consistent with the ecological, social, and economic realities of the day, and that they can be fully implemented. However, in some cases, due to the lack of a clear strategy in the management of natural resources and the change of government, the government's priorities in the management of natural resources and land changed frequently and the continued implementation of the principled policies with has encountered a problem. The research confirms that rangeland specialists believe that policies, management patterns and plans should be tailored to ecological conditions and social characteristics of operators, which is absolutely necessary in a country with a high ecological and social diversity in Iran. Therefore, based on the accurate and updated data of the strategic plan for management of rangelands, we must prepare and implement the ecological diversity and social features of different areas. On the other hand, the government should regulate and define its macro policies in line with the sustainability of natural ecosystems. To achieve this, it is necessary to carry out extensive and in-depth research into the impacts of policies on rangeland management and other natural ecosystems, as well as comprehensive studies on the updating of ecological, social and economic data. In addition, with the presence and cooperation of international institutions, the experiences of other countries that have succeeded in managing rangelands and legislation for natural ecosystems.

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RURAL INFRASTRUCTURE AND PROFITABILITY OF FOOD CROP PRODUCTION IN OYO STATE, NIGERIA

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Abstract. The role of infrastructure in promoting development in Nigeria cannot be overemphasized, given its importance in economic wellbeing of the populace and growth of the economy. This study examined the influence of infrastructure on the profitability of food crop production among rural farming households in Oyo State, Nigeria. Data were collected on six infrastructural facilities (tarred roads, potable water, market, health centre, storage facilities and school). Multi-stage sampling technique was used to select 120 farmers from two Agricultural zones of Oyo state. Data were analyzed using Descriptive statistic, Budgeting Analysis (BA) and Ordinary Least Square regression (OLS). OLS was employed to determine factors affecting profitability of food crop production in the study area and the model was well-fitted. Findings revealed that the majority (70.0%) of the respondents were male with 74.0% of them married and had a mean farm size of 12 ha. Based on the current state of rural infrastructure, 47.5% of the respondents reported that available tarred roads are functioning as against 23.0% of them whom reported that health facilities were not functioning. The study concluded that rural infrastructure is essential to the output of agriculture production in the study area. Rehabilitation of rural roads and the general upgrading of the rural infrastructures which will boost agricultural production across the state is recommended.
Keywords: *budgeting analysis, descriptive statistics, ordinary least square regression, rural infrastructure, Nigeria*

Introduction

In Nigeria, agriculture involves forestry, livestock, fishing as well as farming of food and cash crops such as yams, cassava, maize, cocoa, groundnut and oil palm. The country is largely endowed with natural resources (e.g. abundant land supply, human and forestry) that are necessary for the development of agriculture. The country has a total land area of about 98.3 million ha out of which 71.2 million ha (72.4%) are cultivable but only 34.2 million ha (34.8%) are currently in use (Daramola, 2014). In the country, agriculture faces a number of challenges and the majority of the farmers still depend on subsistence agriculture for their livelihood. More than 64% of people in the rural areas are not able to meet their basic food needs, and well over 50% of women

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still engage in subsistence agriculture for survival (Ale, 2004). Other challenges include under-developed land property rights, infrastructural inadequacies, limited irrigation and inadequate storage facilities (Ashok and Balasubramanian, 2006).

Rural infrastructural is basic physical and organisation structures needed for the operation of a society or enterprises, or the services and facilities necessary for an economy, infrastructure is a set of investments that include rural roads, water supply, rural housing, rural electrification, sanitation, energy and telecommunication and agricultural processing (Anija-Obi, 2001). These facilities enhance the standard of living of rural famers (Onwuemenyi, 2008). The improvement of rural infrastructure is highly related to agricultural production in various ways. For instance, it is is one of the several subject of activities that are essential for rural transformation. Thus, the existence of poor quality or inadequate infrastructure will inevitably have a negative impact on agriculture (Patel, 2014).

Poor access to infrastructural facilities such as healthcare centre, educational institutions, communication gadgets and water supply lead to low agricultural production. This status and development of rural people enhance the quality of rural labour (Pinstrup-Andersen and Shimokawa, 2006). Agricultural development is essential for economic growth, rural development and poverty alleviation in low income and developing countries. Productivity increase in agriculture is an effective driver of economic growth and poverty reduction both within and outside agricultural sectors (Onwuemenyi, 2008). Such productivity increase depends on rural infrastructures, well-functional domestic markets, appropriate institutions and access to appropriate technology. While the state of rural infrastructures varies widely among developing countries, lower income countries including Nigeria suffer severe rural infrastructure deficiencies (Ekong, 2003).

Presently, Nigerian food crop production sub-sector is dominated by weak and inefficient producer-market linkages due to poor infrastructure including lack of improved processing facilities, low farm productivity, poor post-harvest handling and storage, expensive and poor access to inputs (high quality seeds, fertilizing and crop protection products), inadequate market information, lack of transparency among players, low capacity to meet quality standards, and limited efficiency distribution networks. This has declined rice productivity and low income for the rice farmers in Nigeria, especially in Oyo State.

Problem statement

Agriculture infrastructure although involves huge initial capital investments, long gestation periods, high incremental capital output ratio, high risks and low rates of returns on investments and increasing crop yields, thereby promoting agricultural growth (Patel, 2014). Government initiatives to improve the quality and quantity of infrastructure in the rural areas through programmes such as the construction of small dams and boreholes for rural water supply and the clearing of feeder roads for the evacuation of agricultural produce and the supply of electricity to rural areas from large irrigation Dams, the establishment of nine River Basin Development Authorities (RBDAs) in addition to the two existing ones (Sokoto and Rima RBDAs); DFRRI, the Poverty Relief and Infrastructure Investment Fund and the Comprehensive Agricultural Support Programme, have registered limited impact on the lives of many rural people (World Bank, 2006). The effort by government to increase productivity of farmers has been fulfill thus has adversely affected the level of agricultural production and socio-

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economic life of rural farmers because the improvement of these infrastructure are not available in the area of study. Rural infrastructure such as roads, irrigation, transportation, primary markets and weather forecasting services can reduces production cost, transportation cost, storage expenses, dealing cost and operation risk and enhance efficiently. Deficient infrastructures have negative implication on the present state of agriculture.

Agricultural production is still highly dominated by the small holder farming system. The farms are dominated by small scale farmers who are responsible for about 95% of total production (Fasoranti, 2004). This is not unconnected with the unattractiveness of agriculture which is a result of lack of necessary infrastructures in the rural areas which forms the bulk of agricultural zones in the country. In addition, small scale agriculture has in the time past suffered from limited access to credit facilities, modern technology farm inputs and inefficient use of resources. Nevertheless, it is on record that 50% of world's population is dependent on subsistence agriculture. In the words of Akande (2003), food cropping system and the postharvest services in Nigeria encompass a wide range of agricultural activities ranging from land clearing, seed bed preparation, broad casting, fertilizer application, weeding and bird scaring. Others include harvesting, threshing, parboiling, drying, winnowing, bagging and marketing and distribution. These activities are largely executed manually, women and children, the very vulnerable segments of the society are largely involved. Food crop production expansion in Nigeria is therefore bound to reduce drastically the foreign exchange spending on food importation and more importantly it could lead to the transfer to money into hands of the very vulnerable group of the Nigeria economy.

As indicated by Fakayode et al. (2009), the provision of efficient infrastructures is now widely recognize as indispensable to agricultural progress as it is known fact that infrastructure can support economic growth, reduce poverty and make development environmentally sustainable. In any modern society, infrastructure plays a pivotal and often a decisive role in determining the overall productivity and development of a countries economy as well as the quality of life of the citizens. The role of infrastructure such as electricity, transportation networks, safe water, and good health centre in promoting development cannot be overemphasized. The improvement increases the efficiency of production and contributes to standard living (PCU-NFDO, 2005). Rural infrastructure and development have enormous implication on production outcome in agricultural sector and over all significant development of the country. The effort by government to increase productivity of farmers has not been effective, and this has adversely affected the level of agricultural production and socio-economic life of rural farmers because the improvement of these infrastructures is not catered for in the area of study (Fakayode et al., 2009).

Felloni et al. (2001) reported that rural infrastructure such as roads, irrigation, transportation, and primary market and weather forecasting services can decrease production and transportation cost, storage expenses; dealing cost and operation risk and enhance efficiency. Deficient infrastructures have great negative implications on present state of agriculture. The United Nations (2009), estimated that 48.3%t of Nigeria's population live in the urban areas while the majority of the people (51.7%) live in the rural areas and they are largely engaged in agricultural production. Agriculture as the traditional mainstay of Nigeria's economy is a prominent industry in the rural areas, and its development is expected to have positive spill-over effects on food security, rural infrastructural development, and overall rural development. Thus,

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the immense values of the rural communities in overall national development have prompted both past and present administrations to formulate series of policies and programmes aimed at transforming rural-Nigeria. In order to ameliorate this solution, it is important to know the available infrastructures in the area so that more recommendations can be made to enhance farming operations. It is against this background that the study will provide the answers to the following questions:

1. What are the existing rural infrastructures in the study area?
2. What is the current state of existing rural infrastructures?
3. How do these rural infrastructures affect agricultural productivity in the study area?

Aim of the study

This study focused on the effect of rural infrastructure on profitability of food crop production in rural farming households in Oyo state, Nigeria.

The objectives are to

1. Describe the socio-economic characteristics of the respondents
2. Examine current state of the existing rural infrastructure
3. Determine the effect of these infrastructures on farm productivity
4. Estimate the profitability of crop farmers in the study area.

Materials and methods

Methodology

The study was conducted in Oyo State which has its capital in Ibadan (*Figs. 1* and *2*). The study area covers approximately an area of 28,454 km^2 with an estimated population of 5,591,589 people (NPC, 2006). It is an inland state in South-Western Nigeria. It bounded in the north by Kwara state, in the east by Osun State and the south by Ogun State. The income generating activities in the area includes: trading, farming, hunting, blacksmithing, weaving, tailoring and carpentry. The major crops cultivated in the area include yam, maize, cassava, okra, melon, groundnut and cash crops such as mango, cashew and citrus.

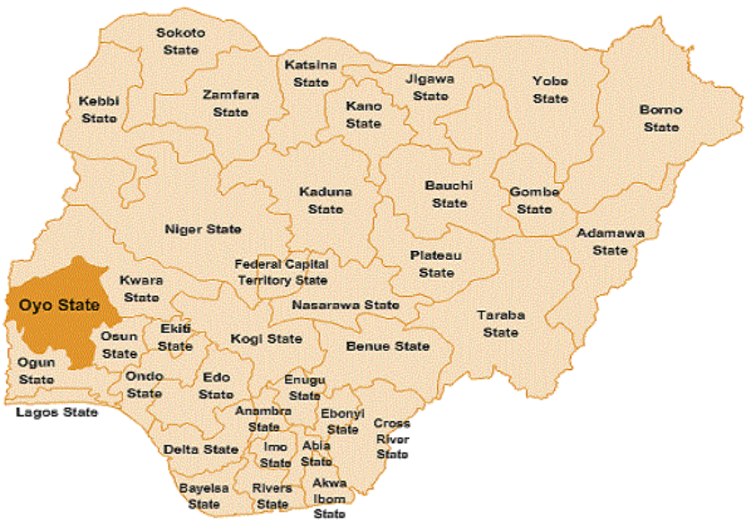

Figure 1. *Map of Nigeria with Oyo State highlighted in orange. (Source: Ndianaefo, 2016)*

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Figure 2. *Map of Oyo State indicating the local government areas and Agricultural Development Programme (ADP) Zones. (Source: Nigerian Muse, 2010)*

Model specification

Ordinary least square regression analysis

$$\text{Ln } y = \beta_1 \text{In } X_1 + \beta_2 \text{In } X_2 + \ldots\ldots.. \beta_n \text{In} X_n + e \qquad \text{(Eq.1)}$$

$$Y = f\,(X_1, X_2, X_3, X_4, X_5 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots X_{14}, U) \qquad \text{(Eq.2)}$$

Y = Gross margin (₦), X_1 = Farm size (hectare), X_2 = cost of hired labour, X_4 = Educational Status, X_5 = Farming Experience (Years), X_6 = Age of household head (Years), X_7 = distance to Potable water (if present, yes = 1, no = 0), X_8 = distance to Tarred Road (if present, yes = 1, no = 0), X_9 = Schools (if present, yes = 1, no = 0), X_{10} = Market (if present, yes = 1, no = 0), X_{11} = Health Centre (if present, yes = 1, no = 0), X_{12} = Storage Facilities (if present, yes = 1, no = 0), e = Error term.

Results and discussion

Demographic characteristics of food crop farmers

The socio-economic distribution of food crop farmers varied for all the parameters evaluated (*Table 1*). For instance, 38.0% of the respondents fall between age group of 20–40 with the mean age of 44 years while about 19.0% of the respondents are above 61 year old. This finding is similar to that of Fasoranti (2004) who reported on cost and return of crop production in Akure, Ondo State, Nigeria. One could infer from this result that crop farmers in the study area are young, active and still in the productive age. Also, the result of socio-economic distributions of the respondents showed that

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majorities (70.0%) of the respondents were male while 30.0% were female. This implies that male are actively involved in farming. This could be attributed to the fact that Nigerian cultures do not permit women free access to and control over land (Rahji, 2007).

Table 1. *Socio-economic characteristics of the respondents. (Source: Field Survey, 2014)*

Characteristics	Frequency	Percentage	Mean
Age			
20–40	45	37.3	
41–50	26	21.8	44
51–60	27	22.5	
61 and above	22	18.4	
Gender			
Male	80	70.0	
Female	40	30.0	
Marital status			
Single	0	0	
Married	89	74.2	
Widowed	19	15.8	
Divorced	12	10	
Educational level			
No formal	54	45.0	
Primary education	35	29.2	
Secondary education	26	21.7	
Tertiary education	5	4.1	
Years of farming			
Less than 10	40	33.6	
10–20	29	24.1	
21–30	21	17.4	22
31–40	10	8.3	
41–50	13	10.8	
Above 50	7	5.8	
Farm size			
Less than 10	93	77.6	
11–20	26	21.6	12
Above 20	1	0.8	
Religion status			
Christainity	67	55.4	
Islam	53	44.6	
Traditional	0	0	
Household size			
1–5	54	43.3	
6–10	50	41.7	6.5
Above 10	18	15.0	
Primary occupation			
Artisan	21	18.2	
Civil servant	2	1.6	
Farming	87	72.0	
Trading	10	8.2	
Total	**120**	**100.0**	

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In terms of formal educational level of food crop farmers, 45.0% of the respondents has no formal education, those with tertiary education were about 4.0% and about 22.0% of the respondents had secondary education. The percentage of uneducated rural farmers is an indication of the level of infrastructure (school) in the study area. This result was in line with Mundluk et al. (2002) who reported that a large proportion of rural households continue to lack access to basic services. Furthermore, the duration of years spent farming indicated that 33.0% have spent between 1–10 years in farming, 24.1% of the rural farmers have spent 11–20 years in farming. Anijah-Obi (2001) corroborated this findings that longer year of farming experience helps the farmers to make rational choices and decision.

Perceived state of rural infrastructure in the study area

The perceived state of the selected rural infrastructure in the study area was evaluated (*Table 2*). Relative to tarred road, 47.5% of food crop farmers indicated that the road present are still functioning while about 4.0% of them reported that the available road are not functioning due bad state of disrepair. Among the respondents, 8.0% of them of had access to functioning potable water as against 5.0% that reported that the available market are been abandoned. Furthermore, 22.0% of the respondents have access to functioning healthcare service in the study area as against about 13.4% that had no access to storage facilities. According to FAO (2005 rural infrastructure plays a crucial role in poverty reduction, economic growth and empowerment for the Africa poor. Farmers efforts to escape poverty and overcome subsistence agricultural levels are been limited by the present of infrastructure in the study area, poor access to market, poor storage facilities and extension contact.

Table 2. *Perceived state of rural infrastructure in the study area. (Source: Field Survey, 2014. Freq = frequency)*

Infrastructure	Functional		Not functional		Partially functional		Abandoned	
	Freq	%	Freq	%	Freq	%	Freq	%
Tarred road	57	47.50	5	4.20	38	31.70	20	16.60
Potable water	74	61.60	11	9.20	25	20.80	10	8.40
Market	79	65.80	22	18.40	13	10.80	6	5.00
School	17	21.20	18	15.00	69	57.50	16	13.30
Health	25	20.80	15	12.50	72	60.00	8	6.70
Storage	21	17.50	58	48.30	25	20.80	10	13.40

Budgeting analysis of food crop farmers in Oyo State

Gross margin was used to estimate the cost and return on food crop production in the study area as presented thus: GM = TR – TVC

$$\text{Benefit cost ratio (BCR)} = \frac{TR}{TC}$$

$$TC = TFC + TVC$$

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where TC = total cost, TR = total revenue, TC = total cost, TR = total revenue, TFC = total fixed cost, TVC = total variable cost, GM = gross margin,
TR = ₦233909.5,
TVC = ₦122053.9,
TFC = ₦9200.0,
TC = ₦131253.9,
GM = ₦233909.5 – ₦131253.9 = ₦102655.6

$$BCR = \frac{233909.5}{131253.9} = 1.8$$

Given that the BCR is greater than 1, it shows that the crop production is profitable in the study area. This implies that for every ₦1.00 invested in agriculture in the study area, an expected return of ₦1.80 is certain with all things been equal.
This conformed with the findings of (Omotayo and Oladejo, 2016; Ekpe and Alimba , 2013) on food crop production in Ebonyi State which has positive gross margin as shown by the study because Total Revenue (TR) is far more than Total Variable cost (TVC). The profitability of crop enterprise and farmers income is expected to increase significantly if more land is put under food crop production.

Regression analysis

Result of the determinant of profitability of food crop production in the study area was presented in *Table 3*. The marginal effects of the independent variables were estimated because they are very important for policy and decision making. Parameter estimate of OLS revealed that farm size, potable water, storage facilities and quantity of agrochemicals used are significantly related to the farmers' profitability. Furthermore, total household size, educational level of the respondent, health, market and school are statistically not significant to farmers' production. This may be due to the availability of extension service, their scale of production is low and majority of them used local storage facilities. This is in line with the result of the work done by Okorie et al. (2011), who noted that farmers with increased household size obtained higher yield due to family labour supply. This reduces the cost of production since family labour is not paid for. The educational qualification of rice farmers bore positive signed coefficient and was not statistically significant.

The positive relationship indicates that the higher the level of education of the farmers, the higher their yield in rice production. This is true because educated farmers are intelligent and calculative in utilization of available resources and are able to adopt innovation on rice products. Unlike uneducated farmers who has low adoption level. Furthermore, it also implies that better education and more farming experience in terms of longer years may improve awareness of potential benefits and willingness to participate in local natural resource management and conservation activities. This result was in conformity with Ekong (2003), which explained that the spread of needed infrastructure and introduction of appropriate technology in rural areas would markedly improve rural economy and their output. Adjusted R^2 value of 0.507 implies that 50.7% variability in farmer's profitability is explained by the independent variables while the remaining 49.3% of the variability is accounted for by the error term and the excluded variables.

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Table 3. *Parameter estimate of ordinary least square (OLS) regression*

Variable	Coeficient	t-ratio	Significant
Constant	2.267	1.897	0.061
Household size	0.008	0.211	0.221
Educational status	0.255	1.569	0.119
Age of household head	0.008	0.516	0.607
Farming experience	-0.009	-0.723	0.471
Farm size	0.082	1.834**	0.049
State of road	-0.106	-0.472	0.638
State of potable water	-0.331	1.666*	0.099
State of market facilities	-0.176	-0.706	0.482
State of health facilities	-0.115	-0.403	0.687
State of storage	0.202	1.673*	0.097
State of school	-0.010	-0.102	0.919
Quantity of agrochemicals	6.230	3.092***	0.003

* significant at 10%;** significant at 5%;*** significant at 1%

Conclusion and recommendation

The analysis of the socio-economic characteristics of the respondents showed that majority of the farmers are old and quite experienced in food crop production. Based on the findings from this study, it is evident that male are more involved in farming enterprise than female in the study area. Majority of the respondents are married, under basic education and mean household size of 12 persons. The study further revealed that food crop production is profitable in the study area with BCR of 1.8 which implies that for every ₦1.00 invested in agriculture in the study area, an expected return of ₦1.80 is certain with all things been equal. The result of the regression analysis showed that farm size, state of potable water, storage facilities and quantity of agrochemical used were statistically significant and had influence on farmers' profitability in the study area. Therefore, government and Non-government organization (NGOs) should collaborate with farmers with a view in providing the needed infrastructure in order of their priorities so as to increase their production in the study area.

1. Government should provide the rural farmers with necessary agricultural inputs at a subsidized level as this will equally enhances their productivity.
2. On basic healthcare facilities, both Government and Non-governmental Organizations (NGOs) should help in developing the rural health sector so as to increase labour availability and at the long run improve the productivity.
3. This research calls for general rehabilitation of the infrastructural facilities in the study area with a view of encouraging more people to agriculture and improving their productivity.
4. Government should work with existing social organization and involve them in distribution of necessary inputs for rice production.
5. Timely provision of necessary farm inputs to enhance rice production.

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WATER DISINFECTION WITH ADVANCED METHODS: SUCCESSIVE AND HYBRID APPLICATION OF ANTIBACTERIAL COLUMN WITH SILVER, ULTRASOUND AND UV RADIATION

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Abstract. The disinfection process is of great importance to the water supply to prevent diseases spread by water. Due to its negative impacts on human health, the number of studies conducted on disinfection systems alternative to chlorination and similar traditional methods has been increased. Successive and hybrid systems that are highly efficient in inactivating the pathogenic microorganisms that may be present in water are being developed. In this study, the disinfection efficiency of the antibacterial filled column, ultrasonic reactor and UV reactor that we developed was increased by the successive and hybrid usage of the methods when *Escherichia coli* bacteria were used to detect the disinfection efficiency of the systems. In single disinfection studies, bacterial inactivation efficiency occurred in UV process was higher than antibacterial column and also, efficiency occurred in antibacterial column was higher than ultrasonic reactor. Triple hybrid usage of these systems resulted in an 8 log microbial inactivation reducing single treatment disadvantages of these systems. Furthermore, the flow speed and temperature effect were examined during the studies, and it was observed that the temperature had a positive effect on the hybrid system.
Keywords: *bacterial inactivation, ultrasound, silver, ultraviolet radiation*

Introduction

Obtaining safe drinking and usage water has been increasingly gaining importance today. The fecal microorganisms that contaminate water generally originate from infected or disease carrying animal and human wastes. When water is contaminated with fecal matter, the disinfection procedure required to remove the microbial load is very important. Chlorination, ozone, ultraviolet radiation (UV), filtration and other methods are widely used for disinfection at the water distribution source. Choosing the right disinfection method is important for achieving the most effective microbial removal in water. The main factors affecting the choice of disinfection method are the efficiency, cost, applicability, pilot study requirement and side effects. The disinfection efficiency is determined by the ability of the method to inactivate the selected microorganisms to the target level. The disinfection method must be practical; and its handling, storage and production/application in the field as well as its monitoring and control must be simple. It must also achieve disinfection targets. The disinfectant should not create a toxic effect on aquatic life, it should not be transported to or bioaccumulate in nutrients, and it should not be transformed into or form carcinogenic, mutagenic or toxic substances (Liviac et al., 2010). Therefore, the side effects of the system used must be researched. When all of these elements come

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together, a compelling argument can be made for using methods such as disinfection with ultrasound and metal ions as an alternative to conventional techniques when these new systems are used successively as hybrids.

Metal ions such as Hg, Au, Ag, Pb and Cu affect living organisms as disinfecting substances (Blanc et al., 2005). Metal ions are effective for the inactivation of bacteria (Fauss et al., 2014), fungi (Das et al., 2013) and viruses (De Gusseme et al., 2011), even at low concentrations. Metal ions inhibit the growth of some algae and fungi. A review of the literature (Chang et al., 2008; De Gusseme et al., 2011; Das et al., 2013; Fauss et al., 2014) shows that the Ag ion has often been used in antibacterial material production (Srinivasan et al., 2013) and water disinfection studies because it is nontoxic and effective for human health, even at low concentrations compared to other metal ions.

It is possible to kill the cells of microbial populations through ultrasound. The bactericidal effect of ultrasound is related to cavitation (Declerck et al., 2010). The formation of bubbles during cavitation usually kills the microbial cells (Naddeo et al., 2014). Small, high energy gas filled bubbles are formed during cavitation. The ultrasound process causes the microscopic bubbles to explode and a mechanical shock occurs (Rutala et al., 2007). High temperature (5500^0 C) and pressure (50 Mpa) points are formed when the molecules around the bubbles collide with each other during explosions (Leighton, 2007). Fragmentation occurs after the destruction of structural and functional units in the cell (Skauen, 1976). The cavitation bubbles blow up when the ultrasound is applied, and OH^- and H + reactive radicals are produced in the liquid media (Birkin et al., 2001). H_2O_2 (hydrogen peroxide) is formed when these radicals react with oxygen. The intense bubble formation prevents temperature and pressure alterations and causes DNA lysis, cell membrane disintegration and cell wall damage when the free radicals are formed (Vasilyak, 2010).

Since the 1900s, radiation emitted from an ultraviolet light source has been used for the disinfection of water sources (Choi and Choi, 2010). UV radiation, initially used for high quality water supplies, has gained importance in recent years in the treatment of waste water (Copperwhite et al., 2012; Nelson et al., 2013). It was determined that ultraviolet radiation at sufficient dosages may be used as a bactericide and virucide without forming any toxic compounds (Xu et al., 2009; Oguma et al., 2013). Radiation at a wavelength of 254 nm prevents cell reproduction when it passes through the cell membrane of the microorganism, leading to its death (Rincon and Pulgarin, 2004). The disinfection efficiency of UV radiation depends on the turbidity of the water (Bergmann et al., 2002). Therefore, a pre-distillation process such as filtration is needed when the organic content of the water is high and when it is turbid (Winward et al., 2008; Abd-Elmaksoud et al., 2013).

In the disinfection studies that were conducted using ultraviolet radiation, ultrasound and metal ions, it was possible to increase the bacterial inactivation efficiency and remove its disadvantages when successive and hybrid methods were used (Summerfelt et al., 2009; Wu et al., 2011; Zhu et al., 2014). Amirsardari, Yu and Williams found that trihalomethanes were decreased by 90% and halides were decreased by 98% when ozonation was used as a pre-treatment method along with UV radiation (Amirsardari et al., 2001). Butkus et al. (2005) determined that one more log removal was obtained compared to single UV radiation application in the inactivation of MS-2 coliphage viruses at 10^9 PFU/mL concentration when UV radiation was used. Naddeo et al. (2009) determined that the disinfection efficiency of the US-UV hybrid system was approximately 96% while the disinfection efficiency of the UV system alone was approximately 80% in the total coliform and E. coli disinfection studies that were conducted using pilot-scaled ultrasonic and UV reactors.

This study aims to develop alternative water disinfection systems with the goal of recovering disadvantages present in conventional water disinfection methods and increasing the efficiency of the disinfection. Except for conventional disinfection methods, the bacterial inactivation efficiencies of UV radiation, ultrasound and Ag^+ ion adulterated antibacterial column methods were examined with the goal of achieving optimal water hygiene standards. The disinfection efficiencies of these methods in dual ((Ag+ US, Ag+UV, US+UV) and triple (Ag+US+UV) successive systems were determined. We concluded that the disinfection efficiency was increased when successive systems were applied.

Materials and methods

This research was conducted in Department of Environmental Engineering, Anadolu University, Eskisehir, Turkey.

In this study, the disinfection efficiencies of antibacterial filled columns, ultrasonic (US) reactors and UV reactors were determined under continuous flow conditions. The inactivation of *Escherichia coli* bacteria was examined after single (Ag, US and UV), dual (Ag+US, Ag+UV and US+UV) and triple (Ag+US+UV) system disinfection.

Water disinfection with the antibacterial filled column

Two mm bead-shaped antibacterial filling material was used for water disinfection in the antibacterial filled column. Brick dust was used as the primary body material to which 25% antibacterial powder was added. The filling materials were then baked at 800 °C. Antibacterial powder (ABP) has an antibacterial effect due to the presence of silver. The calcium and phosphate based body releases the silver in a controlled manner. The reactor column that was used in this study had a height of 30 cm and a diameter of 2 cm.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Varian 720) was used to measure the silver concentrations in treated water to control silver released from ABP because hesitation of silver usage in water has increasing for human health recently. In order to show silver concentration released from ABP is under safe limit, measurements were taken after subjecting the study solution through the antibacterial filled column at different flow rates. The study solutions were put through the reactor one time at different continuous flow rates: 5 mL/min, 25 mL/min, 50 mL/min and 100 mL/min.

Water disinfection with the ultrasonic reactor

An ultrasonic reactor that generates sound at a frequency of 40 kHz referred low-frequency ultrasound was used in the ultrasound disinfection procedure. The ultrasonic reactor was designed by us and has a diameter of approximately 60 mm and a width of 40 mm. There were transducers at two sides of the reactor. 100 watts of power was applied to the system. The disinfection studies were conducted at the5 mL/min, 25 mL/min, 50 mL/min and 100 mL/min. flow rates.

Water disinfection with the UV reactor

The UV disinfection column that was used for inactivating the microorganisms in this study had a diameter of 4.6 cm and a length of 25 cm. The UV lamp that was used here (Philips TUV 8 W UVC) had a wavelength of approximately 254 nm. The studies carried out in the continuous system using 1.3 L/min and 2.6 L/min flow rates.

Water disinfection with successive systems

Water disinfection with successive systems was carried out with the goal of removing disadvantages of the disinfection methods and observing the synergetic effects of these methods. The antibacterial filled column, the ultrasonic reactor and the UV reactor were successively used as dual and triple systems in the successive disinfection studies. The triple hybrid disinfection system yielded the best results and was conducted at 40 °C as well as ambient temperature (25 °C). The goal was to determine the temperature effect.

The disinfection studies where the antibacterial filled column and ultrasonic reactor were successively used were conducted at a 5 mL/min flow rate for the antibacterial filled column and a 25 mL/min flow rate for the ultrasonic reactor.

The disinfection studies where the ultrasonic reactor and UV reactor were successively used were conducted at a 5 mL/min flow rate for the ultrasonic reactor and a 2.6 mL/min flow rate for the UV reactor.

The disinfection study where the antibacterial filled column, ultrasonic reactor and UV reactor were used as a triple hybrid system was conducted using a 5×10^8/mL bacterial concentration. The flow rates for the antibacterial filled column and the ultrasonic reactor were 100 mL/min, and the flow rates for the UV reactor were 1.3 L/min and 2.6 L/min. The study was performed at a 2.6 L/min at a flow rate under 40 °C.

Microbiologic studies

The solution to be used for the disinfection studies was prepared by adding *Escherichia coli* bacteria to sterilized distilled water at the desired concentrations. The desired initial bacterial concentrations were obtained by making necessary dilutions from a bacteria culture that was kept for 1 day at 37 °C. The samples were cultivated in plate count agar (PCA, Merck KGgA, Germany) media and were incubated for 1 day at 37 °C. The microbiologic studies were conducted at ambient temperature inside sterile cabinets (Heraeus KSP- 18 Class II). After incubation, the efficiency of the disinfection systems was determined by counting the colonies. It was assumed that bacteria that were alive equaled a colony in solid media.

The bacterial concentration of initial and effluent water was determined with a simple and well-known plate count method during all disinfection studies using serial dilution of the samples. 1 mL aliquot was taken from treated water and un treated water according to desired time interval depending operation parameters (flow rate, hydraulic retention time or disinfection period). The bacterial concentrations of aliquot taken from system were obtained by making necessary dilutions (10×, 100× and 1000× dilutions) transferring 9 mL sterilized water gradually. 150 µL samples taken from each dilution was inoculated on PCA solid media, and the plates were incubated at 37 °C for 18-24 h. The bacterial concentration of each samples was calculated using viable cells grown in PCA plates and 150 µL sampled volume as colony forming unit per sample volume expressed mL (CFU/mL).

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The results of microbiological studies were expressed as average of three independent experiments with error bar using standard deviation (SD). Each experiment was performed with three parallels. Statistical significance of the difference of the counted bacteria in in plate count agar among each trials was determined by analysis of variance (ANOVA) using one-way post hoc statistical test. In order to show reliability of measurement, p value obtained from one-way ANOVA test was given for each disinfection study.

Results and discussion

The disinfection efficiency with the antibacterial filled column

The disinfection procedure was conducted at a 1×10^5 CFU/mL bacterial concentration using different flow rates with the goal of determining the efficiency of the antibacterial filled column. The experimental results are given in *Figure 1*.

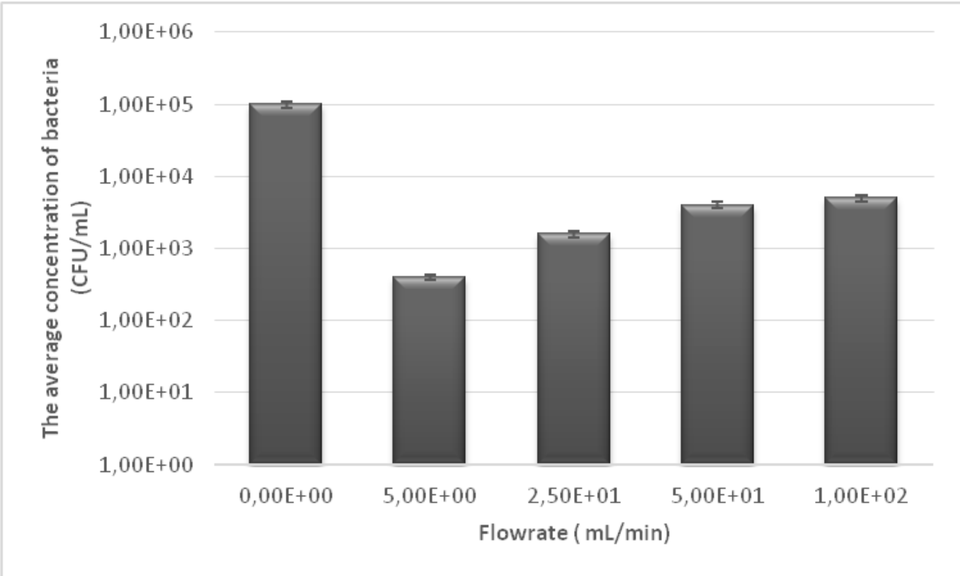

Figure 1. *The effect of different flow rates on bacteria removal at a 1×10^5 CFU/mL initial bacterial concentration in the antibacterial filled column (p value was calculated as 0.0000000003 using one-way ANOVA test. This value showed that there is no significant difference of each measurements among each three disinfection experiment because $p < 0.01$)*

The best bacteria removal was achieved at the 5 mL/min flow rate. As the contact time with the active matter increases with a decrease in flow rate, it is expected that the bacterial inactivation rate also increases. These results are seen in *Figure 1*. 2 log bacterial inactivation was achieved for the 5 mL/min flow rate. The silver concentrations of the study solutions subjected through the antibacterial filled column in a continuous system at different flow rates are given in *Figure 2*.

It was observed that the amount of silver present in the samples at the 5 mL/min flow rate was greater than that at other flow rates, however 5 mL/min was the lowest flow rate used in the study. The silver concentration at 5 mL/min was determined to be 74 µg/L.

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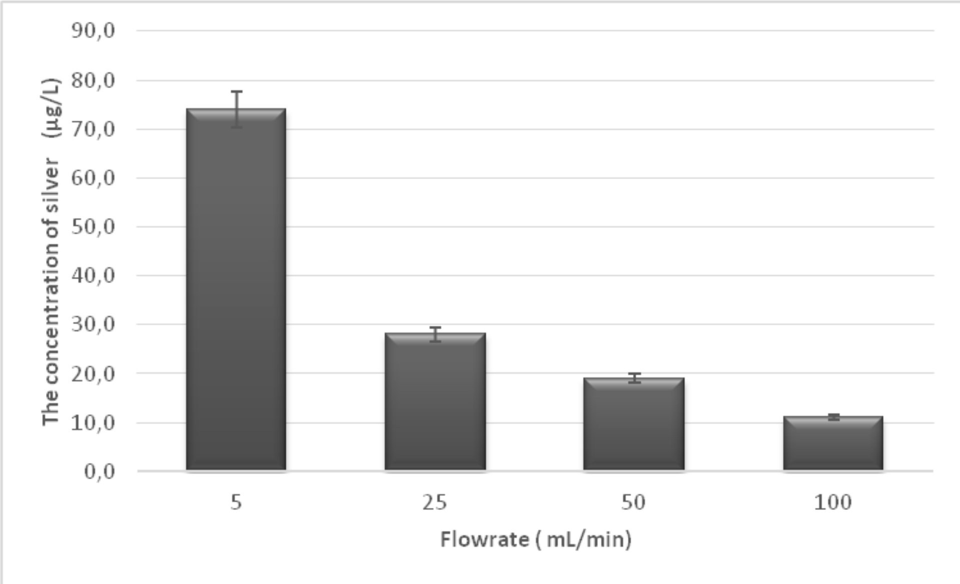

Figure 2. *The silver amounts that are released to study the solution of different flow rates in the antibacterial filled column disinfection procedure (DL: 0.35 µg/L, RSD: 5% and R^2: 0,9967 of ICP-OES calibration)*

Silver ions have the highest level of antimicrobial activity of all the heavy metals and its bacterial inactivation mechanism is well known in the literature. Our study demonstrated that disinfection process conducted with antibacterial filled column achieved sufficient inactivation ratio using silver ions released to water from filling materials surface.

Although, there is no unique or rigid safe limit of silver ion concentration allowed in water according to health and environmental organization, number of studies focused on silver toxicity has been increasing in literature to find out safe limit of silver used in potential implications for human health and the environment (Marambio-Jones and Hoek, 2010). These studies have showed that silver can be present in metallic silver, silver salts (generally silver sulphate), silver complexes and nano-sized silver. Also, researcher has reported silver nano particle is more toxic to fish, algae, some plants, fungi and mammalian cells due to nanosized effect than silver salts compounds (Panyala et al., 2008). Marambio-Jones and Hoek reported toxiccity concentraions of silver between 50 µg/mL-100 µg/mL among rewied reserchs depending toxicity organisms and silver form presented in water.

The results of disinfection studies and ICP-OES analyses showed that increasing hydraulic retention time affected directly disinfection efficiency because silver ion amount released to water increased with lowering water flow rate. ICP-OES measurement showed that silver concentration released from ABT to water was under toxicity level reported in previous studies and released silver concentration could be controlled increasing flowrate. In order to achieve powerful bacterial disinfection in antibacterial column, silver ion concentration should be increased in water according to water and health regulation for silver amount of water to avoid its environmental risks. However, there are two possible problems in the case of demanding more silver concentration to achieve adequate disinfection efficiency for strength microorganisms. These are the increasing cost of silver added to system and increasing silver amount in

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the human bodies consuming the water disinfected with silver due to bioaccumulation. Our study indicates that innovative successive and hybrid disinfection process conducted with ultrasound and UV can be used in order to escape these problems.

The disinfection efficiency of the ultrasonic reactor

The disinfection procedure was conducted using a 1×10^5 CFU/mL bacterial concentration subjected to a 40 kHz ultrasonic frequency with the goal of determining the efficiency of the ultrasonic reactor. The results obtained using different flow rates through the ultrasonic reactor are given in *Figure 3*.

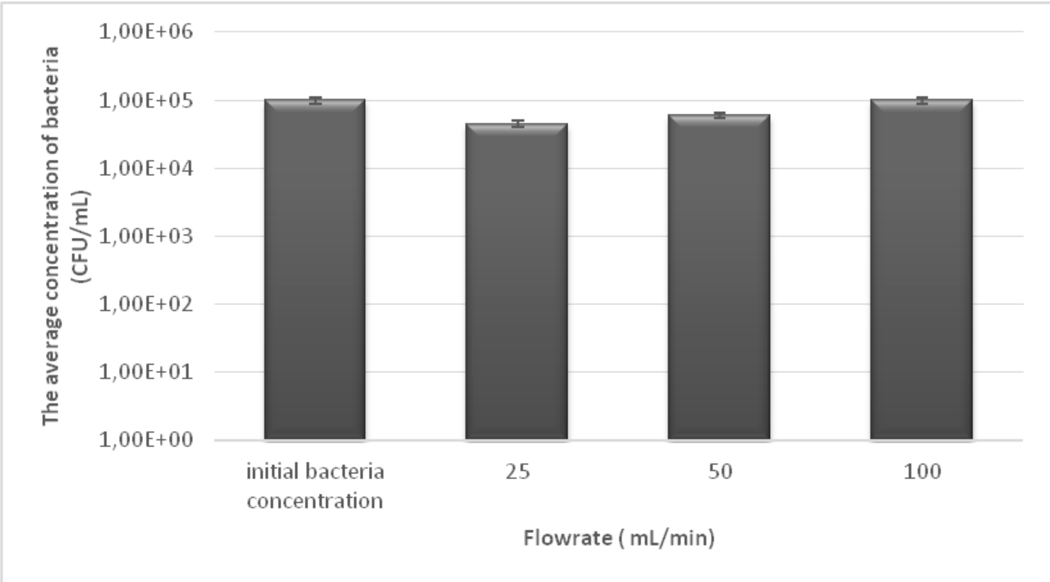

Figure 3. *The effect of bacteria removal at a 1×10^5 CFU/mL initial bacterial concentration in ultrasonic reactors at different flow rates (p value was calculated as 0.000020158 using one-way ANOVA test. This value showed that there is no significant difference of each measurements among each three independent disinfection experiment of three treatment conditions because $p < 0.01$)*

In the ultrasonic reactor, 0.5 log bacterial inactivation was achieved at the 25 mL/min flow rate. Bacterial inactivation increased with a decrease in flow rate. The result of the ultrasonic disinfection studies showed that ultrasound had no significant effect on inactivation of *Escherichia coli* in the case of single application. The mechanical effects of low-frequency ultrasound leading to cell membrane disruption of bacteria were probably insufficient for the inactivation of *Escherichia coli* (Joyce et al., 2003). It was stated that higher bacterial inactivation required higher power supply for ultrasound usage aiming disinfection in literature (Mason et al., 2003). In order to recover this disadvantages of ultrasound for water disinfection various ultrasonic reactor was produced with different frequency, power supply and different configuration. Cerecedo et al. (2018) conducted a disinfection study using rotor-stator device for water disinfection based on hydrodynamic cavitation and they reported that the water was infected with *E. coli* and *E. faecalis* with initial concentrations in the range 5×10^2–1.2×10^6 CFU/ml due to mechanical cavitation's cell membrane disruption.

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The disinfection efficiency of the UV reactor

Escherichia coli disinfection was conducted using an initial bacteria concentration of 3×10^8 CFU/mL with the aim of determining the efficiency of the UV reactor. The results obtained from the UV disinfection procedure conducted at different flow rates are given in *Figure 4*. A 5 log bacterial inactivation was achieved for both of the flow rates used in the studies. The UV reactor effectively disinfected very high initial bacteria concentrations. In single UV disinfection studies, bacterial inactivation efficiency was significantly higher than antibacterial column and ultrasonic reactor.

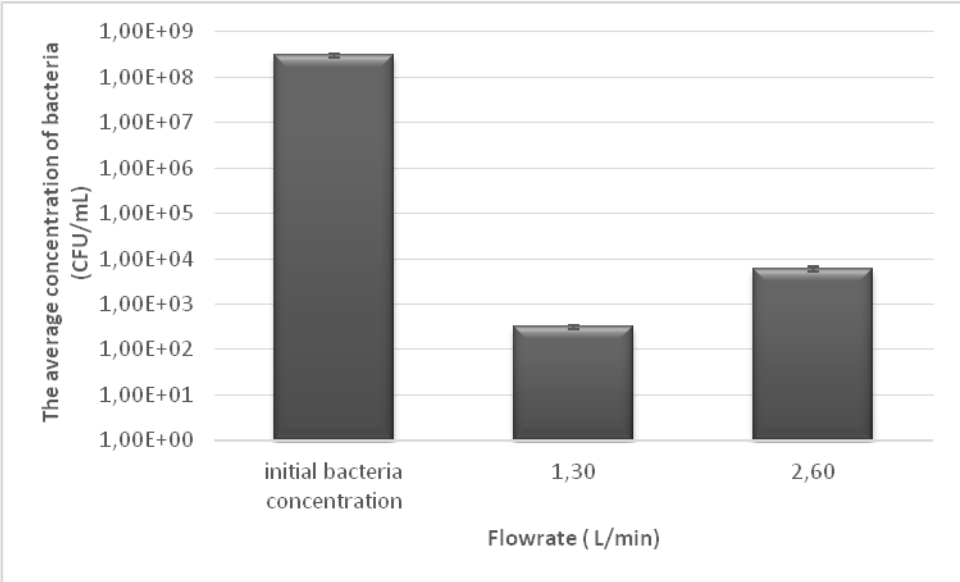

Figure 4. *The effect of bacteria removal at a 3×10^8 CFU/mL initial bacterial concentration in the UV reactor at different flow rates (p value was calculated as 0.000000070 using one-way ANOVA test. This value showed that there is no significant difference of each measurements among each three independent disinfection experiment of two treatment conditions because $p < 0.01$)*

The disinfection efficiency of successive systems

The results obtained from the studies conducted with the goal of determining the effectiveness of the dual antibacterial filled column and ultrasonic systems are given in *Figure 5*.

The silver ions were more evenly distributed inside the liquid media in the ultrasonic systems. Therefore, the efficiency of the disinfection was increased by providing continuous silver-bacteria contact inside the liquid media. Another factor increasing the efficiency of the disinfection was that the ultrasound damaged the cell membrane of the bacteria and the silver ions are much more effective when the cell membrane is damaged. In the studies conducted using the ultrasonic system after the antibacterial filled column all of the bacteria available in the media were inactivated and log 5 inactivation was achieved.

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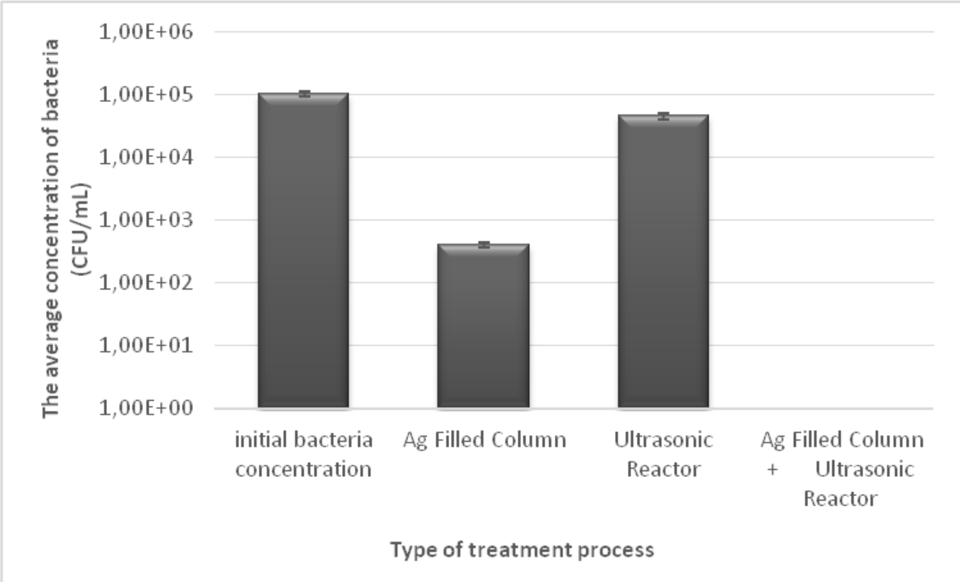

Figure 5. *The effect of the antibacterial filled column and ultrasonic system together at a 10^5 CFU/mL initial bacterial concentration (p value was calculated as 0.0005173 using one-way ANOVA test. This value showed that there is no significant difference of each measurements among each three independent disinfection experiment of three treatment conditions because $p < 0.01$)*

The results of the studies conducted with the goal of determining the efficiency of the dual system where the ultrasound reactor and the UV reactor were successively used are given in *Figure 6*.

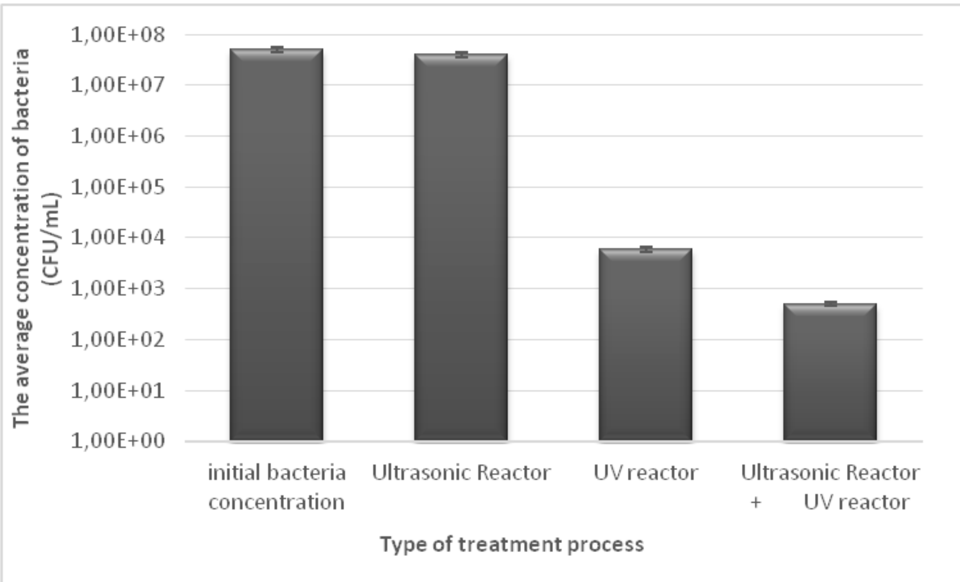

Figure 6. *The effect of the UV and ultrasonic system together at a 1 × 10^8 CFU/mL initial bacterial concentration (p value was calculated as 0.000000070 using one-way ANOVA test. This value showed that there is no significant difference of each measurements among each three independent disinfection experiment of three treatment conditions because $p < 0.01$)*

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This study revealed that the dual ultrasonic and UV reactor system had a stronger disinfection effect than when each was used alone. When these systems were used together, a 6 log bacterial inactivation was achieved. The study using the antibacterial material-filled column and the UV reactor together aimed to examine the disinfection efficiency of the dual system. The results obtained from the studies are given in *Figure 7*. When the antibacterial filled column and the UV reactor were used together the disinfection efficiencies increased. 7 log bacterial inactivation was achieved using this system successively.

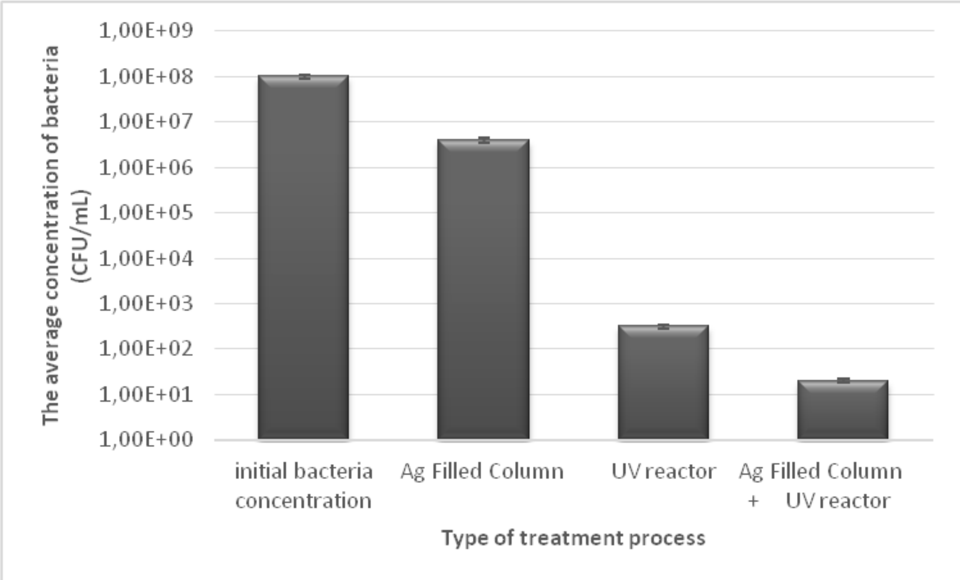

Figure 7. *The successive effect of antibacterial filled column and UV reactor at a 1×10^8 CFU/mL initial bacterial concentration (p value was calculated as 0.000000016 using one-way ANOVA test. This value showed that there is no significant difference of each measurements among each three independent disinfection experiment of three treatment conditions because $p < 0.01$)*

These result demonstrated that successive application of three systems could be successfully performed for *Escherichia coli* disinfection reducing total silver amount released to the water related and increasing mechanical effects of low frequency ultrasound. Also, the efficiency of UV treatment was improved with synergistic effects of ultrasound and silver ion. It was observed that the use of successive systems increases the efficiency of disinfection. The effectiveness of each disinfection system increases when the bacteria has sufficient contact with the disinfectants. The ultrasound reactor contributed to the effectiveness by decreasing the shielding effect in the bacteria. The Ag ion was better distributed inside the system when the ultrasound reactor was used and the disinfection effectiveness of the systems when used together was higher.

The disinfection efficiency of triple successive systems

Triple hybrid studies were conducted using the antibacterial filled column, the ultrasound reactor and the UV reactor together. The results obtained from the study are given in *Figure 8* and post hoc statistic test results of final disinfection study were summarized in *Table 1*.

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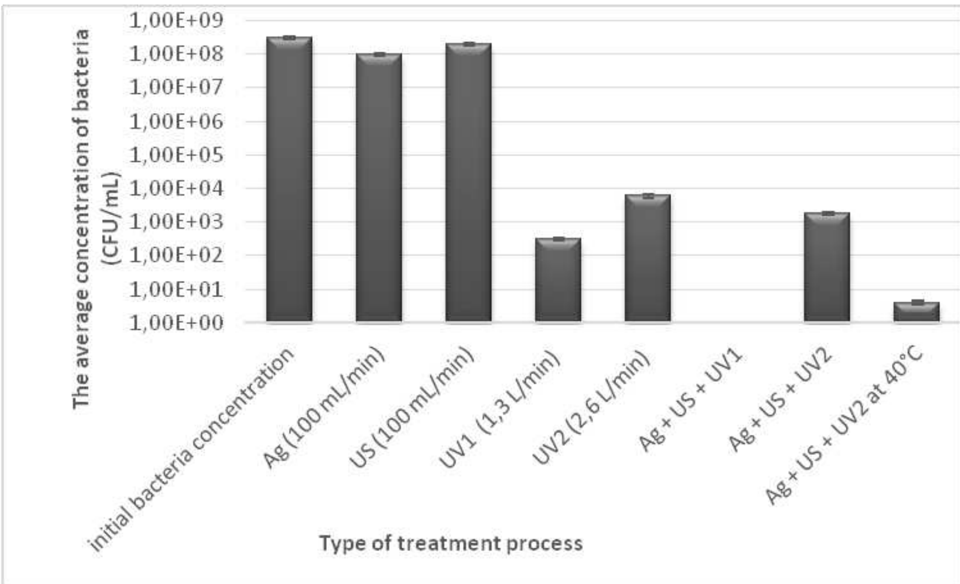

Figure 8. *The triple successive effect of the antibacterial filled column, UV reactor and ultrasonic system at a 5 × 10^8 CFU/mL initial bacterial concentration*

Table 1. *Post hoc statistic test of the triple successive effect of the antibacterial filled column, UV reactor and ultrasonic system at a 5 × 10^8 CFU/mL initial bacterial concentration*

Anova: one-way						
Summary						
Groups	Trials	Sum	Average	Varyans		
Initial bacteria concentration	3.00	9,E+08	3,E+08	9,E+14		
Ag (100 mL/min)	3.00	3,E+08	1,E+08	1,E+14		
US (100 mL/min)	3.00	6,E+08	2,E+08	4,E+14		
UV1 (1.3 L/min)	3.00	1,E+03	3,E+02	6,E+02		
UV2 (2.6 L/min)	3.00	2,E+04	6,E+03	4,E+05		
Ag + US + UV1	3.00	0,E+00	0,E+00	0,E+00		
Ag + US + UV2	3.00	5,E+03	2,E+03	4,E+02		
Ag + US + UV2 at 40°C	3.00	1,E+01	4,E+00	1,E+00		
P-value evolution						
Varyans resource	SS	df	MS	F	P-value	F criteria
Among groups	3,E+17	7.00	4.07E+16	2.32	0.00000000000001	2.66
In-groups	3,E+15	16.00	1.75E+14			
Total	3,E+17	23.00				

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The most effective disinfection was achieved in the successive system consisting of the antibacterial filled column, the ultrasonic reactor and the UV reactor used together. The triple hybrid system studies were conducted at 40 °C with the goal of determining the effect of the temperature. Better results were obtained in the studies conducted at 40 °C with a 2.6 L/min flow rate than at 25 °C. 2 log inactivation increases was observed with the increase in temperature. 8 log bacterial inactivation was achieved by the triple hybrid system. It was determined that the triple hybrid system showed a synergetic effect.

When triple hybrid studies were conducted using the antibacterial filled column, the ultrasound reactor and the UV reactor together, the disinfection efficiency of *Escherichia coli* was 8 log after disinfection period. This indicates that *Escherichia coli* disinfection with triple hybrid studies was performed reducing separate disadvantages of single application of three systems. Although mechanical effects of low-frequency ultrasound were insufficient for disinfection in single treatment, it improved other antibacterial column efficiency with cavitation mass transfer effects of silver ion from filling material surface to bacterial cell membrane. When ultrasound used with UV in successive or triple hybrid system, ultrasound prohibited regrowth of bacteria, which is most common problem in UV treatment, due to mechanical effect on cell membrane. In successive and triple application with antibacterial column, it improved the disinfection efficiency of UV treatment for water samples with high turbidity characteristic. These results indicated that using successive and triple hybrid system application of antibacterial column, UV and ultrasound vanish individual disadvantages of single application of three systems. Temperature effect accelerated the disinfection efficiency for strength microorganisms presented in water to obtain eligible inactivation ratio additionally.

This study was successfully indicated that innovative successive and hybrid disinfection process conducted with silver, ultrasound and UV can be used in order to escape serious problems when faced their single usage.

Conclusion

In this paper, the targeted *Escherichia coli* disinfection efficiency was obtained using successive and triple hybrid application of antibacterial column, ultrasound and UV aiming more sufficient inactivation ratio using lower individual disadvantages of these three treatment methods. The highest *Escherichia coli* inactivation ratio was determined in UV in single treatment application and ultrasound was insufficient for *Escherichia coli* disinfection due to week mechanical effect of cavitation. The result of successive studies demonstrated that UV, ultrasound and silver ion created synergy to achieve adequate water disinfection efficiency. Increasing disinfection efficiency with successive or triple hybrid systems was expected results but single treatment process of both antibacterial column, ultrasound and UV indicated that synergic effect was not only formed of ultrasound. The hybrid disinfection using antibacterial column, ultrasound and UV provided higher bacterial inactivation efficiency in shorter disinfection period than single processes of these treatment methods, especially when need to more powerful disinfection efficiency for strength microorganisms assisting temperature effect. This study demonstrated that hybrid application of antibacterial column, ultrasound and UV could be performed effectively for water disinfection with less environmental risks and reducing individual disadvantages of disinfection methods.

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SOIL WATER RETENTION OF THE ODRA RIVER ALLUVIAL SOILS (POLAND): ESTIMATING PARAMETERS BY RETC MODEL AND LABORATORY MEASUREMENTS

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Abstract. The paper presents water retention of alluvial soils in the Odra valley. Floodplain soils are one of the most fertile intrazonal soils in Europe. The relevant field studies and laboratory tests were carried out during the vegetation seasons (2012 and 2013) on arable lands located in Tworków (south of the Silesian Province, Poland). A three pedotransfer functions (PTFs) parameter van Genuchten type model was used to describe the water retention curves of alluvial soils. Fitted soil water retention curve (SWRC) parameters were regressed linearly with a different number of soil physical properties in the RETC and Rosetta. The first model (PTF-1) uses soil textural classes, consisting of a lookup table that provides parameter averages for each USDA textural class. The second model (PTF-2) uses sand, silt, and clay percentages as input, and the third model (PTF-3) includes bulk density as a predictor. The simulation results were considered for each layer separately and as a whole for all soil profiles. The predicted values of water content of the RETC and Rosetta for each soil layer are close to the measured values (R^2 = 0.825–0.995). Simulated values of water content (all soil profiles) by PTF-3 are very similar to the measured values. The predicted values of the residual soil water content (θr) and the saturated soil water content (θs) by PTF-3 provided good simulation results R^2 = 0.860 and R^2 = 0.667, respectively. The specific alluvial soil conditions affect the high water content in the soil, which is reflected in the content of water available for plants. This information should contribute to the rational management of water resources in the agricultural area, what can be used to mitigate the effects of drought.
Keywords: *water properties of soil, floodplain soil, RETC program, Poland*

Introduction

Soil is a non-renewable natural resource that is essential to life. Water movement, water quality, land use, and vegetation productivity all have relationships with soil (Schoonover and Crim, 2015). Diversity of alluvial soil types has been the subject of much less study than typical mineral soils. Thus, studies focused on physical and hydraulic parameters of alluvial soils are essential for a better understanding of how alluvial ecosystems function (Ľuptáčik et al., 2012).

Alluvial soils have the highest productivity with respect to other soils (Huong et al., 2013). They are present mostly along rivers and are carried by its streams during weathering of rocks. Most vegetable production is on alluvial soil generally succeed. The key role in creating alluvial soil is a high ground water level (Dobrovol'ski et al., 2011; Pirastru and Niedda, 2013; Liu et al., 2015; Dwevedi et al., 2017; Yassoglou et al., 2017).

River alluvial soils are formed from silts deposited chiefly by flood-waters, from here are often called floodplain soils. Soil formation is in this case very long, in consequence, alluvial soils have, as a rule, an undeveloped profile. The natural soils in

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river-valley bottoms are almost wholly used for meadows, pastures and tilled fields (Szafer, 1966). According to Bednarek and Prusinkiewicz (1999) alluvial soils constitute about 5% of Poland's area.

As a result of soil cultivation, farmers can change some of its physical and hydraulic parameters, such as: soil compaction, soil water permeability, relations between water and air (Cameira et al., 2003; Pagliai et al., 2004; Hakl et al., 2007; Nawaz et al., 2013; Bogdał et al., 2014; Kahlon and Khurana, 2017). Soil compaction is a significant problem of agriculture nowadays. It is directly connected with the mechanization of field treatments. Compaction is a physical form of soil degradation: it changes the soil structure, water and air permeability, porosity, and it inhibits penetration by plant roots (Hakl et al., 2007; Nawaz et al., 2013).

Knowledge of the soil hydraulic properties is indispensable to solve many soil and water management problems related to agriculture, ecology, and environmental issues. The most important hydraulic properties of soils are the soil water retention curve (SWRC) and soil water permeability (Shwetha and Varija, 2015; Qanza et al., 2015). Soil hydraulic properties depend mainly on soil texture, organic matter content and bulk density (Hillel, 1998). In scientific research, many methods and types of models are used to determine the hydraulic parameters of soils (Minasny et al., 2004; Šimůnek et al., 2005; Pandey et al., 2006; Nasta et al., 2013). The role of water retention in soil has been analyzed and reviewed in many scientific studies (Van Genuchten et al., 1991; Minasny et al., 2003; Merdun et al., 2006; Wassar et al., 2016; Nguyen et al., 2017).

Modeling water flow in soil requires knowledge of soil hydraulic properties, which are water retention curves. As an alternative to direct measurement, indirect determination of these functions from basic soil properties using pedotransfer functions (PTFs) has attracted the attention of researchers in a variety of fields such as soil scientists, hydrologists, and agricultural and environmental engineers (Merdun et al., 2006; Fashi et al., 2016).

Water retention characteristics are fundamental input parameters in any modeling study on water flow and solute transport. These properties are difficult to measure and for that reason, we usually need to use direct and indirect methods to determine them. An extensive comparison between measured and estimated results is needed to determine their applicability for a range of different soils (Wassar et al., 2016).

The RETC can be useful for estimating the hydraulic parameters from retention data only with laboratory, or simultaneously from observed retention and hydraulic conductivity data and other physical parameters (Van Genuchten et al., 1991).

The objective of this study is to evaluate the water retention of the alluvial soil and the estimation of van Genuchten parameters by the three intelligent pedotransfer models RETC.

Materials and methods

Study area

The study area lies on alluvial soils located in the catchment of the Odra River on arable land in Tworków, in the south of Poland (the Racibórz District, the Silesian Province) (*Fig. 1*).

The average altitude of the object is 190.00 m a.s.l. According to the geographical division by Kondracki (2011), the object is situated in the Central European Lowlands province (31), in the macroregion of the Silesian Lowlands (318.5) and in the

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mesoregion of the Raciborska Basin (318.59), in terms of climate is considered as one of the warmest areas in this region. In general, Poland has mostly temperate climate, in transition between oceanic climate dominating in the north and west of the country, and continental climate in the south and east (Kundzewicz et al., 2017). In the multiannual period 1971–2000, average annual air temperature was 8.5 °C and total precipitation was 616 mm (according to the IMGW – Institute of Meteorology and Water Management – station in Racibórz). Depending on changes in meteorological conditions, there are periodically too high moisture contents in soil. The characteristics of meteorological conditions are shown in *Table 1*.

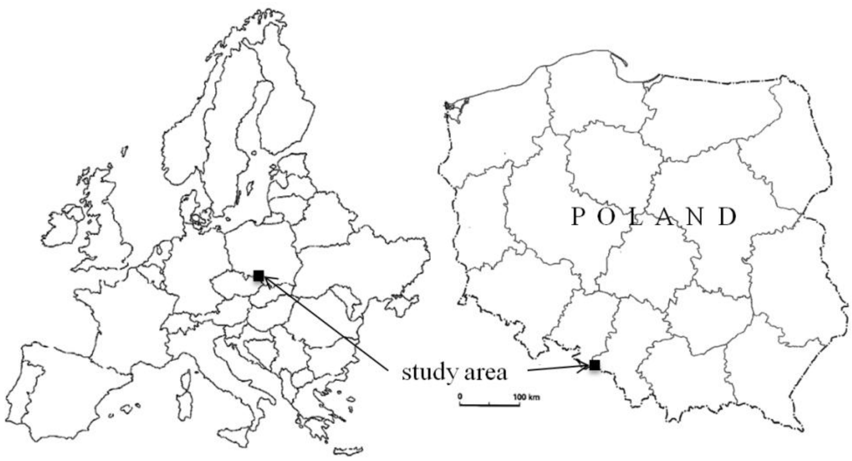

Figure 1. *Location of the study area*

Table 1. *The characteristics of meteorological conditions of the study area*

Period	Months												
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	
Year	**Average monthly precipitation totals [mm]**												**Sum I–XII**
2012	41	24	18	41	35	75	89	69	58	81	37	18	586
2013	44	29	36	21	132	110	14	48	99	24	31	9	597
2014	21	16	23	27	137	75	58	92	127	35	18	16	645
1971–2000	28	26	32	45	67	79	94	74	56	41	40	34	616
	Average monthly air temperatures [°C]												**Average I–XII**
2012	-0.3	-5.6	5.4	9.9	15.3	17.7	19.9	19.1	14.7	9.0	6.5	-1.1	9.2
2013	-2.2	-0.2	0.1	9.0	13.8	16.9	19.7	19.1	12.6	10.8	5.5	2.7	9.0
2014	0.6	4.0	6.9	10.8	13.8	16.3	20.4	17.4	15.6	11.1	7.1	1.6	10.5
1971–2000	-1.3	-0.2	3.8	8.2	13.5	16.1	17.8	17.7	13.6	9.0	3.6	0.2	8.5

Field analysis

The field soil tests were carried out in the two agricultural seasons: 2012 and 2013 on arable lands. The alluvial soils are the predominant soil type here on account of height

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of the region's surface water, which makes them good/very good in terms of soil fertility classes.

In field four soil pits were made up to a depth of 150 cm (*Fig. 2; Table 2*). Undisturbed soil samples were taken from each genetic horizons using Kopecky's cylinders (in 3 replications) to find the soil water content (SWC), bulk density (BD), total porosity and soil water potential. Also, approximately 1 kg, disturbed soil from each genetic horizons to find the soil texture and other laboratory analysis were taken.

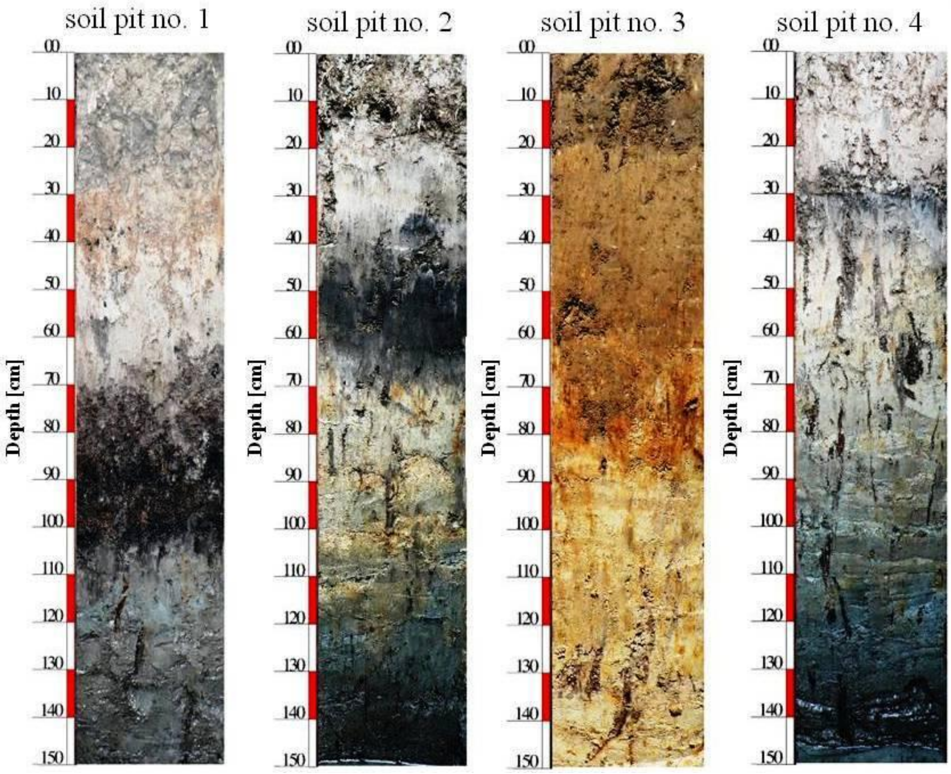

Figure 2. *Characteristics of soil pits for the Tworków object*

Table 2. *Place of soil pits and sampling*

Place of soil pits	Number of soil pits	Position of soil pits	
		N	E
Tworków	1	50°01'02.43"	18°14'20.50"
	2	50°01'03.08"	18°14'17.87"
	3	50°01'25.47"	18°14'25.56"
	4	50°00'57.90"	18°14'21.68"

Laboratory analysis

From the soil samples that were collected the following properties were determined:

- Soil texture by the Bouyoucose-Casagrande areometric method modified by Prószyński. The modification consisted in measurement of suspension of soil by Prószynski's hydrometer, which gives percentages sedimentation fraction content between the two readings in the time. Suspension density is read in the

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terms, which set out in the tables developed by Prószyński. The content of particle size classes (sand, 2.0–0.05 mm; silt, 0.05–0.002 mm; clay, <0.002 mm) is determined according to the Soil Taxonomy system that was made known by the United States Department of Agriculture (USDA) (Soil Survey Staff, 1999).

- Particle density (ρ_s) by the pycnometer method as average density of the mineral grains of the soil (*Eq. 1;* Mocek and Drzymała, 2010; Phogat et al., 2015):

$$\rho_s = \frac{M_s}{V_s}\left(g \cdot cm^{-3}\right) \tag{Eq.1}$$

where M_s is the mass of mineral grains of the soil sample (g) and V_s is the volume of the mineral grains of the soil (cm^3).

- Soil bulk density (ρ_b) by the gravimetric method in Kopecky's cylinders (100 cm^3) as the mass of dry soil per volume. The weight of this soil core is then determined after drying in an oven at 105 °C for about 18–24 h. The dry bulk density for each core sample was then calculated using equation (*Eq. 2;* Hao et al., 2008; Phogat et al., 2015):

$$\rho_b = \frac{M_s}{V_t}\left(g \cdot cm^{-3}\right) \tag{Eq.2}$$

where M_s is the mass of dry soil weight (g) and V_t is the soil volume (cm^3).

- Soil moisture (volume wetnees) by the gravimetric method in Kopecky's cylinders (100 cm^3) as the ratio of the amount of water in the soil sample to the dry weight of the soil, after drying in an oven at 105 °C for about 18–24 h, then calculated using *Equation 3* (Mocek and Drzymała, 2010; Phogat et al., 2015):

$$\theta_v = \left[\frac{V_w}{V_t}\right] \cdot 100\left(cm^3 \cdot cm^{-3}\right) \tag{Eq.3}$$

where V_w is the volume of water in the soil sample (cm^3) and V_t is the volume of soil sample (cm^3).

- Water storage in layer as (*Eq. 4;* Mocek and Drzymała, 2010):

$$S_w = \frac{\theta_V \cdot h}{10}\,(mm) \tag{Eq.4}$$

where θ_v is soil moisture (% cm^3·cm^{-3}), h is thickness of the soil layer (cm), *10* – water conversion from Mg·ha^{-1} to mm water.

- Soil water retention using set for pF determination with ceramic plates in the 5 and 15 Bar Pressure Plate Extractor. The pressure plate equipment used in this study is made by the American Soil Moisture Equipment Corporation. In engineering practice, soil suction has usually been calculated in *pF* units (*Eq. 5;* Schofield, 1935):

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$$pF = log\ h\ (cm\ H_2O) \qquad \text{(Eq.5)}$$

where h is the suction of water (in cm).

In laboratory the soil water potentials were measured at pF: 0.0, 2.0, 2.2, 2.5, 2.7, 3.0, 3.4, 3.7 and 4.2 (*Table 3*). The total available water capacity (TAWC) was determined as a difference between the moisture retained at pF 2.5–4.2. Easily and hardly available water were determined as a difference between the moisture retained at pF 2.5–3.7 and at pF 3.7–4.2, respectively. The field capacity (FC) was considered at pF 2.5 and the permanent wilting point (PWP) at 4.2 (Mocek and Drzymała, 2010).

Table 3. *The pressure applied to determine the pF curves*

Number of measurements	pF	cm H_2O	kPa	bar	mm Hg
1	0.0	0	0.00	0.0000	0.0
2	2.0	100	9.81	0.0981	73.6
3	2.2	159	15.54	0.1554	116.6
4	2.5	316	30.99	0.3099	232.4
5	2.7	500	49.03	0.4903	367.8
6	3.0	1000	98.07	0.9807	735.6
7	3.4	2500	245.17	2.4517	1838.9
8	3.7	5000	490.33	4.9033	3677.8
9	4.2	15849	1554.26	15.5426	11657.9
10	7.0	10 000 000	1 000 000	10 000	735538.1

- Total organic carbon (TOC) content by the Tiurin's method, then converted to soil organic matter content (SOM) according to the formula (*Eq. 6;* Mebius, 1960; Mocek and Drzymała, 2010):

$$SOM = TOC \times 1.724\ (\%) \qquad \text{(Eq.6)}$$

- pH value by means of the potentiometric method in KCl of 1 mol·dm^{-3} concentration in one repetition (Mocek and Drzymała, 2010).
- On the basis of results obtained total porosity was calculated from bulk density and particle density, as (*Eq. 7;* Mocek and Drzymała, 2010; Phogat et al., 2015):

$$f = \left[1 - \frac{\rho_b}{\rho_s}\right] \cdot 100(\%) \qquad \text{(Eq.7)}$$

Soil classification was established according to the Polish Soil Classification (PTG, 2011), the World Reference Base for Soil Resources – IUSS Working Group WRB (2006) and USDA soil taxonomy (Soil Survey Staff, 1999).

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Modeling the soil water retention in the RETC program

The RETC (*Retention Curve Program*) is a computer program which may be used to analyze the soil water retention and hydraulic conductivity functions of unsaturated soils, especially to predict the hydraulic conductivity from observed soil water retention data assuming that one observed conductivity value (not necessarily at saturation) is available (Van Genuchten et al., 1991; Hollenbeck et al., 2000).

The shape of water retention curves (pF) can be characterized by several models, one of them known as the van Genuchten model (1991) (*Eq. 8*):

$$\theta_{(h)} = \theta_r + \frac{\theta_s - \theta_r}{[1 + (\alpha |h|^n)]^m} \quad \text{(Eq. 8)}$$

where, $\theta_{(h)}$ is the soil water content ($cm^3 \cdot cm^{-3}$), θ_r is the soil residual water content at pF > 4.2 ($cm^3 \cdot cm^{-3}$), θ_s is the soil saturated water content at pF 0.0 ($cm^3 \cdot cm^{-3}$), h is soil water potential (kPa), α is a scale parameter inversely proportional to mean pore diameter (cm^{-1}), n and m are the shape parameters of soil water characteristic

$$m = \frac{1}{n};\ 0 < m < 1.$$

Retention curves (pF) were compared against those obtained from the RETC program estimations and laboratory measurements. The water retention parameters according to Van Genuchten (1980) with predicted hydraulic functions were estimated by a published neural networks program *Rosetta Lite v. 1.1.* The necessary input data for the RETC model (e.g. % soil texture and bulk density) were determined in laboratory analysis of soil samples from different soil horizons (*Tables 2, 3* and *4*). The unknown parameters (θ_s, θ_r, n, α) were determined in the RETC program (Van Genuchten et al., 1991) using measured soil water retention data. *Rosetta* offers five pedotransfer functions (PTFs). To estimate of the water retention parameters (θ_r, θ_s, α, n), were used only three of them with limited or more extended sets of predictor. The first model (PTF-1) uses soil textural classes, consisting of a lookup table that provides parameter averages for each USDA textural class. The second model (PTF-2) uses sand, silt, and clay percentages as input, and in contrast to PTF-1, provides hydraulic parameters that vary continuously with texture. The third model (PTF-3) includes bulk density as a predictor (Schaap et al., 1998, 2001).

Statistical analysis

The data set consisted of analytical results of soil samples collected at the four soil pits located in the Odra River valley during two vegetation seasons (2012 and 2013). For statistical analysis, the procedures provided by the program *Statistica PL version 12.5* were used. A 5% significance level was used. A statistical method was chosen after checking data normality (Shapiro-Wilk test). For each analysed physical and water parameters of soil its minimum and maximum values were determined its arithmetic mean, median, standard deviation (SD) and coefficient of variation (CV) were computed. The performances of the RETC model and laboratory measurements in predicting measured data were assessed using a coefficient of determination (R^2).

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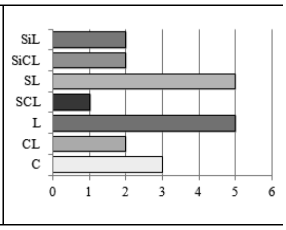
Moreover, the Spearman's correlation relationship (r) between the granulometric composition and the organic matter of soils and their physical and water values were calculated. The correlation strength and direction of a linear relationship between two variables were determined based on a scale (Rumsey, 2016): exactly ±1 – a perfect downhill/uphill (negative/positive) linear relationship; ±0.70 – a strong downhill/uphill (negative/positive) linear relationship; ±0.50 – a moderate downhill/uphill (negative/positive) relationship; ±0.30 – a weak downhill/uphill (negative/positive) linear relationship; 0 – no linear relationship.

***Table 4.** Granulometric composition of soils and basic descriptive statistics*

Profile number	**Depth (cm)**	**Genetic horizons** ***(acc. to PTG)***	**Percentage fraction with diameter (mm)**			**Soil texture group** ***(acc. to USDA)***
			Sand 2.0–0.05	**Silt 0.05–0.002**	**Clay <0.002**	
1	0–30	Ap	18	47	35	Silty clay loam (SiCL)
	30–46	AC	31	26	43	Clay (C)
	46–63	OCg	16	19	65	Clay (C)
	63–94	2O	57	29	14	Sandy clay loam (SCL)
	94–150	3G	19	62	19	Silty loam (SiL)
2	0–18	Ap	25	35	40	Clay loam (CL)
	18–34	AC	18	21	61	Clay (C)
	34–52	G	32	44	24	Loam (L)
	52–85	Cg1	55	33	12	Sandy loam (SL)
	85–150	2G	76	16	8	Sandy loam (SL)
3	0–25	Ap	43	35	22	Loam (L)
	25–47	A/Bw	45	33	22	Loam (L)
	47–75	Bw	58	24	18	Sandy loam (SL)
	75–117	Cg	40	43	17	Loam (L)
	117–150	2Cg	41	45	14	Loam (L)
4	0–30	Ap	22	40	38	Clay loam (CL)
	30–42	O	17	55	28	Silty clay loam (SiCL)
	42–71	Bw	27	53	20	Silty loam (SiL)
	71–100	Cg	54	33	13	Sandy loam (SL)
	100–150	2Cg	67	25	8	Sandy loam (SL)
Basic descriptive statistics						
Index value:		Minimum	16.0	16.0	8.0	
		Maximum	76.0	62.0	65.0	
		Mean	38.0	35.9	26.1	
		Median	36.0	34.0	21.0	
		SD	18.4	12.6	16.3	
		CV (%)	48.2	35.1	62.4	

Results

Physical properties of soils

As shown in *Table 4* and *Figure 2*, the alluvial soils are heterogeneous in terms of texture. In each soil profile, five genetic levels were specified. The granulometric composition of the profiles was classified into to seven soil texture groups (acc. to

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USDA) as: silty loam (SiL), silty clay loam (SiCL), loam (L), sandy clay loam (SCL), sandy loam (SL), clay (C) and clay loam (CL). The high coefficient of variation (CV) values was observed, which is the measure of empirical data deviations from average values. The highest CV was in silt (62.4%). Sand content in the profiles was between 16 and 76%, silt content between 16 and 62%, and clay content between 8 and 65%. Finally, according to the Polish Soil Classification (PTG, 2011), World Reference Base for Soil Resources – IUSS Working Group WRB (2006) and USDA soil taxonomy (Soil Survey Staff, 1999), examined soils were classified as:

- Order 7. Chernozemic soils (Polish: Gleby czarnoziemne; WRB: Chernozems, Phaeozems; ST: Mollisols – Aquolls, Udolls).
- Type 7.4 "Chernoziemic fluvisols" (Polish: Mady czarnoziemne; WRB: Mollic Fluvisol, Endofluvic Phaeozem; ST: Fluvaquentic Endoaquolls)

The soils formed mostly in recent alluvium on flood plains. Many of the soils have been artificially drained and are used as cropland, but some are used as pasture or forest (Soil Survey Staff, 1999).

The basic physical and chemical properties of soils with descriptive statistics for each sites is given in *Table 5*.

Table 5. *Basic physical and chemical properties of soils and descriptive statistics*

Profile number	**Depth**	**Soil moisture**	**Bulk density**	**Particle density**	**Total porosity**	**Soil organic matter**	**pH**	**Water storage in layer**
	(cm)	**($cm^3 \cdot cm^{-3}$)**	**($g \cdot cm^{-3}$)**		**(%)**		**(KCl)**	**(mm)**
1	0–30	0.3087	1.44	2.53	43.08	3.7	6.29	93
	30–46	0.4644	1.12	2.62	57.25	2.4	6.22	74
	46–63	0.4844	1.06	2.43	56.38	4.0	6.02	82
	63–94	0.6684	0.44	1.69	73.96	7.5	5.61	207
	94–150	0.4622	1.40	2.63	46.77	1.8	7.27	259
2	0–18	0.4016	1.32	2.54	48.03	3.3	6.59	72
	18–34	0.4247	1.29	2.56	49.61	2.3	6.45	68
	34–52	0.3740	1.52	2.57	40.86	2.2	6.67	67
	52–85	0.3363	1.75	2.64	33.71	0.7	6.61	111
	85–150	0.3941	1.61	2.63	38.78	1.1	3.71	256
3	0–25	0.2734	1.65	2.53	34.78	1.8	5.83	68
	25–47	0.2330	1.81	2.66	31.95	1.0	6.16	51
	47–75	0.3197	1.66	2.68	38.06	0.4	6.40	90
	75–117	0.3500	1.68	2.70	37.78	0.3	6.42	147
	117–150	0.3678	1.68	2.67	37.08	0.2	6.29	121
4	0–30	0.5145	1.11	2.56	56.64	4.0	6.49	154
	30–42	0.3943	1.51	2.48	39.11	1.9	6.53	47
	42–71	0.4028	1.56	2.65	41.13	0.7	6.37	117
	71–100	0.3241	1.65	2.67	38.20	0.4	6.53	94
	100–150	0.3159	1.62	2.69	39.78	0.8	4.95	158
Basic descriptive statistics								
Index value:	Minimum	0.2330	0.44	1.69	31.95	0.2	3.71	47
	Maximum	0.6684	1.81	2.70	73.96	7.5	7.27	259
	Mean	0.3907	1.44	2.56	44.15	2.0	6.17	117
	Median	0.3841	1.54	2.63	40.32	1.8	6.39	94
	SD	9.71	0.32	0.22	10.31	1.8	0.74	63
	CV (%)	24.85	22.27	8.49	23.36	88.54	12.02	53.81

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High soil moisture values were observed in soil profiles. The average soil moisture content was 0.3907 $cm^3 \cdot cm^{-3}$, which means it is very close to average value of water content at the field's water capacity (*Table 6*). Bulk density (BD) was in range 0.44–1.81 $g \cdot cm^{-3}$ and predominantly increasing with a depth and yielding a very high value for total porosity from 31.95% to 73.96%. Mean particle density value is very similar in whole profiles about 2.56 $g \cdot cm^{-3}$, only in profile no. 1 in fourth genetic horizon (rich in organic matter) it is much smaller (1.69 $g \cdot cm^{-3}$). According to coefficient of variation (CV), the behaviour in the soil profiles can be considered as a low variability (see *Table 5*). Mean organic matter content, about 2.03%, was highest in top layers and decreasing with depth. The pH values were in a wide range from 3.71 to 7.27, which corresponds to very acidic to alkaline soils. The high values of water storage ranged 477–715 mm in soil profiles was observed.

Table 6. *Retention of available water in soil*

Profile number	Depth [cm]	Maximum retentive capacity	Field water capacity	Permanent wilting point	Available water for plants	Easily accessible water	Hardly accessible water
		pF 0.0	pF 2.5	pF 4.2	pF 2.5–4.2	pF 2.5–3.7	pF 3.7–4.2
		($cm^3 \cdot cm^{-3}$)					
1	0–30	0.4995	0.4289	0.2690	0.1599	0.0529	0.1070
	30–46	0.5618	0.5280	0.3166	0.2114	0.1235	0.0879
	46–63	0.6242	0.6119	0.3314	0.2805	0.2247	0.0558
	63–94	0.7936	0.6784	0.3092	0.3692	0.3437	0.0255
	94–150	0.5203	0.4894	0.1915	0.2979	0.2877	0.0102
2	0–18	0.5117	0.4759	0.2466	0.2293	0.0808	0.1485
	18–34	0.5282	0.5177	0.2894	0.2283	0.1486	0.0797
	34–52	0.4687	0.4502	0.2557	0.1945	0.1150	0.0795
	52–85	0.4080	0.3556	0.1327	0.2229	0.1839	0.0390
	85–150	0.4253	0.3467	0.1034	0.2433	0.2145	0.0288
3	0–25	0.4052	0.3770	0.2500	0.1270	0.1214	0.0056
	25–47	0.3211	0.2985	0.1503	0.1482	0.0879	0.0603
	47–75	0.3823	0.3345	0.1576	0.1769	0.1465	0.0304
	75–117	0.3689	0.3484	0.1306	0.2178	0.1858	0.0320
	117–150	0.3962	0.3417	0.0886	0.2531	0.2135	0.0396
4	0–30	0.5760	0.5663	0.2943	0.2720	0.1764	0.0956
	30–42	0.4514	0.4386	0.2870	0.1516	0.1304	0.0212
	42–71	0.4394	0.4225	0.1873	0.2352	0.2131	0.0221
	71–100	0.3948	0.2690	0.0737	0.1953	0.1352	0.0601
	100–150	0.4314	0.3094	0.0649	0.2445	0.2442	0.0003
Basic descriptive statistics							
Index value	Minimum	0.3211	0.2690	0.0649	0.1270	0.0529	0.0003
	Maximum	0.7936	0.6784	0.3314	0.3692	0.3437	0.1485
	Mean	0.4754	0.4294	0.2065	0.2229	0.1715	0.0515
	Median	0.4454	0.4257	0.2191	0.2256	0.1625	0.0393
	SD	0.11	0.11	0.08	0.06	0.07	0.04
	CV (%)	22.64	25.81	42.84	25.72	41.97	74.66

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Hydraulic properties of soils

The data of soil water retention, incuded available water for plants, from laboratory analysis are presented in *Table 6*. The water retention curve for the studied soils showed a mean value of soil moisture of about 0.4754 $cm^3 \cdot cm^{-3}$ at saturation (pF 0.0), 0.4294 $cm^3 \cdot cm^{-3}$ at field water capacity (pF 2.5), and 0.2065 $cm^3 \cdot cm^{-3}$ at permanent wilting point (pF 4.2). Mean share of easily accessible water for plants contributed 77% of the total available water for plants (TAWC), whereas hardly accessible water accounted 23% TAWC.

The determination coefficient (R^2) of observed and fitted values in each layers of soil profiles ranges from 0.825 to 0.995. These results are very satisfactory and show that the estimation procedure works well (*Tables 7, 8* and *9*). *Tables 7, 8* and *9* present different values of the van Genuchten parameters (θ_r, θ_s, α, n) obtained.

Table 7. *Characteristic parameters of soil water content from model PTF-1 with soil USDA textural classes*

Profile number	Depth [cm]	θ_r ($cm^3 \cdot cm^{-3}$)	θ_s ($cm^3 \cdot cm^{-3}$)	α (cm^{-1})	n (–)	R^2
1	0–30	0.0901	0.4820	0.0084	1.5202	0.978
	30–46	0.0982	0.4588	0.0150	1.2529	0.993
	46–63	0.0982	0.4588	0.0150	1.2529	0.987
	63–94	0.0633	0.3837	0.0211	1.3298	0.979
	94–150	0.0645	0.4387	0.0051	1.6626	0.955
2	0–18	0.0792	0.4418	0.0158	1.4145	0.825
	18–34	0.0982	0.4588	0.0150	1.2529	0.995
	34–52	0.0609	0.3991	0.0111	1.4737	0.989
	52–85	0.0387	0.3870	0.0267	1.4484	0.979
	85–150	0.0387	0.3870	0.0267	1.4484	0.965
3	0–25	0.0609	0.3991	0.0111	1.4737	0.969
	25–47	0.0609	0.3991	0.0111	1.4737	0.989
	47–75	0.0387	0.3870	0.0267	1.4484	0.985
	75–117	0.0609	0.3991	0.0111	1.4737	0.987
	117–150	0.0609	0.3991	0.0111	1.4737	0.979
4	0–30	0.0792	0.4418	0.0158	1.4145	0.995
	30–42	0.0901	0.4820	0.0084	1.5202	0.979
	42–71	0.0645	0.4387	0.0051	1.6626	0.981
	71–100	0.0387	0.3870	0.0267	1.4484	0.978
	100–150	0.0387	0.3870	0.0267	1.4484	0.956

Table 8. *Characteristic parameters of soil water content from model PTF-2 with % sand, silt, and clay*

Profile number	Depth [cm]	θ_r ($cm^3 \cdot cm^{-3}$)	θ_s ($cm^3 \cdot cm^{-3}$)	α (cm^{-1})	n (–)	R^2
1	0–30	0.0891	0.4665	0.0096	1.4593	0.978
	30–46	0.0886	0.4484	0.0184	1.2893	0.993
	46–63	0.0990	0.4950	0.0209	1.1936	0.987
	63–94	0.0493	0.3884	0.0217	1.4033	0.979
	94–150	0.0683	0.4340	0.0048	1.6502	0.955

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2	0–18	0.0893	0.4586	0.0136	1.3573	0.825
	18–34	0.0979	0.4899	0.0205	1.2075	0.995
	34–52	0.0710	0.4228	0.0080	1.5222	0.989
	52–85	0.0456	0.3889	0.0189	1.4229	0.979
	85–150	0.0417	0.3841	0.0386	1.5283	0.965
3	0–25	0.0656	0.4064	0.0128	1.4469	0.969
	25–47	0.0654	0.4042	0.0144	1.4294	0.989
	47–75	0.0564	0.3888	0.0240	1.3761	0.985
	75–117	0.0574	0.4019	0.0084	1.5280	0.987
	117–150	0.0518	0.3992	0.0079	1.5455	0.979
4	0–30	0.0896	0.4631	0.0117	1.4001	0.995
	30–42	0.0812	0.4516	0.0068	1.5501	0.979
	42–71	0.0664	0.4215	0.0054	1.6101	0.981
	71–100	0.0477	0.3894	0.0181	1.4251	0.978
	100–150	0.0380	0.3876	0.0355	1.4168	0.956

Table 9. *Characteristic parameters of soil water content from model PTF-3 with % sand, silt, clay and bulk density (BD)*

Profile number	**Depth [cm]**	θ_r **($cm^3 \cdot cm^{-3}$)**	θ_s **($cm^3 \cdot cm^{-3}$)**	**α (cm^{-1})**	**n (–)**	R^2
1	0–30	0.0978	0.5650	0.0112	1.4528	0.978
	30–46	0.1035	0.5774	0.0195	1.3542	0.993
	46–63	0.1146	0.6102	0.0282	1.2495	0.987
	63–94	indicates the parameter cannot be estimated (too low bulk density value)				
	94–150	0.0780	0.5163	0.0046	1.6932	0.955
2	0–18	0.1014	0.5727	0.0156	1.3894	0.825
	18–34	0.1130	0.6064	0.0271	1.2618	0.995
	34–52	0.0807	0.5199	0.0073	1.5678	0.989
	52–85	0.0535	0.4896	0.0115	1.4812	0.979
	85–150	0.0475	0.5235	0.0353	1.4232	0.965
3	0–25	0.0753	0.5160	0.0094	1.5163	0.969
	25–47	0.0749	0.5178	0.0102	1.5004	0.989
	47–75	0.0658	0.5208	0.0159	1.4322	0.985
	75–117	0.0663	0.4917	0.0063	1.6010	0.987
	117–150	0.0602	0.4791	0.0058	1.6215	0.979
4	0–30	0.1000	0.5696	0.0137	1.4136	0.995
	30–42	0.0897	0.5450	0.0073	1.5529	0.979
	42–71	0.0757	0.5068	0.0051	1.6576	0.981
	71–100	0.0577	0.4921	0.0111	1.4871	0.978
	100–150	0.0454	0.5010	0.0231	1.4086	0.956

The graphs of the fitted the van Genuchten model with the RETC to the soil water retention data with laboratory measurements for soil pit no. 1 – from depth of 0–30 cm and for soil pit no. 2 – from depth of 34–52 cm are illustrated in *Figure 3*, as examples. It is observed that both the results of research fit well.

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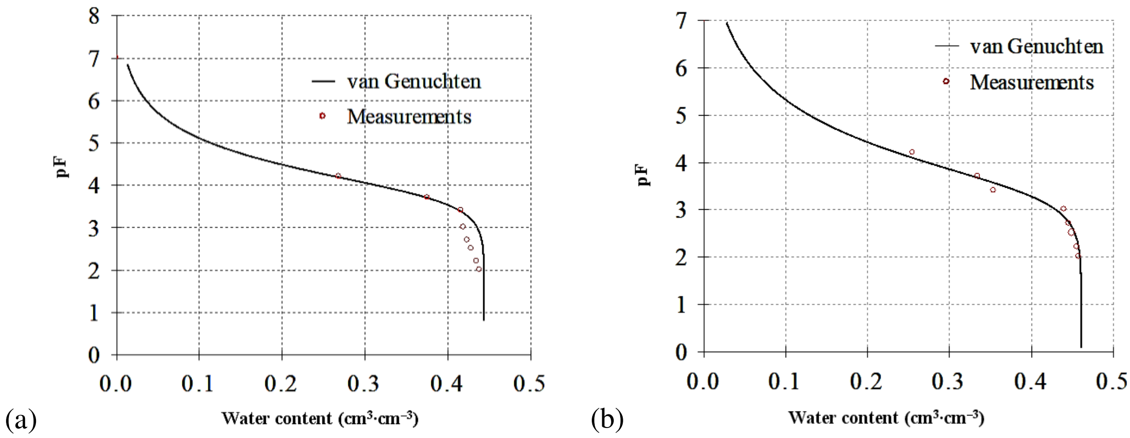

Figure 3. *Water retention curve (pF): a) – soil pit no. 1; b) – soil pit no. 2*

Figures 4 and *5* illustrate the comparison of the predicted water content of the RETC and Rosetta with that of measurement for three pedotransfer models (PTF-1, PTF-2 and PTF-3). The measured results from all soil profiles for θ_s and θ_r were compared with fitted results those of the RETC and Rosetta.

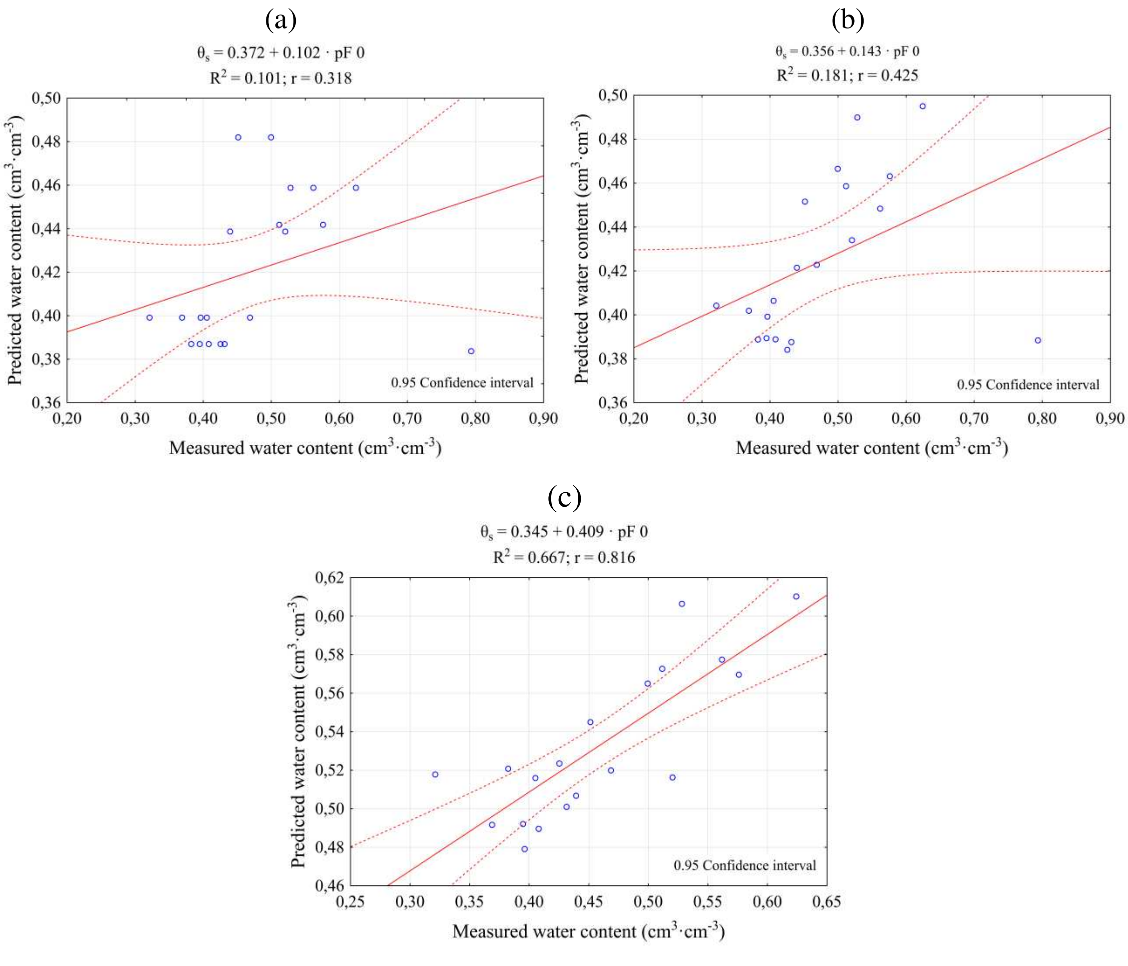

Figure 4. *Estimated vs. measured water contents (θ_s) of the RETC a) for first model (PTF-1), b) second model (PTF-2), c) for third model (PTF-3)*

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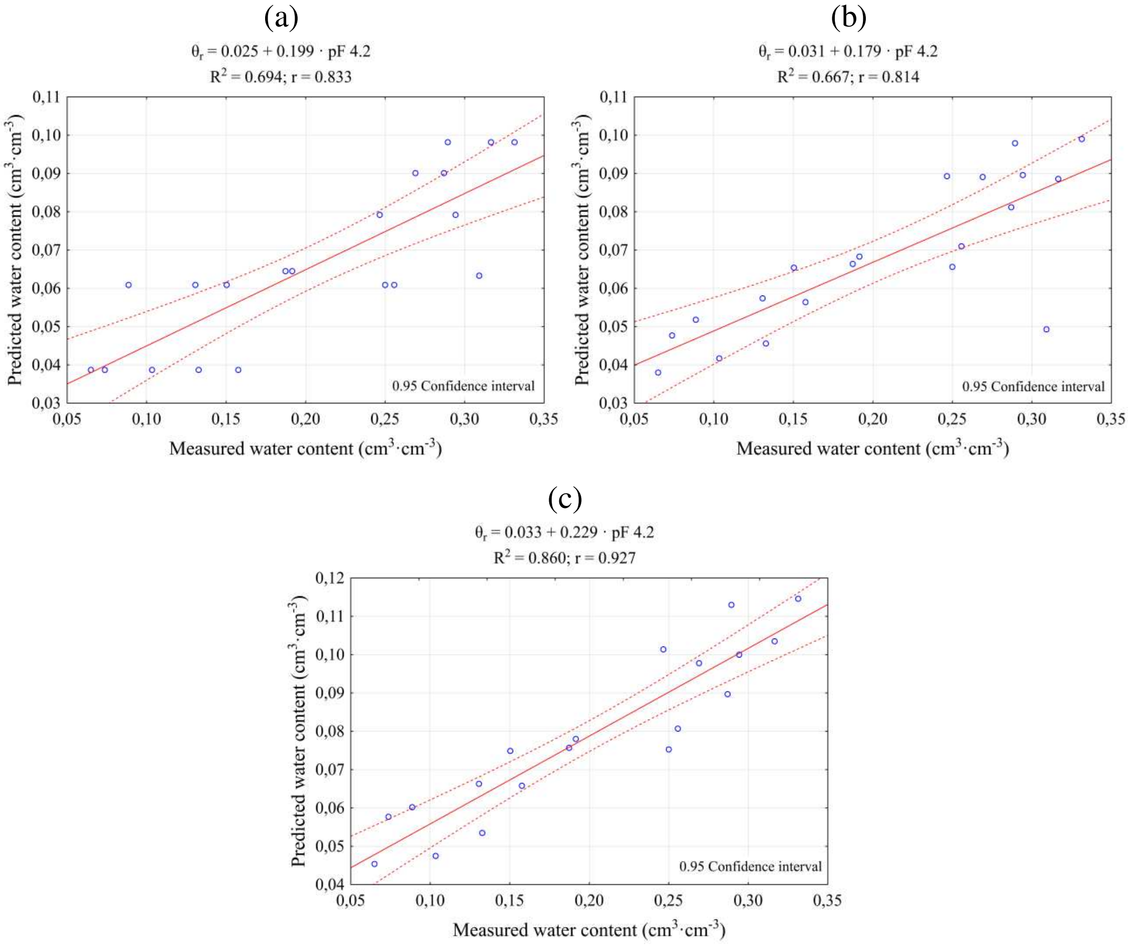

Figure 5. *Estimated vs. measured water contents (θr) of the RETC a) for first model (PTF-1), b) second model (PTF-2), c) for third model (PTF-3)*

The results of the three pedotransfer functions show statistically increase in the R^2 for θ_r (PTF-1 – $R^2 = 0.694$, $p < 0.05$; PTF-2 – $R^2 = 0.667$, $p < 0.05$ and for PTF-3 – $R^2 = 0.860$, $p < 0.05$). On the other hand two neural network models show statistically low R^2 and also no statistically significant for θ_s (PTF-1 – $R^2 = 0.101$, $p > 0.05$ and for PTF-2 – $R^2 = 0.181$, $p > 0.05$) only in case of PTF-3 results was high and statistically significant ($R^2 = 0.667$, $p < 0.05$).

In order to find interrelations of soil properties a correlation R Spearman analysis was conducted. The correlation (*Table 10*) displayed significant correlation between particle size distribution (i.e. sand, silt, clay content) and soil organic matter (SOM) content, and physical and water properties of soil.

Sand content was statistically significance ($p < 0.05$) negatively correlated with field capacity (FC), hardly accessible water and pH, whereas silt content was statistically significance positively correlated with pH. Also clay content was statistically significance positively correlated with FC and hardly accessible water. Soil organic matter was statistically significance positively correlated with water content, total porosity, FC and available water but statistically significance negatively correlated with BD and particle density. The correlation strength between SOM and soil moisture, BD,

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particle density, total porosity and FC was statistically very high ($r > 0.7$), while between SOM and available water was statistically high ($0.5 < r < 0.7$).

Table 10. *Correlation coefficient (r) between the granulometric composition and organic matter of soils and selected physical and water properties*

Variables:	**Percentage fraction with diameter (mm)**			**SOM (%)**
Physical and water properties of soils:	**Sand 2.0–0.05**	**Silt 0.05–0.002**	**Clay <0.002**	
Water content (% $cm^3 \cdot cm^{-3}$)	r = -0.2134 p = 0.366	r = -0.0488 p = 0.838	r = 0.2785 p = 0.235	**r = 0.7597**[*] **p = 0.000**
Bulk density ($g \cdot cm^{-3}$)	r = 0.2540 p = 0.280	r = 0.1771 p = 0.455	r = -0.4236 p = 0.063	**r = -0.9220** **p = 0.000**
Particle density ($g \cdot cm^{-3}$)	r = -0.0108 p = 0.964	r = 0.1185 p = 0.619	r = -0.0795 p = 0.739	**r = -0.8707** **p = 0.000**
Total porosity (% $cm^3 \cdot cm^{-3}$)	r = -0.2545 p = 0.279	r = -0.1965 p = 0.406	r = 0.4392 p = 0.053	**r = 0.8747** **p = 0.000**
pH (–)	**r = -0.6649** **p = 0.001**	**r = 0.6055** **p = 0.005**	r = 0.2810 p = 0.230	r = -0.0278 p = 0.907
Field capacity (pF 2.5)	**r = -0.5138** **p = 0.020**	r = -0.0367 p = 0.878	**r = 0.6079** **p = 0.004**	**r = 0.8723** **p = 0.000**
Available water (pF 2.5–4.2)	r = 0.0551 p = 0.817	r = -0.0984 p = 0.680	r = 0.0140 p = 0.953	**r = 0.5163** **p = 0.020**
Easily accessible water (pF 2.5–3.7)	r = 0.2828 p = 0.227	r = -0.0287 p = 0.904	r = -0.2968 p = 0.204	r = 0.2377 p = 0.313
Hardly accessible water (pF 3.7–4.2)	**r = -0.4477** **p = 0.048**	r = -0.0931 p = 0.696	**r = 0.5770** **p = 0.008**	r = 0.3251 p = 0.162

*Values in bold indicate that correlation relationships are statistically significant ($p < 0.05$)

Discussion

The examined alluvial soils are very specified and diversity in terms of texture (Iqbal et al., 2005; Bullinger-Weber et al., 2007; Ľuptáčik et al., 2012) and showed high water content values (at saturation 0.4754 $cm^3 \cdot cm^{-3}$ and wilting point 0.2065 $cm^3 \cdot cm^{-3}$). This feature may be related to the high silt (35.9%) and clay (26.1%) for all soil profiles and also mean organic matter content about 2.03% (Rawls et al., 2003; Rubio and Poyatos, 2012). Minasny et al. (2003) reported. that the hydraulic parameters are mostly sensitive to sand content and saturated water content. Gama-Castro et al. (2000) found, that the high productivity of alluvial soils is largely due to the plant available water. According to Hong et al. (2013) the most important values for agricultural use is a field water capacity (at pF 2.5), which in this case ranged between 0.2690 and 0.6784 $cm^3 \cdot cm^{-3}$.

The maximum retentive capacity (at pF 0.0) measured in laboratory was slightly different than total porosity calculated from bulk density and particle density. Paluszek (2011) stated that the difference between maximum retentive capacity and total porosity may be especially visible in the soils with high content of swelling clay minerals because maximum retentive capacity was directly determined after capillary rise to the

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state of full saturation, while total porosity was calculated on the basis of the particle density and bulk density.

Bulk density (BD) is one of important physical soil properties that characterizes soil compaction (Reynolds et al., 2002). The BD range of alluvial soils can be very wide – smaller values were observed in the Ap horizons and increased with depth (Gama-Castro et al., 2000). We observed that bulk density was related to organic matter contents, which is not in agreement with the findings of Gama-Castro et al. (2000), where they assumed that the presence of more noncrystalline material due to increased pumice weathering contributes to the low bulk density through the development of a porous soil structure.

Used the pedotransfer models of artificial neural network program Rosetta in this case, produces promising results only for sand, silt, and clay percentages and bulk density as data input. This might be due to the fact that the van Genuchten's parameters. which express the shape of the water retention curve, are sensitively affected by the wide ranges of soil properties as in this alluvial soil. Even though the data used in this study was obtained from a relatively small area, large spatial and temporal variability in physical and hydraulic properties of this alluvial soil may cause such a low performance in predictions. Similar results were observed by Minasny et al. (1999), Nemes et al. (2002) and Merdun et al. (2006).

Conclusions

In conclusion the course of the water retention curves obtained on the basis of non-linear regression analysis (R^2) for each layers of soil profiles indicates a good adjustment of the approximating functions used to the retention capacity obtained from the measurements. The pedotransfer models of artificial neural network program RETC and Rosetta in this case produces promising results and its advantages can be utilized by developing of water hydraulic characteristics of alluvial soils in future studies. The simulation of the RETC using the van Genuchten equation with a percentages of sand, silt and clay and also bulk density entry value was adequate to estimate soil water contents for four soil profiles located in the Odra River valley, in spite of the differences obtained between observed and predicted data.

On the basis of the conducted research it can be concluded that share of easily accessible water for plants was higher of the hardly accessible water for plants. It is very important from the viewpoint of the production and cultivation. Alluvial soil can hold moisture and is very fertile so in dry years plants do not suffer from a shortage of water.

Studies on water retention of alluvial soils should be extended in order to obtain more data that will be used for proper statistical inference. Other properties of alluvial soils, e.g. water permeability, should also be carried out.

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INVESTIGATION OF WILDFIRES AT FORESTED LANDSCAPES: A NOVEL CONTRIBUTION TO NONPARAMETRIC DENSITY MAPPING AT REGIONAL SCALE

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Abstract. Forest fires have been regarded as a common phenomenon that has endangered forests throughout the history of the world. It changes the vegetation and forest floor suddenly and drastically. Kernel density estimation (KDE) is a frequently employed approach in order to transform historical wildfire data into a smooth and continuous 2-D surface during the spatiotemporal analysis of forest fires. The most crucial step in KDE is to choose the appropriate bandwidth parameter. In this study, a novel approach to obtain an appropriate smoothing parameter is introduced by adapting a powerful nonparametric spline fitting methodology, namely, multivariate adaptive regression splines (MARS) into the KDE analysis of wildfires for the first time. Spatial and temporal analysis of wildfires between 2000 and 2017 in Mumcular Forest Subdistrict in Turkey is investigated by using the MARS-based KDE analysis. The proposed methodology in this paper produces a single smoothing parameter, instead of a range of values laying within a certain fixed interval, and it appears more robust to the positional uncertainties in the historical fire data, and more reliable than nonobjective visual analysis of maps generated by using alternating smoothing parameter values.
Keywords: *forest fires, kernel density, kernel bandwidth, MARS, Mumcular*

Introduction

Wildfires may often lead to serious damage to the ecosystems of forested landscapes, as well as to the diversity of flora and fauna. Preparing wildfire risk maps by using temporal fire occurrence data is very important for reducing these negative effects of forest fires. However, a wildfire event is generally recorded with its x and y coordinates by assuming each fire event as a point process, which negatively alters its 2-D exterior characteristics and propagation style. Additionally, temporal wildfire data mostly suffer from inappropriate or inadequate information and precision due to the fact that it is operationally and logistically hard to obtain the real coordinates of locations where a wildfire event starts.

This problematic issue significantly degrades the geolocational accuracy of wildfire data. These errors can eventually propagate wildfire liability estimates and result in unforeseeable negative impacts on the related risk maps. In order to tackle with this problem, two main approaches exist: *i*) to overlay a 10 × 10 km Universal Transverse Mercator (UTM) grid on administrative borderlines and to force the wildfire starting locations (i.e., points) to be confined in a polygonal area demarcated by the 10 × 10 km net, administrative borders and prescribed wild-land sites, or *ii*) to increase the resolution of lattice superimposed over the recorded wildfire starting locations in order

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to decrease geolocational inaccuracies, which, in turn, may often cause complications regarding abstraction and reduction in geospatial variability (Koutsias et al., 2004).

Derivation of a density function by using the locations of recorded observations is often considered as an effective approach to create a continuous raster surface from point data. Kernel density estimation (KDE) is a commonly preferred approach to obtain such a 2-D raster surface. KDE is a nonparametric method and its output is a surface that models the hidden density function (Anderson, 2009). KDE is a widely-recognized methodology and used in wildlife ecology (Horne et al., 2007; Katajisto and Moilanen, 2006; Kie et al., 2010), in geology and geophysics (Kagan and Jackson, 2000; Lasocki and Orlecka-Sikora, 2008), in spatial epidemiology (Anderson and Titterington, 1997; Robertson et al., 2010), in transportation planning and management (Okabe et al., 2009; Xie and Yan, 2008), in crime analysis (Chainey et al., 2008; Nakaya and Yano, 2010), in civil and mechanical engineering (Chen et al., 2000; Worden et al., 2003; Yu and Su, 2012), and in medicine (Rossiter, 1991; Zou et al., 1997).

Preceding studies

Koutsias et al. (2014) conducted a study in order to obtain a map that reveals the fire occurrence zones of Greece at a national scale by using KDE. In the study, they employed 4 different kernel widths as 1000, 2000, 3000 and 4000 m, and statistically compared them by Monte Carlo randomization test. The results indicated that increasing the width of kernels above 1000 m did not have any influence on the density surfaces.

To investigate the relationship between the fuel phenology and the spatiotemporal patterns of wildfire events in Sardinia, Italy, Bajocco et al. (2017) used KDE to convert wildfire ignition points to a 2-D raster density surface. According to the results of the study, wildfire locations were significantly associated to both anthropogenic pressure and to the spatiotemporal variation of fuel conditions in the study area.

In the study of Camarero et al. (2018), the impact of anthropogenic factors and climate systems on historical fire incidence in Mediterranean black pine forests were investigated within a long time interval (i.e., 1800-2000). Three input parameters, i.e., charcoal accumulation rates, historical records of wild fire events and tree-ring data, were collected in Sierra de Gredos (central Spain), and employed in the analysis. The historic fire data in point format were transformed into a continuous 2-D density surface by using KDE approach. The results of the study pointed that extensive grazing and uncontrolled use of forests and grasslands, together with enormous warm spring temperatures could lead to increased wildfire occurrence in Mediterranean pine forests.

The studies of Koutsias et al. (2014), Bajocco et al. (2017) and Camarero et al. (2018) have revealed that KDE is an effective way to convert the temporal wildfire data with inherent point characteristic and a certain degree of uncertainty into a continuous raster surface for defining spatial arrangement of wildfire occurrences at regional dimensions and analyzing their spatial scope, and it has also been applied by many other researchers to investigate the spatiotemporal patterns of such events (Del Hoyo et al., 2011; Gonzalez-Olabarria et al., 2012; Gralewicz et al., 2012; Hély et al., 2010).

Basics of kernel density estimation

KDE is a fundamental data smoothing problem and a nonparametric statistical technique to estimate a real valued function. In order to calculate KDE for a finite data

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sample, *i*) a grid with predefined spatial resolution is overlaid on the study area, *ii*) and the intensity at each crossing of the grid is predicted by putting a symmetrical surface on each of the point event locations (cf. *Fig. 1*). KDE in bivariate form can be defined as (*Eq. 1;* Hastie et al., 2009; Worden et al., 2003):

$$\hat{f}(\mathbf{z}) = \frac{1}{nh^2} \sum_{i=1}^{n} K\left(\frac{\mathbf{z} - \mathbf{Z}_i}{h}\right), \quad \text{(Eq.1)}$$

where $\hat{f}(\mathbf{z})$ is the prediction for the real intensity function, n denotes the number of total event records which are in point data format, h is the width of the kernel window (also referred as Parzen window (Hastie et al., 2009)), which is commonly known as a smoothing parameter, $\mathbf{z}$ gives the vector of coordinates at which the density is calculated (i.e., intersection of overlaid grid), $\mathbf{Z}_i$ represents the geolocation of each event point (i.e., x and y coordinates of wildfire events), and K indicates the kernel function that meets the following requirement (*Eq. 2*):

$$\int K(z)\,dz = 1. \quad \text{(Eq.2)}$$

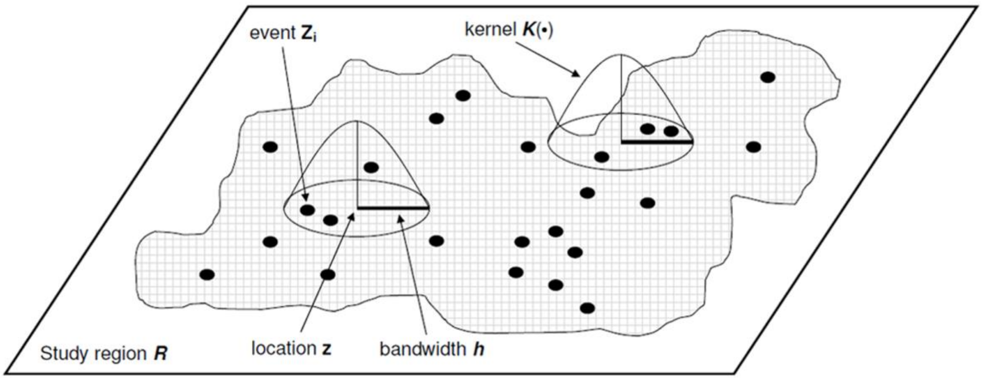

Figure 1. *KDE of a set of point observations in 2-D. (Adapted from Bailey and Gatrell, 1995)*

The choice for K can be made from a range of options such as triweight, uniform and normal. In this study, normal kernel (i.e., Gaussian), the widely recognized and employed kernel function, is preferred and its bivariate form is given as (*Eq. 3;* Wand and Jones, 1993)

$$K(\mathbf{x}) = \frac{1}{2\pi} \exp\left(-\frac{1}{2}\mathbf{x}^T\mathbf{x}\right). \quad \text{(Eq.3)}$$

At this point, it is of value to mention that the type of the kernel function does not have a significant impact; however, the selection of smoothing parameter directly and strongly affects the estimated density values. While small choice of h causes insufficient smoothing and gives spikier density values rather than the underlying density function, large values of h result in over-smoothed estimates that obscure important features of the underlying structure (Bowman and Azzalini, 1997; Wand and Jones, 1993; Worden et al., 2003).

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For KDE analysis, there are several data-driven methods introduced by researchers in order to calculate the bandwidth parameter (Bailey and Gatrell, 1995; Williamson et al., 2008; Worton, 1989), yet enumerating them all with details is out of our scope. Selecting the bandwidth value by visual inspection is another frequently used method in such analysis and this approach can be found satisfactory for many applications (Kie et al., 2010; Wand and Jones, 1995). However, a profound and extensive information and a high level of expertise regarding the area of study are required in this approach, which may not always be satisfied by the analyst.

Main motivation

In our study, as a novel contribution to KDE analysis of wildfire events, a state-of-the-art multivariate adaptive regression splines (MARS) (Friedman, 1991) algorithm is applied to predict a suitable value for smoothing parameter. For this purpose, Mumcular Forest Subdistrict located in City of Muğla, Turkey is selected as study area, and wildfire data of the region between years 2000 and 2017 are used. KDE maps with different bandwidths are produced, and then compared by correlation analysis. Correlation values are plotted against bandwidths for building the MARS model function. By using the generated MARS model function, the appropriate value of smoothing parameter is decided. Then, KDE maps of wildfire events in the study area are produced to expose the behavior of wildfires in both spatial and temporal domains in the region. Then, final density map is obtained and converted into a mean density map representing the average intensity values for each of the administrative polygons in the entire region.

The paper is organized as follows. The next section represents the materials and methods including a brief mathematical background of the MARS approach, study area, the wildfire data and the estimation of suitable smoothing parameter. The results then are discussed in the third section. And finally, the last section concludes our study.

Materials and methods

Multivariate adaptive regression splines (MARS)

In this subsection, a brief introduction to MARS algorithm is given based on Friedman (1991), Hastie et al. (2009), Nalcaci et al. (2018) and Özmen et al. (2018).

MARS is a nonparametric spline regression method and it uses one-dimensional piecewise linear basis functions (BFs) in order to define a relationship between a response variable and its predictors. MARS has many successful implementations in almost every branch of science and engineering (Alp et al., 2011; Çevik et al., 2017; Durmaz et al., 2010; Özmen et al., 2014; Quirós et al., 2009).

The BFs of MARS are in the following form (*Eq. 4*):

$$[x-t]_+ = \begin{cases} x-t, & \text{if } x > t, \\ 0, & \text{otherwise,} \end{cases} \qquad [t-x]_+ = \begin{cases} t-x, & \text{if } x < t, \\ 0, & \text{otherwise,} \end{cases} \tag{Eq.4}$$

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where t is a univariate knot determined using the dataset. The range of each predictor variable is cut into subsets of the full range by using knots which defines an inflection point along the range of a predictor. The MARS model in its general form can be expressed as (*Eq. 5*):

$$Y = \beta_0 + \sum_{m=1}^{M} \beta_m B_m \left(\boldsymbol{X}^m \right) + \varepsilon. \qquad \text{(Eq.5)}$$

In *Equation 5, Y* is the response, β_0 is the intercept, β_m denotes an unknown coefficient of the mth BF, or the constant 1 ($m = 0$), M is given as the number of BFs in the present model, B_m is a BF or product of two or more BFs, $\boldsymbol{X}^m$ is the vector of predictor variables contributing to the function B_m, and finally, ε is an additive stochastic component with zero mean and finite variance.

The basic form of the mth BF is given as (*Eq. 6*):

$$B_m \left(\boldsymbol{X}^m \right) := \prod_{j=1}^{K_m} \left[s_{\kappa_j^m} \cdot \left(x_{\kappa_j^m} - \tau_{\kappa_j^m} \right) \right]_+ . \qquad \text{(Eq.6)}$$

In *Equation 6, K_m* denotes the total number of truncated linear functions multiplied in the mth BF, $x_{\kappa_j^m}$ indicates the input variable corresponding to the kth truncated linear function in the mth BF, $\tau_{\kappa_j^m}$ is the knot location for $x_{\kappa_j^m}$ and, finally, $s_{\kappa_j^m} \in \{\pm 1\}$.

The final MARS model is determined with a two-step process including *forward pass* and *backward pass* stages. Forward pass generally creates an over-fit model. At the backward stage, the over-fitted model is simplified in terms of its complexity without degrading the overall fit to the data by imposing a *lack-of-fit* criterion defined by the following *generalized cross-validation* (GCV) formula (*Eq. 7*):

$$\mathrm{LOF}(\hat{f}_\alpha) = \mathrm{GCV}(\alpha) = \frac{\sum_{i=1}^{N} (y_i - \hat{f}_\alpha(\boldsymbol{X}_i))^2}{(1 - Q(\alpha)/N)^2}, \qquad \text{(Eq.7)}$$

where $Q(\alpha)$ denotes the effective number of parameters in the model, and N is the number of observations.

Study area

In the Mediterranean and Aegean regions of Turkey, forest fire is still one of the greatest natural hazard problems. Forest Subdistrict of Mumcular in Muğla province (cf. *Fig. 2*) located in the south-west Aegean region of Turkey is selected, since this region has experienced large number of wildfire events for the last two decades. The study site covers an area of nearly 322 km^2, and its altitude varies between 0 to 870 m above mean sea level. Forested lands constitute nearly 67% (214 km^2) of the study area, the canopy density of 43% of the forested lands ranges from 41 to 100%. Cultivated lands cover 29% of the area (i.e., 93 km^2). The land classification is derived by using the latest digital stand map of the subdistrict in ArcMap shape file format and can be seen in *Figure 3*. “*Pinus brutia*” is the prevailing tree species in the subdistrict region and it is

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extremely flammable and fire-prone due to its resinous structure. The detailed info on study area is given in *Table 1*.

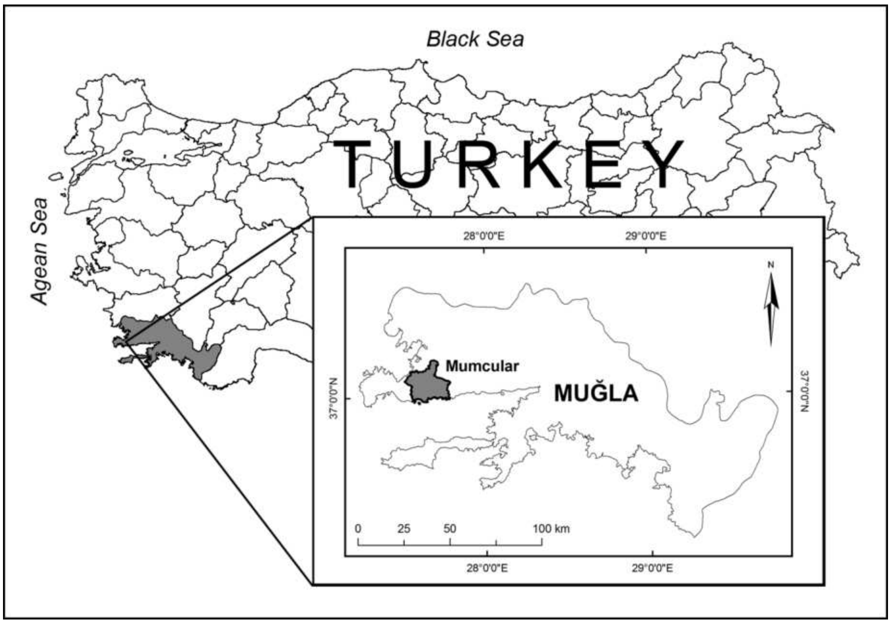

Figure 2. *Study area*

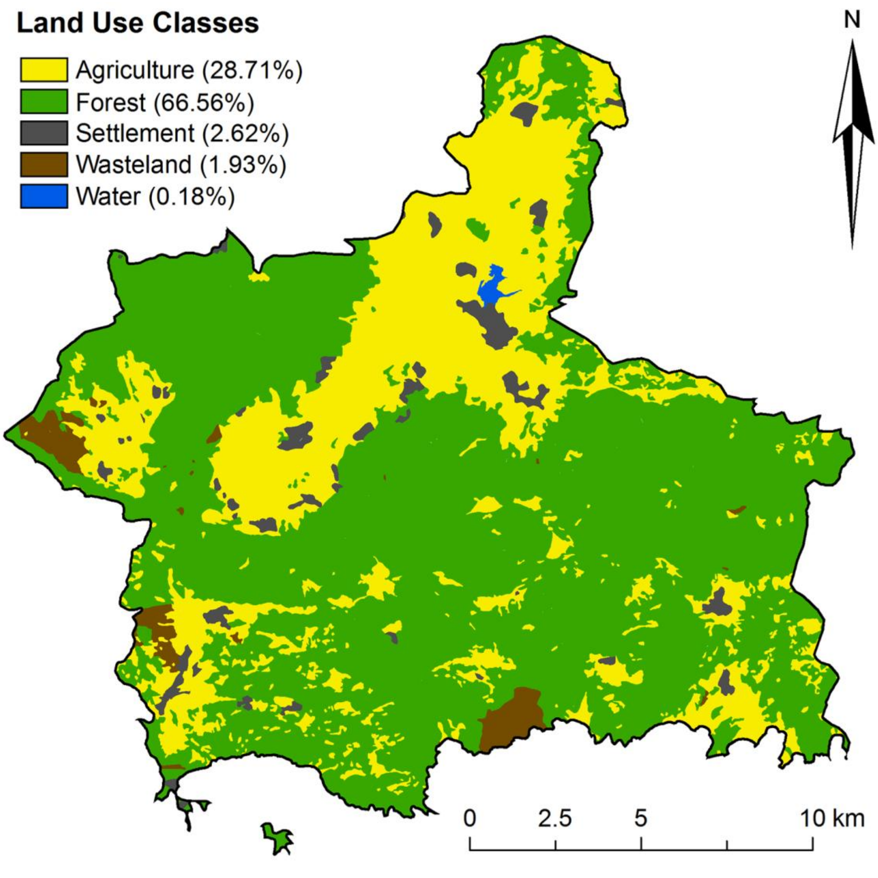

Figure 3. *Land-use map of the study area (Produced from the latest version of the digital stand map of the area supplied by Mumcular Forest Subdistrict)*

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Table 1. *Basic information on the forest subdistrict area*

Surface area	321.7 km^2
Number of compartments (polygons)	373
Average value of wildfire occurrence per compartment	0.582
Number of wildfire events (years 2000-2017)	217
Min. compartment area	0.0029 km^2
Max. compartment area	9.43 km^2
Average compartment area	0.83 km^2

Dataset

The temporal wildfire events data supplied by Mumcular Forest Subdistrict are in spread sheet format and belong to years between 2000 and 2017. There are 217 fire ignition locations in total. The coordinate system of the digital stand map of the subdistrict conforms with WGS84/UTM projection. The map comprises 373 compartments, which can be defined as administrative polygons (cf. *Fig. 4*). As a preliminary preparation prior to the analysis, the complete wildfire data are merged with the digital stand map in *ArcMap 10.3*.

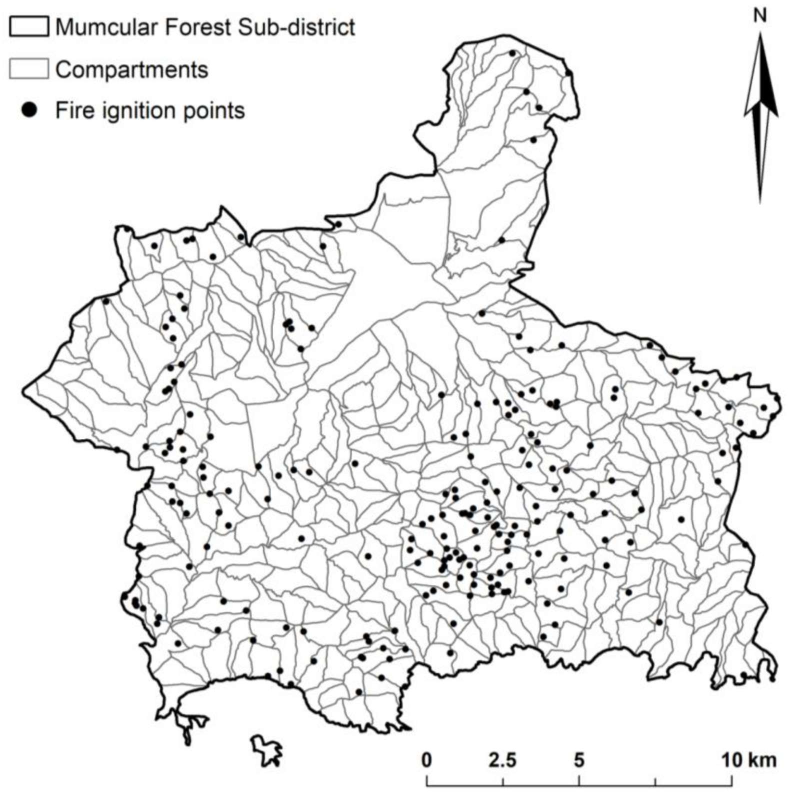

Figure 4. *Compartments of the digital stand map and ignition points*

Choice of KDE smoothing parameter

Although true geolocations of wildfires are not known, the true number of wildfire events is available for each compartment. Therefore, instead of utilizing above

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mentioned traditional methods, three completely unique and distinct random point distributions are created (i.e., R1, R2 and R3), and by this way, exact number of wildfire occurrences in each of the administrative polygons (i.e., compartments) is realized.

This approach lets us not only provide the ability of introducing randomness into the analysis, but also confine the wildfire starting points in relatively smaller administrative areas (i.e., compartments in the stand map) as compared to the traditional method (i.e., superimposing 10 × 10 km net over the area). As a result of this process, an improvement over positional inaccuracies is achieved.

To select an appropriate smoothing parameter (i.e., *h*), a different and a novel strategy is followed. First, KDE maps for various *h* taking values within the set {1,2,…5000} are obtained for each random point distribution, i.e., R1, R2 and R3, which means that 15000 KDE maps are generated on aggregate. Then, mutual comparison between each pair of KDE maps for each value of *h* is carried out via a simple analysis of correlation. Correlation values for each random point distribution for several *h* values (i.e., 100, 500, 2500 and 5000 m) are given in *Table 2*.

Table 2. *Correlation coefficients vs. smoothing parameter h*

Random distributions→ *h*↓	**R1-R2**	**R1-R3**	**R2-R3**
100 m	0.4015275	0.4427352	0.4259254
500 m	0.9225721	0.9245119	0.9205818
2500 m	0.9993016	0.9990487	0.9994646
5000 m	0.9990197	0.9990199	0.9990199

Next, in order to include more stochasticity in the process, random noise is added to the correlation values of each pair of random point distributions. This noise mimics the uncertainties inherited in the coordinates of the wildfire locations which may eventually propagate to the correlation analysis between each pair of point distributions. By this way, extra randomness in the positional inaccuracies of wildfire events is introduced. The standard deviation of the noise added is equal to the standard deviation of the correlation values of that specific pair. The plots of the correlation values with noise with respect to *h* for three random point distributions are depicted in *Figure 5*.

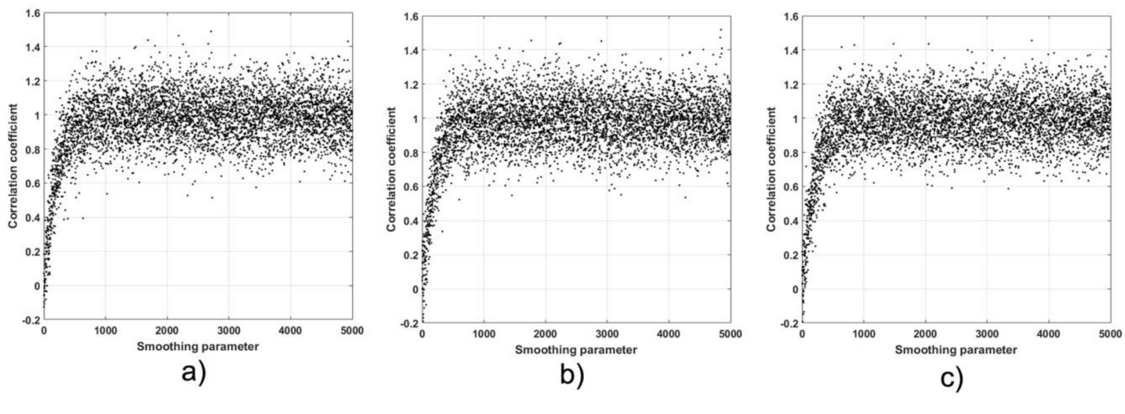

Figure 5. *Correlation coefficients with stochastic noise vs. smoothing parameter for a) R1-R2, b) R1-R3, and c) R2-R3. (Please note that the range of correlation values exceeds the usual range of [0, 1] due to the added stochastic noise)*

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Then, the corresponding MARS model that gives the best fit to the correlation coefficient data with noise for each pair of random point distributions according to GCV criterion given in *Equation 7* is obtained. The graphs of individual MARS models are available in *Figure 6*.

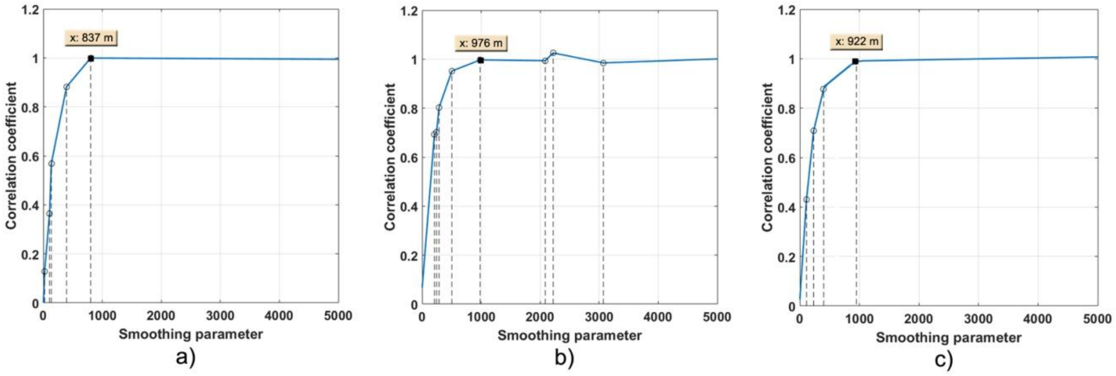

Figure 6. *Plots of MARS model functions for a) R1-R2, b) R1-R3, and c) R2-R3. In the figure, o indicates the location of knot points (i.e., t) between two consecutive BFs, and ■ denotes the position of the knot location where further increase in h results in over-smoothing in the corresponding KDEs*

The obtained MARS models for R1-R2, R1-R3 and R2-R3 are given as:

for R1-R2:
$BF_1 = \max[0, 395 - x_1]$,
$BF_2 = \max[0, 137 - x_1]$,
$BF_3 = \max[0, x_1 - 837]$,
$BF_4 = \max[0, x_1 - 23]$,
$BF_5 = \max[0, x_1 - 107]$ and,
$Y = 1.1146 - 0.00092793 \cdot BF_1 - 0.0056183 \cdot BF_2 - 0.00028838 \cdot BF_3 - 0.0037535 \cdot BF_4 + 0.0040407 \cdot BF_5$,

for R1-R3:
$BF_1 = \max[0, x_2 - 976]$,
$BF_2 = \max[0, 209 - x_2]$,
$BF_3 = \max[0, 503 - x_2]$,
$BF_4 = \max[0, 287 - x_2]$,
$BF_5 = \max[0, 2219 - x_2]$,
$BF_6 = \max[0, x_2 - 2087]$,
$BF_7 = \max[0, x_2 - 2531]$,
$BF_8 = \max[0, 2531 - x_2]$ and,
$Y = 0.97569 - 0.00009659 \cdot BF_1 - 0.00148852 \cdot BF_2 - 0.000631463 \cdot BF_3 + 0.00072293 \cdot BF_4 - 0.00052987 \cdot BF_5 + 0.0003719 \cdot BF6 - 0.00027321 \cdot BF7 + 0.00043687 \cdot BF8$,

and finally for R2-R3:
$BF_1 = \max[0, x_3 - 401]$,
$BF_2 = \max[0, x_3 - 113]$,

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$BF_3 = \max[0, 113 - x_3]$,
$BF_4 = \max[0, x_3 - 233]$,
$BF_5 = \max[0, 922 - x_3]$ and,
$Y = 0.68602 - 0.00050397 \cdot BF_1 + 0.0018125 \cdot BF_2 - 0.0031154 \cdot BF_3 - 0.0013047 \cdot BF_4 - 0.00050019 \cdot BF_5$.

In the above equations, x_1, x_2 and x_3 denote the correlation values of R1-R2, R1-R3 and R2-R3, respectively.

As indicated by the correlation coefficient values in *Table 2*, resemblance between R1, R2 and R3 becomes more obvious when the value of smoothing parameter gets larger. Actually, this is an expected result since over-smoothed KDE estimates are generated with increasing values of *h*, which results in less variability, or i.e., higher similarity between the maps. On the other hand, for narrower choices of smoothing parameter, spikier estimates containing lots of spurious local structure are obtained. This behavior is illustrated in *Figure 7*.

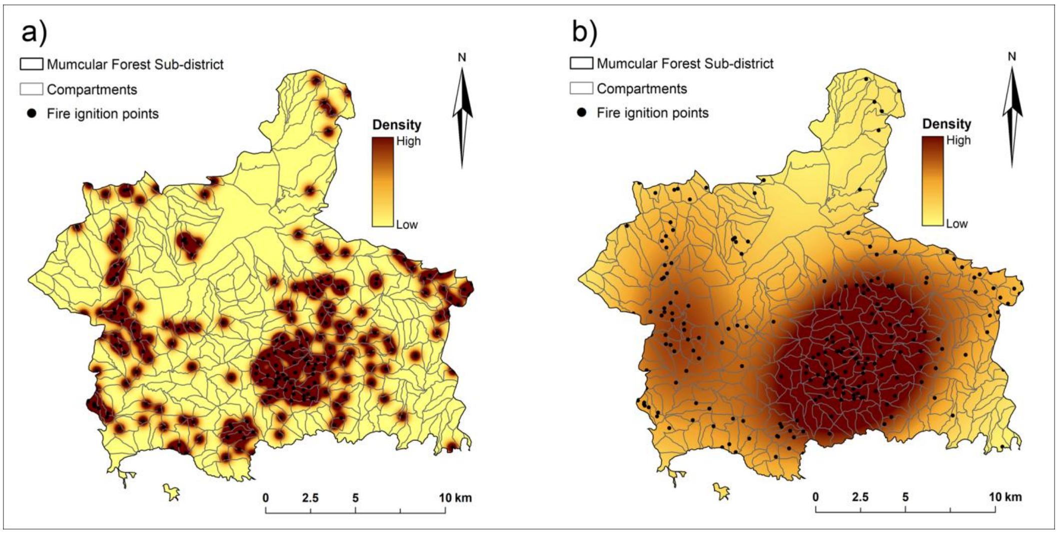

Figure 7. *KDE maps of the study area: a) h = 250 m, and b) h = 2500 m*

Results and discussion

Although the increments between successive correlation values with increasing *h* values are not so distinctive as depicted in *Table 2*, the amount of variation against increasing values of *h* can be observed very clearly in *Figure 6* for R1-R2, R1-R3 and R2-R3. A very sharp increase in the rate of change in correlation values are observed, and then the each curve exhibits a stabilized behavior above a certain *h* value (i.e., over-smoothing prevails).

The advantage of using MARS approach which employs piecewise linear splines in modelling is that the selection of knot locations is completely data-driven, i.e., specific to the dataset. Since the BFs that give smallest increase in the residual sum of squares are omitted from the model at each iterative step, an optimal model is generated at the end. The mean square error (MSE) versus GCV values for each MARS model shown in *Figure 6* is also illustrated in *Figure 8*. The change in MSE and GCV values during the iterations in the backward step can easily be observed in *Figure 8*. For a typical MARS

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model building process, lines of MSE and GCV seem close to each other at the early stages. However, as the number of BFs included in the model increases, GCV diverges from MSE and rises up. The corresponding MSE and GCV values of the final MARS models are given *Table 3*.

Table 3. *MSE and GCV values of the final MARS models*

MARS model	MSE	GCV
R1-R2	0.01804	0.01812
R1-R3	0.01829	0.01842
R2-R3	0.01771	0.01779

As it is obvious in *Figure 6,* the specific knot location between the two consecutive BFs where the MARS model curve forms a kink shape indicates the specific value of *h* for that random point distribution. Further increase in *h* brings no significant change in the correlation values which simply indicates that the over-smoothing effect dominates in that specific KDE.

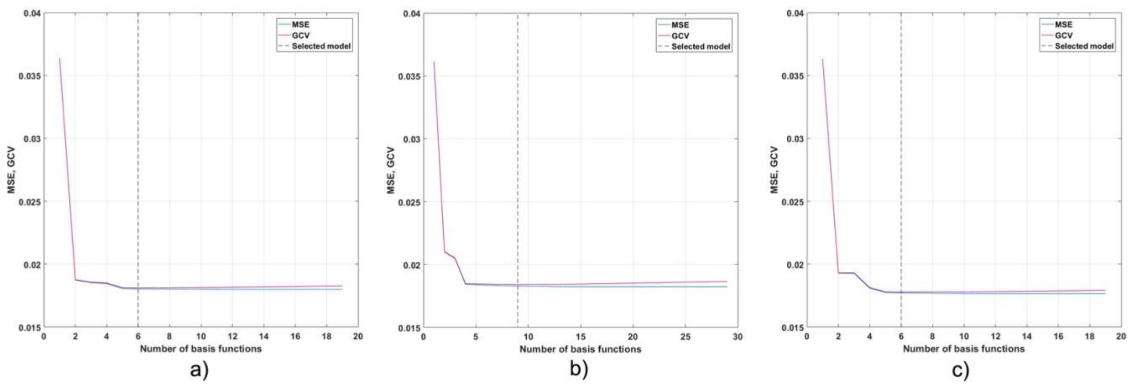

Figure 8. *MSE vs. GCV values for the MARS models of a) R1-R2, b) R1-R3, and c) R2-R3*

According to Silverman (1998), an appropriate choice of smoothing parameter is a crucial factor for the success of a KDE, and plotting several density estimates with various *h* values and choosing the one in accordance with the user's idea about the density often gives quite satisfactory results. This approach is also known as subjective choice of smoothing parameter (i.e., smoothing by eye). However, as again indicated in Silverman (1998), an inexperienced user may need somewhat an automated way to find a value of *h* which can be used as a reliable starting point for further subjective adjustment. In the studies of Koutsias et al. (2014) and Bajocco et al. (2017), smoothing parameter was chosen based on nearest neighbor distance method; however, this approach only generates rough estimates for the value of smoothing parameter when the inevitable positional uncertainties in the wildfire locations are considered.

On the other hand, the use of MARS has unique advantages in the modelling of complex environmental dynamics with uncertainties at some degree. Since the method is nonparametric, it does not make any specific assumption about the underlying functional relationship between the dependent and independent variables. Additionally, the selection of BFs is specific to the dataset in MARS which makes it and adaptive

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procedure to handle large and complex datasets. During MARS-model building, the BFs are directly obtained from the observations and their space partitioning property results in an adaptive model. By this way, the flexible piecewise spline BFs of MARS can smoothly approximate the discrete data with noise and model its inherent characteristics. As it is obvious in *Figure 6*, our KDE approach integrated with MARS gives unique smoothing parameter values which can definitely be employed as reference point for further adjustment by the user.

The specific h values for R1-R2, R1-R3 and R2-R3 are 837, 976 and 922 m, respectively. Very similar h values from the three different random point distributions reveal that even though these three random point distributions are spatially distinctive from each other and their pair wise correlation values include stochastic noise, MARS is able to capture the inherent characteristic of the dataset by using smoothing and data-driven space partitioning properties of the piecewise linear BFs.

Thus, the final smoothing parameter value of the Gaussian KDE for the study area is taken as the average of these three, which is equal to 912 m. The seasonal KDE maps and the final KDE map of the study area are prepared accordingly by using *CrimeStat 3.3* software (Levine, 2004).

When the seasonal KDE maps are analyzed (cf. *Fig. 9*), fire events in the study area temporally and spatially exhibit different cluster patterns.

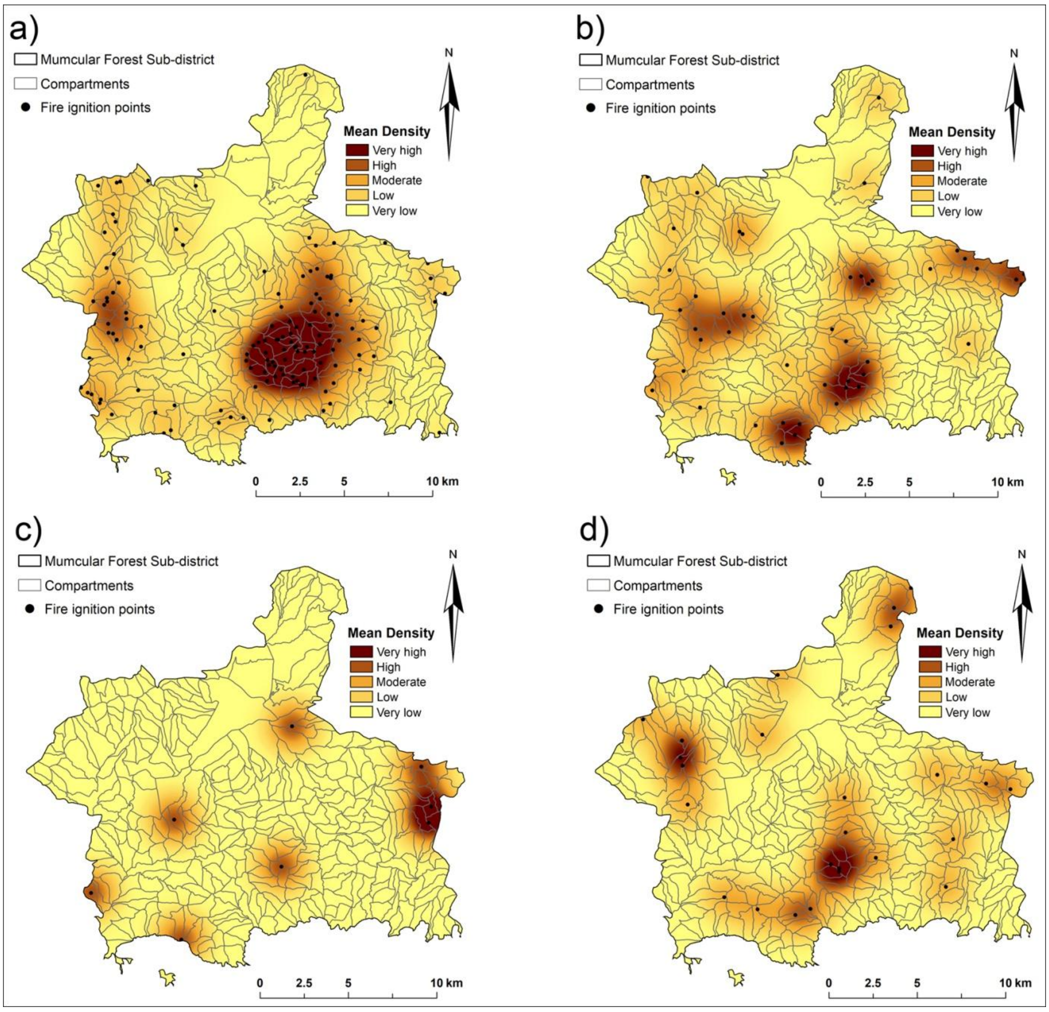

Figure 9. *Seasonal KDE maps: a) summer, b) fall, c) winter, and d) spring*

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The majority of forest fires (i.e., 67.6%) occur in the summer seasons (cf. *Fig. 9a*), which is obviously expected, and they are manly concentrated in the south-east central part, and small clusters are observed along the western part. Fire incidents that take place in summer are mostly located either in dense forested areas with closed canopy structure or around agricultural lands that are also close to settlement areas. Thus, these fire events would be associated with two factors: *i*) higher rate of human activities for recreational purposes during summer season, and *ii*) fires ignited on purpose by farmers for the removal of husk, which is often encountered in agricultural regions of Turkey.

Fire events that happen in fall have a 19.5% share in total incidents and exhibit a bit more homogenous spatial characteristic than summer season events (cf. *Fig. 9b*). They are mainly observed along the west side, in both south central and south parts, and small clusters exist along eastern side. A few events are observed in the dense forested areas; however, most of the incidents are located around the agricultural areas in the west and the north. This kind of spatial structure may arise from the raise in straw fire events.

Fire events in winter have the smallest share in all the seasons (i.e., 2.8%) and only limited number of events is observed in the central region, as well as the middle and upper parts of the western border (cf. *Fig. 9c*). Following the winter, spring fire events have an increasing trend in number (10.1%) and they are clustered in the central-south and eastern areas of the region, a few events are observed at northern cape as well (cf. *Fig. 9d*). Since the harvest season is not started yet in spring, these events can hardly be related with straw fires, but can be attributed to increasing recreational activities.

As the final step, the mean density map that shows the average intensity for each administrative polygon (i.e., compartment) is generated by employing the final intensity map of the area (cf. *Fig. 10*).

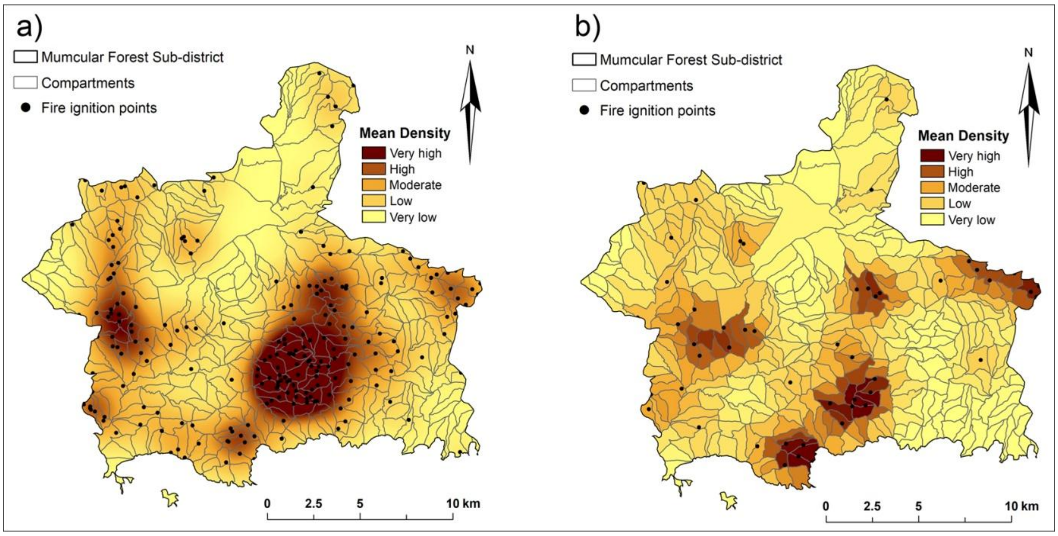

Figure 10. *a) Final density map, and b) mean density map of the study area*

Conclusion and outlook

Fire management planners always demand for elaborately prepared maps, which reveal the relation between wildfire events and several contributing characteristic attributes of the area of study, like topography, demographic structure, meteorological factors etc., for proper analysis of forest fire risk. However, historical fire data used in

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this kind of analysis are mostly in point format and seriously suffer from positional inaccuracies. KDE method is often preferred for converting such data into continuous raster surface, and finding an appropriate smoothing parameter value that suits to the area under investigation would be of vital importance.

In this study, above mentioned points are addressed within the frame of Mumcular Forest Subdistrict case, and quite effective solutions are introduced in order to deal with them. These methods can easily be applied on different geographical areas.

The method employed to reduce the positional uncertainties is simply based on utilizing the temporal wildfire records at a finer spatial resolution, i.e., compartments in the stand map. Even though the geolocation of wildfire starting points at this spatial resolution may still include some level of uncertainty, an improvement is achieved by confining the wildfire starting locations into smaller administrative polygons.

In order to determine an appropriate smoothing parameter value h, a novel methodology is proposed by utilizing MARS for the first time in the KDE-based analysis of historical wildfire data. The results of our study indicate that MARS is an effective method for modelling dynamic and complex environmental processes, as in the case of wildfires, particularly, when expert judgment is not readily available. The results show that MARS is a proper choice to select suitable value of smoothing parameter for the KDE of wildfire events, and we definitely consider that the effectiveness of the MARS approach for dynamic modelling of wildfires should be further investigated by other case studies.

MARS provides important results on modelling the KDE of wildfires in Mumcular Forest Subdistrict in a 18-year period. It can enable us to obtain valuable and reliable information on spatiotemporal patterns of wildfires. This information is quite important for fire management planners' analytic studies on short- and long-term planning. As illustrated in this study, incorporating effective application of modern applied mathematics within geospatial analysis tools would have a significant contribution to fire departments and help them take more effective and timely measures for more risky areas.

In future researches, this study can be extended in order to produce a comprehensive wildfire assessment map of the study area by the inclusion of significant contributors to wildfires such as topographical, meteorological and anthropogenic factors together with KDE analysis. Within this phase of the study, topographic factors such as elevation, slope and aspect would be derived from the digital elevation model of the area. Meteorological data (i.e., temperature, rainfall and wind) would be acquired from the local ground stations in the area and interpolated by using kriging technique in order to produce the corresponding 2-D raster surfaces. For anthropogenic factors, distance from road networks and residential areas with certain population densities would be considered and realized by creating associated buffer zones.

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STUDY ON THE SPATIAL EXPANSION OF GARDEN CITY UNDER THE ECOLOGICAL GREEN SPACES PROTECTION (QUJING AREA, CHINA)

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Abstract. With the acceleration of urbanization in China, the phenomenon of occupying ecological green space is becoming more serious in the process of urbanization, especially in Chinese Nationally Designated Garden Cities. The study simulates the dynamic change of Qujing in Yunnan Province of the 1993-2015 period. According to different ecological green space protection intensity, four kinds of scenarios were set up to predict the urban expansion in 2030. The spatial and temporal characteristics of urban expansion under different scenarios are quantitatively studied by using the Urban Expansion Intensity Index (*UEII*) and Urban Expansion Speed Index (*UESI*). The results show that the SLEUTH model has strong applicability and can better reflect the spatial and temporal characteristics of the urban expansion in Garden City. In four scenarios, the urban area shows an increasing trend, and the urban expansion speed and urban expansion intensity decrease with the increase of ecological protection intensity. Comparing four scenarios, we found that the urban development trend predicted by the ecological development scenario is more similar to the urban planning, and this conclusion has been confirmed by the distribution of urban areas of 2018. In ecological development scenario, the urban ecological land has been effectively protected and the development of the city has not been affected, which is beneficial to the sustainable development and the protection of the ecological environment. For urban planning, the suitable measures of ecological green space protection can restrain the urban expansion, and the ecological protection-oriented development mode accords with the development trend of Garden City.
Keywords: *ecological green space, urban expansion, SLEUTH model, dynamic change, sustainable development*

Introduction

Urban expansion is the inevitable result of social and economic development (Hansen et al., 2005). With the acceleration of urbanization in China, a large number of ecological green spaces were occupied in the process of urban construction. This phenomenon is more prominent in Chinese Nationally Designated Garden Cities, leading to a large number of urbanization problems, such as heat island effect, city haze, soil erosion and so on (Black, 1999; Peck, 2005). Most urban development researches attach importance to the solution of two problems: one is about ensuring the sustainable development of cities, another concerns minimizing the impact on ecological environment during urban expansion (López et al., 2001; Roberts and Diederichs, 2016; Boulos, 2016).

In recent years, many researchers have established a large number of models to simulate and predict the spatial expansion of cities. On the basis of two experiments conducted in San Francisco and the Washington area, Clarke (1997) and Silva (2002) pointed out that with its high accuracy, the SLEUTH model not only can be used to simulate urban evolution by making use of historical data, but also can be applied to predicting urban expansion from a macro perspective and a meso perspective (Silva and Clark, 2005). Pijanowski (2002) used the Land Transformation Model (LTM) to explore the consequences of future urban changes of 2020 and 2040 with the help of non-urban sprawl and urban-sprawl trends. Results of this work have significant implications for the Lake Michigan Lake Area Management Plan developed by the United States Environmental Protection Agency recently. But the model did not embody the influences of terrain and policy on the urban expansion. The Cellular Automata (CA) model can be used to simulate different urban forms and developments in the planning of sustainable cities (Yeh et al., 2001). However, due to the fact that the conversion rules of cell can not be quantified in practical application, the accuracy of model simulation is low. Yea explored the simulation of urban growth by applying SLEUTH model to Yilan, Luodong and Suao in the Yilan Delta area of northeast Taiwan, so that he can make use of some historical data and study the dynamics of the region. He found that the result of the model's application for predicting city development in the Yilan Delta area shows that the cities of Yilan and Luodong are developing and expanding more evenly from the city center (Yea and Yang, 2007). It is found that when CA model is applied to simulating the dynamic evolution of complex urban expansion, the transformation rules and parameters of the model are difficult to design quantitatively and it is difficult to explain the physical meaning of the model parameters.

SLEUTH model can simulate and forecast urban expansion based on cellular automata. It can predict the possibility of urban expansion according to traffic, topography and urban development constrains because pixels of the current city fucntion as seed points to present the future development of the city through their diffusion (Herold et al., 2003; Xian and Crane, 2005; Silva and Clark, 2005). Compared with the traditional CA model, the most important feature of SLEUTH model is the optimal parameter combination for urban expansion process calculated by Monte Carlo iterative simulation. Because of its high simulation precision, easy access and portability, the model has been widely used in the study of urban expansion simulation and prediction (Dietzel and Clarke, 2004). At present, the SLEUTH model is often used in dynamic simulation studies of large cities. However, there are few researches on Garden Cities with higher greening coverage (Laura et al., 2008; Dietzel and Clarke, 2007; Mc and Justin, 2013). Based on the existing theory of urban expansion and the application of SLEUTH model, this paper takes the Nationally Designated Garden Cities (Qujing) as the research area. According to the actual situation of the research area, four kinds of scenarios are set up based on the protection intensity of different ecological green space, and the spatial expansion of the city is simulated and forecasted. The spatial and temporal characteristics of urban sprawl in different scenarios are quantitatively studied by using the Urban Expansion Intensity Index (*UEII*) and Urban Expansion Speed Index (*UESI*). Comparing four scenarios with urban planning of 2030, the most similar scenario with urban development trends was identified, and the conclusion was confirmed by the distribution of urban areas of 2018. Basic laws and trends of urban spatial expansion in the research area are revealed, which provide

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references for the sustainable development and urban planning of Nationally Designated Garden Cities in China.

Materials and methods

Study area

Qujing, located in the eastern part of Yunnan Province, China, is an important industrial and commercial city and transportation hub in Yunnan province. It is adjacent to Guizhou province and Guangxi Zhuang Autonomous Region. With the rapid urbanization construction, the whole city has been increased from 81.27 km^2 in 1997 to 253.8 km^2 in 2016, but its urban green space area has also reduced (Ding, 2010). Located in the low latitude area, Qujing has a subtropical plateau type monsoon climate. The annual average temperature is 14.5 °C, and the annual rainfall at the urban area is over 1,008 mm. The difference between summer temperature and winter temperature in most areas is small, which means the climate is very mild and suitable for human habitation. The average forest coverage is about 45%, and the urban green coverage is 36.16%, it is a typical Nationally Designated Garden City in the west of China. In this paper, the study area is the central area of Qujing, which covers the northern Qiling District and southern Zhanyi District. The research area lies between longitude 103°42’18” to 103°54’54” east and latitude 25°24’18” to 25°38’24”north, the total area is about 1061.9 km^2 (Yuan et al., 2017). The research area locates in the plain area; the terrain is low in the middle: the middle is flat dam area, the east-west side is mountainous. The spatial pattern shows the area’s transition from the city core to the suburb (*Fig. 1*).

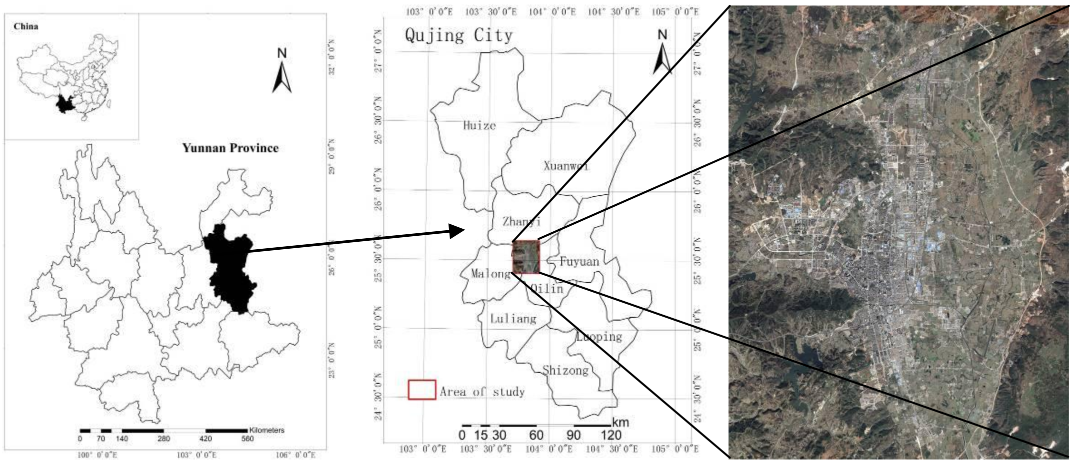

Figure 1. *Location map and high-resolution image of the research area. (Image photos from Google Earth in September 2017)*

Data acquisition and preprocessing

The basic data of the study area were gathered from 1993 Landsat TM images, 2000 Landsat ETM images, 2009 Landsat TM images and the Landsat OLI images in 2015 and 2018 (*Fig. 2*), which were collected in September to December. The cloud cover was less than 5% and they can be used for visual interpretation (Morf, 2011). In

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addition, the data of digital elevation model (DEM) for 2015 in the research area, the urban road traffic map of the corresponding year and the urban planning map of the research area were collected. The classification of remote sensing images was based on supervised classification method, through which researchers classified the images in accordance with six land use types including urban land, bare land, farmland, forestland, grassland and waters. Then we used the Government Gazette of Qujing and the data of field survey to test the precision of classification results. The total precision data of the classification resulting in four periods were 83.17%, 86.52%, 85.45% and 87.89% respectively. Additionally, the kappa coefficients of these periods were higher than 0.79, which can meet the needs of this paper. According to the operational requirements of the SLEUTH model, the traffic road layer, slope layer, shadow layer, city range layer and exclusion layer were made by ArcGIS (Rafiee et al., 2009; Feng et al., 2008).

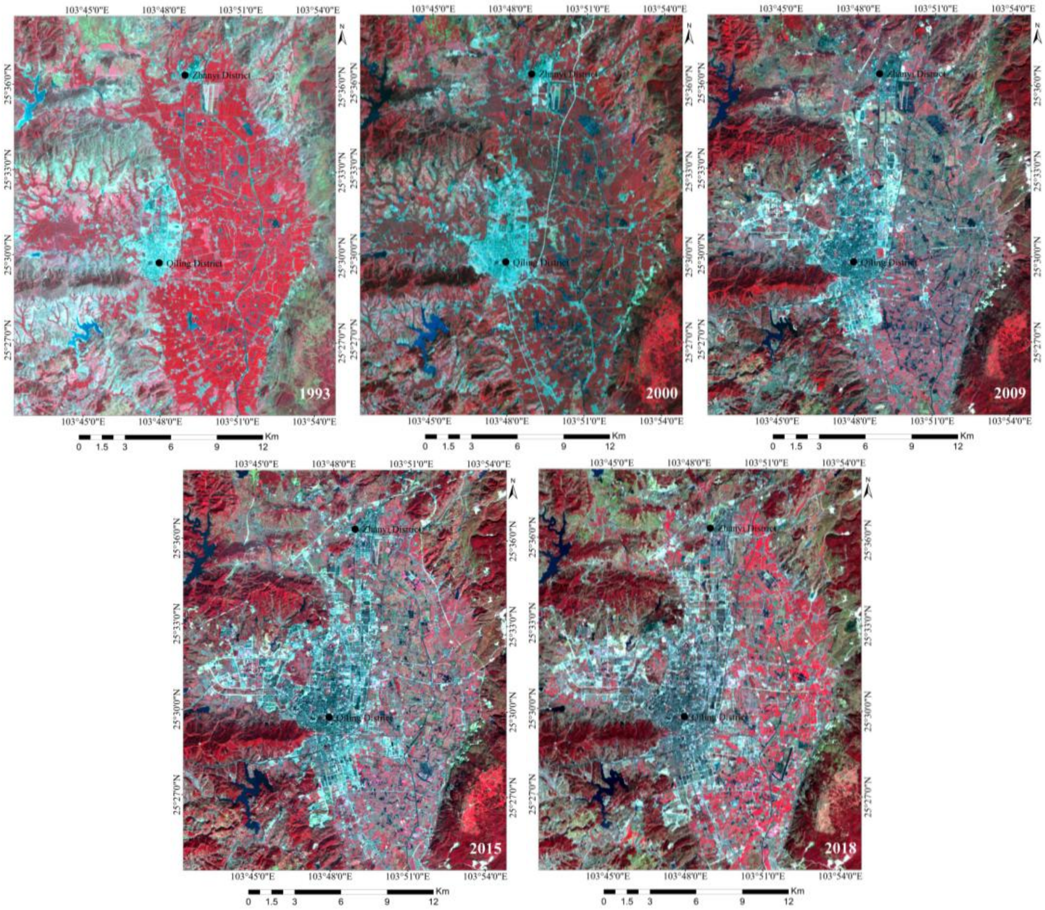

Figure 2. *Remote sensing images from 1993 (Landsat 5), 2000 (Landsat 7), 2009 (Landsat 5), 2015 (Landsat 8) and 2018 (Landsat 8) in the central areas of Qujing. (The false color composite of Landsat5 (RGB)432, Landsat7 (RGB)432 and Landsat8 (RGB)543)*

Based on the urban road traffic map of the year, the layer of the traffic road was created by digital processing. According to the classification of road grade, they were given different weights, among which the main urban road was given a weight value of 100, the urban secondary road was given a weight value of 50, and the non-road

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assignment is 0. Researchers collected the urban range vector data through the supervision of the classification of the years and then convert them to different spatial resolution grids with the use of the ArcGIS Toolbox and the classification of the generation of research areas of the city map. Upon finishing this work, the urban layer was created in the fourth period of remote sensing image prepossessing. The slope layer was generated by the re-projection and re-sampling of DEM data, which was expressed as a percentage slope value. The shadow layer was the background of the model image output which improves the spatial visual effect of the simulated urban spatial expansion process. The shadow layer was embedded as a background layer and was not involved in the operation of the model. The exclusion layer, whose range is 0-100, was a constrained layer that restricted the simulated expansion of the urban area. When this value tends to be 0, the probability of urbanization is higher, and when this value tends to be 100, the probability of urbanization is lower. All of the input layers were converted to 8-bit grayscale raster images projected by horizontal Mercator (UTM_WGSl984 48N), and the data format was required to be in GIF format.

SLEUTH model and the setting of multiple scenarios

The SLEUTH model was developed by Professor Clark of the University of California. The model is capable of predicting the dynamics of urban growth. The basic urban growth procedure of SLEUTH is a cellular automaton, in which urban expansion is modeled in a GIS environment (Chaudhuri and Clarke, 2014; AlShalabi, 2013). The major characteristic of the model is that the computer can automatically judge the coincidence degree between the simulation result and the actual situation. It can find the combination scheme with the least error, and works out the best parameter combination. The model assumes that the future phenomenon could be simulated by the past real evolution trend, while assuming that the historical growth trend is sustained. The model can simulate and predict urban dynamics through the application of four growth rules: spontaneous growth, new expansion centers, marginal growth, and Road-Influenced, which are controlled by five parameters: diffusion, breed, spread, slope and road gravity (Silva and Clarke, 2002). In order to enable the simulation result to be more accordant with the real situation, the model can adjust the growth coefficient through the self-adjusting function (Sangawongse et al., 2005). By altering the self-organization constraints or changing parameter values that affect the expansion of the urban area, scenarios for different planning goals can be transplanted into the SLEUTH model, and patterns of future urban area differing in quantity and location may be generated (Solecki et al., 2004; Feng et al., 2008; Liu and Ying, 2012). SLEUTH model has now been updated to the 3.0 beta version, and it is available on the United States Geological Survey website. The new version SLEUTH model can better simulate the historical trend of urban growth and improve the calibration efficiency of the model compared to the previous version in the calibration module (Watkiss, 2008). The city simulation module of the model was applied to this investigation and was debugged and compiled by using Cygwin software in the Windows system before running.

The SLEUTH model cannot reflect the influence of government decision and social economy on urban expansion, for this reason different scenarios are often introduced to simulate the future growth of the city (Xiang and Clarke, 2003; Feng et al., 2012; Dezhkamet al., 2014). When setting up the forecast scenarios, the future of urban planning and the actual situation were taken into consideration. According to “The Urban Master Planning of Qujing from the Year 2016 to 2030” (CAUPD and QCPB,

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2016), the eastern region will be protected in the course of future urbanization, because of the plenty of protected ecological land in that region. According to the existing research results and the actual situation of the research area, four scenarios were determined and assigned to the local classes in this investigation, and the specific assignment is shown in *Table 1*. In general, the existing cities appeared in the exclusion layer were assigned 0, some of the lakes and waters were assigned 100, while woodland, arable land, bare land, and grassland were assigned a value of 0-100 according to the study. We mostly assigned greater value to the ground classes that need to be protected, so that these areas are less likely to be urbanized, and the protected areas designed in this investigation are based on urban planning (Jantz et al., 2004). In the scenarios of natural development, it was assumed that the future expansion of urban space would be based on the existing urban space, and urban spatial expansion is not affected by planning, policy, ecological environment and so on, only assigning 100 to waters. In the planning-oriented scenario, it was assumed that the future cities would took the construction of urbanization as the dominant factor, some farmland, grassland and the forest land may be urbanized. In the ecological development scenario, it was assumed that the future city would be in the protection of the existing urban ecological pattern on the basis of expansion, so as to avoid the land occupancy and protect the cultivated land during urbanization. In the scenario of ecological protection, by increasing the protection of ecological green space and reducing the speed of urbanization in the future, the waters, forestland and key protected areas in the research area were designed as being forbidden to be urbanized, and the protection level of bare land, farmland and grassland were designed to be higher correspondingly (*Fig. 3*).

Table 1. *Excluded assignments of scenarios 1-4*

Scenario settings	Urban land in 2015	Waters	Bare land	Farm land	Grassland	Forest land	Protected land
Scenario 1 (Natural development)	0	100	0	0	0	0	–
Scenario 2 (Planning-oriented)	0	100	0	25	25	50	–
Scenario 3 (Ecological development)	0	100	50	25	25	100	100
Scenario 4 (Ecological protection)	0	100	50	50	50	100	100

Spatial-temporal analysis indicators of urban expansion

Urban Expansion Speed Index (*UESI*) and Urban Expansion Intensity Index (*UEII*) are the common indices for the study of urban expansion quantitative characteristics. They are used to characterize the speed and degree of urban expansion (Al-Sharif et al., 2014; Wang et al., 2016). These two indexes simply describe and analyse the change of material space from the result of urban expansion by comparing the speed and strength of urban expansion in different periods, studying the characteristics of the change of urban expansion form, and revealing the spatial and morphological evolution law of urban expansion (Terry, 1995; Salvati et al., 2014). In order to explore the temporal and spatial patterns of urban land use growth in Qujing, the two analytical indices are introduced in this paper.

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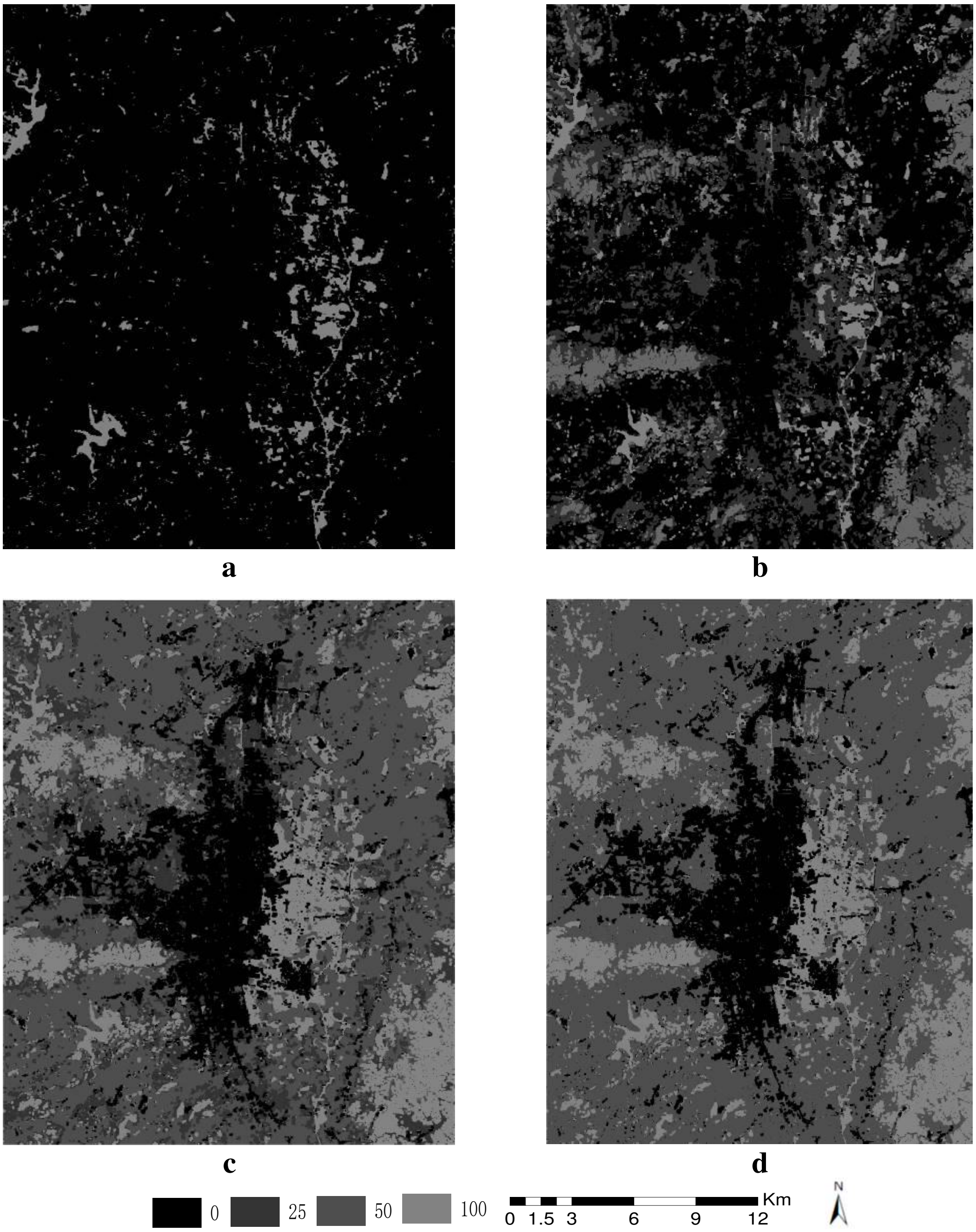

Figure 3. *The exclusion layer of scenario 1-4.* ***a*** *Scenario 1,* ***b*** *scenario 2,* ***c*** *scenario 3,* ***d*** *scenario 4*

(1) Urban expansion speed index

The *UESI* indicates the range of urban land area change at a certain time, which can reflect the overall scale and trend of urban expansion, and the formula is shown as follows (*Eq. 1*):

$$UESI = \frac{\left(UA_{n+i} - UA_{i}\right)}{n} \tag{Eq.1}$$

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(2) Urban expansion intensity index

The *UEII* is used to reflect the percentage of the total urban land area in a certain period. If the index increases, it means that the urban area expands at a faster rate in this period. The formula is shown as follows (*Eq. 2*):

$$UEII = \frac{\left(UA_{n+i}\text{-}UA_i\right)}{UA_i} \quad \text{(Eq.2)}$$

In the above formula: *UESI* represents the expansion rate of urban land use, *UEII* represents the expansion intensity of urban land use. UA_{n+i}, UA_i is the $n+i$ year and i year urban area, n represents the time interval in years.

Results and discussion

The correction result of SLEUTH model and validation of the simulation results

In general, the Compare Index and Lee-Sallee Index were often used to evaluate the precision of the simulation results (Clarke et al., 1997; Silva and Clarke, 2002). In this experiment, the Compare Index is 0.83 and Lee-sallee Index is 0.67, which indicate that the total number of urbanized pixels and urban boundary in the simulated year coincide with the actual situation. In the previous study, the Compare Index can generally reach more than 85%, Lee-sallee Index is generally from 0.3 to 0.7. But to some cities in the western part of China, these two indices may be less affected by topography and ecological environment (Herold et al., 2003; Mc and Justin, 2013; Zhao et al., 2014). The calibration process and parameters are shown in *Table 2*. The optimal parameter combination was obtained by final correction, and the walking coefficient and propagation coefficient were 15 and 18, which indicated that the effect of the study area on the urban development was small. The diffusion coefficient was 86, which indicated that the urban expansion was autonomous by marginal growth. The slope coefficient was 20 while the road coefficient was 72. It is indicated that the slope has limited restriction in the process of urban expansion. On the contrary, the increasing effect of road gravity is obvious, which is consistent with the results of other applications in China (Yuan et al., 2011; Feng et al., 2012; Zhao et al., 2014).

Table 2. *The correction result of SLEUTH model*

Growth factors	**Rough correction**		**Accurate correction**		**Final correction**		**Final reference value**
Monte Carlo iteration	10		20		30		
	Range	Step	Range	Step	Range	Step	
Diffusion coefficient	1-100	25	0-50	10	10-20	1	15
Breed coefficient	1-100	25	0-50	10	0-25	2	18
Spread coefficient	1-100	25	50-100	10	80-90	1	86
Slope coefficient	1-100	25	0-50	10	10-25	1	20
Road coefficient	1-100	25	50-100	10	70-85	1	72
Compare Index	0.75		0.79		0.83		
Lee-Sallee Index	0.55		0.61		0.67		

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In order to verify the simulation results of the SLEUTH model, we simulated the urban expansion process from 1993 to 2015 with the parameters obtained from the final correction. The data of simulation results and the real situation were gathered in 2000, 2009, and 2015 (the starting year is 1993, so the data of 1993 are not included in *Fig. 4* and *Table 3*).

Table 3. *Comparison between simulation results and actual situation*

Year	2000	2009	2015
Number of urban pixels in the actual situation /unit	13741	19802	24098
Number of urban pixels in the simulated result /unit	16021	23870	31976
Precision of simulation /%	85.77	82.96	75.36

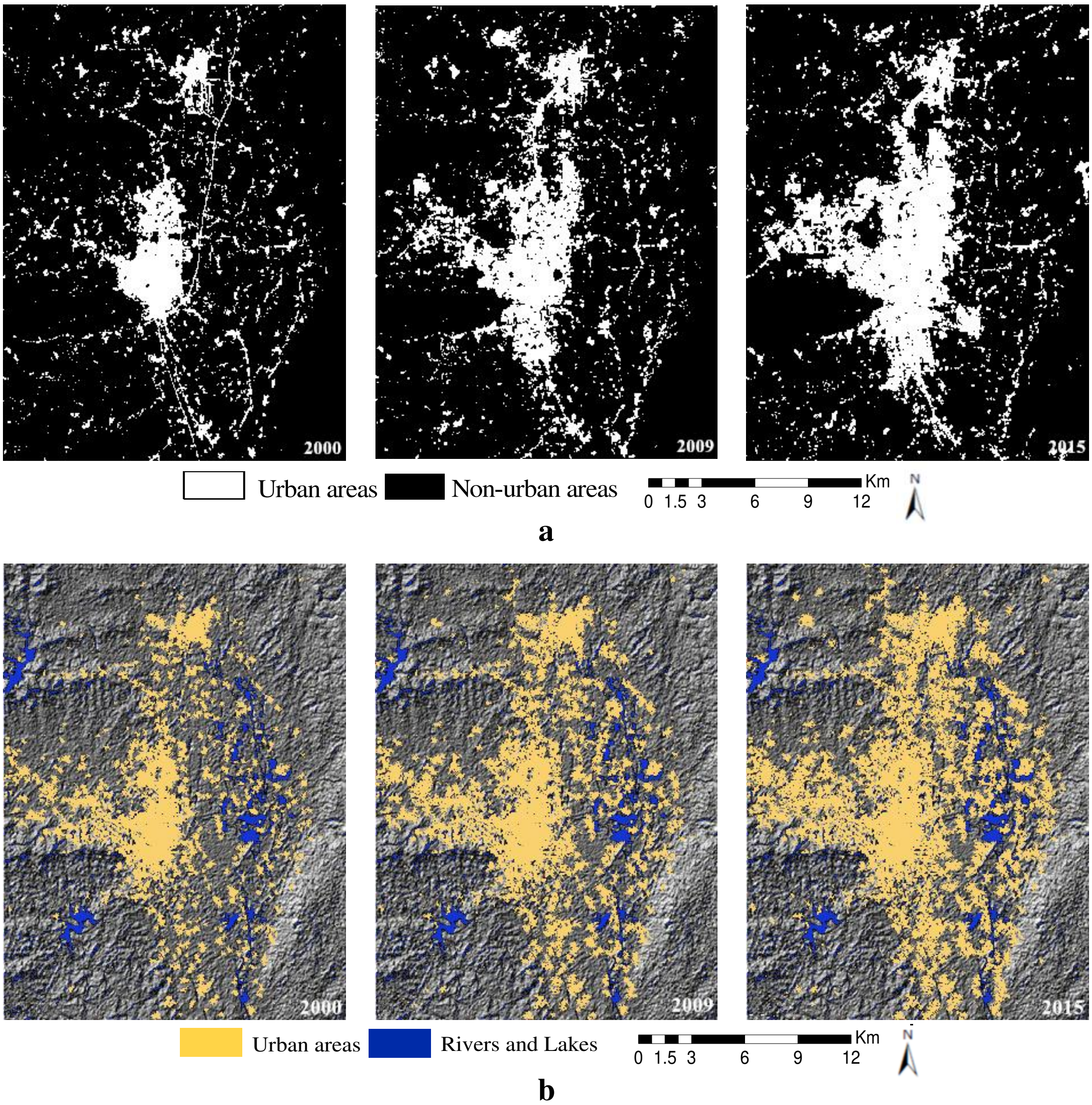

Figure 4. *Comparison of simulation results of SLEUTH model with actual situation in Qujing city.* ***a*** *The actual situation and* ***b*** *the simulated results of Qujing city in 2000, 2009 and 2015*

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As can be seen from *Figure 4,* the simulation results are similar to the actual spatial distribution: the simulated urban areas mainly extend along the road, and the old town is centered around Qujing, which accords with the characteristics of the development along the road and the edge growth. However, the difference between the two is very prominent, because the government prohibits the occupation of the large amount of ecological land in the eastern region, and as a result the expansion of the city in the eastern region is limited. However, in the simulation of urban settlements, a large scale of expansion also appeared in the eastern part, which is mainly caused by the high spread coefficient (Mc et al., 2013; Rafiee et al., 2009). The result of the simulation can not fully reflect the transfer of urban development center caused by the change of government behavior, because the cell is very dependent on the neighboring state, which is easy to expand outwards on the basis of the existing city, and the new diffusion center is not easy to appear (Salvati et al., 2014; Liu et al., 2010; Chaudhuri and Clarke, 2014).

We not only used the Compare Index and Lee-scale Index to evaluate the accuracy of simulation results, but also compared the actual situation with simulation results. Additionally, data about the number of pixel points and the precision of simulation are collected (*Table 3*). It can be seen that the precision is higher than 75% in three periods, which indicates that the simulation results are in accordance with the actual situation. The precision of simulation is 85.77%, 82.96 and 75.36% in 2000, 2009 and 2015 respectively. With the increase of the simulated year, the precision decreases gradually, while similar situations have occurred in other studies (Clarke and Gaydos, 1998; Liu et al., 2010). This is mainly due to the shift of the city center during the expansion, which was not taken into account in the process of urban simulation (Aerts et al., 2009). Therefore, it is necessary to integrate urban planning factor into urban expansion data. The exclusion layer of sleuth model in urban expansion forecast can solve this problem effectively.

The prediction results of the urban expansion

The reference value of each coefficient is observed in the final correction. 100 times Monte Carlo iteration is used to predict the urban spatial expansion while the 2016 to 2030 forecast years were designed. The results are shown in *Figure 5 and Table 4*.

Table 4. *The prediction results of scenario 1-4*

Urban area	Scenario 1	Scenario 2	Scenario 3	Scenario 4
Current urban area (yellow)/km²	213.27	213.27	213.27	213.27
Urbanization rate 70-100% (red)/km²	105.98	74.86	51.48	20.21
Urbanization rate 40-70% (green)/km²	19.65	20.60	21.37	19.12
Urban area in 2030/km²	338.9	308.73	286.12	252.6

From *Figure 5a* it can be seen that in the natural development scenario, the development of the city is almost free from any restrictions. Cities occupy a large amount of ecological green space in the process of expansion, which is more prominent in the suburbs of the eastern part of the city. In this scenario, the urban land area would be a total of 125.63 km² in 2030 with an average annual growth rate of 3.93%. Besides,

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a large number of urbanized pixels can be seen from the eastern part of the study area, which means the probability of urbanization ranging from 70% to 100% by 2030. Most of the newly added urban land was originally the outskirts of the city, which led to the fragmentation of the spatial pattern and aggravate the contradiction between urban development and ecological protection.

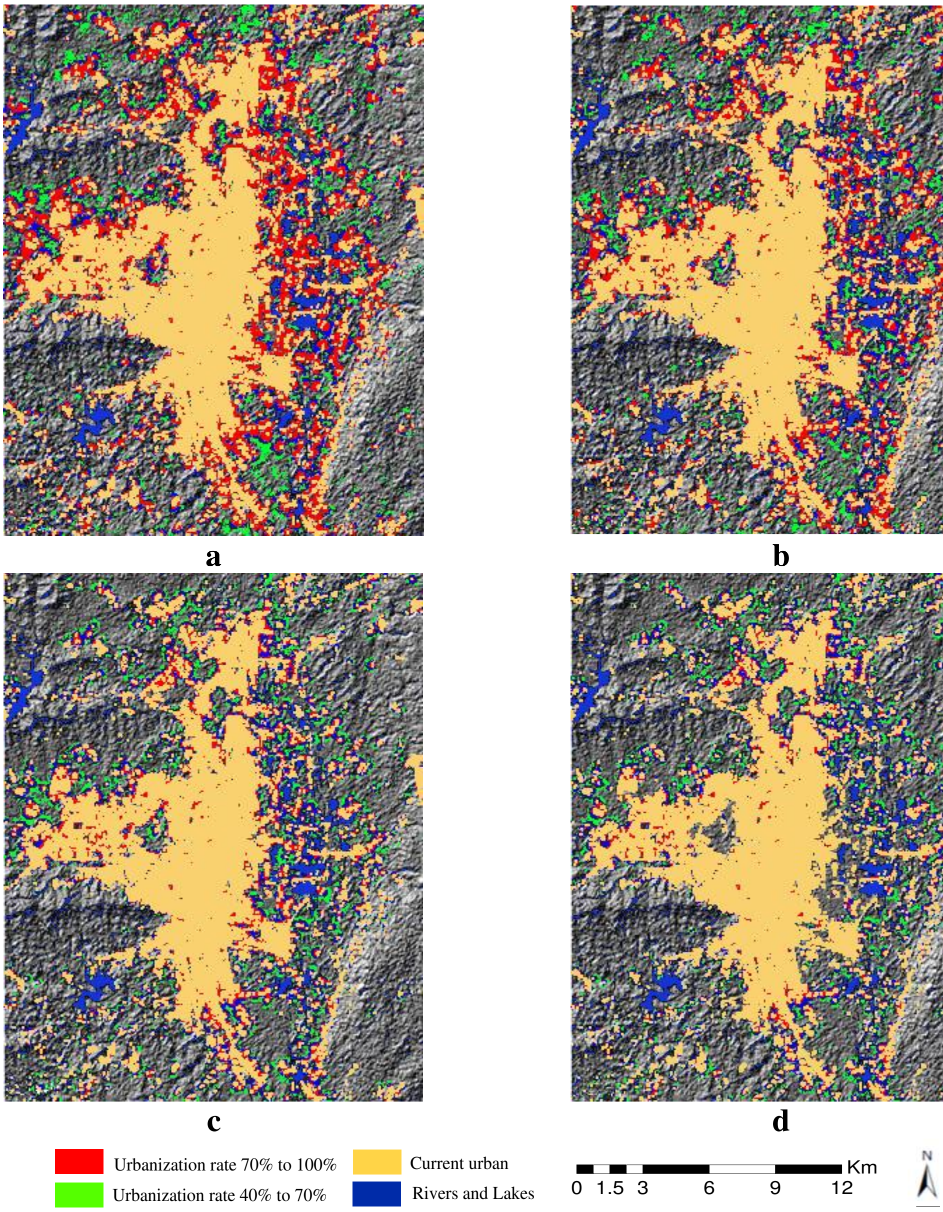

Figure 5. *Urban expansion prediction of scenario 1-4.* ***a*** *Scenario 1,* ***b*** *scenario 2,* ***c*** *scenario 3,* ***d*** *scenario 4*

Figure 5 (b and *c)* shows that the cities mainly extend to the north, the south and the west. The model which predicts the spatial pattern of urban development is basically consistent with the established urban planning, realizing the integrated urban structure system. The distribution of newly added city image element is obviously concentrated on the traffic road and the existing city periphery. It indicates that the road has played a

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major role in urban expansion (Rafiee et al., 2009; Dezhkam et al., 2014). However, in the planning-oriented scenario, there is still a large amount of ecological green spaces occupied in the process of urban expansion. This phenomenon is more prominent in the Qusheng Highway which lies in the east of the Daihe and Huangjiazhuang area. In the scenario of ecological development, the city carries out ecological construction at the same time and focuses on building the ecological cultural belt of the urban area with Mount Liaokuo and Nanpan River as the core. In this scenario, the city has realized the long-term ecological development plan of ecological landscape green corridor, which leads to the sustainable and harmonious development of the society, economy and culture. In this situation, through a series of the ecological landscape green corridor and other measures, the "one city, one river, one mountain" mode and the long-term blueprint of the scenic area have been achieved, which benefits the city's sustainable and coordinated development. *Figure 5d* shows that in the ecological protection scenario, due to improved protection of forests and farmlands, rapid urban expansion has been effectively controlled and urban land growth rate has dropped to 1.23%. But this restriction has obviously restricted the development of the city, which means by 2030 the area of the city would only increase by 39.33 km². From the forecast results of four scenarios, it can be seen that the future cities mainly expand by marginal growth and road traction growth while the new urban center would hardly come into formation, and the impact of the slope is insignificant. The results are similar in other experiments (Dietzel and Clarke, 2007; Al-Shalabi et al., 2013). The inclusion of the exclusion layer can better guide the city development according to the established urban planning, so as to avoid the disordered urban development and the destruction of ecological green space. The four kinds of forecast scenarios can well reflect the predefined planning policy (Aerts et al., 2003; Jantz et al., 2004).

The comparison of prediction results in four scenarios

From 1993 to 2030, the urban land of the study area has always been increasing. The urban land growth rate remains at above 3%. This phenomenon can be identified from *Figure 6 (a* and *b)* indicates that the study area has maintained a rapid development since 1993. The growth rate of the urban area increased first and then decreased in the period of 2000-2015, which indicates that the study area was at a time of rapid development before 2009, and the urban land increase rate was flat after 2009. However, according to forecast results, all the four kinds of scenarios present the growth tendency of the urban area.

According to the UESI (*Fig. 6c*), *UESI* has been more than 3.3 since 1993 and presented a growing trend. During the rapid expansion period of 1993-2009, the expansion rate slowed from 2009 to 2015 with the 2015 *UESI* being 6.34, and the city was in the middle-speed expansion stage (Wang et al., 2016). In the four scenarios, the *UESI* was gradually decreasing with the increase of urban ecological protection intensity. In the natural development scenario, *UESI* is as high as 8.37 in 2015-2030, much higher than *UESI* of the historical period; the city will be at a rapid growth rate of annual 8.37 km². In the planning-oriented scenario, the city basically maintains the existing urban expansion speed and it is in the middle-speed expansion stage. In this scenario, *UESI* and the previous stage are quite. In the ecological development scenario, the city is at a low-speed expansion period, and the expansion speed drops slightly (Al-Sharif, 2014). In the ecological protection scenario, the *UESI* is only 2.62, the urban expansion is at the low-speed expansion stage, the growth rate declines to 2.62 km²

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every year, and the urban expansion is obviously reduced compared with the historical periods.

According to *Figure 6d,* the *UEII* has been more than 3.4% since 1993; during 1993-2009, the *UEII* reached 4.9%. However, it dropped to 3.6% during 2009-2015. This is mainly because the government has made a local adjustment to the urban master plan, which restricts the construction and development of some ecological protection areas. In the four scenarios, the *UEII* gradually decreases with the increase of urban ecological protection intensity. In the natural development scenario, *UEII* is basically flat compared with the previous period from 2015 to 2030, the city will continue to maintain the strength of the strong expansion of urban development (Wang, 2016; Salvati et al., 2014). In planning-oriented scenarios and ecological development scenarios, the future expansion intensity of the city will be slightly lower than that of the previous period, but the *UEII* still maintain 2.3% to continue the development of the city. In the ecological protection scenario, the *UEII* descends to 1.23%, which is far below the level of the recorded extended intensity o and the future expansion of the city will be obviously restricted.

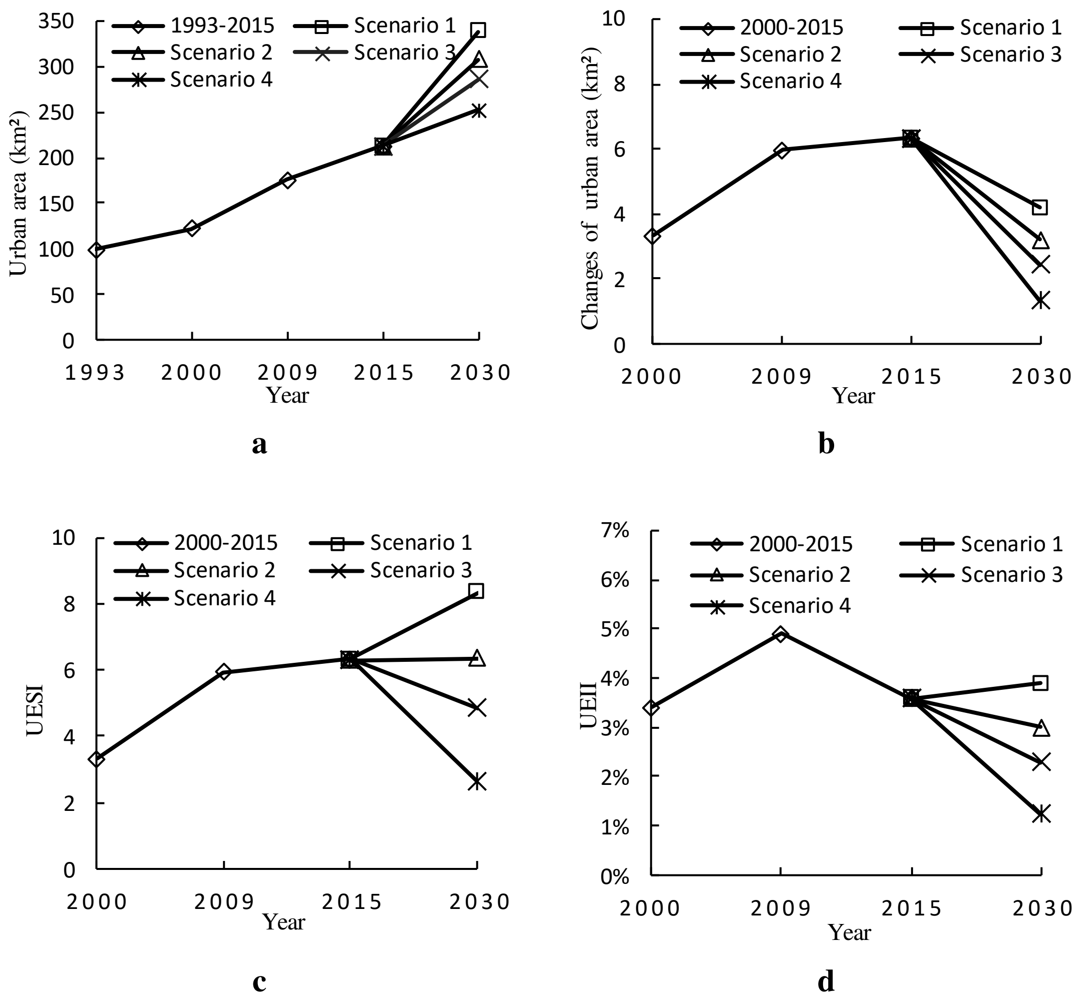

***Figure 6.** The statistics of urban area changes in different scenario. Changes in **a** total urban area, **b** urban area in each period, **c** urban expansion speed index and **d** urban expansion intensity index*

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In order to choose the best forecast scenario, the predicted results of four scenarios were compared with the Urban Planning Project of 2030 (CAUPD and QCPB, 2016). We found that the predicted results of the ecological development scenarios were in accordance with the urban development requirements, especially in the urban spatial distribution. To prove this assumption, we extracted the urban land area data through the remote sensing image, with the image preprocessing and classification method being the same as other years'. The urban land of the research area is 225.5 km^2 in 2018. It can be seen from *Figure 7a* that the urban area is closest to the result of the ecological development scenario. During this period, the urban area increased 12.23 km^2, which exceeded the results of the four scenarios (*Fig. 7b).*

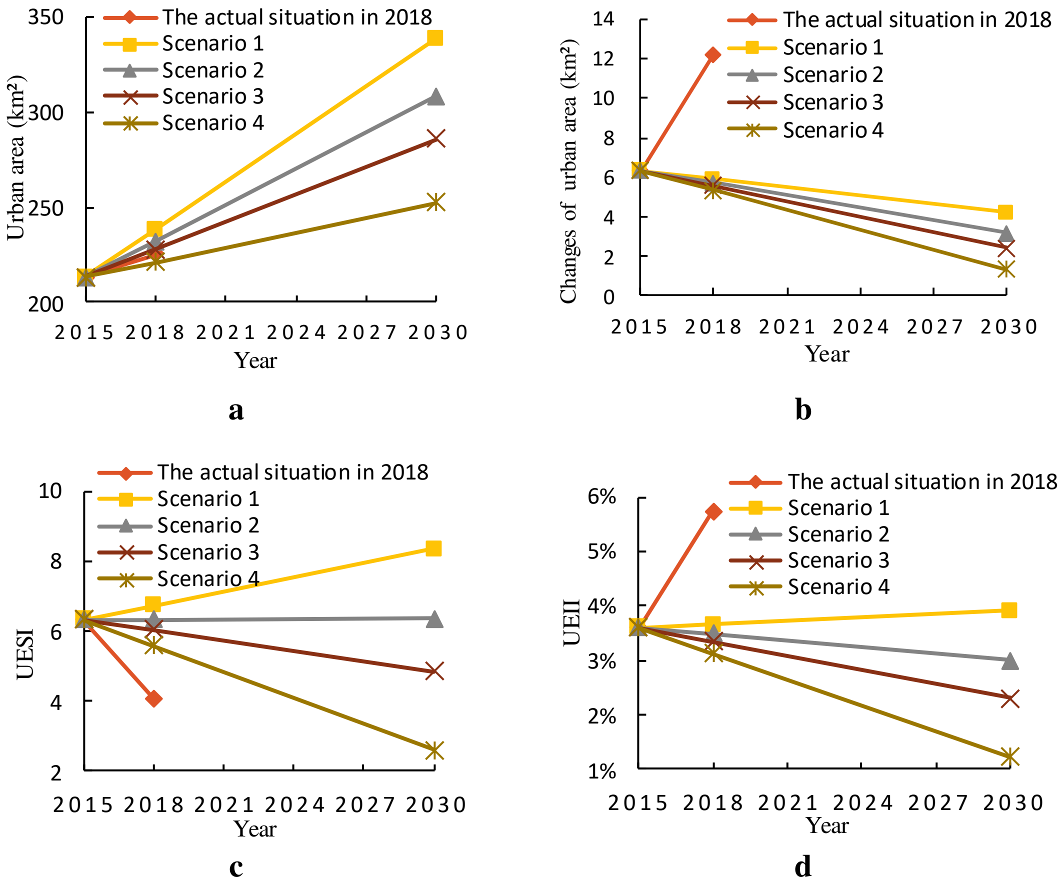

Figure 7. *The comparison between the actual situation of research area and four scenarios. Comparison of* ***a*** *total urban area,* ***b*** *urban area in each period,* ***c*** *USEI and* ***d*** *UEII*

The speed of urban expansion is limited to 4.07 (*Fig. 7c*), which is below the average velocity level of four scenarios (The average of four scenarios is 6.18). However, the urban expansion strength reached 5.3, which is much higher than the four scenarios predicted results (*Fig. 7d*). We speculate that because the government has strengthened the protection of ecological green space, the urban construction transfer from the blind expansion to high-quality development, and the rapid urbanization process was restricted. Therefore, from the perspective of the whole city, the speed of urban expansion has decreased. However, a large number of construction activities have

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occurred in some local areas of the city, which mainly occur in the fringe of urban settlements in the eastern part of the city; for example, people build large numbers of new houses and occupy farmland for commercial activities. These human activities explain why the growth of *UESI* slowed down but the *UEII* increased in the process of urban expansion.

Conclusion

This investigation integrates GIS, RS and SLEUTH model to simulate and predict the urban spatial expansion of the typical Chinese Nationally Designated Garden City (Qujing), and to explore the urban expansion trend under different ecological green space protection. Comparing the urban evolution laws in different scenarios, the results show that the SLEUTH model excels with a higher spatial matching degree with historical data, which enjoys better applicability and portability. The model can be used in Nationally Designated Garden Cities with dense vegetation and it can provide a reference for the sustainable development and urban planning of Nationally Designated Garden Cities in China. Under the protection intensity of different ecological green space, the urban area is obviously increased in four scenarios, and the other land types are occupied. This result indicates that the increase of urban land use is affecting the future space use pattern. By increasing the protection intensity of the ecological land around the city, the urban spatial expansion will be obviously restricted. It is mainly manifested by the decrease of *UESI* and *UEII*. Comparing the results of different scenarios, it is found that the future cities will spread around the present urban area in the natural development scenario. In the other three scenarios, the trend of urban expansion is expanding to the north, the south and the west, and would ultimately achieve the spatial integration of the Qiling and Zhanyi District, which is consistent with the requirements of urban master planning. Comparing with four scenarios, we found that the urban development trend predicted by the ecological development scenario is more similar to the urban planning, and this conclusion is confirmed by the 2018 distribution of urban areas. In the ecological development scenario, the urban ecological land has been effectively protected and the development of the city has not been affected, which is beneficial to the sustainable development of the city and the protection of the ecological environment. The ecological development scenario can minimize the impact on the urban ecological environment by increasing the protection of natural environment, controlling the development intensity and clarifying the direction of urban development. It is beneficial to the sustainable development of the city and the protection of the ecological environment.

We propose to use the SLEUTH model to reflect the spatial and temporal characteristics of urban expansion, and forecast urban expansion from both macroscopic and medium perspective (Silva and Clarke, 2005; Watkiss, 2008). In this case, the main factors affecting urban expansion, such as road, topography, lake and river are integrated into the urban expansion process. In this way, the results are more in accordance with the actual urban change. At the same time, the temporal resolution and spatial resolution of the image data will have certain influences on the prediction result of the model (Xiang and Clarke, 2003; Al-Shalabi et al., 2013; Chaudhuri and Clarke, 2014). This study selects four periods of city-wide data to test, and each designed period interval is between 6 to 9 years and the image space resolution is 30 m. The application of higher resolution remote sensing imagery and increased urban base period data

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would help to improve the accuracy of urban expansion simulations. In addition, the setting of the exclusion layer is the key link that directly affects the prediction results (Aerts et al., 2003; Clarke et al., 1997). How to embed the factors suitable for population, greening coverage, built-up area greening, per capita public green area based on SLEUTH model, and how to comprehensively consider the influence of various factors in the process of urban expansion will be the focus of the next research.

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AN ASSESSMENT OF THE INFLUENCE OF SELECTED HERBICIDES ON THE MICROBIAL PARAMETERS OF SOIL IN MAIZE (*ZEA MAYS*) CULTIVATION

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Abstract. Application of herbicides inhibit development of weeds, but at the same time they can negatively influence soil microbial activity. The microbiological indicators allow estimating changes in soil contaminated with herbicides. The aim of the study was to assess the influence of selected post-emergence herbicides: Stellar 210 SL + Olbras, Maister Power 42.5 OD, Laudis 44 OD, Collage 064 OD, Hector Max 66.5 WG + Trend 0.1 % and Arigo 51 WG + Trend 0.1% on the microbial activity of soil under maize plantation. The research included assessment of the count of selected groups of microorganisms, the activity of soil enzymes and soil fertility (BIF), as well as soil sensitivity (RS) to the preparations tested in the experiments. The results of study show that in some cases the count of the groups of microorganisms under study increased (the total bacterial count, the count of copiotrophs), especially immediately after the herbicide treatment, i.e. at the first term of analyses. The study showed that sixty days after the herbicide treatment all the xenobiotics exhibited strongly negative influence on the dehydrogenase activity (DHA). The activity of alkaline phosphatase and catalase became significantly reduced thirty days after the soil treatment with all of the herbicides used in the experiment. The highest values of biological index of fertility (BIF) were noted thirty days after the herbicide treatment and were strictly correlated with DHA and PAC.
Keywords: *post-emergence herbicides, total bacteria, fungi, dehydrogenase, phosphomonoesterases, BIF*

Introduction

For many years chemical crop protection products have been commonly used in farming practice to achieve the highest possible yield and desirable quality of crops. Intensive agricultural production as well as large numbers of pests and weeds cause increased use of pesticides (Baćmaga, 2007a). Herbicides are a group of crop protection products. They are considerably diversified and provide highly effective protection of particular crops. Long-term use of herbicides results in their accumulation in soil and it may cause a wide range of side effects (Walker et al., 2001). At present there are new EU regulations concerning integrated crop protection methods (introduced in Poland in 2014), which prefer non-chemical to chemical crop protection. The same active substances cannot be used consecutively in order to reduce the negative effect of agriculture on the environment.

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Modern herbicides biodegrade rapidly and they are effective even at small doses or at divided doses. Nevertheless, whenever the active substance of the pesticide is applied, it may affect the population and species composition of soil microorganisms. It may also indirectly influence the biochemical processes in which these microorganisms take part (Baćmaga et al., 2013). When herbicides are applied, the microbial biomass of soil may increase, decrease or remain the same (Singh and Ghoshal, 2010). Both the active substance and its decomposition products may be more toxic than the input products. They also influence the population and bioactivity of microorganisms (Arias-Estévez et al., 2008).

The count of soil microorganisms and the soil enzymatic activity are the indicators which enable assessment of changes in soil pollution and fertility (Baćmaga et al., 2007b).

The aim of the study was to assess the influence of selected post-emergence herbicides: Stellar 210 SL + Olbras, Maister Power 42.5 OD, Laudis 44 OD, Collage 064 OD, Hector Max 66.5 WG + Trend 0.1 % and Arigo 51 WG + Trend 0.1% on the microbial activity of soil under maize plantation. The research included assessment of the count of selected groups of microorganisms (total bacteria, actinobacteria, moulds, oligotrophs and copiotrophs), the activity of soil enzymes (dehydrogenase, acid and alkaline phosphatase, catalases) and soil fertility (BIF), as well as soil sensitivity to the preparations tested in the experiments.

Material and methods

Between 2015 and 2016 experiments were conducted in the village of Swadzim (52°26 N, 16°45 E) in fields belonging to the Experimental and Educational Station Gorzyń, Poznań University of Life Sciences, Poland.

The experiments were conducted in plots on *Haplic Luvisols* (according to the FAO/WRB classification [IUSS Working Group WRB, 2007]), derived from loamy sands. (*Table 1*).

The soil texture was determined using sieving (sand fraction) for silt and clay fraction (Van Reeuwijk, 2002).

Table 1. *The texture of soil material sampled from 0-25 depth*

Percentage content of soil fractions %				Texture class
Fraction	sand 2 – 0.05 mm	silt 0.05-0.002 mm	clay < 0.002 mm	
	78	18	4	LS

LS – loamy sand

P9175 maize cultivar (FAO 280) was sown in plots of 28 m^2, in four replications for each treatment. The cultivar is highly resistant to water deficit and high temperatures.

Soil samples were collected at three terms, which differed in the number of days following the application of individual herbicides. The samples were collected at the following terms:

1st term –3 days after herbicide treatment,
2nd term – 30 days after herbicide treatment,
3rd term – 60 days after herbicide treatment.

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Individual experimental samples were collected from seven variants, which differed in the type of herbicides applied. One variant did not receive any chemical protection and was used for reference (*Table 2*).

Table 2. *The field experiment scheme*

Treatment	Activity substances of herbicide	Doses of herbicide
1. Control	without herbicide	without herbicide
2. Stellar 210 SL + Olbras	Dicamba, topramezon + adjuvant	1.25 l/ha + 1 l/ha
3. Maister Power 42.5 OD	Iodosulfuron-methyl-sodium, foramsulfuron, methyl thiencarbazone	1.25-1.5 l/ha
4. Laudis 44 OD	Tembotrione	2-2.5 l/ha
5. Collage 064 OD	Nicosulfuron, thifensulfuron-methyl	1 l/ha
6. Hector Max 66.5 WG + Trend 0.1 %	Nicosulfuron, rimsulfuron, dicamba + adjuvant	330-440 g/ha
7. Arigo 51 WG + Trend 0.1 %	Mesotrione, nicosulfuron, rimsulfuron + adjuvant	330 g/ha

Weather conditions

It is generally accepted that climatic conditions found throughout Poland are suitable for maize cultivation. This species needs approx. 500 mm annual precipitation to grow. The weather conditions during the experiment were characterised with Sielianinov hydrothermal coefficient K (Skowera, 2004). Selyaninov hydrothermal coefficient K was calculated according to the formula $K = (P \cdot 10)/(T \cdot L)$, where K Sielianinov hydrothermal coefficient, P – total monthly precipitation, T – average monthly temperature, L – number of days in the month (*Fig. 1*). In all the years mean air temperature ranged from 8.6 to 10°C and it was comparable to the multiannual mean. A crucial element of optimal growth and biomass yielding conditions is also total precipitation, particularly its distribution. Variability of weather conditions in the years of the study was reflected in the values of the Sielianinov coefficient. More advantageous moisture conditions for maize were found in the year 2016 than in the drier 2015 ($K = 0.9$).

Interpretation of the Sielianinov hydrothermal coefficient:

$K > 1.5$ – excessive moisture for all plants

$K = 1.0$-1.5 – sufficient moisture

$K = 0.5$-1.0 – insufficient moisture

$K < 0.5$ – moisture level below the requirement for most plants – drought.

Soil enzymatic activity

The analyses of the enzymatic activity of soil in different variants (each in four replication) were based on the colorimetric method applied to measure the dehydrogenase activity (EC 1.1.1. DHA), where 1% TTC (triphenyl tetrazolium chloride) was used as a substrate. The measurement took place after a 24-hour

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incubation at a temperature of 30°C and a wavelength of 485 nm and it was expressed as µmol TPF (triphenyl formazane)·24 h^{-1} g^{-1} dm of soil (Thalmann, 1968).

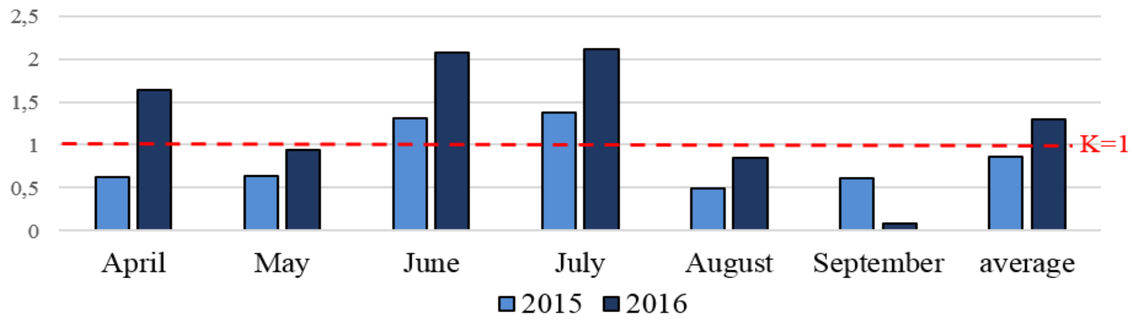

Figure 1. *Humidity characteristic of the months of the vegetation period of 2015–2016 according to the value of the Sielianinov hydrothermal coefficient (K)*

Apart from that, the acid and alkaline phosphomonoesterase (EC 3.1.3.2, PAC/PAL) activity were analysed with the method developed by Tabatabai and Bremner (1969). The activity was analysed using disodium p-nitrophenyl phosphate tetrahydrate as a substrate after 1 h incubation at a temperature of 37°C and wavelength of 400 nm. The results were converted into µmol (p-nitrophenol) PNP h^{-1} g^{-1} dm of soil.

The catalyse activity (EC 1.11.1.6) was analysed manometrically with the method developed by Johnsons and Temple (1964), where 0.3% H_2O_2 was used as a substrate. After a 20 minute incubation at room temperature (20°C) it was titrated with 0.02 M $KMnO_4$ until its colour was light pink. The value was expressed as mmol $H_2O_2g^{-1}$ d.m. min^{-1}.

Measurement of biological index of fertility (BIF)

The biological index of fertility (BIF) was measured using the dehydrogenase activity (DHA) and catalyse activity (CAT) in the formula: (DHA +kCAT)/2, where k is the proportionality factor amounting to 0.01 (Saviozzi et al., 2004).

Microbial analyses

Soil samples were collected at a depth of 0-20 cm from interrows in the maize plantation. Volume of the samples to microbiological and biochemical tests was 700 - 800 g, which was sampled by using Egner's sticks from 10 points of each variant plot.

The serial dilution method developed by Koch was applied to measure the count of microorganisms on appropriate agar mediums (in five replicates). The average count of colonies per dry mass of soil was calculated:

- the total bacterial count was measured on ready Merck-Standard count agar after 5 days of incubation at 25°C;
- moulds were measured on a Martin medium after 5 days of incubation at 24°C;
- actinobacteria were measured on a Pochon medium after 5 days of incubation at 25°C;
- copiotrophs were measured on an NB (Nutrient Broth) medium (Ohta and Hattori, 1980) after 5 days of incubation at 25°C;

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- oligotrophs were measured on a DNB (Dilution Nutrient Broth) medium (Ohta and Hattori, 1980) after 5 days of incubation at 25°C.

The formula (*Eq. 1*) proposed by Orwin and Wardle (2004) was used to estimate soil resistance (RS) to contamination with the herbicides under analysis:

$$RS = 1 - \frac{2\left| D_0 \right|}{C_0 + \left| D_0 \right|} \quad \text{(Eq.1)}$$

where:
C_0 – soil resistance under natural conditions over time t_0,
P_0 – resistance of soil subjected to pressure over time, $D_0 = C_0 - P_0$.

Statistical analysis

Two-way analysis of variance (α = 0.05) was used to compare the mean values of biological parameters at individual terms of analyses and the influence of selected herbicide combinations. Next, post-hoc Tukey HSD test was applied. Principal Component Analysis (PCA) was used to analyse the bioactivity date at individual maize development phases in consequence of herbicide treatment.

Results

Microbial number

The results of microbial analyses are mean values of the two years of the research. Our results in general were consistent in the years of study so we decided to make a synthesis from years. The two-way analysis of variance showed that the factors under study significantly influenced the total bacterial count as well as the count of actinobacteria, copiotrophs and oligotrophs. There was no significant dependence between the factors and the count of moulds (*Table 3*).

Table 3. *F test statistics and significance levels of two-way analysis of variance for the number of selected groups of microorganisms associated with herbicides and terms research fixed factors (*** p = 0.001. ** p = 0.01. * p = 0.05. ns - not statistically significant)*

Parameter	Time	Treatment	Interaction
Total bacterial count	23.31^{***}	5.77^{***}	8.15^{***}
Moulds	0.15^{ns}	0.80^{ns}	1.21^{ns}
Actinobacteria	77.02^{***}	3.34^{**}	10.20^{***}
Copiotrophs	29.34^{***}	2.29^{*}	2.87^{**}
Oligotrophs	97.19^{***}	22.05^{***}	15.60^{***}

The research showed the influence of individual herbicides on the total bacterial count (*Table 4*). In comparison with the control variant, the xenobiotic treatment with Stellar 210 SL + Olbras (a.s. dicamba, topramezone + adjuvant), Laudis 44 OD (a.s. tembotrione) and Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl) caused an

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increase in the total bacterial count at all the three terms. When Maister Power 42.5 OD (a.s. iodosulfuron-methyl-sodium, foramsulfuron, thiencarbazone-methyl) was applied, the total bacterial count was similar to the count in the control variant during the whole period of the research. When Hector Max 66.5 WG + Trend 0.1% (a.s. nicosulfuron, rimsulfuron, dicamba + adjuvant) and Arigo 51 WG + Trend 0.1% (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant) were applied, the count of bacteria was greater than in the control variant at the first and third term. However, at the second term the bacterial count was respectively about 60% and 70% lower than in the soil which had not been treated with the herbicides.

The research revealed that the herbicides caused considerable fluctuations in the count of oligotrophs. Three days after the application (the first term) of Stellar 210 SL + Olbras (a.s. dicamba, topramezone + adjuvant), Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl) and Hector Max 66.5 WG + Trend 0.1 % (a.s. nicosulfuron, rimsulfuron, dicamba + adjuvant) the crop protection products stimulated the growth of oligotrophs, as their count was greater than in the control variant *(Table 4)*. At the second term of analyses, i.e. thirty days after the herbicide treatment, the count of oligotrophs increased in the variants where Stellar 210 SL + Olbras (a.s. dicamba, topramezone + adjuvant), Maister Power 42.5 OD (a.s. iodosulfuron-methyl-sodium, foramsulfuron, thiencarbazone-methyl) and Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl) had been applied. Sixty days after the herbicide treatment the count of these microorganisms dropped in all the variants, as compared with the control sample.

Like oligotrophs, the count of copiotrophs fluctuated during the whole period of the research. It depended on the chemical product and the time after the treatment.

Table 4. *The effect of herbicides on the number of microorganisms*

Experimental combination	Term of analysis		
	3 days after herbicides application	30 days after herbicides application	60 days after herbicides application
Total bacteria count (cfu x g^{-1} d.m. soil x 10^{-5})			
Control	2.71 a ± 0.98	14.93 c-h ± 5.81	5.81 a-d ± 1.78
Stellar 210 SL + Olbras	3.43 ab ± 1.09	17.46 f-h ± 4.26	8.10 a-f ± 2.02
Maister Power 42.5 OD	3.90 ab ± 1.86	18.04 h ± 3.52	5.60 a-c ± 2.79
Laudis 44 OD	8.34 a-g ± 2.56	17.38 f-h ± 6.42	12.08 a-h ± 2.80
Collage 064 OD	12.44 b-h ± 2.49	18.01 gh ± 6.34	15.27 d-h ± 4.86
Hector Max 66.5 WG + Trend 0.1 %	12.50 b-h ± 1.76	5.98 a-d ± 2.20	17.97 gh ± 3.94
Arigo 51 WG + Trend 0.1 %	6.52 a-e ± 2.55	4.92 ab ± 2.62	16.08 e-h ± 2.68
Actinobacteria (cfu x g^{-1} d.m. soil x 10^{4})			
Control	2.09 a ± 1.44	2.41 a ± 1.36	13.05 a-d ± 5.54
Stellar 210 SL + Olbras	2. 78 a ± 1.06	5.16 a ± 2.03	6.29 ab ± 5.23
Maister Power 42.5 OD	2.17 a ± 2.06	12.14 a-c ± 3.30	3.83 a ± 2.83
Laudis 44 OD	2.38 a ± 1.24	2.06 a ± 0.89	6.89 a-c ± 4.75
Collage 064 OD	4.89 a ± 3.73	3.17 a ± 0.64	13.75 d ± 4.55
Hector Max 66.5 WG + Trend 0.1 %	1.72 a ± 1.46	3.83 a ± 1.66	16.18 d ± 4.19
Arigo 51 WG + Trend 0.1 %	1.54 a ± 1.04	3.51 a ± 1.16	11.39 bc ± 2.41

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Moulds (cfu x g^{-1} d.m. soil x 10^4)			
Control	4.61 a ± 1.36	3.39 a ± 1.56	2.30 a ± 0.20
Stellar 210 SL + Olbras	4.46 a ± 1.20	6.07 a ± 1.38	3.06 a ± 0.50
Maister Power 42.5 OD	4.08 a ± 1.48	4.31 a ± 1.18	3.83 a ± 0.95
Laudis 44 OD	2.47 a ± 0.19	3.02 a ± 1.33	3.99 a ± 0.27
Collage 064 OD	2.64 a ± 1.08	1.83 a ± 0.40	4.47 a ± 0.96
Hector Max 66.5 WG + Trend 0.1 %	4.11 a ± 0.63	1.77 a ± 0.09	3.61 a ± 0.31
Arigo 51 WG + Trend 0.1 %	2.03 a ± 0.95	4.85 a ± 0.09	1.58 a ± 0.60
Oligotrophic bacteria (cfu x g^{-1} d.m. soil x 10^5)			
Control	26.03 de ± 1.10	14.66 a-d ± 5.68	13.24 a-d ± 2.76
Stellar 210 SL + Olbras	42.93 f ± 5.82	38.06 ef ± 3.70	10.68 ab ± 1.41
Maister Power 42.5 OD	7.49 ab ± 0.96	25.33 a-c ± 4.89	4.13 a ± 2.33
Laudis 44 OD	22.04 bc ± 6.70	9.37 ab ± 2.43	11.41 a-d ± 2.41
Collage 064 OD	57.58 g ± 5.35	16.27 a-d ± 3.5	8.95 ab ± 3.64
Hector Max 66.5 WG + Trend 0.1 %	37.99 ef ± 6.10	9.01 ab ± 3.11	11.13 a-c ± 2.00
Arigo 51 WG + Trend 0.1 %	18.07 a-d ± 4.14	5.43 a ± 2.57	7.32 a ± 1.87
Copiotrophic bacteria (cfu x g^{-1} d.m. soil x 10^5)			
Control	19.97 b-e ± 4.34	17.53 a-e ± 6.41	11.45 a-d ± 1.47
Stellar 210 SL + Olbras	21.29 b-e ± 4.98	25.13 a-d ± 7.38	11.02 a-d ± 1.92
Maister Power 42.5 OD	12.48 a-d ± 6.22	13.03 de ± 3.88	6.89 ab ± 5.09
Laudis 44 OD	18.84 a-e ± 0.55	24.51 a-d ± 3.40	12.13 a-d ± 2.92
Collage 064 OD	30.21 e ± 0.55	10.13 a-d ± 5.34	13.61 a-d ± 6.82
Hector Max 66.5 WG + Trend 0.1 %	23.92 de ± 3.46	20.54 b-e ± 5.32	7.31 a-c ± 2.47
Arigo 51 WG + Trend 0.1 %	23.74 c-e ± 3.84	25.34 de ± 5.83	2.79 a ± 1.43

Note: data are represented as means of five replications. Values after means are standard deviations ±; mean values ± standard errors; a, b, c, d, e – homogenous groups according to Tuckey's test; different letters denote statistical differences at level $\alpha = 0.05$; n = 5

In comparison with the control soil variant, immediately after treatment with the crop protection products and thirty days after the treatment the count of copiotrophic bacteria increased in the variants with Stellar 210 SL + Olbras (a.s. dicamba, topramezone + adjuvant), Hector Max 66.5 WG + Trend 0.1 % (a.s. nicosulfuron, rimsulfuron, dicamba + adjuvant) and Arigo 51 WG + Trend 0.1 % (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant). However, sixty days after the treatment there was an inverse dependence – the count of copiotrophic bacteria decreased. When Maister Power 42.5 OD (a.s. iodosulfuron-methyl-sodium, foramsulfuron, thiencarbazone-methyl) was applied, it inhibited the count of copiotrophs at each of the three terms of analyses, as compared with the soil which was not treated with herbicides *(Table 4)*. The count of copiotrophs in the variants where Laudis 44 OD (a.s. tembotrione) and Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl) were applied fluctuated continuously during the whole research period.

Actinobacteria were another group of microorganisms under study *(Table 4)*. At the first term of soil sample collection the count of actinobacteria in most of the herbicide variants was similar to the count in the control variant. Only treatment with Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl) caused the count of actinobacteria to increase by more than 100%. Thirty days after the herbicide treatment Maister Power

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42.5 OD (a.s. iodosulfuron-methyl-sodium, foramsulfuron, thiencarbazone-methyl) had the most stimulating effect on actinobacteria as their count was almost 5 times greater than in the control variant. At the last term of analyses, i.e. sixty days after the herbicide treatment, only Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl) and Hector Max 66.5 WG + Trend 0.1% (a.s. nicosulfuron, rimsulfuron, dicamba + adjuvant) caused an increase in the count of actinobacteria, whereas the other herbicides resulted in a decrease. The greatest decrease in the count of actinobacteria at the last term of analyses was noted in the Maister Power 42.5 OD variant (a.s. iodosulfuron-methyl-sodium, foramsulfuron, thiencarbazone-methyl).

Like all groups of microorganisms, moulds also responded to the herbicide treatment with changes in their count in soil (*Table 4*). At the first term each of the crop protection products decreased the population of these microorganisms. Arigo 51 WG + Trend 0.1% (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant) was the most toxic as it decreased the count of moulds by more than 56%, as compared with the control variant. Thirty days after the herbicide treatment Stellar 210 SL + Olbras (a.s. dicamba, topramezone + adjuvant), Maister Power 42.5 OD (a.s. iodosulfuron-methyl-sodium, foramsulfuron, thiencarbazone-methyl) and Arigo 51 WG + Trend 0.1% (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant) caused an increase in the count of moulds. Sixty days after the herbicide treatment only the Arigo 51 WG + Trend 0.1% variant (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant) had negative effect on the population of these microorganisms.

Soil biochemical activity

The herbicides had significant influence on the enzymatic activity of alkaline phosphatase, catalase and BIF. They did not have significant influence on the activity of dehydrogenase or acid phosphatase (*Table 5*).

Table 5. *F test statistics and significance levels of two-way analysis of variance for the enzymes activity associated with herbicides and terms research fixed factors (*** p = 0.001. ** p = 0.01. * p = 0.05. ns - not statistically significant)*

Parameter	Time	Treatment	Interaction
Dehydrogenase	0.03^{ns}	0.82^{ns}	1.89^{*}
Alkaline phosphatase	495.52^{***}	87.40^{***}	82.66^{***}
Acid phosphatase	32.59^{***}	0.65^{ns}	0.62^{ns}
Catalase	19051.80^{***}	4312.67^{***}	2691.16^{***}
BIF	39.00^{***}	8.77^{***}	9.05^{***}

As can be seen in *Figure 2* the greatest fluctuations in the dehydrogenase activity were observed thirty days after the herbicide treatment. Maister Power 42.5 OD (a.s. iodosulfuron-methyl-sodium, foramsulfuron, thiencarbazone-methyl), Laudis 44 OD (a.s. tembotrione) and Arigo 51 WG + Trend 0.1% (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant) caused increased activity of these enzymes. The last variant increased the dehydrogenase activity nearly 5 times. Sixty days after the treatment the activity of these enzymes in all the variants with herbicides dropped below the value noted in the control variant.

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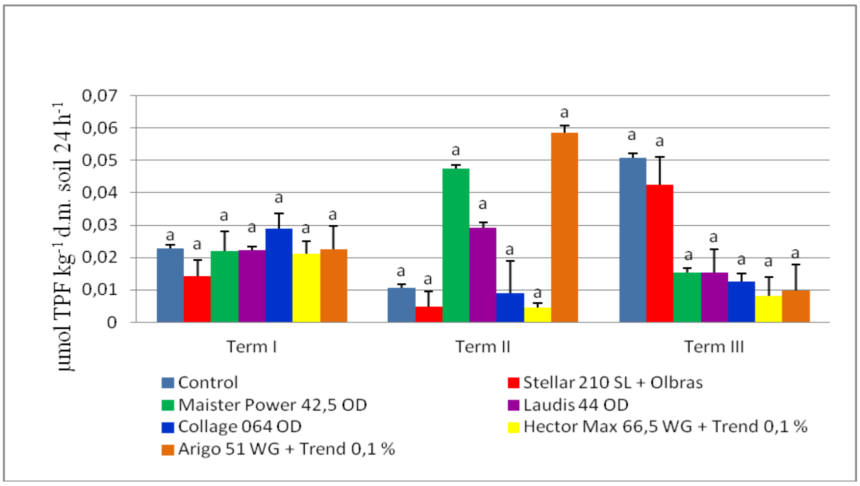

Figure 2. *The effect of herbicides on dehydrogenase activity. Note: data are represented as means of five replications. Vertical bars are standard deviation ±; mean values ± standard errors; a, b, c, d, e – homogenous groups according to Tuckey's test; different letters denote statistical differences at level α = 0.05; n = 5*

The alkaline phosphatase activity in the samples collected three days after the herbicide treatment was similar in all the variants of the experiment (*Fig. 3*). At the second term of analyses the activity of these enzymes was lower in the variants treated with herbicides than in the control soil sample. Sixty days after the treatment with xenobiotics the alkaline phosphatase activity increased only in the Stellar 210 SL + Olbras (a.s. dicamba, topramezone + adjuvant) and Maister Power 42.5 OD variants (a.s. iodosulfuron-methyl-sodium, foramsulfuron, thiencarbazone-methyl). Laudis 44 OD (a.s. tembotrione) was the most toxic herbicide to alkaline phosphatase at that term of analyses. In comparison with the control variant the activity of this enzyme was reduced by more than 80%.

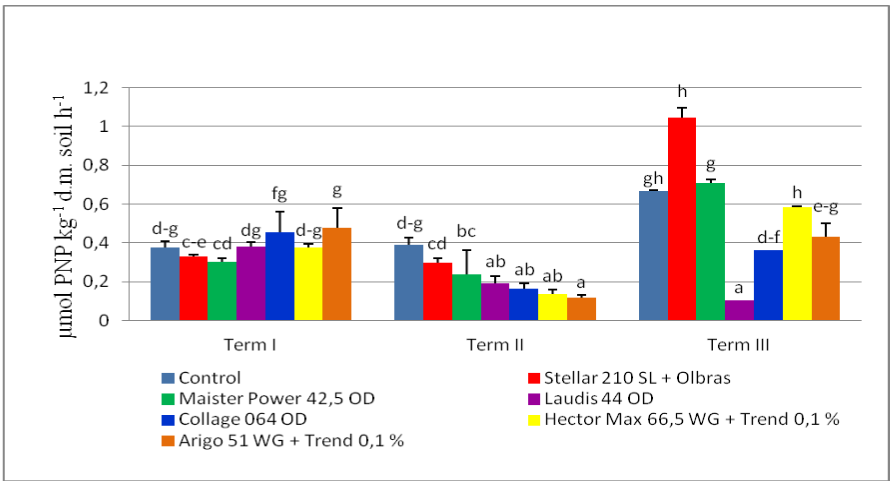

Figure 3. *The effect of herbicides on alkaline phosphatase activity. Note: data are represented as means of five replications. Vertical bars are standard deviation ±; mean values ± standard errors; a, b, c, d, e – homogenous groups according to Tuckey's test; different letters denote statistical differences at level α = 0.05; n = 5*

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The results in *Figure 4* show that only at the second term of analyses all the herbicides caused an increase in the acid phosphatase activity, as compared with the control variant. The highest acid phosphatase activity was observed when Arigo 51 WG + Trend 0.1% (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant) was applied. At the other terms there were slight fluctuations in the enzyme activity, as compared with the control variant.

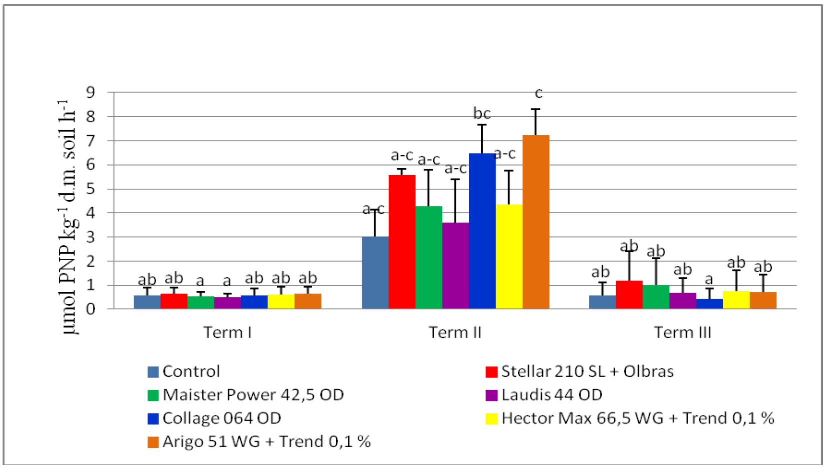

Figure 4. *The effect of herbicides on acid phosphatase activity. Note: data are represented as means of five replications. Vertical bars are standard deviation ±; mean values ± standard errors; a, b, c, d, e – homogenous groups according to Tuckey's test; different letters denote statistical differences at level α = 0.05; n = 5*

The catalase activity was also measured to assess the microbial state of soil. The data in *Figure 5* show that only at the first term of analyses the activity of this enzyme was stable and remained at a high level. There were no significant changes in comparison with the control sample. After thirty days of the experiment most of the herbicides reduced the catalase activity, as compared with the control sample. Nevertheless, the catalase activity in the variants with Arigo 51 WG + Trend 0.1% (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant) and Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl) increased 9 and 2 times, respectively. At the last term of analyses, i.e. sixty days after the treatment with xenobiotics, the catalase activity in the variants with Arigo 51 WG + Trend 0.1% (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant) and Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl) was higher than in the control variant.

The research also enabled measurement of the biological index of fertility (BIF) in all the variants (*Fig. 6*). The index value was based on the dehydrogenase and catalase activity. The highest values of the index were noted thirty days after the herbicide treatment (2nd term). The treatment with Arigo 51 WG + Trend 0.1% (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant), Maister Power 42.5 OD (a.s. iodosulfuron-methyl-sodium, foramsulfuron, thiencarbazone-methyl) and Laudis 44 OD (a.s. tembotrione) resulted in higher values of the index. At the last term the results noted in the variants treated with the herbicides were lower than in the control variant.

The sensitivity value (RS) in *Table 6* shows that the soil enzymes differed in their response to pollution with the herbicides. This index of resistance is bounded by -1 and +1, where the value of +1 shows that disturbance had no effect (maximum resistance),

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whereas lower values indicate stronger effects (lower resistance). The lowest RS value for dehydrogenase in the soil was noted immediately after the treatment with Stellar 210 SL + Olbras (a.s. dicamba, topramezone + adjuvant) and Arigo 51 WG + Trend 0.1% (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant). At the consecutive terms of analyses, i.e. thirty and sixty days after the herbicide treatment, the RS value for dehydrogenase was close to ‘1’ and it ranged from 0.785 to 0.983 and from 0.887 to 0.904, respectively. The sensitivity of alkaline phosphatase to the herbicides was different during the experiment. The lowest RS value was noted thirty days after the herbicide treatment. It amounted to -0.029 after the treatment with Hector Max 66.5 WG + Trend 0.1% (a.s. nicosulfuron, rimsulfuron, dicamba + adjuvant). Sixty days after the herbicide treatment the highest sensitivity of the enzyme was observed in the Laudis 44 OD (a.s. tembotrione) and Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl) variants *(Table 6).*

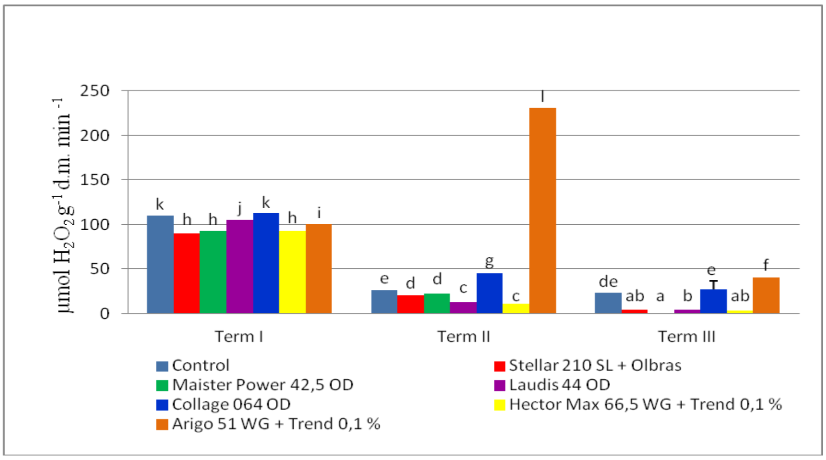

Figure 5. *The effect of herbicides on catalase activity. Note: data are represented as means of five replications. Vertical bars are standard deviation ±; mean values ± standard errors; a, b, c, d, e – homogenous groups according to Tuckey’s test; different letters denote statistical differences at level α = 0.05; n = 5*

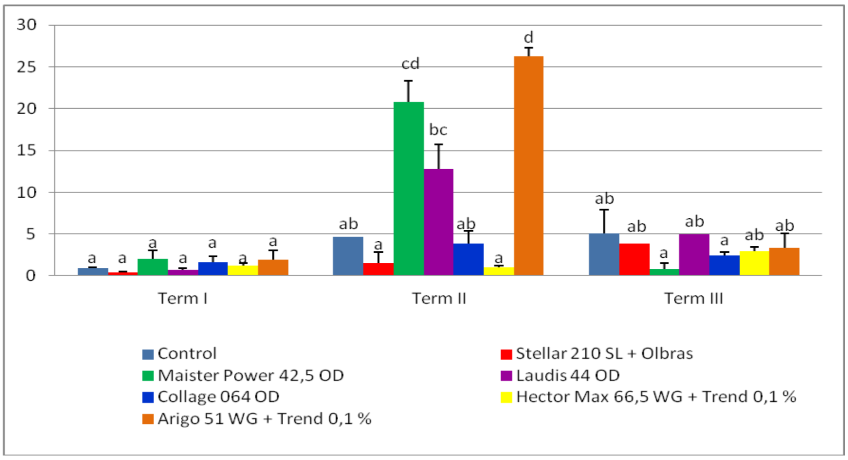

Figure 6. *The effect of herbicides on BIF. Note: data are represented as means of five replications. Vertical bars are standard deviation ±; means values ± standard errors; a, b, c, d, e – homogenous groups according to Tuckey’s test; different letters denote statistical differences at level α = 0.05; n = 5*

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Table 6. *Resistance of soil enzymes to contamination with herbicides. (PAC – acid phosphatase activity; PAL – alkaline phosphatase activity; DHA – dehydrogenase activity)*

Experimental variant	**Term of analysis**		
	3 days after herbicides application	**30 days after herbicides application**	**60 days after herbicides application**
RS index of DHA			
Stellar 210 SL + Olbras (a.s. dicamba. topramezon + adjuvant)	-0.153	0.958	0.876
Maister Power 42.5 OD (a.s. iodosulfuron-methyl-sodium, foramsulfuron, methyl thiencarbazone	0.365	0.833	0.889
Laudis 44 OD (a.s. tembotrion)	0.769	0.887	0.904
Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl)	0.403	0.983	0.903
Hector Max 66.5 WG + Trend 0.1 % (a.s nicosulfuron, rimsulfuron, dicamba + adjuvant)	0.530	0.947	0.895
Arigo 51 WG + Trend 0.1 % (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant)	0.288	0.785	0.887
RS index of PAC			
Stellar 210 SL + Olbras (a.s. dicamba, topramezon + adjuvant)	0.363	0.554	0.464
Maister Power 42.5 OD (a.s. iodosulfuron-methyl-sodium, foramsulfuron, methyl thiencarbazone	0.692	0.815	0.434
Laudis 44 OD (a.s. tembotrion)	0.799	0.784	0.340
Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl)	0.657	0.450	0.346
Hector Max 66.5 WG + Trend 0.1 % (a.s nicosulfuron, rimsulfuron, dicamba + adjuvant)	0.785	-0.029	0.500
Arigo 51 WG + Trend 0.1 % (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant)	0.699	0.231	0.534
RS index of PAL			
Stellar 210 SL + Olbras (a.s. dicamba, topramezon + adjuvant)	0.767	0.629	0.279
Maister Power 42.5 OD (a.s. iodosulfuron-methyl-sodium, foramsulfuron, methyl thiencarbazone	0.895	0.553	0.329
Laudis 44 OD (a.s. tembotrion)	0.740	0.703	0.0483
Collage 064 OD (a.s. nicosulfuron, thifensulfuron-methyl)	0.763	0.802	0.437
Hector Max 66.5 WG + Trend 0.1 % (a.s nicosulfuron, rimsulfuron, dicamba + adjuvant)	0.741	0.859	0.502
Arigo 51 WG + Trend 0.1 % (a.s. mesotrione, nicosulfuron, rimsulfuron + adjuvant)	0.693	0.910	0.643

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The results concerning the influence of the herbicides on the soil bioactivity were illustrated by means of principal component analysis (PCA) (*Fig. 7*). The analysis showed highly significant dependences occurring in the soil environment under maize plantation after the herbicide treatment. There was a high positive correlation between the biological parameters such as the total bacterial count and the count of actinobacteria and the alkaline phosphatase activity sixty days after the herbicide treatment. On the other hand, the dehydrogenase activity, the biological index of fertility (BIF) and the count of moulds were negatively correlated with the effect of the crop protection products at all the terms of the analyses.

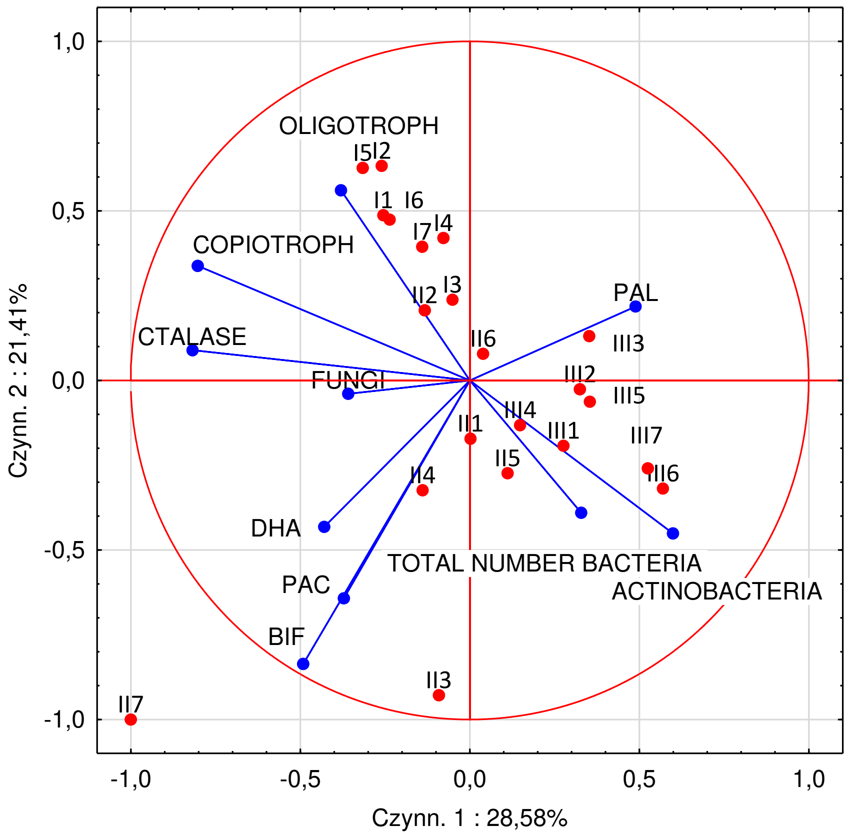

Figure 7. *The dependence between the population of microorganisms and soil enzymatic activity for all variants with herbicides at the terms of analyses; I, 3 days after herbicides application; II, 30 days after herbicide treatment; III, 60 days after herbicide treatment; 1, control; 2, Stellar 210 SL + Olbras; 3, Maister Power 42.5 OD; 4, Laudis 44 OD; 5 Collage 064 OD; 6 Hector Max 66.5 WG + Trend 0.1 %; 7 Arigo 51 WG + Trend 0.1 %; PAC - acid phosphatase; PAL- alkaline phosphatase; DHA - dehydrogenase; RS - soil resistance*

Discussion

The count of microorganisms and the enzymatic activity are important biological indicators of soil. The dynamics of changes in the count of microorganisms may be caused by cultivation, fertilisation and chemical crop protection. Herbicides may influence the count of soil bacteria, which participate in the processes responsible for soil fertility.

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The results of our study show that the crop protection products used during maize growing had diversified influence on the groups of soil microorganisms under analysis, which depended on the products and the term of analyses. Apart from that, there was high diversification in the populations of different microorganisms colonising the soil environment after the application of the chemical crop protection products. The research results showed that the products significantly disturbed the microbial balance, causing changes in the count of oligotrophic and copiotrophic bacteria, actinobacteria, moulds and the total bacterial count.

Hector Max 66.5 WG + Trend and Arigo 51 WG + Trend significantly reduced the total bacterial count thirty days after the treatment. The same herbicides significantly reduced the population of oligotrophs thirty and sixty days after the treatment, whereas Maister Power 42.5 OD had significant negative influence on the development of actinobacteria also at the last term of analyses.

Sixty days after the herbicide treatment the count of actinobacteria and oligotrophs may have been reduced because of the high toxicity of metabolites formed in consequence of the decomposition of the active substances in the herbicides. When pesticides are applied, they undergo various processes in the environment, such as: transformation, degradation, sorption-desorption, volatilisation, uptake by plants, runoff to surface water and transport to groundwater (Chowdhury et al., 2008). Transformation and degradation are two of the major processes regulating the state of the soil environment. Pesticides are transformed into secondary metabolites or they are completely mineralised (Singh et al., 2006). Although abiotic degradation plays a significant role in many cases, the biodegradation of pesticides by microorganisms is usually the most important and predominant process (Karpouzas and Walker, 2000; Chen et al., 2012a, b; Cycoń et al., 2014; Silva et al., 2015). The negative influence of crop protection products on the proliferation of microorganisms was also observed by Sebiamo et al. (2011), who studied atrazine, primextra, paraquat and glyphosate. Niewiadomska et al. (2018) made similar observations.

The results of our study concerning the influence of the active substance in herbicides show that in some cases the count of the groups of microorganisms under study increased (the total bacterial count, the count of copiotrophs), especially immediately after the herbicide treatment, i.e. at the first term of analyses. There are publications (Fečko et al., 2010) which confirm this reaction of the soil microflora and stress the fact that some species of microorganisms use herbicides as a source of carbon and nutrients. Due to the fact that crop protection products have been used in agriculture for decades, microorganisms have adapted to the polluted environment. They have developed natural mechanisms of biodegradation of toxic substances. The capacity of some microorganisms to biodegrade pesticides partly reduces the toxic substances which might affect the yield. It also improves the biological properties of soil. Many studies proved that strains such as *Pseudomonas* (Fernandes et al., 2014), *Acinetobacter* (Singh et al., 2004*)*, *Rhodococcus* (Batra et al., 2009; Fazlurrahman et al., 2009), *Arthrobacter* (El Sebaï et al., 2011), *Bacillus* (Wang et al., 2014), *Variovorax itp.* (Zhang et al., 2012) were capable of total degradation of atrazine (Zhang et al., 2012) and glyphosate. However, in most cases the effectiveness of biodegradation of herbicides by bacteria is significantly correlated with the soil pH, temperature and availability of building elements such as carbon and nitrogen.

The soil enzymes which participate in the biodegradation of natural and anthropogenic compounds are often used as indicators of changes in the soil

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environment caused by the use of crop protection products, including herbicides (Wang et al., 2009; Baćmaga et al., 2012).

The study showed that the enzymes under analysis were sensitive to the herbicides applied in the experiment. Sixty days after the herbicide treatment all the xenobiotics exhibited strongly negative influence on the dehydrogenase activity. The negative influence was also observed thirty days after the treatment with Stellar 210SL + Olbras and Hector Max 66.5 WG + Trend 0.1%. There were similar observations made by Cycoń et al. (2010, 2013), who studied the influence of napropamide and linuron in herbicides, and by Baćmaga et al. (2014), who researched the influence of metazachlor on the dehydrogenase activity.

Our study also showed that alkaline phosphatase exhibited high sensitivity. The activity of this enzyme became significantly reduced thirty days after the soil treatment with all of the herbicides used in the experiment. This phenomenon may have been caused by the low content of organic matter in the soil and by the inhibitory effect of herbicides on local moulds, which are known to produce alkaline phosphatase in soil (Cycoń et al., 2013). On the other hand, at the same term of analyses the acid phosphatase activity in the soil variants treated with the herbicides was higher than in the control variant.

Pesticides and their metabolites may also influence physiological processes in microorganisms such as cell lysis and changes in the cell membrane, thus modifying the activity of soil enzymes (Hussain et al., 2009; Romero et al., 2010).

The soil ecosystem is regarded as stable if it can resist stress factors and regain balance shortly (Griffiths and Philippot, 2013; Orwin and Wardle, 2004). Soil stability is usually assessed on the basis of resistance (RS) and resilience (RL). In our study the RS value was calculated by estimating the resistance of soil to contamination with the herbicides and adjuvants (*Table 6*).

The RS values calculated in our study show that immediately after the herbicide treatment alkaline phosphatase was the least sensitive enzyme, whereas dehydrogenase was the most sensitive. However, alkaline phosphatase exhibited the highest sensitivity thirty days after the treatment. The herbicides caused greater disturbance to the phosphatase activity than to the dehydrogenase activity. Griffith and Philippot (2013) stress the fact that when the soil ecosystem is exposed to long-lasting stress factors, it becomes more stable due to the development of defence mechanisms responsible for its biostability.

Conclusion

Tested post-emergence herbicides applied to maize caused a statistically significant biological imbalance in the soil, reducing the actinobacteria as well as the number of moulds at the beginning of vegetation, three days after the treatment. The research revealed very high sensitivity of dehydrogenases and alkaline phosphatase to the soil contamination caused by the application of herbicides. The field doses of the xenobiotics used in the research significantly reduced the dehydrogenase activity in sixty days after the herbicide treatment. The dehydrogenase activity values were strictly correlated with BIF and PAC.

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MORPHO-PHYSIOLOGICAL RESPONSES OF STEVIA (*STEVIA REBAUDIANA* BERTONI) TO VARIOUS PRIMING TREATMENTS UNDER DROUGHT STRESS

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Abstract. Poor germination capacity of stevia is a major problem in its cultivation. Moreover, drought stress is one of the most major environmental constraints, which influences seed germination and early seedling growth of many crops. The aim of this research was to evaluate the impact of seed priming with salicylic acid (SA), zinc (Zn) and iron (Fe) on some germination parameters and physiological attributes of stevia seedlings under drought stress induced by polyethylene glycol (PEG) 6000 (0, – 3, – 6 and – 9 bars). The results revealed that germination traits (germination percentage, germination rate, mean germination time, germination value, seedling length, and seedling vigor index) and chlorophyll (Chl) content were negatively affected by drought stress. However, the reduction of germination parameters in seedlings exposed to drought stress in most cases was moderated by seed priming, which also increased the Chl content at all levels of drought stress as compared with the control. Drought stress also increased the proline accumulation and the enzymatic activity of catalase (CAT) and peroxidase (POD) in all priming treatments, but these enhancements were significantly higher in primed seedlings than those in unprimed ones. Among all priming treatments, priming with SA + Fe + Zn was found to be more effective than other treatments to improve growth and physiological characteristics under normal and drought stress conditions. Thus, we suggest that seed priming with SA, Fe, Zn and particularly the integrated application of these three agents at a suitable concentration can promote the poor germination performance of stevia and improve the seedling growth by increasing the antioxidant capacity under drought conditions.
Keywords: *antioxidant enzymes, chlorophyll, proline, salicylic acid, seed germination*

Introduction

Stevia (*Stevia rebaudiana* Bert.), a member of the Asteraceae (Compositae) family, is an herbaceous perennial and short-day species (Yadav et al., 2011; Ucar et al., 2016). Naturally, this plant grows between 22–24° south and 53–56° west in South America (Paraguay and Brazil), where it is called ka'ahe'ê (or sweet herb) (Lemus-Mondaca et al., 2012). Stevia is widely grown for its non-caloric sweet compounds. The sweetness of the stevia plant is related to the presence of *ent*-kaurene-type diterpene glycosides (stevioside and rebaudiosides), which can be 300 times sweeter than sucrose (Hajihashemi and Ehsanpour, 2014; Aghighi Shaverdi et al., 2018). Since the sweet herb has self-incompatible flowers, its pollination is probably carried out by insects and wind (Ucar et al., 2016; Shahverdi et al., 2017). As a result, stevia produces often few pollinated flowers which have low germination percentage and poor establishment (Yadav et al., 2011; Lemus-Mondaca et al., 2012). In general, the poor germination

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capacity of stevia continues to be a major impediment for the plantation of this plant on a large-scale (Abdullateef et al., 2015).

Plants are frequently subjected to a lot of abiotic environmental stresses such as drought, high salinity, heavy metals, high/low temperatures and, or heat (Brito et al., 2016). Drought stress is considered as the most major environmental problem that severely affects crop growth and development worldwide, especially in arid and semi-arid regions (Askari et al., 2018). Among various stages of the plant's life cycle, seed germination and seedling emergence are the most sensitive stages to environmental stresses (Patade et al., 2011). Because of the reduction in water potential and water absorption during drought stress, seed germination may be limited (Farooq et al., 2009) and with increasing intensity of water deficit, the complete inhibition of seedling growth can occur (Kaya et al., 2006). Moreover, some investigations have pointed out that drought stress can accelerate the generation of reactive oxygen species (ROS) in plants (Zhang et al., 2015), resulting in reduced growth, cellular damage and eventually plant death (Al Hassan et al., 2017). To improve drought tolerance in plants, one of the most effective methods is the adoption of physiological improvement approaches like seed priming (Zheng et al., 2016). It has been reported that different priming treatments have a good potential to enhance germination uniformity, germination rate, seed vigor, as well as stronger seedling growth under stressed conditions (Patade et al., 2011; Paparella et al., 2015). The higher tolerance of primed seeds against environmental stresses can be occurred due to the activation of free radical scavenging enzymes such as SOD, POD, CAT, and the accumulation of osmoprotectants (e.g. proline) (Rouhi et al., 2012).

Under environmental stresses, seed priming with various plant growth regulators (such as SA) is a common and effective technique for modulating the destructive effects of stress (Ashraf and Foolad, 2005; Hussain et al., 2016). SA (2-hydroxybenzoic acid), a phenolic compound, is known as an endogenous signal hormone that activates a wide range of diverse physiological and biochemical processes in plant cells in response to stressful conditions (Kabiri et al., 2014; Najafabadi and Ehsanzadeh, 2017). In this regard, SA has been successfully applied to alleviate the adverse effects of drought stress on wheat germination parameters (Movaghatian and Khorsandi, 2013).

Micronutrients are essential for normal growth and development of plants, and most of the arable soils in Iran have the severe deficiency of micronutrients, especially Zn and Fe (Mirshekari et al., 2012). Among various priming techniques, seed priming with micronutrients (nutri-priming) has been reported to be a physiological beneficial method for overcoming the micronutrient deficiency in seeds which improves seedling emergence (Farooq et al., 2012). Various studies have revealed that this technique significantly improves germination rate, seed quality, early seedling growth and stress tolerance in different plants (Mirshekari et al., 2012; Imran et al., 2017; Shahverdi et al., 2017; Reis et al., 2018).

According to the seed germination problem in stevia and on the other hand the lack of sufficient information about the role of SA and micronutrients on the improvement of germination behavior and early seedling growth of stevia, the present study was conducted to examine the effect of seed priming with SA and micronutrients (Fe and Zn) on germination indices and biochemical characteristics of stevia under control and drought stress conditions.

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Materials and methods

The present experiment was carried out at the Seed Science and Technology Laboratory of Agricultural College, Shahed University of Tehran, Iran in 2017.

Seed material and storage

New mature seeds of stevia (Var. Bertoni) used in the experiment were purchased in late November 2017 from stevia production fields located in Fars province, Firoozabad city, Iran (34° 37' N, 54° 45' E and 1790 m ASL). The average of 100-seed weight was 27.7 ± 0.5 mg and seed moisture content was around 8.64% (on a dry weight basis). To keep the seed vigor during the course of experimentation, the seed samples were stored in paper bags at 4 ± 1°C and 20% relative humidity before being utilized for experiments. The 1.5-month seed samples were used in this research.

Priming and drought stress treatments

The study was a factorial experiment based on a completely randomized design (CRD) with three replications in which the experiment factors included four levels of drought stress induced by PEG 6000 (0, – 3, – 6 and – 9 bar) and seven combinations of priming (SA, Fe, Zn, SA + Fe, SA + Zn, Fe + Zn, SA + Fe + Zn), and unprimed dry seeds were considered as control. In this experiment, Fe and Zn were supplied from sources of iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$, 26% Fe) and Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$, 21% Zn) respectively, as well as SA (2-hydroxybenzoic acid, Sigma Aldridge Company Ltd.) in powder form, was used.

Germination process

Prior to conducting the experiment and based on three separate tests, the best duration and concentration of pretreatment for each of the priming agents (Fe, Zn and SA) were optimized. The factors evaluated in these experiments were five priming durations (0, 6, 12, 18 and 24 hours) and six concentrations of micronutrients (0, 0.25%, 0.5%, 1%, 1.5% and 2%) and SA (0, 0.25, 0.5, 1, 1.5 and 2 mM), which were tested separately. According to the results, the best duration and concentration of seed priming with micronutrients (Fe, Zn) and SA were 24 hours at the concentration of 0.5% and 24 hours at the concentration of 1 mM, respectively. These data were used in the experiment (data not shown).

To conduct the germination test, first, all used equipment and seeds were thoroughly disinfected. Stevia seeds were sterilized in 70% (v/v) ethanol for 1 min and 20% (v/v) sodium hypochlorite solution for 15 min and then were rinsed three times with sterile distilled water (Hajihashemi and Ehsanpour, 2013). The priming treatments were prepared in distilled water. Stevia seeds were entirely immersed in determined concentrations of priming media (1 mM SA, 0.5% Fe, 0.5% Zn, 1 mM SA + 0.5% Fe, 1 mM SA + 0.5% Zn, 0.5% Fe + 0.5% Zn and 1 mM SA + 0.5% Fe + 0.5% Zn) at 15 °C in darkness (Bradford, 1986). After the end of the priming process, the treated seeds were surface washed with distilled water and then dried at room temperature (about 21 °C) for 24 h back to the initial moisture content. After that, 100 primed and unprimed (control) seeds were counted and placed between double layers of sterilized Whatman paper (No. 2) in 12-cm-diameter Petri dishes and based on various levels of drought stress, 7 mL of PEG solution was added to each Petri dish. The osmotic

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potentials of – 3, – 6 and – 9 bars were obtained by adding 148.01, 219.54 and 274.65 g of PEG 6000 in 1000 mL of distilled water, respectively. The required amount of PEG 6000 was calculated by Michel and Kaufmann's (1973) formula (*Eq. 1*):

$$\Psi s = -\left(1.18\times10^{-2}\right)\times C - \left(1.18\times10^{-4}\right)\times C^2 + \left(2.67\times10^{-4}\right)\times CT + \left(8.39\times10^{-7}\right)\times C^2T \quad \text{(Eq.1)}$$

Ψs, C, and T are osmotic potential (bars), the concentration of PEG (g L^{-1} of distilled water) and temperature (°C), respectively. The distilled water potential is zero, so it was used as the control treatment (without drought stress). Petri dishes were wrapped with impermeable parafilm to avoid the loss of moisture and evaporation. Subsequently, all dishes were transferred in a programmed germination chamber at 23 ± 2 °C, 16/8 h light/darkness and 75% relative humidity (Liopa-Tsakalidi et al., 2012). The germinated seeds were daily counted, and the germination test was ended when the germination was not observed for three consecutive days. The seeds which had radicles with 2 mm long or more were considered as germinated seeds (ISTA, 2010). Eventually, at the end of the germination period (14 days), germination percentage, germination rate, mean germination time, seedling vigor index and germination value were calculated based on the relationships presented in *Table 1*.

Table 1. *The computing relations of the parameters studied in the experiment*

Parameters	Formula	Reference
Germination percentage	GP = (N × 100)/M	Liopa-Tsakalidi et al., 2012
Germination rate	$GR = \sum_{i=1}^{n} \frac{si}{Di}$	Maguire, 1962
Mean germination time	$MGT = \frac{\sum(Dn)}{\sum n}$	Salehzade et al., 2009
Germination value	GV = GP × MDG	Czabator, 1962
Seedling vigor index	SVI = GP × Mean (SL) /100	Abdul-Baki and Anderson, 1973

N: the sum of germinated seeds at the end of the experiment, M: the total number of planted seeds, S_i: the number of germinated seeds in each enumeration, D_i: the number of day from the start of the test to the enumeration of n^{th}, D: the time from the start of the experiment to the i^{th} observation, n: the number of germinated seeds at time D, MDG: mean daily germination, SL: mean of seedling (root + shoot) length (mm)

After two weeks of growth, the seedlings from each replicate were collected and immediately frozen in liquid nitrogen and stored in the ultra-low freezer at –80 °C for biochemical studies.

Antioxidant enzymes assay

To evaluate the activity of antioxidant enzymes, 0.5 g of frozen samples were homogenized in 5 mL of cool extraction buffer (50 mM potassium phosphate buffer (pH = 7.5)), containing 1 mM ethylenediaminete-traacetic acid (EDTA), 1 mM dithiothreitol (DTT) and 2% (w/v) polyvinylpyrrolidine (PVP)). The homogenate was centrifuged at 15,000 × *g* for 25 min and obtained supernatants were used as the enzyme source for CAT and POD assays. The whole extraction process was done at 4 °C.

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Peroxidase assay

The POD activity (EC 1.11.1.7) was determined following the method of MacAdam et al. (1992) with some modification. In this method, The enzymatic activity was assayed by adding 50 µL of enzyme extract to the reaction mixture (3 mL), containing 0.1 M potassium phosphate buffer (pH 6.0), 50 µL guaiacol and 50 µL H_2O_2 (3%), and absorption alterations were immediately recorded at 436 nm for 3 min per 15 s by spectrophotometer. The control contained 3 mL of 0.1 M potassium phosphate buffer, 50 µL guaiacol and 50 µL H_2O_2.

Catalase assay

The CAT activity (EC.1.11.1.6) was assayed according to the method of Chance and Maehly (1955) which is briefly described here. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH = 7.0), 15 mM H_2O_2, and 25 µL of the enzyme extract in a total volume of 3 mL. The absorbance at 240 nm for 1 min at 25 °C was recorded spectrophotometrically. One unit of CAT was defined as the amount of enzyme that decomposes 1 µmol of H_2O_2 per min.

Proline determination

The free proline content was measured using the method described by Bates et al. (1973). Frozen samples (0.5 g) were homogenized in 10 mL of 3% (w/v) sulpho salicylic acid and centrifuged at 4000 × g for 10 min. After centrifugation, this homogenized solution was filtered with Whatman's paper (No. 2) and then 2 mL of filtrated solution was mixed with acid-ninhydrin (2 mL) and glacial acetic acid (2 mL) in a test tube. The mixture was placed at 100 °C for 1 h in a water bath and immediately transferred to an ice bath for a few minutes. After cooling, the reaction mixture was thoroughly mixed with toluene (4 mL), and then the absorbance was measured at 520 nm.

Total chlorophyll content

The Chl content was measured using the methods of Lichtenthaler (1987). In this method, chlorophyll was extracted in chilled 80% acetone in dark. After centrifugation at 5000 × *g* for 10 min, the total chlorophyll content was determined at 663.2 and 646.8 nm with a spectrophotometer. The total chlorophyll content was calculated according to *Equation 2:*

$$\text{Total Chl} = 7.15\ (\text{A}663.2) + 18.71\ (\text{A}646.8) \qquad \text{(Eq.2)}$$

where A is absorbance at the specific wavelength.

Statistical analysis

After checking the data distribution normality (Kolmogorov-Smirnov and Shapiro-Wilk test) assumption, the studied traits were statistically analyzed by the Statistical Analysis System software (SAS Institute, Cary, NC, USA, Version 9.4). The differences among means were separated using least significant difference test (LSD) at 0.05 statistical probability level and the graphs were drawn by MS–Excel.

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Results

Germination percentage (GP)

The effects of drought stress due to PEG, priming treatments and the interplay between them were significant ($P \leq 0.01$) on GP parameter (*Table 2*). The results of stevia seed germination under drought stress after various priming treatments are shown in *Table 3*. The highest GP was observed in seeds primed with SA + Fe + Zn and Fe + Zn under control conditions (without drought stress), as well as seeds primed with SA + Fe + Zn at – 3 bar (66.66%, 60.33% and 60.33%, respectively). GP was drastically affected by drought stress, so that the minimum GP was obtained from unprimed seeds at – 9 bar (72.4% reduction compared to unprimed seeds under the osmotic potential of 0 bar). The priming treatments improved seed germination of stevia under normal and drought stress conditions. At the highest level of drought stress (– 9 bar), the maximum GP (33.6 and 32.33%) was achieved from primed seeds by Fe + Zn and SA + Fe + Zn, respectively (*Table 3*). The listed treatments improved the GP parameter by 57.4% and 55.7% as compared to the control seeds (without priming) at this level.

Germination rate (GR)

Drought stress, priming and the interaction of these two factors had highly significant effects ($P \leq 0.01$) on GR trait (*Table 2*). The results showed that the GR of stevia seeds was significantly decreased with increasing drought stress levels (*Table 3*). Nevertheless, the amount of this trait was higher in the primed seeds than in unprimed ones under normal and drought stress conditions. The most value of GR (18.52 seed per day) belonged to seeds which were treated by SA + Fe + Zn under normal conditions. The lowest amounts of GR were found in unprimed seeds and primed seeds by SA, Fe, and Zn under the osmotic potential of – 9 bar (2.11, 2.52, 3.02 and 3.01 seed per day, respectively). The results clearly indicated that the integrated application of priming agents was more effective than their separate application to alleviate the drought-induced damaging effects on GR (*Table 3*).

Table 2. *Analysis of variance for the effect of priming treatments and drought stress on stevia seed germination indices*

Sources of variance	df	Mean squares and significance					
		GP	GR	MGT	GV	SVI	SL
Drought (D)	3	5489.5^{**}	576.08^{**}	29.1^{**}	18.04^{**}	808.3^{**}	1420.8^{**}
Priming (P)	7	561.6^{**}	25.1^{**}	0.24^{ns}	1.77^{**}	162.9^{**}	334.1^{**}
D × P	21	33.9^{**}	1.9^{**}	0.33^{**}	0.08^{ns}	10.11^{**}	13.31^{**}
Experimental error	64	15.1	0.81	0.11	0.057	2.91	4.67
Coefficient variation (%)	-	9.18	10	5.99	16.52	18.16	11.11

ns, * and **: non-significant, significant at 5% and 1%, respectively. df: degrees of freedom, GP: germination percentage, GR: germination rate, MGT: mean germination time, GV: germination value, SL: seedling length, SVI: seedling vigor index

Mean germination time (MGT)

As shown in *Table 2*, drought stress and the interaction of drought stress and priming treatments had highly significant effects on MGT parameter ($p \leq 0.01$). In response to

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drought stress, MGT was significantly increased so that the maximum values of MGT were observed at the highest level of drought stress (– 9 bar) while the lowest required times for germination (4.12, 4.15, 4.18, 4.56 and 4.65 days) were recorded in the absence of stress (0 bar) in seeds treated with Fe, SA, SA + Fe + Zn, SA + Fe and Fe + Zn, respectively. At – 9 bar, those seeds primed with SA + Fe + Zn had the shortest required time for germination (6.68 days), which was significantly lower than unprimed seeds at this level.

Table 3. *Mean comparison of the interaction between different priming treatments and drought stress for stevia seed germination indices*

Treatments		GP (%)	GR (seed/day)	MGT (day)	SL (mm)	SVI
Drought (bars)	Priming					
0	Control	52c..f	13.44cd	4.71i..l	20.5fg	10.82ef
	SA	51.33c..f	14.12bc	4.15m	25.9c.e	13.32de
	Fe	56.66bc	15.38b	4.12m	24.8c.e	14.09b.d
	Zn	54.66b..e	13.96b.d	4.71i..l	19.3gh	10.56ef
	SA + Fe	58.66b	15.04b	4.56k.m	26.8b.d	15.79b.d
	SA + Zn	59.00b	14.51bc	4.89h..k	27.3bc	16.18bc
	Fe + Zn	60.33ab	15.32b	4.65j..m	27.8bc	16.8b
	SA + Fe + Zn	66.66a	18.52a	4.18lm	37.4a	25.01a
–3	Control	43.33h.j	7.43ij	6.46cd	16.86h.j	7.34g..j
	SA	43.66g..j	8.53hi	5.58e.g	24.4c.e	10.67ef
	Fe	50d..g	10.05fg	5.46fg	20.8fg	10.42f
	Zn	48f..i	9.48gh	5.45fg	15.4i.k	7.4g..j
	SA + Fe	56b.d	11.88e	5.14f...j	23.7d.f	13.3de
	SA + Zn	55.33b..e	11.25ef	5.42f.g	24.8c.e	13.77cd
	Fe + Zn	55.33b..e	11.8e	5.13f…j	27.9bc	15.5b.d
	SA + Fe + Zn	60.33ab	12.57de	5.24f..i	38.2a	23.11a
–6	Control	15.66op	3.06no	5.46fg	12.4k.m	1.98l..o
	SA	28.66lm	5.92kl	5.06g…k	18.6g.i	5.29i.k
	Fe	38jk	7.27i.k	5.56e.g	16.1h.j	6.24h..k
	Zn	37.33jk	7.03jk	5.63ef	11.6l.n	4.32k.m
	SA + Fe	43.33h.j	8.22h.j	5.62ef	19.03gh	8.28f.h
	SA + Zn	42.66ij	8.67g.i	5.34f.h	23.1ef	9.99fg
	Fe + Zn	42.66ij	8.10h.j	5.68ef	17.4g..j	7.48h.j
	SA + Fe + Zn	49.33e..h	8.41h.j	6.09de	30.1b	14.97b.d
–9	Control	14.33p	2.11o	7.52a	5.76p	0.85o
	SA	17.33op	2.52no	7.23ab	8.14n.p	1.43no
	Fe	21.66no	3.02no	7.32a	7.76op	1.7m.o
	Zn	20n.p	3.01no	7.04ab	5.8op	1.19no
	SA + Fe	25.33mn	3.66mn	7.19ab	9.07m..p	2.3l..o
	SA + Zn	24.66mn	3.67mn	7.12ab	9.36m.o	2.23l..o
	Fe + Zn	33.66kl	5.00lm	7.18ab	11.49l.n	3.83k..n
	SA + Fe + Zn	32.33kl	5.18l	6.68bc	14.4j.l	4.67j.l

Means in each column with the same alphabetical letter (s) are not significantly different at 0.05 probability level according to LSD test. GP: germination percentage, GR: germination rate, MGT: mean germination time, SL: seedling length, SVI: seedling vigor index

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Seedling length (SL)

Based on the analysis of variance, drought stress, priming treatments and the interaction among them had highly significant effects ($P \leq 0.01$) on SL trait (*Table 2*). Drought stress was found to severely limit the seedling growth of stevia. The longest SL pertained to the seeds primed by SA + Fe + Zn at – 3 and 0 bar (control) levels of drought stress (38.2 and 37.4 mm, respectively), and the shortest SL was observed at the highest level of drought stress (– 9 bar) in unprimed seeds (5.76 mm) (*Table 3*). Under severe drought stress, the SL in SA + Fe + Zn treatment was 60% higher compared to the control.

Seedling vigor index (SVI)

According to the results, the effect of drought stress, priming treatments and the interaction among them was highly significant ($p \leq 0.01$) on SVI (*Table 2*). Depending on the reduction in SL and GP under drought stress, SVI was gradually reduced with increasing drought stress levels, so that the lowest means were recorded at – 9 bar (*Table 3*). On the other hand, priming treatments, especially the integrated application of SA, Fe, and Zn increased SVI under drought and normal conditions compared to unprimed seeds. The highest amount of this trait was detected in primed seeds with SA + Fe + Zn under the osmotic potentials of 0 and – 3 bar (25.01 and 23.11, respectively) (*Table 3*).

Germination value (GV)

Variance analysis of data revealed that the GV parameter was significantly affected by drought stress and priming agents ($p \leq 0.01$), but the interplay of seed priming × drought stress on this trait was not significant (*Table 2*). Comparison of mean values at different levels of drought stress showed that the increase of PEG concentration from the control to the highest level (– 9 bar) resulted in an 81.85% reduction in GV of stevia seeds. Presented results in *Table 4* showed that all the seed priming treatments except SA treatment had a significant positive effect on GV compared with unprimed seeds. In comparison with the control (without priming), various priming treatments enhanced the GV of stevia seeds between 10.78 – 56.03%. The best result in this respect (with an average of 2.07) was achieved by applying the composition of SA, Fe and Zn (*Table 4*).

Free proline content

The proline content was significantly affected by drought stress and tested priming compounds ($p \leq 0.01$). Drought stress due to PEG at all the studied concentrations gradually increased the accumulation of free proline in stevia seedlings, so that the highest and the lowest content of proline were observed at the osmotic potentials of – 9 and 0 bar (control) (0.363 and 0.233 μmol g^{-1} FW respectively) (*Table 4*). Also, the proline content in primed seeds was significantly higher as compared to the control seeds (without priming). Among all studied priming treatments, the highest amount of the proline content belonged to seeds which were treated by SA + Fe + Zn, Fe + Zn and SA + Zn, respectively. The proline content in SA, Fe, Zn, SA + Fe, SA + Zn, Fe + Zn and SA + Fe + Zn treatments was increased by 48.56, 53.26, 53.72, 56.79, 59.02, 59.14 and 59.6% compared with the control (*Table 4*).

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Table 4. *Mean comparison of the effect of drought stress and various priming treatments on germination value (GV) and proline content*

Drought stress (bars)	GV	Proline content (µmol g^{-1} FW)
0 (Control)	2.37a	0.233d
– 3	1.92b	0.282c
– 6	1.06c	0.327b
– 9	0.43d	0.363a
Priming treatments		
Control	0.91d	0.143f
SA	1.02d	0.278e
Fe	1.37c	0.306d
Zn	1.27c	0.309cd
SA + Fe	1.63b	0.331bc
SA + Zn	1.60b	0.349ab
Fe + Zn	1.72b	0.350ab
SA + Fe + Zn	2.07a	0.354a

Means in each column with the same alphabetical letter (s) are not significantly different at 0.05 probability level according to LSD test

Total chlorophyll content

Data recorded in *Table 5* shows that the total Chl content was significantly affected by drought stress, priming agents ($p \leq 0.01$) and the interaction between them ($p \leq 0.05$). The Chl content was significantly decreased with increasing PEG concentration in all studied treatments when compared with the control conditions (without drought stress). However, priming treatments alleviated effectively the damaging effect of drought stress on Chl content. The lowest amount of Chl content was recorded at the highest level of drought stress (– 9 bar) in unprimed seeds. The highest values of total Chl content were recorded in the combination of three priming agents (SA + Fe + Zn) and the combination of Fe and Zn under normal conditions (2.22 and 2.04 mg/g FW, respectively). At all levels of drought stress, the most effective priming treatments with the highest total Chl content were SA + Fe + Zn and Fe + Zn (*Fig. 1*).

Table 5. *Analysis of variance for the effect of priming treatments and drought stress on physiological traits of stevia seedling*

Sources of variance	df	Mean squares and significance			
		Total Chl	Proline	CAT	POD
Drought (D)	3	5.13^{**}	0.075^{**}	6.16^{**}	196.42^{**}
Priming (P)	7	1.44^{**}	0.063^{**}	0.376^{**}	14.73^{**}
D × P	21	0.022^{*}	0.0006^{ns}	0.085^{*}	1.102^{*}
Experimental error	64	0.012	0.0008	0.046	0.628
Coefficient variation (%)	-	9.05	9.40	17.56	9.76

ns, * and **: non-significant, significant at 5% and 1%, respectively, df: degrees of freedom, Chl: chlorophyll content, CAT: catalase activity, POD: peroxidase activity

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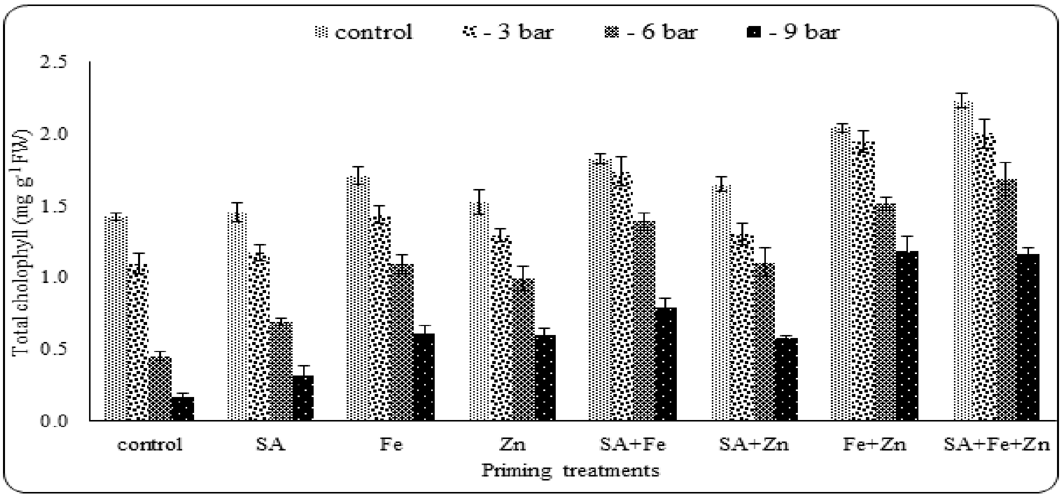

Figure 1. *Total Chlorophyll content in primed and unprimed seedlings of stevia under normal and drought stress conditions (LSD 5%= 0.185). Error bars indicate standard error (SE)*

CAT activity

Statistical analysis demonstrated that the enzymatic activity of CAT varied significantly in response to drought stress, priming treatments ($p \leq 0.01$) and the interaction between them ($p \leq 0.05$) (*Table 5*). Data regarding the CAT activity in primed and unprimed seedlings of stevia under drought stress are shown in *Figure 2*. The CAT activity was significantly increased in primed and unprimed seedlings with increasing drought stress. On the other hand, seed priming promoted the CAT activity in the stevia seedlings under drought stress, so that the maximum values of enzymatic activity were obtained from primed seeds with SA + Fe + Zn at the osmotic potential of – 9 bar (2.23 Unit/mg protein.min, respectively). In comparison to the control, under severe drought stress (– 9 bar), the enzymatic activity of CAT was significantly increased in the seedlings which were raised from primed seeds by SA + Fe + Zn, Fe + Zn, SA + Zn, SA + Fe and Fe (*Fig. 2*).

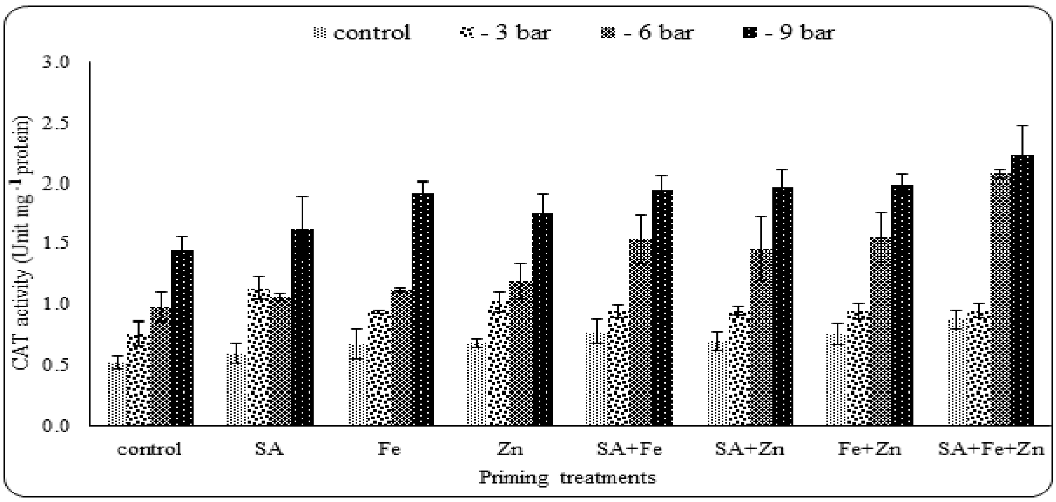

Figure 2. *The catalase (CAT) activity in primed and unprimed seedlings of stevia under normal and drought stress conditions (LSD 5%= 0.35). Error bars indicate standard error (SE)*

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POD activity

According to the results, drought stress, priming treatments ($p \leq 0.01$) and the interaction between them ($p \leq 0.05$) had significant effects on the POD activity (*Table 5*). Results regarding the POD activity of stevia seedlings under the influence of drought stress and different priming treatments are presented in *Figure 3*. The enzymatic activity of POD was considerably enhanced with elevating of drought stress in all studied treatments, so that the lowest POD activity for all treatments was observed under the controlled conditions. The average values revealed that the priming treatments caused an increase in the POD activity of the stevia seedlings compared to the control seeds. The maximum activity of POD was recorded in seedlings primed with SA + Fe + Zn and Fe + Zn at the osmotic potential of – 9 bar (14.04 and 13.15 Unit/mg protein.min, respectively). Compared with the control seeds (without priming), the SA + Fe + Zn, Fe + Zn, SA+ Fe, Zn and Fe were the most effective treatments regarding the POD activity at the highest level of drought stress (*Fig. 3*).

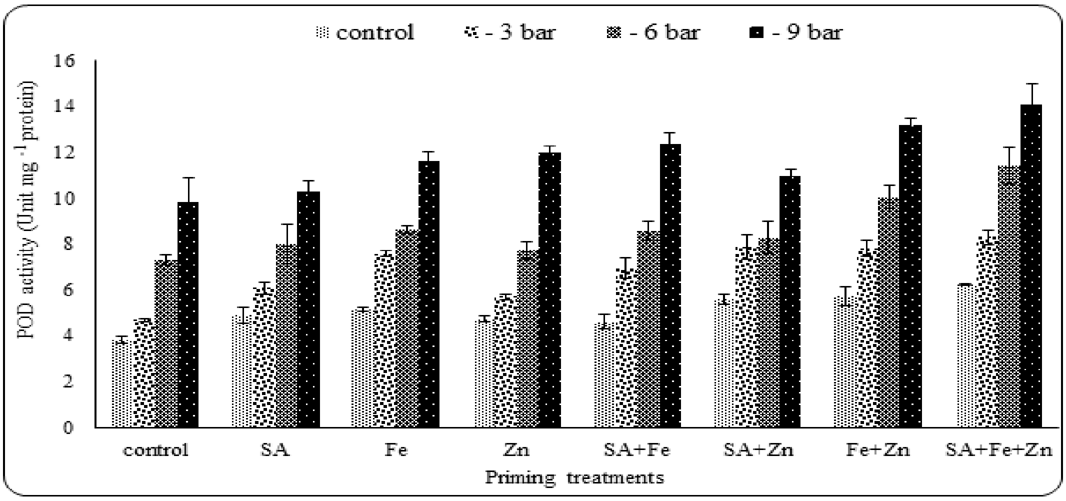

Figure 3. *The peroxidase (POD) activity in primed and unprimed seedlings of stevia under normal and drought stress conditions (LSD 5%= 1.29). Error bars indicate standard error (SE)*

Discussion

Evaluation of drought stress effect on some seed germination and physiological characteristics of stevia seedlings was one of the objectives in the present investigation. In recent years, drought stress is known as an important environmental factor which seriously impacts the productivity of many crops and can retard growth and development of them (Lipiec et al., 2013). The results of our study showed that the seed germination indices of stevia were affected by the deleterious effects of drought stress induced by PEG, so that GP, GR, and GV were significantly decreased along with the increase of PEG concentration (*Table 3*). In the present study, it was found that the average of GP was dropped to below 24% at the highest PEG concentration (– 9 bar). Poor and erratic germination might be attributed to the lower water uptake by seeds and the elevation of ROS levels under water deficit (Kaya et al., 2006). It has been reported that under stress conditions, the alteration of some enzymes and hormones found in the seed could lead to the reduction of final germination (Botía et al., 1998). Also in this study, SL and SVI (the product of SL and GP) which are the important traits in the

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primary establishment of seedling were obviously reduced with increased drought stress (*Table 3*). This might be due to the reduction of water content in plant tissues under water stress which declines turgor pressure in cells, and consequently inhibits cell enlargement, cell division and plant growth (Farooq et al., 2009). These results are consistent with the findings presented in some previous research in other plants (Patane et al., 2013; Zaher-Ara et al., 2016). Additionally, the decrease of water potential was accompanied by a significant increase of MGT (*Table 3*), because seeds under water deficit require more time to reach an adequate level of hydration to initiate germination (Steiner et al., 2017). The increase of required time for germination under drought stress has been reported in the research done by Steiner et al. (2017) in wheat and black oat.

In our study, the comparative performance of different priming treatments for improving drought tolerance at germination and early growth stages of stevia was also evaluated. The results showed that seed priming with SA and micronutrients (Fe and Zn) not only improved the measured germination parameters and seedling growth of stevia in both normal and stress conditions but also alleviated drought stress damages. In this regard, it was observed that the integrated application of priming agents in most of the cases was more effective than their separate application. The beneficial effects of seed priming on germination might be related to the stimulation of pre-germination metabolic procedures, the increment in protein synthesis, the repair and the build-up of nucleic acids, the repair of membranes and osmotic adjustment (Ibrahim et al., 2016). As can be seen in *Table 3*, at all drought stress levels, the highest values of GP, GV as well as SL and SVI were noticed in seeds primed by SA + Fe + Zn. The reason of the increment in seedling weight and length as a result of nutri-priming might be due to the role of these elements in increased cell division, cell expansion and meristematic growth, which caused an increase of plant growth (Shahverdi et al., 2017). It has been reported that in the presence of zinc, hormones such as auxin are increased. Therefore, it seems that the increase of auxin in seed due to the presence of zinc increases the growth of seedling (Laware and Raskar, 2014). In this regard, Munawar et al. (2013) reported that treating seeds of *Daucus carota* L. with Zn (1.5%) and Mn (1.5 and 2%) resulted in the highest mean values of the shoot and root length. Begum et al. (2014) concluded that canola seed priming with $ZnSO_4$ and $CuSO_4$ leads to higher GP, GR and SL under NaCl stress. Previously, various studies on dill (Mirshekari et al., 2012), maize (Imarn et al., 2017) and wheat (Reis et al., 2018) have reported the enhanced germination and yield after priming with micronutrients. On the other hand, several studies have revealed that pre-soaking of seeds in the optimal concentrations of plant growth regulators such as SA could improve the germination of crops, particularly under stress conditions (Ansari and Sharifzadeh, 2012; Arbaoui et al., 2015; Kumari et al., 2017). Sakhabutdinova et al. (2003) reported that SA prevents the reduction of Indole acetic acid (IAA) and cytokinin levels in plant tissues, and alleviates the inhibitory effects of water stress on plant growth. The findings of Wang et al. (2016) also indicated that seed priming with SA improved the seedling emergence and growth of rice.

Chl is one of the main components of chloroplast in photosynthesis. Under water stress, the reduction of Chl content has been considered a usual symptom of oxidative stress (Fathi and Tari, 2016). In our study, the total Chl content was significantly diminished with an increase in the level of applied PEG as compared to the control seedlings. The priming treatments (especially Fe + Zn, SA + Fe + Zn and SA + Fe) did not only moderate the adverse effect of drought stress on the Chl content, but also had a significant stimulatory effect on the biosynthesis of Chl, so that under severe drought

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stress (– 9 bar), the maximum amount of Chl was recorded in the seedlings which were raised from primed seeds by Fe + Zn and SA + Fe + Zn (*Fig. 1*). The decrement of Chl content under drought stress is mainly due to damage to the chloroplast membrane and Chl degradation as result of the activity of ROS (Madany and Khalil, 2017). These findings are in agreement with previous results that drought stress reduced the total Chl content, whereas priming treatments minimized the deleterious effects of stress conditions (Hussain et al., 2017; Madany and Khalil, 2017). Zn is an essential element for Chl synthesis, pollen function, and germination (Cakmak, 2008). It has been reported that Zn element by the protection of the sulfhydryl group keeps the Chl content (Latef et al., 2017). Fe is an essential element required for the maintenance of chloroplast structure and is involved in Chl synthesis (Rout and Sahoo, 2015). The limitation of this element has a remarkable effect on the productivity of photosynthetic organisms (Tewari et al., 2013). Shahverdi et al. (2017) reported that stevia seed priming with Fe alone and the integrated application of selenium (Se) and boron (B) significantly increased the total Chl content under salinity stress. In another research, Li and Zhang (2012) concluded that rice seed priming with SA increases the photosynthetic activities in this plant.

In plant cells, ROS are generated in both normal and stress conditions, but in response to different environmental stresses such as drought stress, the production of ROS is significantly increased which could result in the progressive oxidative damages (Sharma et al., 2012). Plants usually have several defensive mechanisms to overcome the oxidative stress (Lipiec et al., 2013), which include enzymatic and non-enzymatic (such as proline) antioxidants and as well as reparation systems that orchestrate stress signaling and block the adverse effects of ROS (Demidchik, 2015). Proline is known to act as an osmolyte /osmoprotectant agent under drought stress (Lehmann et al., 2010) and has the important role in the osmotic pressure adjustment, scavenging free radicals, stabilizing sub-cellular structures (e.g. membranes and proteins) and storing carbon and nitrogen (Bartels and Sunkar, 2005). The results of our study demonstrated a significant increase in the amount of proline content under drought stress. According to the results, all priming treatments (especially SA + Fe + Zn, Fe + Zn and SA + Zn) significantly enhanced (48.5-59.6%) the accumulation of free proline in stevia seedlings (*Table 4*). The better ability of primed seeds is attributed to a wide range of metabolic and physiological improvements (Shehab et al., 2010). In agreement with our results, Kabiri et al. (2014) reported that the seedlings derived from primed seeds of fennel with SA had the higher values of the proline content than unprimed seeds under drought stress. Fallah et al. (2018) have documented that the various priming treatments such as $ZnSO_4$ (0.5%) increase the proline content in *Nigella sativa* L. under drought stress. Also, it has been reported that seed priming by micronutrients increases the proline content under salinity stress (Shahverdi et al., 2017). Hayat et al. (2010) reported that SA treatment enhances the proline accumulation with a concomitant induction of different stress enzymes.

CAT and POD are described to be two of the most important antioxidant enzymes that protect plants against cell oxidative damages caused by drought and other environmental stresses (Huang et al., 2016). These enzymes play an important role in scavenging of H_2O_2 (Hussain et al., 2016). In our study, drought stress activated the activity of CAT and POD in stevia seedlings, so that the lowest activities were observed under the controlled conditions. The increasing of the enzymatic activity, as seen in our study has been considered a part of seed strategy to overcome free radicals (Chiu et al.,

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2005). Also, in primed seedlings, the activities of CAT and POD were higher than those in unprimed ones which eventually helped in alleviating the effect of oxidative stress on germination indices. The increased antioxidant activity in the primed seedlings, resulting in the better growth might be related to their higher ROS scavenging ability under stress conditions (Hussain et al., 2016). Generally, among studied priming treatments, SA + Fe + Zn had the most positive effect in increasing the activity of these enzymes (*Figs. 2* and *3*). Zn is used in numerous physiological and biochemical processes in plant cells, including protein synthesis, membrane function, and gene expression. It is a metal component of many important enzymes, acting as a regulatory cofactor for many enzymes, and has the major role in defense systems of plants against stress conditions (Cakmak, 2000; Reis et al., 2018). In this connection, Aboutalebian and Nazari (2017) mentioned that osmopriming with $ZnSO_4$ increased the activity of SOD, POD and CAT enzymes in canola under chilling stress. Fe element also is well known as an important co-factor for many antioxidant enzymes (such as CAT and POD) (Kusvuran et al., 2016). Ruiz et al. (2000) reported that the activities of the CAT and POD enzymes were correlated with the Fe content in leaves. SA has been found to act as the endogenous signal molecule which participates in the regulation of many physiological processes in plants in response to stressful conditions (Kabiri et al., 2014). Pouramir-Dashtmian et al. (2014) stated that seed priming with SA could ameliorate the destructive effects of oxidative stress caused by the generation of ROS by increasing the antioxidant defense system in rice. In this regard, Hara et al. (2012) mentioned that SA in low concentration could increase the oxidative capacity in plants. Similar findings were also reported by Sheykhbaglou et al (2014), who claimed that seed priming by SA increased the antioxidant activity and led to the improvement of germination parameters in sorghum, which coherent with the present study.

Conclusion

It is concluded from findings of our research that PEG-induced drought stress had inhibitory effects on all germination parameters studied in stevia, nevertheless, seed priming effectively promoted germination characteristics, seedling growth, the total Chl content and the antioxidant capacity under different levels of drought stress as compared to the unprimed ones. The better germination performance and vigorous seedling growth in stevia through seed priming treatments under drought stress could be related to the enhanced enzymatic activities of POD and CAT and the higher accumulation of free proline. Accordingly, our results suggest that seed priming with SA and micronutrients (Fe and Zn) particularly the concurrent application of them at the appropriate concentration can improve drought tolerance of stevia in germination stage. Nevertheless, confirmatory trials under natural field conditions over several years are needed to ensure whether studied priming treatments would result in the improvement of seedling emergence and vegetative growth of stevia as well as its resistance to drought stress on the farm. Also, it is recommended to develop more comprehensive results and evaluate the effect of seed priming with other micronutrients in combination with different plant hormones on germination behavior and the physiological and biochemical changes of stevia during unfavorable conditions. Besides, further researches at transcriptomic and proteomic levels are required to decipher the molecular mechanisms of seed priming-induced drought tolerance in stevia plants.

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EFFECTS OF NANO AND CHEMICAL FERTILIZERS ON PHYSIOLOGICAL EFFICIENCY AND ESSENTIAL OIL YIELD OF *BORAGO OFFICINALIS* L.

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Abstract. This study was conducted to compare the effects of nano and chemical fertilizers on physiological efficiency and essential oil yield of *Borago officinalis* L. during 2013 and 2014 crop years. The different levels of fertilizers were used as main factors in 11 levels including iron-sulphate and nano-iron, zinc and nano-zinc, urea and nano-urea, potassium sulphate and nano-potassium, micro-complete and micro-complete nanosuper and control. The application methods of fertilizers were considered as secondary factor (soil application, foliar application and combined application). Physiological efficiency of nitrogen, phosphorous, potassium, zinc and iron, dry and fresh weight of aerial parts, number of secondary branches, chlorophyll content, 100 grains and essence yield were evaluated. Our findings showed that chemical fertilizers had no beneficial effects in comparison to nano fertilizers ($P > 0.05$). In addition, nano-urea and urea fertilizers increased essential oil yield because of increased wet and dry weight of aerial parts and number of secondary branches. In conclusion, nano-fertilizers can be used in order to improve the essence production and also as environmentally friendly fertilizers.
Keywords: *environmentally friendly fertilizers, foliar application, nano-fertilizers, essential oil production, Borago officinalis* L.

Introduction

Medicinal plants are used in order to treat some incurable chronic disorders and by about 80% of the people of the world are dependent on medicinal plants for health and treatment (Ibrahim et al., 2013). There are increasing interest to use the medicinal plants because of side effects of synthetic drugs. Borage (*Borago officinalis* L.*)* is belonging to *Boraginaceae* family and it is traditionally used for therapeutic uses. Borage oil seed is rich source of essential fatty acids such as linoleic acid and γ-linolenic acid which can have beneficial effects on human health (Mhamdi et al., 2007). Some medicinal plants are known to have antioxidant and anti-inflammatory properties (Balasubramanian and Palaniappan, 2001) which are attributed to their essential oils. However, yield and content of essential oils are dependent on climate and geographical conditions, harvest time and application of fertilizers (Baranauskiene et al., 2003). Considering increased needs medicinal plants and effect of fertilizers on quantity and quality of essential oils, it is essential to use the best fertilizers in order to increase the efficiency of medicinal plants. Studies have reported that fertilizers increase the production of medicinal plants (Das et al., 2007; Sharma and Kumar, 2011). There are increasing interest to use the

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nanoparticles for industrial and pharmaceutical purposes (Siddiqui and Al-Whaib, 2014). Nanoparticles have high surface/volume ratio that increase biochemical activities and reactivity (Lee et al., 2008, 2010). It is still unknown interaction between biological systems and nanoparticles (Barrena et al., 2009). Nanotechnology is a strategy in order to increase the value of agriculture products and decreasing environmental problems (Elfeky et al., 2013). Kottegoda et al. (2011) have produced slow-released nanoparticles and nano powders. Studies have shown beneficial effects of nanoparticles on germination, growth and development in plants (Roghayyeh et al., 2010). Elfeky et al. (2013) reported positive effects of nano-iron on chlorophyll content, total carbohydrate, essential oil content and growth characteristics of *Ocimum basilicum*.

Nitrogen and potassium are nutrients influencing on production and quality in plants. Nitrogen modulates in chlorophyll synthesis and photosynthetic efficiency, but potassium regulates cell wall permeability and activity of different elements (Akamine et al., 2007). Potassium and nitrogen fertilizers increased turmeric production but had not significant effects on curcumin production (Akamine et al., 2007).

Regarding the use of chemical fertilizers in start of season, part of chemical form may be converted to other forms and/or deleted, resulting in increased economical harmful. In order to increase the efficiency of nutrients intake, fertilizers must provide essential nutrients during long times. Application methods can have significant effects on plants yield. Foliar application of some fertilizer may have more beneficial effects in comparison to soil application. It seems that the use of nano fertilizers may increase efficiency of essential oil production (Kennedy et al., 2004).

Physiological efficiency of elements can be defined as production of dry matter/unit absorbed nutrient element (Moll et al., 1982). The efficiency of element absorption is controlled by growth and morphology of root system in plants (Barbieri et al., 2008). Application of nitrogen fertilizers can have positive effects on grain yield and also develop procedures in order to improve the management of nitrogen (Flowers et al., 2004). Fertilizers efficiency can be applied in order to investigate the yield/per unit (Delbert and Ulter, 1989). Increased absorption efficiency and the use of nitrogen in grain yield are required in order to efficient processes for absorption, transformation, assimilation and nitrogen redistribution. The relative relation of processes and genotypic differences in nitrogen efficiency can be changed among genetic populations and different climates (Moll et al., 1982). Medicinal plants are required because of their high advantages. However, high production by nano fertilizer can be beneficial and environmentally friendly. To the best our knowledge, any study has not been conducted to compare of chemical and nano fertilizers on performance, essential oil yield and physiological efficiency of medicinal plants. Thus, the present study was conducted to evaluate the effects of chemical and nano fertilizers by methods of foliar and soil application on essential yield and physiological efficiency of elements in borage.

Materials and methods

This experiment was conducted during 2013 and 2014 crop years in Agriculture station of Islamic Azad University, Tabriz-Iran in the north-west of Iran (37° 24’ N, 46° 17’ E; 1360 m). Soil pH was ranged from weak alkaline up to medium alkaline and the threat of salinity was not considered (*Table 1*). Seeds were prepared from Pakan Institute, Isfahan-Iran. This experiment was conducted in spilt plot trial based on randomized block completely design with 3 replications. The different levels of

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fertilizers were considered as follows; 1-iron-sulphate fertilizer, 2-nano-iron 10% (2-3 kg or 4 L/ha in irrigation form and/or foliar form in 2 ppm concentration), 3- zinc sulphate, 4-nano-zinc 20% (1-2 kg or 2-4 L/ha in irrigation form and/or foliar form in 2 ppm concentration, 5-urea, 6-nano-urea (2-3 kg or 4 L/ha in irrigation form and/or foliar form in 2 ppm concentration), 7- potassium sulphate, 8-nano-potassium 23% (1-2 kg or 2-4 L/ha in irrigation form and/or foliar form in 2 ppm concentration), 9- micro-complete, 10- nano super micro-complete (2-3 kg or 4 L/ha in irrigation form and/or foliar form in 2 ppm concentration) and 11-non-application of fertilizer (control). The method of fertilizer application was considered as secondary factor (soil application, foliar application and combination of soil and foliar application). Experimental treatments were as follows:

1) Soil+foliar application of zinc
2) Soil application of zinc
3) Foliar application of zinc
4) Soil+foliar application of nano-zinc
5) Soil application of nano-zinc
6) Foliar application of nano-zinc
7) Soil+foliar application of complete
8) Soil application of complete
9) Foliar application of complete
10) Soil+foliar application of complete nano
11) Soil application of complete nano
12) Foliar application of complete nano
13) Soil+foliar application of potassium
14) Foliar application of potassium
15) Soil application of potassium
16) Soil+foliar application of nano potassium
17) Soil application of nano potassium
18) Foliar application of nano potassium
19) Soil+foliar application of iron
20) Foliar application of iron
21) Foliar application of iron
22) Soil+foliar application of nano- iron
23) Soil application of nano- iron
24) Foliar application of nano- iron
25) Soil+foliar application of urea
26) Soil application of urea
27) Foliar application of urea
28) Soil+foliar application of nano-urea
29) Soil application of nano-urea
30) Foliar application of nano-urea
31) Control

Number of experimental plots was 99 plots with length of 4 m, width 3 m with 4 rows planting and distance 75 cm. The seeds were planted in distances of 15 cm. Secondary plots were consecutively planted and a line was considered as non-planting. In order to prepare the land for planting, sowing and plotting were conducted. Fertilizer application was conducted on basis 150 kg/ha for urea fertilizer and 200 kg/ha for

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phosphorous and potassium fertilizers. The levels of iron, zinc, copper, boron and manganese were essential for growth requirements (*Table 1*).

Table 1. *Some of physicochemical characteristics of field soil*

	Depth (cm)	Iron	Copper	Zinc	Manganese	Total N%	Phosphorous	Potassium	Boron
		(ava) ppm					(ava) ppm		
2013	0-30	0.43	0.38	0.8	1.15	0.11	39.72	352	2.12
2014	0-30	2.04	0.42	1.02	1.82	0.086	47.5	381	2.36

The seeds were planted in 3 up to 5 cm depths of soil. Field irrigation was conducted by soil humidity condition and environmental conditions. After planting, foliar application was conducted. Foliar application of iron-sulphate, zinc sulphate (concentration of 5 ppm), potassium sulphate and urea fertilizer (5 ppm), complete fertilizer (2 ppm) and nano fertilizers (2 ppm) were conducted. Dry and fresh weight of aerial parts, 100 grain weight, chlorophyll index content, stem and leaf dry weight, leaf number, harvest index and plant height were measured. While growing plant, samples were collected from per plot (Mhamdi et al., 2007). While flowering plant, 5 plants were harvested for preparation of essential oil. Samples were then evaluated in order to determine the elements. Physiological efficiency of elements was calculated based on *Equation 1*.

$$\text{PE}\left(\text{kg.kg}^{-1}\right) = \left(\text{BYf - BYu}\right)/\left(\text{Pf - Pu}\right) \qquad \text{(Eq.1)}$$

where BYf is biological function (grain+straw) in fertilized plot (kg), BYu is biological function (grain + straw) in non-fertilized plot (kg), Pf is fertilizer absorption (grain+ straw) in fertilized plot (kg) and Pu is fertilizer absorption (grain+ straw) in non-fertilized plot (kg).

Crop efficiency can be resulted from economical production/absorption unit of nutrients based on *Equation 2*.

$$\text{APE}\left(\text{kg.kg}^{-1}\right) = \left(\text{Gf - Gu}\right)/\left(\text{Pf - Pu}\right) \qquad \text{(Eq.2)}$$

where Gf is grain yield/fertilized plot (kg), Gu is grain yield/non-fertilized plot, Pf is fertilizer absorption (grain+straw)/fertilized plot and Pu is fertilizer absorption (grain+straw)/non-fertilized plot.

The collected data was analyzed and the means were compared by using Duncan test at levels of 5%. The SAS software was used to analyze the data and figures were illustrated by Excel software.

Results and discussion

The data for analysis of variances of the evaluated traits are presented in *Table 2*.

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Table 2. *Analysis of variance of traits evaluated in borage*

Sources of variation	df	Fresh weight of aerial parts	Dry weight of aerial parts	Number of secondary branches	Chlorophyll index content	Leaf area	100 grain	Essential oil (%)
Year	1	157.57^{ns}	18.44^{ns}	96.57**	2601.11^{ns}	9365641.37**	0.074^{ns}	0.01^{ns}
Replication (year)	4	623.57**	9.03**	2.04*	404.50**	117984.60^{ns}	0.028**	1.09**
Fertilizer type	10	257.77**	3.08*	9.25*	755.13^{ns}	1425064.83^{ns}	0.019**	7.0005**
Year×fertilizer type	10	72.46**	0.95**	2.96*	507.50**	715342.16**	0.001^{ns}	1.03*
Replication×fertilizer type (year)	40	28.37**	0.68**	1.49**	61.31^{ns}	40311.93^{ns}	0.012^{ns}	0.49**
Application type	2	16.05^{ns}	0.47^{ns}	0.33^{ns}	88.11*	1095386.67^{ns}	0.006*	0.01^{ns}
Fertilizer type×application type	20	37.61**	0.48^{ns}	2.50^{ns}	253.91**	648198.75*	0.017**	0.362^{ns}
Year×application type	2	11.49^{ns}	0.10^{ns}	1.87^{ns}	4.98^{ns}	186842.71^{ns}	0.00021^{ns}	0.184^{ns}
Year×application type×fertilizer type	20	11.42^{ns}	0.24*	1.74**	28.27^{ns}	309860.44**	0.0030^{ns}	0.32**
Error	88	6.42	0.141	0.65	51.99	6646.61	0.008	0.147
CV (%)	-	7.55	9.87	8.17	22.06	19.96	5.17	20.21

ns: non- significant, ** and * significant at the 1% , 5% probability levels, respectively

Number of secondary branches

The variance analysis of data showed significant interaction between fertilizer type and application method (*Fig. 1*) (P < 0.01). The most number of secondary branches (13.29 & 13.14) were observed in soil application + foliar application of nano-urea and soil application of nano micro-complete. A combination of soil and foliar application of nano-urea fertilizer increased 1.14% number of secondary branches in comparison to nano micro-complete; indicating that similar efficiency the both fertilizers. The lowest number of secondary branches was firstly observed in foliar application of iron (8.85), followed foliar + soil application of urea (8.95), foliar application of potassium (9.00) and foliar application of nano-zinc (9.04). However, application of complete nano, nano-potassium and nano-urea increased secondary branches compared with control group (P < 0.05). The increased branch numbers in nano-urea treatment may be attributed to slow-degraded of urea and its role in growth and development. On the other words, increased branches in nano-urea treatment can be explained by increased penetration rate of required nutrients for growth reactions. Increased nitrogen rate could increase secondary branches in *Matricaria chamimilla* L. (Dastborhan et al., 2010). Increased nano treatment can be explained by increasing permeability nano fertilizers into leaf tissue and increasing nutrient penetration which increases growth parameters. Controlling elements is one of nanotechnology advantages (Hassani et al., 2015). The lowest number of branches was found in iron treatment. Iron is essential for plants growth but its application may have adverse effects on growth and development in plants, because it induces oxidative stress in plants (Briat et al., 2010). In addition, phyroferitine degrades and releases iron, produces hydroxyle iones (Zhang et al., 2013) and finally decreases growth.

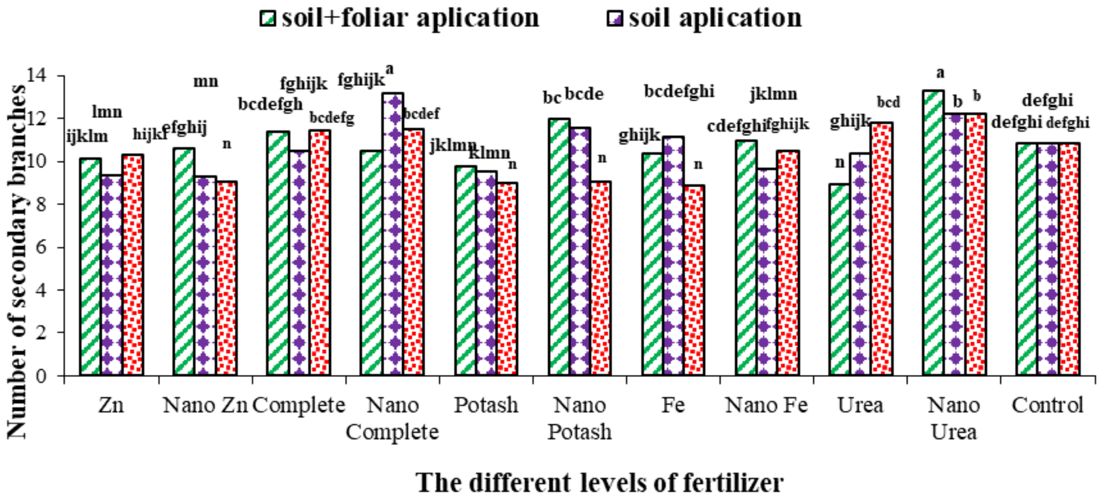

Figure 1. *Effects of fertilizer and application type on number (n) of secondary branches in borage*

Wet and dry weight of flowering aerial parts

The highest fresh weight of flowering aerial parts was observed in foliar and soil application treatment of urea. Our findings showed that foliar application of urea increased weight by 51.1% in comparison to control group (*Fig. 2*). Urea, nano-urea, nano-iron, iron and nano-potassium had higher weight in comparison to control group.

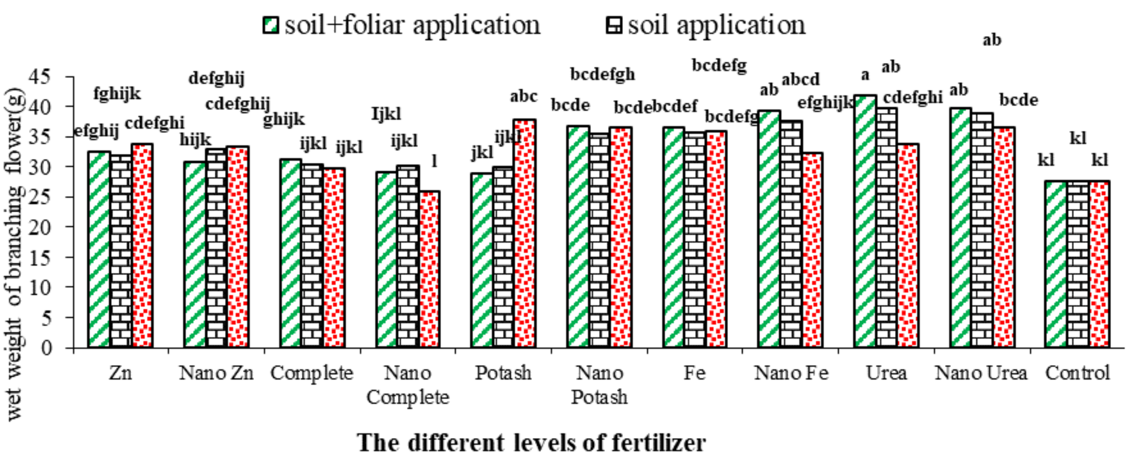

Figure 2. *Effects of fertilizers type and utilization methods on fresh weight (g) of the branching flower*

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The mean comparison of fertilizer types showed that the highest dry weight of aerial parts was observed in urea treatment (4.20 g). Application of urea could cause significant differences in comparison to nano-zinc, micro-complete and complete nano. The lowest dry weight of aerial parts was observed in control group (2.87 g) and application of urea increased dry weight of aerial parts in comparison to control group by 46% (*Fig. 3*).

The comparison of chemical and nano fertilizers did not show significant differences between chemical and nano fertilizers for dry weight of aerial parts, but nano fertilizers showed more efficiency compared to chemical fertilizers (P > 0.05). Except zinc fertilizers, application of nano-zinc in combined method, complete, complete nano and potassium in combined application and soil application could show better efficiency in comparison to control group. Nitrogen is an essential nutrient in order to increase the growth parameters in medicinal plants (Akamine et al., 2007). Deficient nitrogenous fertilizers decreased chlorophyll contents in leaves and immature death in plants. Chlorophyll level was appropriate in urea and nano-urea groups and it was no observed difference between urea and nano-urea for chlorophyll in combined application method. Nanofertilizers may prevent to loss nitrogen and longtime use of microorganisms. Slow-released of nitrogen in nano-urea fertilizers may decrease toxic effects in high application (Gendy et al., 2013). Nitrogen not only participates in structure of purines, pyrimidines and enzymes but also participates in chlorophyll and cytochrome structure (Gendy et al., 2013). However, results showed that urea and nano-urea fertilizers had most efficiency. This result shows that nanoparticles are absorbed by root apex, cell wall and vascular system in plants (Lv et al., 2015).

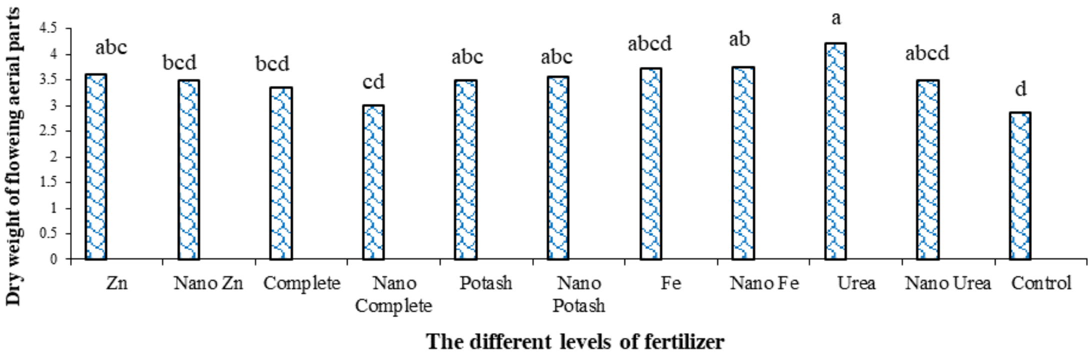

Figure 3. *Effects of fertilizers type and application methods on dry weight (g) of aerial parts in borage*

Leaf area

Interaction between different levels and application methods of fertilizers on leaf area was significant (*Table 2*). The highest leaf area (1706 cm^2) was observed in soil + foliar application of nano-urea and foliar application of urea (*Fig. 4*). Application of zinc fertilizers in soil application, foliar + soil application of zinc, combined application of complete, soil application of complete fertilizers respectively increased leaf area 64%, 3%, 37% and 50% in comparison to control group (*Fig. 4*). It seems that nano-urea fertilizer is slowly released in soil and increases leaf area and photosynthetic activity in plants (Kottegoda et al., 2011). Increased efficiency of nitrogen is one of critical factors in order to decrease environment pollutants. Increased leaf area not only

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depends on genetically factors but also leaf nitrogen. Nitrogen supplement can have significant effects on leaf activity. On the other hand, nitrogen modulates in growth and development in plants. Increased number and leaf area increased the chlorophyll structure, involved enzymes and increasing essential oil yield (Briat et al., 2015).

Chlrophyll contents index

Analysis of variance showed significant interaction between the different levels of fertilizer and application method on chlorophyll index (*Table 1*). The mean comparison showed that highest chlorophyll index was observed in soil application of complete (CCI = 50.91) and nanozinc (CCI = 48.35) (*Fig. 4*). The lowest chlorophyll index (CCI = 18.4) was observed in control treatment (*Fig. 6*). Zinc, nanozinc, complete, nano complete, potassium, nano potassium, urea and nano-urea fertilizers had chlorophyll indexes in comparison to control group. Application of urea and biological fertilizers increased chlorophyll contents in guar (Gendy et al., 2013). They have also reported increased chlorophyll contents can be attributed to levels of nitrogen and its role in chlorophyll synthesis. Nitrogen participates in chlorophyll synthesis and photosynthetic efficiency. Potassium regulates permeability of cell wall and activity of different cells (Akamine et al., 2007). Amujoyegbe et al. (2007) reported that nitrogen and magnesium can be released by chemical fertilizers and participate in porphyrine ring of chlorophyll molecule.

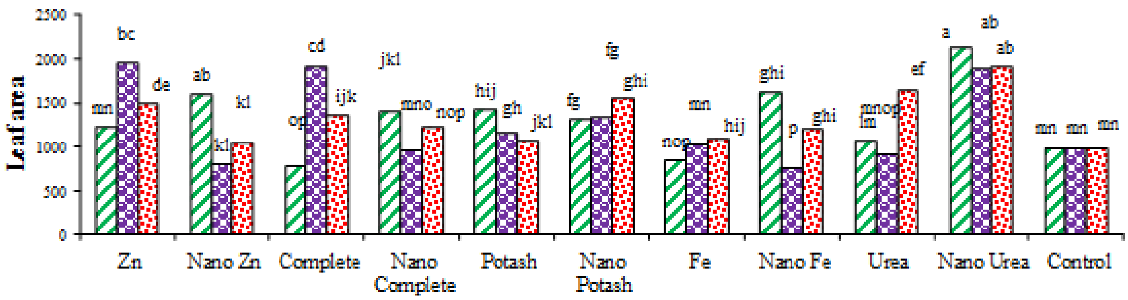

Figure 4. *Effects of fertilizers type and application methods on leaf area (mm^2) of borage*

Essence percentage

Mean comparison of essence showed that nano-urea fertilizer and control treatments had most and lowest essence contents, respectively. Nano-urea treatment could increase essence percentage in comparison to zinc and control treatments 200% and 191.4%, respectively. There are conflicts reports in relation with nitrogenous fertilizers. Biesiada and Kucharska (2008) reported increased use of nitrogenous fertilizers increased the essence and phenolic contents. Lucy et al. (2004) have reported a reverse relation between essence percentage and nitrogen content. Gendy et al. (2013) showed that use of nitrogenous fertilizers and biological fertilizers increased essence percentage in guar. Fhatuwani (2008) showed increased essence yield and phenol production by nitrogenous fertilizers.

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Essence yield

The mean comparison showed significant effect of different fertilizers on essential oil yield during 2013 years (P < 0.05, *Fig. 5*). Nano-urea fertilizer (10.96 mg/ha) and control treatment showed highest and lowest essential oil yield, respectively (*Fig. 6*). Results showed that application of nano-urea fertilizers did not have significant difference with nano potassium and micro-complete (P > 0.05). The mean comparison of treatments showed that application of nano-zinc fertilizer increased essential oil yield in comparison to chemical fertilizers, but the difference was not significant. Application of nano-zinc fertilizer increased essential oil yield (105.4%) in comparison to control group (*Fig. 5*).

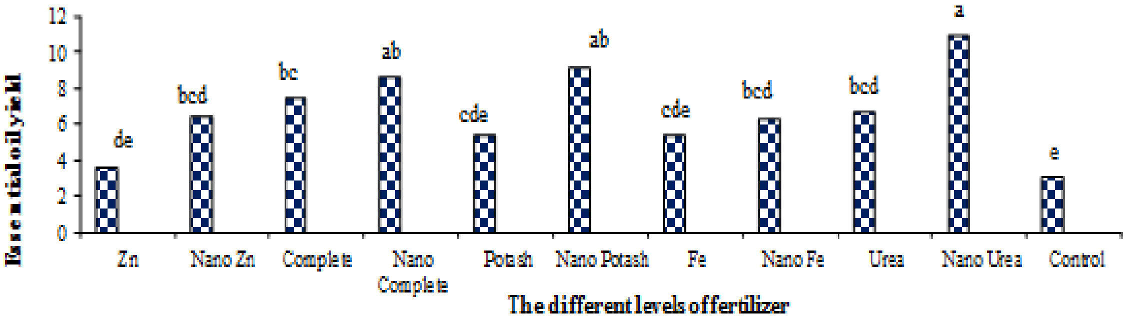

Figure 5. *Effects of different fertilizers on essential oil yield (%) during 2013 year*

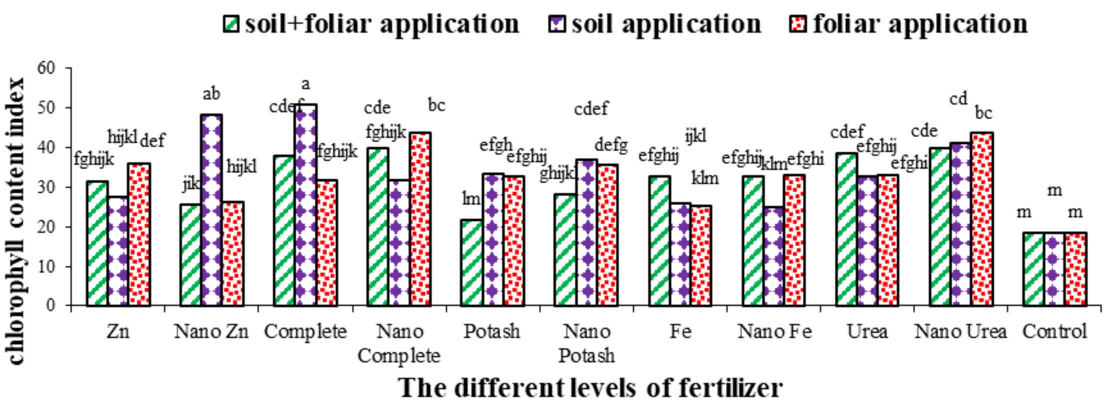

Figure 6. *Effects of fertilizers type and application methods on chlorophyll content index in borago*

Micro-complete and potassium fertilizers increased essential oil yield 15.4% and 71.5% in comparison to micro-complete and potassium fertilizers, respectively. It was no observed significant difference between nano potassium fertilizer in comparison to nano micro-complete (*Fig. 5*). Results showed that nano fertilizer had most essential oil yield but it was no observed significant difference with nano potassium and complete nano. With regards to the different levels of chemical fertilizers, micro-complete treatment (7.5 mg/ha) showed most essential yield in comparison to control group (*Fig. 5*). Results also showed that micro-complete treatment increased essential oil yield rather than potassium (39.6), iron (37.3) and urea (11.9) fertilizers (*Fig. 5*). Nanozinc,

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complete, nano-complete, nano-potassium, nano-iron, urea and nano-urea increased essential oil yield in comparison to control group. Nano-potassium rather than potassium, nano-urea rather than urea showed significant increase in essential oil yield.

Our findings showed significant interaction between fertilizer type and application method on essential oil yield during 2014 years (*Table 3*). Results showed that the highest essential oil yield was observed in combined application of nano iron (12.17 mg/ha), followed foliar application of nano-urea (12.07 mg/ha), soil application of nano-urea (11.92 mg/ha), combined application of nano-urea (11.83 mg/ha) (*Fig. 7*). Results showed that foliar + soil application of nano iron, foliar application of nano-urea, soil application and combined application of nano-urea and soil application of nano iron had not significant effects on essential oil yield (*Fig. 7*). Results showed that soil application of nano-zinc increased essential oil yield by 25.5% and 25.1% in comparison to foliar+soil application of nano-zinc and soil application of nano-zinc (*Fig. 6*). Soil application of nano potassium increased essential oil yield in comparison to foliar application of nano potassium, combined application of potassium and foliar application of nano potassium. Mean comparison of iron fertilizer showed that most essential oil yield was observed in combined application and soil application of nano iron (*Fig. 6*). Nano-urea fertilizer showed significant difference with urea fertilizer (*Fig. 6*).

Table 3. *The mean comparison of year, fertilizer type and fertilizer application during two year*

	Dry weight of aerial parts		Essential oil	
	2013	**2014**	**2013**	**2014**
Soil+foliar application of zinc	3.62^{abcd}	3.66^{fghij}	1.21^{fgh}	1.27^{hijk}
Soil application of zinc	3.49^{abcd}	3.76e^{fghi}	1.01^{fgh}	1.69^{fghij}
Foliar application of zinc	3.69^{abcd}	4.05^{cdefg}	0.75^{h}	1.23^{hijk}
Soil+foliar application of nano-zinc	3.49^{abcd}	4.17^{bcdef}	1.66^{cdefgh}	2.00^{bcdefghi}
Soil application of nano-zinc	3.34^{bcd}	4.13^{bcdefgh}	1.79^{cde}	2.67^{abcde}
Foliar application of nano-zinc	3.62^{abcd}	4.48^{bcde}	2.00^{bcdefg}	2.48^{abcdef}
Soil+foliar application of complete	3.60^{abcd}	3.73^{efghi}	2.11^{bcdef}	2.32^{abcdefg}
Soil application of complete	3.30^{bcd}	3.30^{hij}	2.08^{bcdefg}	2.36^{abcdef}
Foliar application of complete	3.18^{bcd}	3.63^{fghij}	2.57^{abcde}	2.38^{abcdef}
Soil+foliar application of complete nano	3.13^{bcd}	5.42^{a}	2.92^{ab}	2.57^{abcdef}
Soil application of complete nano	3.08^{bcd}	3.74^{efghi}	2.89^{ab}	2.60^{abcdef}
Foliar application of complete nano	2.82^{d}	3.45^{ghij}	2.91^{ab}	2.99^{a}

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Soil+foliar application of potassium	3.34^{bcd}	4.01^{cdefgh}	1.57^{fgh}	1.26^{hijk}
Soil application of potassium	3.35^{bcd}	3.61^{fghij}	1.42^{fgh}	0.95^{k}
Foliar application of potassium	3.81bcd	3.78^{defghi}	1.61^{efgh}	1.17^{ijk}
Soil+foliar application of nano potassium	3.50^{abcd}	4.45^{bcde}	2.59^{abcde}	2.87^{ab}
Soil application of nano potassium	3.66^{abcd}	4.56^{bc}	2.68^{abc}	1.74^{efghij}
Foliar application of nano potassium	3.54^{abcd}	4.20^{bcdef}	2.62^{abcde}	1.29^{hijk}
Soil+foliar application of iron	3.60^{abcd}	3.14^{ij}	1.63^{defgh}	1.04^{ijk}
Soil application of iron	3.93^{ab}	4.43^{bcde}	1.49^{fgh}	0.62^{k}
Foliar application of iron	3.61^{abcd}	4.26^{bcde}	1.29^{fgh}	1.32^{hijk}
Soil+foliar application of nano-iron	3.92^{ab}	4.83^{ab}	1.40^{fgh}	3.08^{a}
Soil application of nano- iron	3.91^{ab}	4.51^{bcd}	1.88^{cdefgh}	2.77^{abcd}
Foliar application of nano- iron	3.45^{abcd}	4.14^{bcdefg}	1.71^{cdefgh}	1.40^{ghijk}
Soil+foliar application of urea	4.29^{a}	4.87^{ab}	1.61^{efgh}	$1.71^{efghijk}$
Soil application of urea	4.37^{a}	4.50^{bcd}	1.52^{fgh}	$1.83^{defghij}$
Foliar application of urea	3.95^{ab}	4.7^{6abc}	1.66^{cdefgh}	$1.87^{cdefghij}$
Soil+foliar application of nano-urea	3.70^{abcd}	5.41^{a}	2.65^{abcd}	$2.18^{abcdefgghi}$
Soil application of nano-urea	3.61^{abcd}	5.29^{a}	3.38^{a}	2.44abcdef
Foliar application of nano-urea	3.22^{bcd}	4.51^{bcd}	3.16^{a}	2.82^{abc}
Control	2.87^{cd}	3.03^{j}	1.05^{gh}	1.17^{ijk}

Means by the uncommon letter are significantly different according to Duncan tests ($p<0.05$).

Biesiada and Kucharska (2008) have reported increased nitrogen could enhance essential oil yield and phenolic contents. Studies have reported reverse relation between essential oil yield and nitrogen content (Lucy et al., 2004). Najafi Vafa et al. (2015) stated that the use of nano-zinc increased essential oil yield in savory. El Gendy et al. (2013) showed that the use of nitrogenous fertilizers and biological fertilizers increased essential oil percentage in guar. Fhatuwani (2008) related essential oil yield with increased phenolic compounds by nitrogenous fertilizers. Regarding increased leaf area,

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it can be stated that photosynthesis and photosynthetic productions have direct relation with essential oil production. With regards to correlation between photosynthesis and essential oil production, it can be stated that carbon dioxide and glucose are essential oil for essential oil production. Considering the states mentioned, it seems that increased of leaf area, number of pores and glucose are essential for energy supply. Potassium and phosphorous improve effect of nitrogen on photosynthesis by oligodynamical role or participation in energy transformation, activating kinases and participating in osmotic absorption. Comparing nano-urea, urea and control show that as chlorophyll index increases, photosynthesis increases which can have major effect on essential oil production. Nitrogen modulates in cell division and development of new cells and also participates in chlorophyll structure and involves in carbon metabolism and essential oil yield (Briat et al., 2015). It seems that fresh weight of branches can have major impacts on essential oil yield.

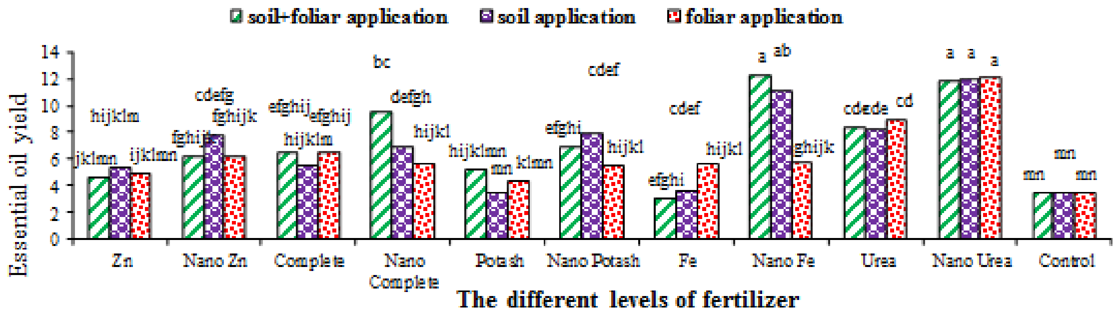

Figure 7. *Interaction between type and application procedure of fertilizers on essential oil yield (%) during 2014 year*

Nitrogen physiological efficiency

Analysis of variance of nitrogen physiological efficiency showed that year, fertilizer type and application method had no significant effect on the mentioned parameters (*Table 4*). Physiological efficiency can be influenced by geographical condition. On the other hand, the loss of nitrogen in environment is known as reason for decreased nitrogen efficiency (Fallah and Tadayyon, 2010). The lack of difference among groups may be attributed to similar loss in all the groups. Some studies have shown inappropriate release of nitrogen regarding plant needs as reason for difference among groups (Russo et al., 2009). Increased the traits depends on ability the use of absorbed nitrogen, so that as plant produces more grain yield, physiological efficiency increases. Thus, one of efficient factors in increasing nitrogen physiological efficiency is chlorophyll production and produced dry matter. It can be stated that similar production of chlorophyll and produced dry matter can be reason for similar nitrogen physiological efficiency.

Phosphorous physiological efficiency

Analysis of variance for phosphorous physiological efficiency during 2013 and 2014 years showed that fertilizer type and application method had no significant effect on phosphorous physiological efficiency. As phosphorous efficiency increases, phosphorous can be fixed in soil (Bahl and Toor, 2002). It seems that fixation have not occurred in the current study.

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Potassium physiological efficiency

Analysis of variance for potassium physiological efficiency during 2013 year showed that fertilizer type, application method and its interaction on the mentioned parameters were not significant ($P > 0.05$). Results indicated a significant interaction between fertilizer type and application method on potassium physiological efficiency (*Table 4*). Analysis of variance for potassium physiological efficiency showed a significant interaction between fertilizer type and application type in 2014 year ($P < 0.05$). Results showed that the most potassium physiological efficiency was observed in combined application and/or foliar application of nano iron (0.70 kg/kg), potassium (0.63 kg/kg) and soil application of nano-urea (0.58 kg/kg) and nano-iron (0.56 kg/kg). Foliar and combined application of nano-iron increased potassium physiological efficiency in comparison to soil and combined application of iron fertilizer by 91%. It seems that application of potassium fertilizer increases efficiency which can be attributed to provide the nutrients for plant growth.

Table 4. *Analysis of variance of physiological efficiency (kh/kg) in borage*

Sources of variation	df	Nitrogen physiological efficiency	Phosphorous physiological efficiency	Potassium physiological efficiency	Zinc physiological efficiency	Iron physiological efficiency
Year	1	5.15^{ns}	3.36^{ns}	0.699^{ns}	0.962^{ns}	0.001^{ns}
Replication (year)	4	19.005^{**}	25.29^{**}	1.52^{**}	1.62^{ns}	0.00024^{**}
Fertilizer type	9	1.19^{ns}	11.83ns	0.207^{ns}	4.28^{ns}	0.000092^{ns}
Year×fertilizer type	9	3.43^{ns}	9.56^{ns}	0.079^{ns}	3.93^{ns}	0.000065^{**}
Replication×fertilizer type (year)	36	3.12^{ns}	9.15^{**}	0.06^{**}	6.93^{ns}	0.000023^{ns}
Application type	2	8.41^{ns}	10.35^{ns}	0.126^{**}	8.85^{ns}	0.000074^{ns}
Fertilizer type×application type	18	2.02^{ns}	6.53^{ns}	0.143^{**}	3.82^{ns}	0.000073^{ns}
Year×application type	2	2.20^{ns}	3.30^{ns}	0.0008^{ns}	8.47^{ns}	0.000036^{ns}
Year×application type×fertilizer type	18	4.59^{ns}	4.77^{ns}	0.024^{ns}	3.63^{ns}	0.000019^{ns}
Error	80	2.91	4.02	0.031	4.88	0.000046
CV (%)	-	330.39	163.03	59.87	2117.34	176.96

ns: non- significant, ** and * significant at the 1% , 5% probability levels, respectively

Results also showed that foliar application of nano-zinc (0.48 kg/kg) had the most potassium physiological efficiency but it had not significant difference with foliar application of nano-zinc (0.27 kg/kg) and soil application of nano-zinc (0.33 kg/kg). Foliar and combined application of complete fertilizers increased potassium physiological efficiency. Results showed that soil application of complete fertilizer and

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foliar application of nano-complete decreased potassium physiological efficiency but it was no observed significant difference between the mentioned treatments. It can be stated that potassium fertilizers had highest potassium physiological efficiency in comparison to other treatments. Results also showed that soil application of nano-iron (3.84 kg/kg) increased potassium physiological efficiency, but the difference was not significant with soil application of nano-iron (3.48 kg/kg). Results showed that application of nano-iron could show significant difference with iron chemical fertilizer. Results showed that the lowest potassium physiological efficiency was observed in foliar application of urea. The most potassium physiological efficiency was observed in application of urea (2.70 kg/kg).

Zinc physiological efficiency

Analysis of variance showed that fertilizer types and their application had no significant effects on zinc physiological efficiency during 2013 year. There was significant interaction between fertilizer types and fertilizer application during 2014 year. The data for analysis of variance showed that year, fertilizer type, application method and their interactions on zinc physiological efficiency were not significant (*Table 4*).

Iron physiological efficiency

Analysis of variance showed that different levels of fertilizer on iron physiological efficiency was significant ($P < 0.01$). Mean comparison of iron physiological efficiency showed that highest iron physiological efficiency was observed in zinc fertilizer (0.016 kg/kg). The lowest iron physiological efficiency (0.003 kg/kg) was observed in complete nano and iron which had no significant difference with other treatments. Results also indicated significant interaction between year and fertilizer type on iron physiological efficiency ($P < 0.01$). The data for mean comparison showed that highest iron physiological efficiency (0.016 kg/kg) was observed in zinc fertilizer in first year.

Conclusion

This study was conducted to investigate the effects of chemical and nano fertilizers on crop physiological efficiency and essential oil production under foliar and soil application during 2013 and 2014 years. Our findings showed that nano-urea fertilizer had the most essential oil yield in comparison control group. Results showed that highest potassium physiological efficiency was observed in soil application and combined application of nano-iron and the most zinc physiological efficiency was observed in zinc fertilizer. It can be stated that urea and nano-urea fertilizers increase essential yield by increasing chlorophyll index, leaf area and increased fresh weight. For increasing the essential oil yield, use of nano-urea can be suggested.

Conflict of Interest. The authors report that they have no financial or personal relationships that could inappropriately influence or bias the content of the paper.

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VEGETATION STRUCTURE AND THREATS TO MONTANE TEMPERATE ECOSYSTEMS IN HINDUKUSH RANGE, SWAT, PAKISTAN

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Abstract. This phytosociological study conducted during 2013-2017 summed up 15 communities including 5 each for trees, shrubs and herbs. Quantitative attributes of vegetation were recorded by using quadrate method i.e. 10 m^2 trees, 5 m^2 for shrubs and 1 m^2 quadrate for herbs. Edaphic attributes were enumerated by employing standard methods. Among tree species highest Family Importance Value was recorded for Pinnaceae (190.85) and lowest for Anacardiaceae (2.39). For shrubs Rosaceae (101.44) had highest FIV and lowest for Rubiaceae (1.89). For herbaceous members highest FIV was recorded for Poaceae (182.15) and lowest for Paeoniaceae (0.42). Three communities viz. *Pinus- Diospyros- Quercus* community, *Indigofera- Cotoneaster- Jasminum* community and *Apluda-Cynodon-Heteropogon* community were established at Lower Chail site. While Upper Chail comprised *Pinus-Quercus-Juglans*, *Rubus-Isodon-Ricinus* and *Apluda-Heteropogon-Poa* communities. Community structure was quite different at Shanku site which established *Quercus-Abies-Pinus*, *Sarcococca-Daphne-Berberis* and *Cynodon-Apluda-Bergenia* communities respectively. Similarly, *Cedrus-Abies-Pinus*, *Parrotiopsis-Vibernum-Berberis* and *Chrysopogon-Themeda-Cynodon* communities were established at Dabargai. At Bishigram, *Picea-Pinus-Abies*, *Parrotiopsis-Cotoneaster-Indigofera* and *Cenchrus-Medicago-Rumex* communities were present. Soils were mildly acidic and had low organic matter content. Vegetation was found to be under severe biotic stress. Deforestation is threatening these ecosystems and conservation of montane temperate forests in this zone is need of the day.
Keywords: *phytosociology, edaphic parameters, maturity index, similarity index, moist temperate vegetation*

Introduction

Study of plant communities and their classification is termed as phytosociology (Mishra et al., 2012; Ahmad and Shaukat, 2012). Primarily phytosociology helps in understanding the multilateral relationships between plants and their environment. It deals with quantitative, qualitative and synthetic attributes of plant communities (Badshah et al., 2016; Ali et al., 2015). Urbanization and industrialization coupled with anthropogenic influences have resulted in forest degradation at an alarming rate across the globe (Sarmah et al., 2011; Nagendra and Utkarsch, 2003). In Pakistan, montane temperate forests have coniferous vegetation with sparsely distributed broad-leaved species (Hussain and Illahi, 1991). Altay et al. (2012) recorded the plant communities in

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urban habitats of Istanbul, Turkey. They documented 13 plant communities and studied the impact of physico-chemical characteristics of soil on these vegetation types. Digiovinazzo et al. (2010) reported that phytosociological thresholds provide guidelines for conservation strategies. Wahab et al. (2008) assessed vegetation structure at 5 monitoring sites of Dangam District, Afghanistan. Vegetation structure of Amazonian Ecuador was studied by Maestre et al. (2006). In Mwanihana forests of Tanzania, Lovett et al. (2006) used phytosociological techniques to conclude that higher species diversity is found at high altitudes. Gould et al. (2006) assessed the structure and composition of vegetation along an elevation gradient in Puerto Rico. Angassa (2005) documented shrubby and grass vegetation in Borna Rangeland ecosystem. Dondeyne et al. (2004) studied the vegetation and soil attributes in woodlands of Southeastern Tanzania. They reported profound impact of edaphic variables on vegetation structure. Vegetation structure of Maasai Mara National Reserve was studied by Salvatori et al. (2003). They also explained the impact of fire and grazing on vegetation structure. Luis et al. (2002) recognized two major vegetation types in Cufada Lagoon in Guinea-Bissau. Grillini et al. (2001) carried out phytosociological studies in Chambura Gorge, Queen Elizabith National Park. Some phytosociological works from Pakistan are presented here. Urooj et al. (2016) conducted vegetation analysis on wetland area around Mangla Dam, Azad Kashmir. Noor and Khatoon (2013) studied vegetation patterns and soil attributes in Astor Valley, Gilgit Baltistan. Shaheen and Qureshi (2011) assessed vegetation of Lesser Himalayan Subtropical forests in Bagh, Azad Kashmir. They also recorded anthropogenic impacts on vegetation structure. Phytosociology and soil characteristics of Quercus forests of Chitral was studied by Khan et al. (2010). Siddiqui et al. (2010) documented the phytosociology of moist temperate Himalayan forests in Pakistan. They classified the vegetation into 3 major groups. Ahmad et al. (2010) presented a review on status of vegetation analysis in Pakistan. Hussain et al. (2009) recorded plant communities from Nurpur reserved forest, District Chakwal. Wazir et al. (2008) identified 5 vegetation types in Chapursan Valley, Gilgit. Ahmad et al. (2008) studied the spatio-temporal effects on plant associations in Soone Valley. From Khyber Pakhtunkhwa province studies of community ecology are summed up here. Ilyas et al. (2015) studied the vegetation structure of Kabal valley, Swat. Wahab et al. (2010) studied vegetation structure in 25 forest types in District Dir. Shah and Hussain (2009) documented the vegetation structure in Hayatabad, Peshawar, establishing 5 plant communities. Hussain et al. (2005) described 7 plant communities from Ghalegy hills, Swat. Hussain and Badshah (1998) documented the vegetation of Pirghar hills, South Waziristan. Review of literature clearly indicates that no comprehensive study is available on vegetation structure of Chail, Shinku, Bishigram and Shinku, hence present study will fill the research gap in these areas which have rich floral diversity. Present study was carried out to map vegetation and to assess the threats to montane temperate forests in Hindukush range, Swat, Pakistan.

Materials and methods

Study area

The research work was carried out in Hindukush range, District Swat. Swat is located from 34° 34’ to 35° 55’ North latitudes and 72° 08’ to 72° 50’ East longitudes. The study area spreads over an area of 24148 acres, including villages of Chail, Shinku,

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Dabargai and Bishigram (*Fig. 1*). Most part of the area falls under moist temperate zone with typical flora and fauna.

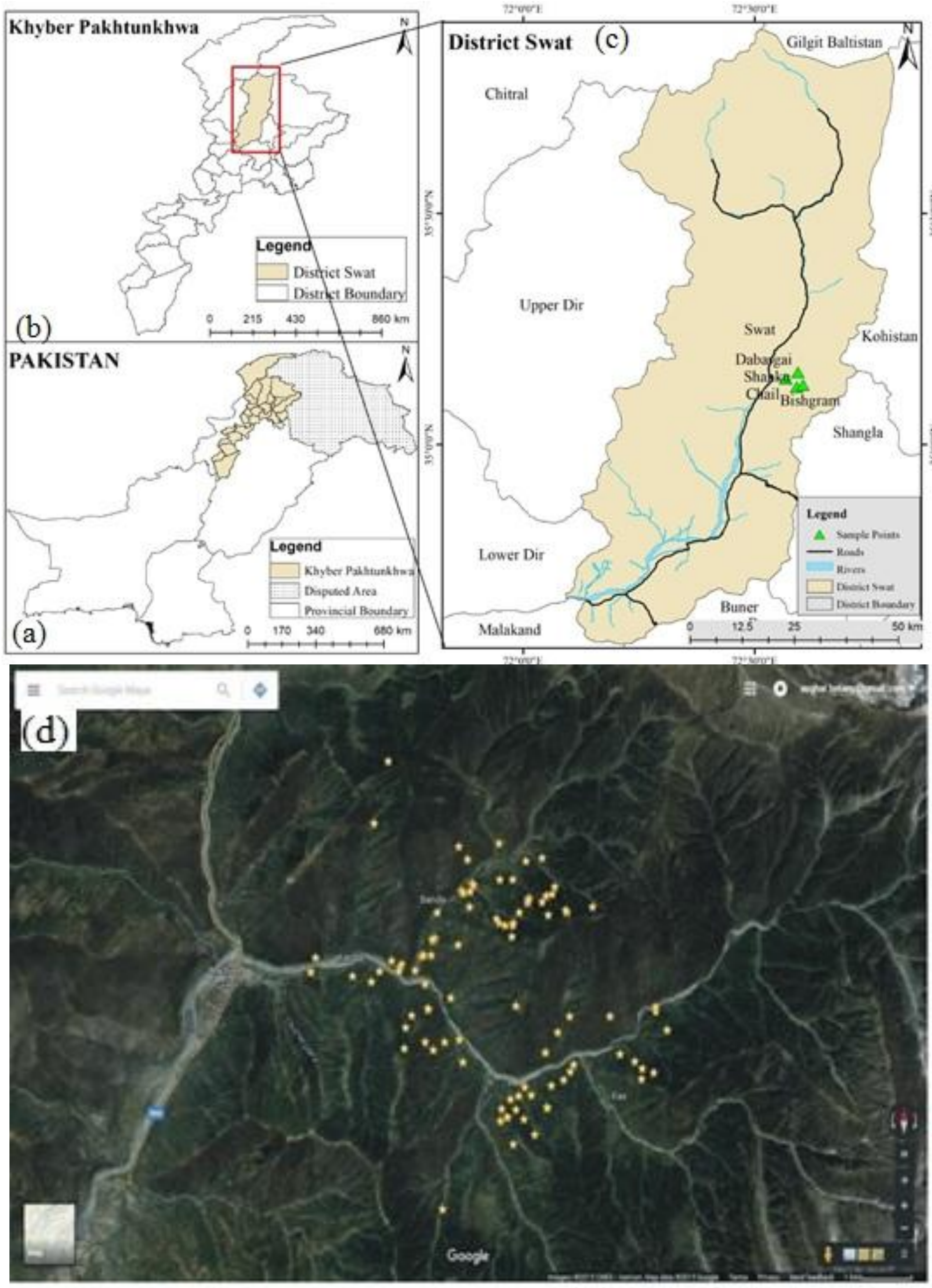

Figure 1. (a) Map of Pakistan. (b) Map of Khyber Pakhtunkhwa Province. (c) Enlarged map of District Swat and monitoring sites. (d) Map of the study area showing all the sampling sites

Phytosociology

Phytosociological studies were conducted in 5 monitoring sites. These sites were chosen based on species composition, habitats, and physiognomic contrast. Vegetation was analyzed by using 5 (10 x 10 m) quadrats for trees, 10 (5 × 5 m) quadrats for shrubs and 15 (1 × 1 m) quadrats for herbs at each monitoring site. Density, cover and

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frequency of each species were measured and values were changed to relative values to calculate IV (Importance value) and FIV (Family importance value). The plant communities were established based on highest importance values following Ahamd and Shaukat (2012), Hussain (1989).

Maturity index

Community maturity index was obtained by Pichi-Sermolli (1948) method.

Similarity index

Similarity index was calculated by using Sorensen's index (Sorensen, 1948) as modified by Motyka et al. (1950).

Soil analysis

Standard methods were applied to estimate Soil texture (Brady, 1990; Bouyoucos, 1936), pH (Jackson, 1962), Organic matter, $CaCO_3$ (Rayan et al., 1997), Electrical conductivity (Rhoades, 1996), Sodium absorption ratio and Sulphates (Richard, 1954). Nitrogen was determined by Kieldahl method of Bremner and Mulvaney (1982). Phosphorous, Potassium, Calcium and Magnesium were enumerated by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

Results

Present study was conducted in Hindukush range of District Swat, Pakistan during 2013-2017. Five monitoring sites viz. Lower Chail, Upper Chail, Shinku, Dabargai and Bishigram were established. The study area displays a unique floristic and vegetation structure. A total of 202 species were recorded in sampling units, of which trees were represented by 33 species, shrubs by 36 species and herbs by 133 species. Based on importance value (IV), three communities respectively of trees, shrubs and herb layer were established at each monitoring site. Tree communities comprised of 16 families, with Pinnaceae as the leading family with an FIV of 190.85 followed by Fagaceae (64.97), Juglandaceae (45.51), Ebenaceae (38.54), Moraceae (31.64). Rosaceae (30.41), Simoroubaceae (22.36) and Salicaceae with 19.86. Oleaceae, Taxaceae, Punicaceae, Meliaceae and Ulmaceae had importance values ranging from 16.8 to 5.13. Shrubby vegetation was represented by 21 families, dominated by Rosaceae, with highest FIV value of 101.44. It was followed by Papilionaceae (59.27), Hammelidaceae (41.62), Berberidaceae (33.47), Thymelaceae (33.44), Oleaceae (31.91), Buxaceae (30.21), Myrsinaceae (27.14), Lamiaceae (26.16) and Caprifoliaceae (23.43). Similarly, Buddlejaceae, Euphorbiaceae, Elaeagnaceae, Grossulariaceae, Rhamnaceae, Acanthaceae, Urticaceae, Rutaceae and Plumbaginaceae were the associated families having FVI ranging from 16.98 to 4.96. Herbaceous layers were represented by 47 families with Poaceae as the leading family with FIV value of 182.15. Asteraceae (43.06), Lamiaceae (27.55), Papilionaceae (24.06), Polygonaceae (23.15), Saxifragaceae (17.4), Brassicaceae (14.24), Euphorbiaceae (14.09), Rosaceae (13.82) and Apiaceae (12.35) were the sub dominant families. Other important families of herbaceous layer included Plantaginaceae, Ranunculaceae, Caryophyllaceae, Sapotaceae, Solanaceae, Amaryllidaceae, Scrophulariaceae, Urticaceae and Oxalidaceae (*Tables 1* and *2*).

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Table 1. *Family importance values*

S.No	TREES Family	FIV	S.No	Family	FIV
1	Pinaceae	190.85	5	Polygonaceae	23.15
2	Fagaceae	64.97	6	Saxifragaceae	17.4
3	Juglandaceae	45.51	7	Brassicaceae	14.24
4	Ebenaceae	38.54	8	Euphorbiaceae	14.09
5	Moraceae	31.64	9	Rosaceae	13.82
6	Rosaceae	30.41	10	Apiaceae	12.35
7	Simaroubaceae	22.36	11	Plantaginaceae	9.95
8	Salicaceae	19.86	12	Ranunculaceae	9.88
9	Oleaceae	16.8	13	Caryophyllaceae	9.43
10	Taxaceae	15.73	14	Sapotaceae	8.54
11	Punicaceae	5.91	15	Solanaceae	8.01
12	Meliaceae	5.82	16	Amaryllidaceae	7.62
13	Ulmaceae	5.13	17	Scrophulariaceae	6.8
14	Plantanaceae	3.6	18	Urticaceae	5.99
15	Aceraceae	3.07	19	Oxalidaceae	5.97
16	Anacardiaceae	2.39	20	Cannabaceae	5.54
S.No	**SHRUBS**	**FIV**	21	Gentianaceae	5.5
1	Rosaceae	101.44	22	Colchicaceae	4.38
2	Papilionaceae	59.27	23	Chenopodiaceae	3.67
3	Hammelidaceae	41.62	24	Rubiaceae	3.39
4	Berberidaceae	33.47	25	Amaranthaceae	3.14
5	Thymelaceae	33.44	26	Covolvulaceae	3.13
6	Oleaceae	31.91	27	Malvaceae	2.82
7	Buxaceae	30.21	28	Cucutaceae	2.68
8	Myrsinaceae	27.14	29	Primulaceae	2.35
9	Lamiaceae	26.16	30	Papaveraceae	2.16
10	Caprifoliaceae	23.43	31	Iridaceae	2.08
11	Euphorbiaceae	16.98	32	Campanulaceae	1.79
12	Buddlejaceae	15.65	33	Liliaceae	1.7
13	Elaeagnaceae	12.16	34	Podophyllaceae	1.66
14	Grossulariaceae	8.59	35	Boraginaceae	1.62
15	Rhamnaceae	7.6	36	Hypericaceae	1.53
16	Acanthaceae	7.09	37	Fumariaceae	1.02
17	Urticacaeae	6.21	38	Violaceae	0.92
18	Rutaceae	5.75	39	Crassulaceae	0.78
19	Plumbaginaceae	4.96	40	Valerianaceae	0.78
20	Celastraceae	4.63	41	Verbenaceae	0.78
21	Rubiaceae	1.89	42	Portulacaceae	0.7
S.No	**HERBS**	**FIV**	43	Zygophyllaceae	0.51
1	Poaceae	182.15	44	Orchidaceae	0.5
2	Asteraceae	43.06	45	Dioscoraceae	0.46
3	Lamiaceae	27.55	46	Gereniaceae	0.42
4	Papilionaceae	24.06	47	Paeoniaceae	0.42

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Table 2. *IV data for plant species*

		LC	UC	S	D	B			LC	UC	S	D	B
	Trees	PDQ	PQJ	QAP	CAP	PPA		**Trees**	PDQ	PQJ	QAP	CAP	PPA
1	*Abies pindrow* Royle.	-	-	18.6	16.01	12.36	***23***	*Prunus persica* (L.) Batsch.	-	3.93	-	-	-
2	*Acer cappadocicum* Gled.	-	-	-	-	3.07	***24***	*Punica granatum* L.	-	5.91	-	-	-
3	*Ailanthus altissima* (Mill.) Swingle	9.21	6.42	1.89	2.37	2.47	***25***	*Pyrus pashia* Ham. *ex* D. Don	-	-	4.16	-	-
4	*Cedrela serrata* Royle	-	-	-	2.91	-	***26***	*Pyrus pyrifolia* (Burm.) Nak.	-	-	4.01	-	2.56
5	*Cedrus deodara* (Roxb. ex Lamb.) G. Don	-	-	4.02	22.07	2.92	***27***	*Quercus baloot* Griffth	-	15.52	-	3.88	-
6	*Celtis caucasica* Willd.	-	-	-	2.62	2.51	***28***	*Quercus dilatata* Royle	15.62	-	-	3.02	2.79
7	*Crataegus songarica* G. Koch.	4.49	3.21	2.04	2.47	-	***29***	*Quercus incana* Roxb.	-	-	18.91	-	2.47
8	*Diospyros kaki* L.	-	-	3.64	-	-	***30***	*Quercus semecarpifolia* Sm.	-	-	-	-	2.76
9	*Diospyros lotus* L.	23.38	2.87	1.86	2.71	4.12	***31***	*Salix babylonica* L.	-	-	-	2.47	-
10	*Ficus carica* L. *ssp carica*	8.94	4.23	1.76	4.41	3.6	***32***	*Salix denticulata* Anderson	-	6.66	4.19	-	2.61
11	*Juglans regia* L.	12.93	13.36	11.59	3.27	4.36	***33***	*Taxus fauana* (Zucc.) Pilger	-	-	8.88	4.06	2.79
12	*Malus pumila* Mill.	-	3.54	-	-	-		**Shrubs**	ICJ	RIR	SDB	PVB	PCI
13	*Melia azedarach* L.	-	-	-	2.91	-	***1***	*Andrachne cordifolia* (Wall.ex Dcne.) Muell. Arg.	-	-	-	2.1	3.25
14	*Morus alba* L.	-	-	1.81	3.02	-	***2***	*Berberis lycium* Royle	-	3.67	13.6	12.26	3.94
15	*Morus nigra* L.	-	3.87	-	-	-	***3***	*Buddleja crispa* Bth.	9.6	-	6.05	-	-
16	*Olea ferruginea* Royle	-	8.42	-	5.53	2.85	***4***	*Cotoneaster nummularia* Fisch. & Mey.	13.25	-	7.3	2.47	14.82
17	*Picea smithiana* (Wall.) Boiss.	-	-	-	2.8	22.07	***5***	*Daphne mucronata* Royle	6.84	5.49	17.29	1.96	1.86
18	*Pinus roxburghii* Sargent	-	-	-	-	3.07	***6***	*Debregeasia salicifolia* (D. Don) Rendle	-	6.21	-	-	-
19	*Pinus wallichiana* A.B. Jackson	25.39	18.06	12.56	13.38	14.54	***7***	*Elaeagnus umbellate* Thunb.	6.31	-	3.93	-	1.92
20	*Pistacia chinensis* Bunge *ssp. Integerrima*	-	-	-	-	2.39	***8***	*Himalrandia tetrasperma* (Roxb.) Yamazaki	-	-	-	-	1.89
21	*Platanus orientalis* L.	-	-	-	-	3.6	***9***	*Indigofera heterantha* Wall. Ex Brandis var. *gerardiana*	13.36	7.31	-	7.08	-
22	*Populus alba* L.	-	3.93	-	-	-	***10***	*Indigofera heterantha* Wall. ex Brandis var. *heterantha*	-	-	10.24	2.1	11.95

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		LC	UC	S	D	B			LC	UC	S	D	B
	Shrubs	ICJ	RIR	SDB	PVB	PCI		**Shrubs**	ICJ	RIR	SDB	PVB	PCI
11	*Isodon rugosus* (Wall. ex Bth.) Codd	-	13.67	-	3.86	3.44	***33***	*Spiraea canescens* D. Don	10.01	-	-	-	4.47
12	*Jasminum humile* L.	10.23	-	6.68	3.46	4.6	***34***	*Strobilanthes urticifolia* Wall. ex Kuntze	-	-	-	2.41	-
13	*Jasminum officinale L.*	-	6.94	-	-	-	***35***	*Viburnum cotinifolium* D. Don	4.81	-	-	-	-
14	*Justicia adhatoda* L.	-	4.68	-	-	-	***36***	*Viburnum grandiflorum* Wall. ex DC.	-	-	-	13.08	-
15	*Lespedeza juncea* (L. f.) Pers. var. *juncea*	-	3.89	-	-	3.34		**Herbs**	ACH	AHP	CAB	CTC	CMR
16	*Limonium cabulicum* (Boiss.) O. Kuntze	-	-	-	-	4.96	***1***	*Achillea millefolium* L.	-	1.09	-	-	-
17	*Lonicera asperifolia* (Decne.)	-	3.62	-	-	-	***2***	*Achyranthes aspera* L. var. *pubescens* (Moq.) C.C. Townsend	-	-	-	0.42	-
18	*Lonicera obovata* Royle ex Hk. f. & Thoms.	-	-	-	1.92	-	***3***	*Agrimonia eupatoria* L.	-	-	-	-	0.42
19	*Maytenus royleanus* (Wall. ex Lawson) Cuf.	-	-	-	4.63	-	***4***	*Agrostis viridis* Gouan	-	-	-	1.99	0.5
20	*Myrsine africana* L.	7.34	8.9	6.62	4.28	-	***5***	*Ajuga bracteosa* Wall. *ex* Bth.	-	-	-	1.08	-
21	*Parrotiopsis jacquemontiana* (Dcne.) Rehder	-	-	-	25.51	16.11	***6***	*Ajuga parviflora* Bth.	-	-	-	-	0.74
22	*Phlomis spectabilis* Falc. ex Bth	-	-	-	1.93	3.26	***7***	*Amaranthus viridis* L.	-	1.69	-	1.03	-
23	*Ribes himalense* Dcne.	-	-	-	4.05	-	***8***	*Ammi visnaga* (L.) Lam.	-	-	-	0.51	-
24	*Ribes orientale* Desf.	-	-	-	-	4.54	***9***	*Anagallis arvensis* L. *var. coerulea* (L.) Gouan.	-	-	1.31	0.42	0.63
25	*Ricinus communis* L.	-	11.63	-	-	-	***10***	*Angelica glauca* Edgew.	-	-	-	-	1.19
26	*Rosa webbiana* Wall. ex Royle	-	-	3.51	1.96	1.55	***11***	*Arthraxon prionodes* (Steud.) Dandy	3.61	-	-	-	-
27	*Rubus ellipticus* Smith	6.43	-	-	-	-	***12***	*Apluda mutica* L.	14.57	9.81	12.47	1.85	0.54
28	*Rubus fruiticosus* L.	7.23	-	5.71	1.97	4.84	***13***	*Arenaria serpyllifolia* L.	-	-	-	-	1.07
29	*Rubus ulmifolius* Schott	-	15.92	-	-	-	***14***	*Aristida cyanantha* Nees ex Steud.	-	-	-	-	3.12
30	*Sageretia thea* (Osbeck) M.C. Johnston var. *thea*	4.52	-	-	-	3.08	***15***	*Artemisia absinthium* L.	-	-	-	2.36	-
31	*Sarcococca saligna* (D. Don) Muell.Arg.	-	8	19.01	-	3.2	***16***	*Artemisia scoparia* Waldst. & Kit.	-	5.19	-	-	-
32	*Skimmia laureola* (DC.) Sieb. & Zucc. ex Walp	-	-	-	2.88	2.87	***17***	*Artemisia vulgaris* L.	4.93	-	6.46	-	-

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		LC	UC	S	D	B			LC	UC	S	D	B
	Herbs	ACH	AHP	CAB	CTC	CMR		**Herbs**	ACH	AHP	CAB	CTC	CMR
18	*Avena fatua L.*	-	-	3.53	3.58	-	***40***	*Convolvulus arvensis L.*	-	1.42	-	0.93	0.78
19	*Bergenia ciliata (Haw.) Sternb. var. ciliata Yeo*	5.07	-	6.88	3.09	1.29	***41***	*Conyza Canadensis (L.) Crongquist*	-	1.02	-	0.93	-
20	*Bistorta amplexicaulis (D. Don) Green*	2.54	-	-	0.93	-	***42***	*Cuscuta europaea L.*	1.39	-	0.42	-	-
21	*Brachiaria reptans (L.) Gardner & Hubbard*	-	-	-	-	0.42	***43***	*Cuscuta reflexa Roxb.*	-	0.87	-	-	-
22	*Brassica rapa L. ssp. Campestris*	-	2.09	-	-	-	***44***	*Cynodon dactylon (L.) Pers.*	13.35	-	13.97	8.64	3.4
23	*Buplerum falcatum L.*	2.2	-	2.03	-	-	***45***	*Dactylis glomerata L.*	-	-	-	0.42	0.42
24	*Bupleurum longicaule Wall. ex DC. var. ramosum Nasir*	-	-	-	0.98	-	***46***	*Datura innoxia Mill.*	-	1.63	-	-	-
25	*Calanthe tricarinata Lindl.*	-	-	-	-	0.5	***47***	*Dicanthium annulatum (Forssk.) Stapf*	-	-	-	0.46	-
26	*Caltha alba Camb. var. alba*	-	-	0.66	2.11	-	***48***	*Dioscorea deltoidea Wall. ex Kunth*	-	-	-	0.46	-
27	*Campanula pallida Wall. var. pallida*	-	1.79	-	-	-	***49***	*Eragrostis minor Host*	-	-	-	-	1.31
28	*Cannabis sativa L.*	-	1.78	-	2.39	1.37	***50***	*Erodium ciconium (L.) L' Herit ex Aiton*	-	-	-	-	0.42
29	*Capsella bursa-pastoris (L.) Medik.*	-	1.67	-	1.67	1.44	***51***	*Eryngium coeruleum M-Bieb.*	-	1.74	-	-	1.33
30	*Cenchrus pennisetiformis Hochst. & Steud. Ex Steud.*	-	-	-	-	18.92	***52***	*Euphorbia helioscopia L.*	-	-	1.59	-	0.78
31	*Centaurea iberica Trev. ex Spreng.*	-	-	-	0.42	-	***53***	*Euphorbia peplus L.*	-	1.81	-	2.18	0.46
32	*Ceratocephala falcata (L.) Pers.*	-	-	-	-	0.42	***54***	*Euphorbia prostrata Ait.*	2.75	-	1.44	0.79	-
33	*Chenopodium album L.*	-	2.83	-	0.84	-	***55***	*Foeniculum vulgare Mill.*	-	0.47	-	-	-
34	*Chrysopogon fulvus (Spreng.) Chiov.*	-	-	-	10.89	-	***56***	*Fragaria vesca L.*	5.82	-	6.74	0.42	-
35	*Chrysopogon serrulatus Trin.*	-	-	-	-	0.42	***57***	*Fumaria indica (Hausskn.) Pugsley*	-	0.47	-	0.55	-
36	*Cichorium intybus L.*	1.32	-	1.12	0.88	-	***58***	*Gagea elegans Wall. ex Royle*	-	-	-	0.42	-
37	*Clematis grata Wall.*	2.53	-	2.21	-	-	***59***	*Galium aparine L.*	-	2.61	-	-	0.78
38	*Clinopodium umbrosum (M. Bieb.) C. Koch.*	3.68	-	-	0.55	-	***60***	*Heliotropium strigosum Willd. ssp. brevifolium (Wall.) Kazmi*	-	-	-	-	0.74
39	*Colchicum luteum Baker*	-	1.94	-	1.37	1.07	***61***	*Heteropogon contortis L.*	6.64	8.34	-	-	-

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		LC	UC	S	D	B			LC	UC	S	D	B
	Herbs	ACH	AHP	CAB	CTC	CMR		**Herbs**	ACH	AHP	CAB	CTC	CMR
62	*Hypericum oblongifolium* Choisy	-	-	0.23	-	-	***84***	*Oxalis corniculata* L.	-	-	2.41	-	0.82
63	*Hypericum perforatum* L.	-	-	0.42	0.88	-	***85***	*Paeonia emodi* Wall. ex Royle	-	-		0.42	-
64	*Iris germanica* L.	-	-	-	-	1.11	***86***	*Papaver dubium* L.	-	-	-	-	1.19
65	*Iris hookeriana* Foster	-	-	-	0.97	-	***87***	*Papaver somniferum* L.	-	0.97	-	-	-
66	*Lactuca orientalis* Boiss.	-	-	-	0.46	-	***88***	*Pennisetum orientale* L.C. Rich.	1.96	2.62	-	-	-
67	*Lepidium apetalum* Willd.	-	-	-	-	1.03	***89***	*Phalaris minor* Retz.	-	6.54	-	2.13	-
68	*Lepidium sativum* L.	-	-	-	0.42	-	***90***	*Plantago lanceolata* L.	-	-	0.42	1.3	1.81
69	*Lolium temulentum* L.	-	-	-	1.3	-	***91***	*Plantago himalaica* Pilger	2.37	-	-	0.46	0.7
70	*Notholirion thomsonianum* (Royle) Stapf	-	-	-	0.46	-	***92***	*Plantago major* L.	-	2.89	-	-	-
71	*Malva neglecta* Wall.	-	-	-	-	0.74	***93***	*Poa annua* L.	-	7.92	4.22	-	3.8
72	*Malvastrum coromendelianum* L.	-	-	0.42	0.42	1.24	***94***	*Podophyllum emodi* Wall. ex Royle	-	-	-	-	1.66
73	*Medicago lupulina* L.	-	-	-	-	11.87	***95***	*Portulaca oleracea* L.	-	-	-	-	0.7
74	*Medicago minima* (L.) Grufb.	-	2.51	-	0.69	-	***96***	*Pseudognaphalium affine* (D. Don.) Anderb.	-	-	-	-	0.46
75	*Medicago polymorpha* L.	2.92	1.79	0.66	-	0.99	***97***	*Ranunculus laetus* Wall. ex Hk. f. & Thoms.	-	1.44	-	-	-
76	*Mentha longifolia* (L.) L.	-	2.34	-	-	-	***98***	*Ranunculus sceleratus* L.	-	-	-	-	0.78
77	*Micromeria biflora* (Buch-Ham.ex D. Don) Bth.	-	-	-	0.46	-	***99***	*Rumex dentatus* L.	-	-	0.84	1.11	-
78	*Myosotis alpestris* F. W. Schmidt var. *albicans* (H. Riedl) Y. Nasir	-	-	-	-	0.46	***100***	*Rumex hastatus* D. Don	2.45	-	4.63	1.68	8.97
79	*Narcissus tazetta* L.	-	2.56	3.16	1.12	0.78	***101***	*Salvia lanata* Roxb.	-	1.74	-	0.51	-
80	*Oenanthe javanica* (Blume) DC.	-	-	-	0.42	-	***102***	*Salvia moorcraftiana* Wall. Ex Bth.	3.32	-	2.43	3.04	-
81	*Onosma hispida* Wall. ex G. Don	-	-	-	0.42	-	***103***	*Sanguisorba minor* Scop.	-	-	-	0.42	-
82	*Origanum vulgare* L.	4.3	-	-	-	-	***104***	*Saussurea albescens* (DC.) Sch. Bip.	3.92	-	4.11	0.51	-
83	*Oxalis acetosella L.*	-	1.09	-	0.74	0.91	***105***	*Saxifraga moorcroftiana* (Ser.) Sternb.	-	-	-	-	1.07

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	Herbs	ACH	AHP	CAB	CTC	CMR		**Herbs**	ACH	AHP	CAB	CTC	CMR
106	*Scandix pecten-veneris* L.	-	-	1.48	-	-	***120***	*Teucrium stocksianum* Boiss. var. *incanum* (Aitch. & Hemsley) Hedge & Lamon	-	2.81	-	-	-
107	*Sedum hispanicum* L.	-	-	-	-	0.78	***121***	*Themeda anathera* (Nees ex Steud.) Hack.	-	-	-	9.33	-
108	*Silene conoidea* L.	-	-	2.05	-	-	***122***	*Thymus linearis* Bth. ssp. *linearis* Jalas	-	-	-	0.55	-
109	*Silene viscosa* (L.) Pers.	-	-	-	0.93	0.46	***123***	*Tragopogon gracilis* D.Don	-	-	-	-	0.42
110	*Silene vulgaris* (Moench) Garcke	-	1.49	-	-	-	***124***	*Tribulus terrestris* L.	-	-	-	0.51	-
111	*Sisymbrium irio* L.	-	-	-	2.46	2.83	***125***	*Trigonella emodi* Bth.	-	-	-	-	0.46
112	*Solanum nigrum* L. var. *nigrum*	-	1.42	0.7	0.88	1.98	***126***	*Tulipa stellata* Hk. f.	-	-	-	-	0.82
113	*Solanum pseudocapcicum* L.	-	-	0.66	-	-	***127***	*Urtica dioca* L.	-	3.81	-	-	2.18
114	*Solidago virga-aurea* L.	2.13	-	2.99	0.55	-	***128***	*Valeriana jatamansi* Jones	-	-	-	-	0.78
115	*Sonchus asper* (L.) Hill	2.62	-	3.03	0.93	0.42	***129***	*Verbascum thapsus* L.	-	3.71	-	3.09	-
116	*Stellaria media* (L.) Vill.	-	-	1.03	1.9	0.5	***130***	*Verbena officinalis* L.	-	-	-	-	0.78
117	*Swertia ciliata* (G. Don) B. L. Burtt	3.54	0.99	-	-	-	***131***	*Vicia monantha* Retz.	-	-	1.33	0.42	0.42
118	*Swertia cordata* (G. Don) Clarke	-	-	-	0.97	-	***132***	*Viola canescens* Wall. ex Roxb.	-	0.92	-	-	-
119	*Taraxacum officinale* Wigg.	-	-	2.03	0.84	0.46	***133***	*Withania somnifera* (L.) Dunal	-	-	-	-	0.74

KEY:
LC-Lower Chail, UC-Upper Chail, S-Shinku, D-Dabargai, B-Bishigram
PDQ-*Pinus-Diospyros-Quercus* community, ICJ-*Indigofera-Cotoneaster-Jasminium* community, ACH-*Apluda-Cynodon-Heteropogon* community, PQJ-*Pinus-Quercus-Juglans* community, RIR-*Rubus-Isodon-Ricinus* community, AHP-*Apluda-Heteropogan-Poa* community, QAP-*Quercus-Abies-Pinus* community, SDB-*Sarcococca-Daphne-Berberis* community, CAB-*Cynodon-Apluda-Bergenia* community, CAP-*Cedrus-Abies-Pinus* community, PVB-*Parrotiopsis-Vibernum-Berberis* community, CTC-*Chrysopogon-Themeda-Cynodon* community, PPA-*Picea-Pinus-Abies* community, PCI-*Parrotiopsis-Cotoneaster-Indigofera* community, CMR-*Cenchrus-Medicago-Rumex* community

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Lower Chail (Site-I)

Plant communities at this site were established at Southern and South-Eastern aspects at an elevation of 1390 m up to 1480 m. Soil at this locality was loamy-sand, with 9.2% clay particles, 53.2% silt and 37.6% sand particles. Nitrogen content of the soil was estimated to be 0.1035%, phosphorous 470 µg/g, potassium 6398 µg/g, calcium 34448 µg/g and magnesium 22188 µg/g. sodium absorption ratio (SAR) of the soil was 0.52 millieq/L while sulphates were found to be 516 µg/g. Soil pH at this monitoring site was recorded as 5.2 which reflected the podzolic nature of the soil. Organic matter was found to be 2.07% which is typical to the podzolic soils. $CaCO_3$ content was low at this site i.e. 2.5% while electrical conductivity was 0.43 dsm^{-1} (*Table 3*).

Table 3. *Edaphic variables, mineral content and plant communities at 5 monitoring sites*

	Parameters	Units	PDQ ICJ ACH	PQJ RIR AHP	QAP SDB CAB	CAP PVB CTC	PPA PCI CMR	Mean
			LC	UC	S	D	B	
1	Soil texture							
	Clay	% age	9.2	5.2	7.2	7.2	9.2	7.6
	Silt	% age	53.2	47.2	65.2	49.2	37.2	50.4
	Sand	% age	37.6	47.6	27.6	43.6	53.6	42
2	pH	-	5.2	5.4	5.4	5.8	6	5.56
3	OM	% age	2.07	2.76	1.38	2.08	2.48	2.154
4	$CaCO_3$	% age	2.5	7.5	3	2.5	10	5.1
5	EC	dsm^{-1}	0.43	0.08	0.026	0.03	0.023	0.1178
6	Sulphates	µg/g	516	556	450	496	661	535.8
7	SAR	millieq/L	0.52	0.2	0.3	0.13	0.21	0.272
8	N_2	% age	0.1035	0.138	0.069	0.1104	0.1242	0.10902
9	P	µg/g	470	430	494	385	475	450.8
10	K	µg/g	6398	10634	8925	4162	13059	8635.6
11	Ca	µg/g	34448	16722	24148	39159	14568	25809
12	Mg	µg/g	22188	15433	17056	25127	13738	18708.4

Key: **PDQ**-Pinus-Diospyros-Quercus community, **ICJ**-Indigofera-Cotoneaster-Jasminium community, **ACH**-Apluda-Cynodon-Heteropogon community, **PQJ**-Pinus-Quercus-Juglans community, **RIR**-Rubus-Isodon-Ricinus community, **AHP**-Apluda-Heteropogan-Poa community, **QAP**-Quercus-Abies-Pinus community, **SDB**-Sarcococca-Daphne-Berberis community, **CAB**-Cynodon-Apluda-Bergenia community, **CAP**-Cedrus-Abies-Pinus community, **PVB**-Parrotiopsis-Vibernum-Berberis community, **CTC**-Chrysopogon-Themeda-Cynodon community, **PPA**-Picea-Pinus-Abies community, **PCI**-Parrotiopsis-Cotoneaster-Indigofera community, **CMR**-Cenchrus-Medicago-Rumex community, **LC**-Lower Chail, **UC**-Upper Chail, **S**-Shinku, **D**-Dabargai, **B**-Bishigram, **OM**-Organic matter, **EC**-Electrical conductivity, **SAR**-Sodium Absorption Ratio, **N_2**-Nitrogen, **P**-Phosphorous, **K**-Potassium, **Ca**-Calcium, **Mg**-Magnesium

1. Pinus-Diospyros-Quercus community (PDQ)

This plant community was recorded from South facing slope at an elevation of 1480 m. *Pinus wallichiana* was the dominant species with IV of 25.39. Second dominant was found to be *Diospyros lotus* with an IV of 23.38, forming a close

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association with *Pinus wallichiana* at lower parts of the slopes. As the plant yield edible fruits with medicinal value the population density of *Diospyros lotus* was high with a considerably uniform dispersion. *Quercus dilatata* with an IV of 15.62, was mostly dispersed among *Pinus wallichiana* members at higher altitudes. Other prominent members of this community included *Juglans rigia* (12.93 IV), *Ailanthus altissima* (9.21 IV), *Ficus carica* (8.94 IV) and *Crataegus songarica* (4.49 IV). Common herbs growing around this community included *Rumex hastatus, Solidago virga-aurea, Fragaria vesica, Plantago himalayica, Medicago polymorpha, Sonchus asper, Cuscuta europaea* and *Salvia moorcroftiana.*

2. Indigofera-Cotoneaster-Jasminum community (ICJ)

On South facing slope this shrubby community was recorded. Dominant species was *Indigofera heterantha* var. *gerardiana* with an IV of 13.36 followed by *Cotoneaster nummularia* with an IV of 13.25. These shrubs made thick randomly dispersed patches on mostly tree-less aspect of the cliffs. Co-dominant was found to be *Jasminum humile* with an IV of 10.23. The rest of the shrubs included *Spiraea canescens* (10.01 IV), *Buddleja crispa* (9.6 IV), *Myrsine africana* (7.34 IV), *Rubus fruiticosus* (7.23 IV) and *Daphne mucronata* (6.84 IV). Other members of the shrub layer in this locality were *Rubus ellipticus, Elaeagnus umbellate, Vibernum cotinifolium* and *Sageretia thea*.

3. Apluda-Cynodon-Heteropogon community (ACH)

This community was recorded from South-eastern aspect at an elevation of 1390 m. In Lower Chail area therophytes dominated the landscape. This community was dominated by *Apluda mutica* with an IV of 14.57, almost uniformly distributed in the locality making strong association with the second dominant *Cynodon dactylon* with an IV of 13.35. Just around the shrubby vegetation *Apluda mutica* formed mixed patches with third dominant *Heteropogon contortis* with an IV of 6.64. The other associated species were *Fragaria vesca* (5.82 IV), *Bergenia ciliata* (5.07 IV), *Artemisia vulgaris* (4.93 IV) and *Origanum vulgare* (4.3 IV). Remaining species such as *Arthraxon prionodes, Bistorta amplexicaulis, Buplerum falcatum, Cichorium intybus, Clematis grata, Clinopodium umbrosum, Cuscuta europaea, Euphorbia prostrata, Medicago polymorpha, Pennistum orientale, Plantago himalaica, Rumex hastatus* and *Salvia moorcroftiana* had IV values ranging between (1.32 to 3.92) (*Table* 2).

Upper Chail (Site-II)

Plant communities established at this monitoring site were found on an elevation of 1620 m. Soil analysis displayed a contrasting picture with reference to adjoining Lower Chail area. Soil was silt-loam at this site with 5.2% clay particles, 47.2% silt and 47.6% sand particles. Soil reaction was mildly acidic with a soil pH of 5.4. Organic matter content was estimated as 2.76%. Nitrogen content was 0.138% followed by phosphorous 430 µg/g, potassium 10634 µg/g, calcium 16722 µg/g and magnesium 15433 µg/g. Sodium absorption ratio of the soil was estimated as 0.2 millieq/L and sulphate content was recorded as 556 µg/g. Floods of recent past have eroded soil in the Upper Chail to a huge extent and main water tributary of the valley, Chail stream, is still a big threat to remaining vegetation cover in the valley floor. Highest value for $CaCO_3$ were recorded from upper Chail i.e. 7.5% while electrical conductivity was 0.08 dsm^{-1} (*Table 3*).

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1. Pinus-Quercus-Juglans community (PQJ)

This community was recorded at an elevation of 1620 m, *Pinus wallichiana* was found to be dominated with an IV of 18.06. This was associated with *Quercus baloot* having an IV of 15.52. *Juglans regia* with an IV of 13.36 was next in abundance. These were followed by *Olea ferruginea* (8.42 IV), *Salix denticulata* (6.66 IV), *Alianthus altissima* (6.42) and *Punica granatum* (5.91 IV). In addition to this *Ficus carica, Populus alba, Prunus persica, Morus nigra, Malus pumiula, Crataegus songarica* and *Diospyros lotus* had IV values ranging between 4.32 to 2.87. *Phalaris minor, Achillea millefolium, Cannabis sativa, Viola canescens, Fumaria indica, Urtica dioca, Capsella bursa-pastoris, Chenopodium album* and *Plantago major* were the common herbaceous flora intermingled with trees association.

2. Rubus-Isodon-Ricinus community (RIR)

Rubus ulmifolius with an IV of 15.92 dominated this community with uniform distribution. It had thick patches of *Isodon rugosus* as second dominant with an IV of 13.67. But on low elevation *Ricinus communis* formed randomly dispersed clumps with an IV of 11.67. These were followed by *Mrsine Africana* (8.9 IV), *Sarcococca saligna* (8 IV), *Indigofera heterantha* var. *gerardiana* (7.31 IV), *Jasminum officinale* (6.94 IV) and *Debergeasia salicifolia* (6.21 IV). Other shrubby members of the community such as *Daphne mucronata, Justicia adhatoda, Lespedeza juncea, Berberis lyceum* and *Lonicera asperifolia* had IV ranging between 5.49 and 3.62.

3. Apluda-Heteropogon-Poa community (AHP)

This community of Upper Chail was dominated by *Apluda mutica* with an IV of 9.81. Though herbs were not forming a uniform cover, due to severe soil erosion by Chail stream in past few years, but still thick patches of herbs were recorded on banks of Chail stream. Second dominant species was *Heteropogon contortis* with an IV of 8.34 which formed association with *Poa annua* having an IV of 7.92. These dominants were followed by *Phalaris minor* (6.54 IV), *Artemisia scoparia* (5.19 IV), *Urtica dioca* (3.81 IV), *Verbascum thapsus* (3.71 IV), *Plantago major* (2.89 IV) and *Chenopodium album* (2.83 IV) (*Table 2*).

Shinku (Site-III)

Plant communities at this site were established at elevation of 2150 m on Northern and North-Western slopes. Soil analyses indicated that soils at this site are silt-loam with 7.2% clay particles, 65.2% silt particles and 27.6% sand particles. Soil pH was recorded as 5.4 and organic matter was estimated as 1.38%. Nitrogen content was low i.e. 0.069%. Phosphorous was estimated as 494 µg/g while potassium was found to be 8925 µg/g. Calcium content was a bit higher i.e. 24148 µg/g and magnesium was 17056 µg/g in the soils. Sodium absorption ratio was also a little low in comparison with Lower and Upper Chail i.e. 0.3 millieq/L. Sulphates were estimated as 450 µg/g. Soil had only 3% lime and electrical conductivity remained at 0.026 dsm^{-1} (*Table 3*).

1. Quercus-Abies-Pinus community (QAP)

Quercus-Abies-Pinus community of trees was recorded at an elevation of 1495 m up to 1680 m on Northern and North-Western slopes in Shinku area. In this community the

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dominant member was *Quercus incana* with an IV of 18.91. Second dominant was *Abies pindrow* with an IV of 18.6, making strong association with *Quercus incana* on North-Western aspect and *Pinus wallichiana* with IV of 12.56. The other important contributors to this community were *Juglans regia* (11.59 IV), *Taxus fauna* (8.88 IV), *Pyrus pashia* (4.16 IV) and *Cedrus deodara* (4.02 IV). *Taxus fauna* was more abundant on North-Western aspect than on Northern aspect. On lower elevations, mixed patches of *Diospyros kaki* (3.64 IV), *Crataegus songarica* (2.04 IV) and *Ailanthus altissima* (1.89 IV) were recorded. Some notable herbaceous members found within this community were *Oxalis corniculata, Euphorbia prostrata, Anagallis arvensis, Scandix pecten-veneris, Avena fatua, Taraxacum officinale, Stellaria media, Silene conoidea, Hypericum perforatum, Poa annua* and *Clematis grata*.

2. Sarcococca-Daphne-Berberis community (SDB)

Shrubby layer was much prominent making thick strips from Northern to North-Western aspect. This community was found at an elevation of 1491 m up to 1516 m. *Sarcococca saligna* with an IV of 19.01 was the dominant making thick uniformly dispersed clumps with next dominant *Daphne mucronata* with an IV of 17.29. *Berberis lyceum* with an IV of 13.6, was common to Northern slopes. Other important members of this community included *Indigifera heterantha* var. *heterantha* (10.24 IV), *Cotoneaster nummularia* (7.3 IV), *Myrsine africana* (6.62 IV) and *Buddleja crispa* (6.05 IV). *Rosa webbiana* was randomly distributed at higher elevations with an IV of 3.51.

3. Cynodon-Apluda-Bergenia community (CAB)

Herbaceous community was found at elevation of 1490 to 1610 m. *Cynodon dactylon* appeared to be dominant with an IV of 13.97, followed by *Apluda mutica* with an IV of 12.47 as co-dominant. *Bergenia ciliata* appeared as the next dominant with an IV of 6.88 near small water falls on both aspects. This community also included *Fragaria vesca* (6.74 IV), *Artemisia vulgaris* (6.46 IV), *Rumex hastatus* (4.63 IV), *Poa annua* (4.22 IV), *Sassurea albescense* (4.11 IV), *Avena fatua* (3.53 IV) and *Nacrissus tazetta* (3.16 IV) (*Table* 2).

Dabargai (Site-IV)

Plant communities at this site were established at an elevation of 2371 m on North-Eastern and Southern aspects. Soil was loam with 7.2% clay particles, 49.2% silt particles and 43.6% sand particles. Soil pH was recorded as 5.8 and it was evident from a higher degree of podzolization in the soils of the study area with considerably low organic content i.e. 2.08%. Nitrogen content was estimated as 0.1104%. Phosphorous and potassium were recorded as 385 µg/g and 4162 µg/g respectively. Calcium content was found to be 39159 µg/g while magnesium content was 25127 µg/g. Sodium absorption ratio was 0.13 millieq/L and sulphates were only 496 µg/g. $CaCO_3$ was estimated to be 2.5% and electrical conductivity was 0.03 dsm^{-1} (*Table 3*).

1. Cedrus-Abies-Pinus community (CAP)

This tree community was found between elevation ranges of 1935 to 12371 m. *Cedrus deodara* with an IV of 22.07 was the abundantly growing tree. *Abies pindrow*

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and *Pinus wallichiana* with an IV of 16.01 and 13.38 respectively were the second and third dominants. In addition to these dominant forms other important members of this community included *Olea ferruginea* (5.53 IV), *Ficus carica* (4.41 IV), *Taxus fauna* (4.06 IV), *Quercus baloot* (3.88 IV), *Juglans regia* (3.27 IV), *Moras alba* (3.02 IV) and *Cedrela serrata* (2.91 IV). Notable herbaceous elements of this community were *Thymus linearis, Ammi visnaga, Apluda mutica, Dactylis glomerata, Centaurea iberica, Notholirion thomsonianum, Dioscorea deltoidea, Amaranthus viridis, Verbascum thapsus, Gagea elegans, Micromeria biflora* and *Sisymbrium irio*.

2. Parrotiopsis-Vibernum-Berberis community (PVB)

Shrubby layer was randomly dispersed on North-Eastern and Southern slopes. *Parrotiopsis jacquemontiana* was dominant with an IV of 25.51 making randomly dispersed thick patches mostly on North-Eastern aspect. *Vibernum grandiflorum* was second dominant with an IV of 13.08 making continuous strips with *Berberis lyceum* (IV 12.26). Some other important contributors to this community included *Indigifera heterantha* var. *gerardiana* (7.08 IV), *Maytenus royleanus* (4.63 IV), *Myrsine Africana* (4.28 IV), *Ribes himalense* (4.05 IV), *Isodon rugosus* (3.86 IV) and *Jasminum humile* (3.46 IV).

3. Chrysopogon-Themeda-Cynodon community (CTC)

This community is dominated by *Chrysopogon fulvus* with an IV of 10.89. *Themeda anathera* with an IV of 9.33 was more common to Southern aspect on lower elevations. *Cynodon dactylon* with an IV to 8.64 was the next dominant. Some other important members of this community included *Avenia fetua* (3.58 IV), *Bergenia ciliata* and *Verbascum thapsus* both with IV of 3.09, *Salvia moorcroftiana* (3.04 IV), *Sisymbrium irio* (2.46 IV), *Cannabis sativa* (2.39 IV) and *Artemisis absinthium* (2.36 IV) (*Table 2*).

Bishigram (Site-V)

Plant communities at this monitoring site were established at an elevation of 2698 m on Northern and Southern aspects. Soils in the area were loamy-sand in texture with 9.2% clay particles, 37.2% silt particles and 53.6% sand particles. Soil pH was 6 and organic matter content was estimated as 2.48%. Nitrogen content was 0.1242% which was better than Lower Chail, Upper Chail and Shinku areas displaying a healthy rate of saprophytic activity in the soils. Phosphorous content was 475 μg/g, potassium 13059 μg/g and calcium 14568 μg/g. Magnesium was recorded as 13738 μg/g. Sodium absorption ratio was low i.e. 0.21 millieq/L and sulphates were abundant i.e. 661 μg/g. $CaCO_3$ content was highest at this site i.e. 10% and electrical conductivity was recorded as 0.023 dsm^{-1} (*Table 3*).

1. Picea-Pinus-Abies community (PPA)

This tree community was found at an elevation range of 2475 to 2698 m on both aspects. *Picea smithiana* with an IV of 22.07 followed by *Pinus wallichiana* (14.54 IV) were the leading trees in this community. On higher elevations *Picea smithiana* formed an association with *Abies pindrow* with an IV of 12.36. Other notable contributors were *Juglans rigia* (4.36 IV), *Diospyros lotus* (4.12 IV) and *Ficus carica*

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(3.6 IV). On Southern aspect there was a mixed patch of *Platanus orientalis* (3.6 IV), *Acer cappadocicum* and *Pinus roxbergii* (each with 3.07 IV). In addition to this *Cedrus deodara* made randomly dispersed clumps on Northern aspect with IV of 2.92. On higher elevations at Northern aspect *Quercus dilatata* and *Taxus fauna* were found with an IV of 2.79 each, delimiting a thin strip of *Quercus semicarpifolia* with an IV of 2.76. At this monitoring site the common herbs included *Arenaria serpyllifolia, Brachiaria reptans, Eryngium coeruleum, Pseudognaphalium affine, Eragrostis minor, Angelica glauca, Calanthe tricarinata, Portulaca oleracea, Apluda mutica* and *Podophyllum emodi*.

2. Parrotopsis-Cotoneaster-Indigofera community (PCI)

Parrotiopsis jacuemontiana with an IV of 16.11 making a very prominent stretched patch on Northern slopes and clumped pattern on Southern aspect. It was accompanied by *Cotoneaster nummularia* with an IV of 14.82. *Indigofera heterantha* var. *heterantha* was the companion shrub with an IV of 11.95. It was more common to Southern aspect than to Northern slopes. *Cotoneaster nummularia* displayed a uniformed dispersion on both slopes. Other important contributors to this community were *Limonium cabulicum* (4.96 IV), *Rubus fruiticosus* (4.84 IV), *Jasminum humile* (4.6 IV), *Ribes orientale* (4.54 IV), *Spiraea canescens* (4.47 IV) and *Berberis lyceum* (3.94 IV). Notable shrubs but with lesser IV included *Isodon rugosus, Lespedeza juncea, Phlomis spectabilis, Andrachne cordifolia, Sarcococca saligna, Skimmia laureola* and *Himalrandia tetrasperma.*

3. Cenchrus-Medicago-Rumex community (CMR)

Herbaceous community was found to have rich diversity with *Cenchrus pennisetiformis* as dominant, having an IV of 18.92 forming uniform strips on both slopes. It was found making good association with *Medicago lupulina* having an IV of 11.87. While on lower elevations *Rumex hastatus* with an IV of 8.97 was dominating both the aspects. This community also had *Poa annua* (3.8 IV), *Cyanodon dactylon* (3.4 IV), *Aristida cyanantha* (3.12 IV), and *Sisymbrium irio* (2.83 IV) (*Table 2*).

Maturity index

Maturity index was calculated according to Pichi-Sermolli (1948). At Lower Chail, tree community had 48.57 maturity index, shrubs had 31.66 and herbaceous community had a 28.61 maturity index. The mean value for the site was 36.28. At Upper Chail, tree community showed a maturity index of 34.28, followed by shrubby layer with a value of 23.07 and herb layer having 16.06. The mean value for these three communities was 24.47. In Shinku, tree community had a maturity index of 44. Shrub community with a maturity index value of 27.27 while herb community had only 19.62. Mean value for maturity index at this site was 30.29. Maturity index for tree community at Dabargai area was found to be 28.88 followed by 16.31 value of shrub community and 12.73 value for herbaceous community. Mean value for the maturity index at this site was 19.30. At Bishigram, tree community had a maturity index of 27. At this site shrub had a maturity index of 20 followed by herb layer having a maturity index of 10.86. Mean value of maturity index at this site was 19.28 (*Table 4*).

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***Table 4.** Maturity index*

	Lower Chail	Upper Chail	Shinku	Dabargai	Bishigram
Trees	48.57	34.28	44	28.88	27
Shrubs	31.66	23.07	27.27	16.31	20
Herbs	28.61	16.06	19.62	12.73	10.86
Mean	**36.28**	**24.47**	**30.29**	**19.30**	**19.28**

Index of similarity

1. Tree communities

According to Motyka's index of similarity (Motyka et al., 1950), PDQ tree community of Lower Chail was found to be 16.09% similar, to PQJ community of Upper Chail. The similarity between PDQ community and QAP community at Shinku was found to be 10.56%. The analysis showed that similarity between PDQ community and that of CAP community at Dabargai area was 10.62%. Lowest value of similarity was observed between PDQ community and PPA community of Bishigram, i.e. 10.54%. Similarity between PQJ tree community of Upper Chail and that of QAP community at Shinku was 11.96%. PQJ community was 12.61% like tree community CAP at Dabargai area. Only 11.09% similarity was observed between PQJ community and PPA community of Bishigram area. QAP tree community at Shinku area showed more similarity with CAP tree community of Dabargai area i.e. 16.42%. QAP community was 16.04% like PPA community at Bishigram. CAP tree community at Dabargai area showed maximum similarity with PPA tree community at Bishigram i.e. 18.11% (*Table 5*).

***Table 5.** Similarity indices of tree communities*

PDQ	PQJ	QAP	CAP	PPA
X	X	X	X	X
16.09	X	X	X	X
10.56	11.96	X	X	X
10.62	12.61	16.42	X	X
10.54	11.09	16.04	18.11	X

2. Shrub communities

Shrubby vegetation showed little similarities among communities based on Motyka's index. ICJ community at Lower Chail site was 6.71% like RIR community at Upper Chail. ICJ shrub community was found 10.45% similar to SDB shrub community at Shinku. Similarity between ICJ community and PVB community at Dabargai area was 7.07%. The ICJ community was least similar to PCI community at Bishigram i.e. 5.31%. Shrub community at Upper Chail i.e. RIR was 10.36% similar to SDB shrub community at Shinku. Same RIR community was 6.95% similar to PVB community of shrubs at Dabargai. A 5.17% similarity was observed between RIR community and PCI shrub community at Bishigram. Comparison of shrub community at Shinku area i.e. SDB community and PVB community at Dabargai showed 11.81% similarity while between SDB community and PCI community at Bishigram 9.73% similarity was

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observed. Shrub communities at Dabargai i.e. PVB and that of Bishigram i.e. PCI community showed maximum similarity i.e. 14.6% (*Table 6*).

Table 6. *Similarity indices of shrubby communities*

ICJ	RIR	SDB	PVB	PC1
X	X	X	X	X
6.71	X	X	X	X
10.45	10.36	X	X	X
7.07	6.95	11.81	X	X
5.31	5.17	9.73	14.6	X

3. Herbaceous communities

Among herbaceous communities, ACH community at Lower Chail was 7.06% similar to AHP herb community at Upper Chail. The ACH community was found to be 19.64% similar to CAB community at Shinku while its similarity with CTC community at Dabargai site was 8.27%. The herb community ACH was least similar to Bishigram herb community with a similarity index of just 3.03%. The herb community of Upper Chail i.e. AHP was 4.57 same as CAB herb community at Shinku, 6.13% similar to CTC community at Dabargai and 5.87% similar to CMR community at Bishigram. The CAB herb community of Shinku was 10.68% like CTC community at Dabargai and 6.40% similar to CMR community at Bishigram. Similarty between CTC community at Dabargai and CMR community at Bishigram was found to be 6.88% (*Table 7*).

Table 7. *Similarity indices of herbaceous communities*

ACH	AHP	CAB	CTC	CMR
X	X	X	X	X
7.06	X	X	X	X
19.64	4.57	X	X	X
8.27	6.13	10.68	X	X
3.03	5.87	6.40	6.88	X

Discussion

Vegetation was classified into tree, shrub and herbaceous communities which is in line with the works of Ahmad et al. (2016), Dad (2016), Ali et al. (2015), Saurav and Das (2014), Sharma et al. (2014) Saglam (2013), Ahmad et al. (2010), Digiovinazzo et al. (2010), Hussain et al. (2010), Mohler et al. (2006) Munhoz et al. (2006) and Padalia et al. (2004). At Site-I, PDQ-ICJ-ACH communities were established for trees, shrubs and herbs respectively. Distribution of trees showed clumping rather than a uniform pattern of distribution due to lavish wood cutting in the area. Scanty trees and comparatively thick shrub cover near the foothills indicated the disturbed nature of habitat which is under tremendous biotic stress. Increasing population and construction of residential units is the main reason behind the species loss in this locality. Similar communities are reported from their respective study areas by Ahmad et al. (2016), Urooj et al. (2016), Haq et al. (2015), Ilyas et al. (2015), Dad (2016), Shaheen and

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Qureshi (2011), Ahmad et al. (2010), Ahmad (2009), Hussain et al. (2010) and Jabeen and Ahmad (2009). Site-II had PQJ-RIR-AHP communities. Tree and shrub members showed a clumped dispersion pattern at this monitoring site. Impacts of soil erosion were more visible at Site-I and Site-II where eroded soils had herbaceous members like *Rumex hastatus, Plantago himalayica, P. major, Salvia moorcroftian and Verbascum thapsus* which are indicator species of eroded soils. Similar communities but with different IV are reported by Ahmad et al. (2016), Dad (2016), Khan et al. (2016), Akhtar and Bergmeier (2015), Shaheen et al. (2015), Ilyas et al. (2015), Sharma et al. (2014), Hussain et al. (2010) and Khan et al. (2010). Site-III exhibited QAP-SDB-CAB communities. This site had a uniformly spread thick Oak forest mixed with conifers. Deforestation was also not that alarming as compared to that of Lower and Upper Chail monitoring sites. At higher elevations the forest was almost undisturbed however the habitat will face biotic stress, especially the anthropogenic pressure from increasing population in the low laying villages in next few years. *Apluda mutica* was observed to form randomly dispersed thick patches all along the Northern slopes at this monitoring site. These findings are backed by works of Bokhari et al. (2016), Dad (2016), Ali et al. (2015), Ilyas et al. (2015), Shaheen et al. (2015), Siddiqui et al. (2015), Ahmad et al. (2010) and Ahmad (2009). CAP-PVB-CTC communities were found at site-IV. *Cedrus-Abies* forest was much thicker on North-Eastern aspect and showed degradation on Southern slope. *Chrysopogon fulvus* had a uniform dispersion on North-Eastern aspect than on Southern slopes. This is line with findings of Ahmad et al. (2016), Dad (2016) Bokhari et al. (2016), Khan et al. (2016), Ali et al. (2015), Akhtar and Bergmeier (2015), Ilyas et al. (2015), Shaheen et al. (2015), Sharma et al. (2014) and Ahmad et al. (2010), Hussain et al. (2010), Ahmad (2009) Jabeen and Ahmad (2009). At site-V, PPA-PCI-CMR communities were recorded. Forest at this site was found in good health with the exception on Northern slopes where deforestation was quite evident due to fuel wood extraction by locals. These results are in line with the works of Ahmad et al. (2016), Bokhari et al. (2016), Khan et al. (2016), Akhtar and Bergmeier (2015), Haq et al. (2015), Sharma et al. (2014), Khan et al. (2012), Ahmad et al. (2010) and Hussain et al. (2010) and Ahmad et al. (2006).

Conclusion

This study reveals results of vegetation mapping based on 150 sampling units at 5 monitoring sites. Results of soil analysis cover 12 parameters. Spatial variation among plant communities at different monitoring sites is attributed to variations in edaphic variables, temperature, moisture and slope. Vegetation in the study area is threatened and needs conservation before it is too late. Anthropogenic influences, over grazing, natural calamities and erosion are reducing the vegetation cover. Expansion in agricultural fields is also causing a damage to forest cover in addition to overgrazing. Extraction of wood from forest, especially during harsh winters by the local communities, is not only reducing the tree density but is also increasing the rate of soil erosion. Climate change has not spared Hindukush Range vegetation and floral biodiversity is reducing with every passing year. Coniferous forests in this part of the world are threatened habitats because of accelerated deforestation. Impacts of anthropogenic pressure are evident from low scores for maturity index. Highest maturity index value was recorded for tree community at Site-I i.e. 48.57 followed by tree community at Site-III i.e. 44. Highest maturity index for shrub layer was recorded

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at Site-I i.e. 31.66 followed by Site-III with a value of 27.27. Similar trend was recorded for herbaceous communities. Low values for maturity index indicate inability of the plant communities to cope with anthropogenic, edaphic and climatic stresses. Similarly, low values for similarity index shows the heterogeneity among plant communities. Reforestation programs need to be initiated in the area to protect and conserve the indigenous floristic wealth. But extreme care in this regard is suggested so that no alien plant species is introduced as it will further increase stress on plants in the locality. Well trained *Ecologists* should be invited to chart out a plan for conservation of biodiversity in the study area. Vegetation structure is suggestive that local flora is fighting a war of its survival. Once covered beneath a thick vegetation blanket now bare patches are visible on mountain slopes. Open canopy has enabled some shrub species to make well developed layers. Provision of alternative sources of energy to the locals will reduce pressure on populations of *Pinus roxburghii, P. wallichiana, Cedrus deodara, Picea smithiana, Abies pindrow* and other tree species. This will in turn reduce the extant of deforestation in the locality. This study strongly recommends detailed studies on fuelwood consumption in these monitoring sites to quantify the extent of fuelwood consumption per-annum. Proper grazing management should be ensured for regeneration of understory plants. Influential elders of the area can play a vital part in protection of climax communities as well as in enforcing moderate and rotational grazing routines. Soil in the study area had low organic matter content. Acidic soils show a slow rate of humification which is the reason behind low organic matter in the soil.

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THE EFFECTS OF APPLICATION OF BIOLOGICAL FERTILIZERS AND DIFFERENT AMOUNTS OF UREA FERTILIZER SOURCES UNDER LOW WATER STRESS CONDITIONS ON PHYSIOLOGICAL TRAITS OF MEDICINAL PLANT (*CALENDULA OFFICINALIS* L.)

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Abstract. In order to evaluate the effect of application of biological fertilizers and different amounts of urea fertilizer under low water stress conditions on physiological traits of *Calendula officinalis* L. an experiment was conducted during two years of cultivation of 2015-2016 and 2016-2017 at the research farm of Islamic Azad University (IAU), Varamin-Pishva Branch, Iran. The experiment was split-split plot based on randomized complete block design with three replications. The experimental treatments included different levels of dehydration as the main factor in two levels (lack of water stress (control), irrigation interruption during boiling stage), levels of biological fertilizers in four levels as a sub-agent (no use of biological fertilizer, application of *Azotobacter chroococcum*, application of *Azospirillum brasilense*, combination of Azotobacter and Azospirillum), and urea fertilizer sources at four levels as a sub-subtype (non-consumption (control), recommended amount of urea fertilizer with sulphur coating, 75% of the recommended amount of urea fertilizer with sulphur coating was recommended as the most common form of urea fertilizer (157 kg/ha based on soil test). The results showed that irrigation in boiling stage increased activity of the enzyme di-hydroxy Guanuzyn, glutathione peroxidase, superoxide dismutase also, production of the total phenol, total flavonoid and biomarker of di-tyrosine. In this study, drought stress and application of Nitroxin fertilizer resulted in increased malondialdehyde in the first year. In both years, the application of Nitroxin with the recommended amount of urea fertilizer with sulphur coating resulted in an increase in total flavonoid and Glutathione Peroxidase enzymes. The purpose of this study was to investigate the effect of bacteria in the plant, along with the amount and sources of nitrogen fertilizer to reduce nitrogen fertilizer application on plant yield, as well as sustainable agriculture management through integrated nutrition in different plant conditions.
Keywords: *azospirillum, azotobacter, biomarker, total phenol*

Introduction

Calendula officinalis is a plant native to southern Europe and can be found in countryside fields. Calendula is an annual or sometimes biennial plant with erect stems up to 40-70 cm tall and deep taproot. It belongs to the Asteraceae family, its English name is Marygold. It is one of the well-known medicinal plants today, flowers and essential oils are used in pharmaceutical and cosmetic industries. Pharmacological studies have confirmed that flowers have a large amount of biological effects and pharmacological activity of the liver and antispasmodic protection (Arora et al., 2013).

Medicinal plants have high economical value due to the presence of effective compounds in them. An agricultural crop is economically valuable medicinal plant when secondary metabolites have reached the optimum level and the purpose of this plant is to exploit the effective ingredients in flowers, especially petals. Nitrogen is one of the elements that is lacking in most arid and semi-arid regions, because the amount of organic matter in these areas is very low as the main source of nitrogen storage or it decomposes rapidly due to high heat (Saneoka, 2004). If available nitrogen is toxic or deficient for the plant, the vital processes of the plant cause a disorder that may occur in various forms such as high growth, reduced transpiration, or even cessation of reproductive growth (Saneoka, 2004). Since most nitrogen fertilizers are wasted shortly after consumption, nitrogen management as well as crop should be done accurately during the growing season. In the meantime, Sulphur Coated Urea fertilizer (S.C.U.) on the one hand is a supplier of nitrogen and on the other hand, it is very important to pay attention to the valuable role of sulphur in plant nutrition and the improvement of agricultural soils, especially in our country, which accounts for over 70% of calcareous agricultural land and high pH (Moallem and Eshghizadeh, 2007). One of the basic pillars of sustainable agriculture is the use of bio-fertilizers in crop ecosystems with a view to eliminating or reducing the use of chemical organs (Shubbra et al., 2004). These bacteria, in addition to the role of elemental absorption, reduce disease, improve soil structure, stimulate more plant growth, increase product quality and quantity, and increase resistance to environmental stress (Nagananda et al., 2010). Drought is the most important limiting factor for plant growth and crop production around the world (Abedi and Pakniyat, 2010).

Dehydration also reduces water absorption by plant root system, reducing transpiration, reducing Stomatal conductance and photosynthesis, as well as breaking the hormonal balance of the plant (Auge et al., 2015). Superoxide dismutase is very important for plant tolerance to oxidative stress, which has been reported by many researchers (McKersie et al., 2000). Therefore, superoxide dismutase seems to be at the forefront of oxidative stress protection and the increase of superoxide dismutase is correlated with increasing the protection of damages from environmental stresses (Pang et al., 2005; Sigaud-Kutner et al., 2002). In a study, drought stress resulted in an increase in antioxidants in an *Calendula officinalis* plant (Sedghi et al., 2012). Oxidative stress during drought stress and increased free radicals by reducing the antioxidant defense results in damage to tissues, lipids, proteins and nucleic acids, and the concentration of biomarkers such as malondialdehyde, di-tyrosine and di-hydroxy Guanuzyn increased (Jose et al., 1999). Data were analyzed using (SAS) statistical software (v.9.12) and the meanings were compared by Duncan's multiple range tests at the 5% level. Draw charts with Excel (2010) software. Considering the above, the purpose of this study was to investigate the effect of application of Bio-fertilizers along with fertilizers of the hunchback on physiological characteristics of an ever-spring medicinal plant under stressed water conditions.

Materials and methods

In order to evaluate the effect of application of biological fertilizers and different amounts of urea fertilizer under low water stress conditions on physiological

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characteristics of *Calendula officinalis* L. an experiment was conducted during two years of cultivation of 2015-2016 and 2016-2017 at the research farm of Islamic Azad University, Varamin-Pishva Branch in Tehran province (*Table 1*).

Table 1. *Plan*

S1																**S2**																R2
B1				**B2**				**B3**				**B4**				**B2**				**B3**				**B4**				**B1**				
N1	N3	N2	N4	N3	N2	N1	N4	N4	N2	N1	N3	N4	N2	N3	N1	N1	N3	N2	N4	N4	N2	N3	N1	N4	N2	N1	N3	N3	N4	N2	N1	
S1																**S2**																R1
B4				**B2**				**B1**				**B3**				**B2**				**B1**				**B4**				**B3**				
N4	N2	N1	N3	N1	N3	N2	N4	N1	N3	N2	N4	N1	N3	N2	N4	N4	N2	N1	N3	N4	N2	N3	N1	N3	N4	N2	N1	N4	N2	N3	N1	
S2																**S1**																R3
B3				**B1**				**B4**				**B2**				**B4**				**B2**				**B1**				**B3**				
N4	N2	N3	N1	N3	N4	N2	N1	N4	N2	N3	N1	N1	N3	N2	N4	N1	N3	N2	N4	N1	N3	N2	N4	N1	N3	N2	N4	N4	N2	N1	N3	

N1: non-consumption (control), N2: 75% of the recommended amount of urea fertilizer with sulphur coating, N3: recommended amount of urea fertilizer with sulphur coating, N4: (common form) recommended amount of urea fertilizer, S1: (control) lack of water stress, S2: irrigation interruption during boiling stage, B1: (control) no use of biological fertilizer, B2: Azotobacter, B3: Azospirillum, B4: Azot and Azos

Test site located at latitude 35.19 degrees east longitude 51.39 and the altitude is 1050 m above sea level, with dry and desert weather conditions, and steppes with an average annual precipitation of about 175 mm. The experiment was split-split plot based on randomized complete block design with three replications. The experimental treatments included different levels of dehydration as the main factor in two levels (lack of water stress (control), irrigation interruption during boiling stage) levels of biological fertilizers at four levels as a sub-agent (no use of biological fertilizer, application of *Azotobacter chroococcum*, application of *Azospirillum brasilense*, combination of Azotobacter and Azospirillum (Nitroxin) and urea fertilizer sources in four levels as a subtype (non-consumption (control), recommended amount of urea fertilizer with sulphur coating, 75% of recommended amount of urea fertilizer with sulphur coating, was recommended as the most common form (mentioned area) of urea fertilizer (The amount of fertilizer usage based on fertilizer suggestion, 157 kg/ha^{-1}).

Inoculation of seeds took place with bio-fertilizer by (CFU) method (Bapat et al., 2006). Before planting, to determine the physical and chemical characteristics of the soil, from 0-30 cm depth and 30 to 60 cm soil, two locations for sampling were carried out, the full specifications of which are given in *Table 2*. After the leveling of the land, a 6×2 m plot was made, each plot having six rows and a row spacing of 30 cm and a spacing of 10 cm. The cultivation took place in the first week of June. The first drip irrigation was carried out after planting and until the emergence stage, irrigation was carried out with 3-day courses. After emergence of the seeds, the irrigation interval increased to 5 days and continued to 7-8 leaf stage. After the shrubs were fully deployed, at the beginning of budding, the irrigation was stopped for 10 days (Varamin district, Tehran province). The amount of fertilizer application was based on soil test.

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After harvest, the specimens were transferred to the laboratory to measure the traits and there are traits such as total phenol, total flavonoid, di-hydroxy Guanuzyn enzyme, glutathione peroxidase, superoxide dismutase, malondialdehyde biomarker and di-tyrosine were measured. To determine the amount of superoxide dismutase, Sairam et al. (2001) method was used. To prepare the reaction mixture of 13 ml of methionine, 25 µM of Nitroblutterazolium, 6 µM of 0.5 (MEDTA) solution, 1.5 ml of a solution of 1 M buffer phosphate (pH = 7.8), 60 mM Molybucent riboflavin and 50 mM sodium bicarbonate. Then, 2.9 ml of the resulting mixture was poured into a sterilized tube, immediately after adding 2 µM of riboflavin and 0.1 ml of the enzyme extract, for 15 min, a fluorescence lamp of 15 × 2 watts was placed.

Table 2. *Soil field characteristics*

B (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	Fe (ppm)	K (ppm)	P (ppm)	N (%)	OM (%)	OC (%)	TNV (%)	pH	EC ($dS.M^{-1}$)	Silt (%)	Sand (%)	Clay (%)	Type of experiment
1.22	9.74	0.96	0.56	3.44	406.6	12.8	0.06	1.16	0.68	21.4	7.78	1.97	42	36	22	Soil sample

C: Clay, S: Sand, S: Silt, EC: Electrical Conductivity, TNV: Total Neutralising Value, OC: Organic Carbon, OM: Organic Matter, N: Nitrogen, P: Phosphorus, K: Potassium, Fe: Iron, Zn: Zink, Cu: Copper, Mn: Manganese, B: Boron

To determine the amount of activity of the superoxide dismutase enzyme in the mixture, Spectrophotometer was measured at 560 nm at 23 ± 2 °C spectrophotometry (to measure, 5 leaves from each line was cultivar, in the morning they were harvested from the field). The amount of enzyme changes was determined by Paglia (1997) to calculate the Glutathione Peroxidase enzyme (2 leaves of plant from each line). Di-hydroxy Guanzine assay was performed according to the method (fresh plant tissue (leaf) was homogenized by homogenization and then centrifuged at 5000 rpm for 60 minutes with a centrifugal machine) (Bogdanov and Bical, 1999). Measurement of malondialdehyde and di-tyrosine was determined by the method (The extract used to measure 8-oH-dG is based on the thiobarbetabic acid method with MDA) (Steven and Joseph, 1978). The absorption rate of Flavonoids was measured by Krizek et al. (1993) at three wavelengths of 300, 330 and 270 using the (RAY LEIGH UV 1601) spectrophotometer (24 g samples of dried petals were needed). Also, total phenol measurements were dried by extraction of flower samples and at a rate of 2 g of dried matter *Calendula officinalis* (Extraction of polyphenols from flowers collected using the company protocol Naturalin (Professional of Natural Ingredients)). Data were analyzed using (SAS) statistical software (v.9.12) and the meanings were compared by Duncan's multiple range tests at the 5% level. Draw charts with Excel (2010) software.

Results and discussion

Total phenol

In both years of experiment, the effect of drought stress (S), biological fertilizer (B) and urea fertilizer (N) on this trait was significant at 1% level (*Table 3*). Drought stress in both years of experiment led to a significant increase in this trait. So that the Total Phenol in stress conditions increased 8.3% and 8% in the first and second years respectively (*Table 4*). The application of Nitroxin biological fertilizer in both years led to a significant increase in Total Phenol the Total Phenol in terms of Nitroxin

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application increased 19.2% and 14.8% in the first and second year respectively (*Table 5*). Also, application of recommended amount of urea fertilizer with sulphur coating and recommended amount of urea fertilizer in both years of experiment resulted in significant increase of Total Phenol (*Table 6*). High antioxidant Phenolic compounds such as anthocyanin and Flavonoids play a major role in plant resistance to stress and reduce the damage caused by it (Nasibi, 2005; Prasad, 2002).

Table 3. *Analysis of variance of the effect of biological fertilizers and different amounts of urea sources under irrigated conditions of Calendula officinalis L plant during two years of experiment, 2015-2016 and 2016-2017*

Sources of changes	Degrees of freedom	Average of squares: Total phenol		Di-hydroxy Guanuzyn enzyme		Total flavonoid	
		First year	Second year	First year	Second year	First year	Second year
Repeat	2	14.46^{**}	11.18^{**}	3.26^{**}	1.61^{**}	0.57^{ns}	1.06^{**}
Drought stress (S)	1	124.74^{**}	118.99^{**}	29.06^{**}	30.92^{**}	109.68^{**}	68.12^{**}
Main error	2	0.04	0.35	0.11	0.14	0.67	0.05
Biological fertilizer (B)	3	95.45^{**}	88.66^{**}	7.41^{ns}	2.26^{ns}	122.73^{**}	119^{**}
S×B	3	1.9^{ns}	0.49^{ns}	0.47^{ns}	0.09^{ns}	0.88^{ns}	1.3^{ns}
Sub-error	12	3.36	0.67	3.57	1.6	0.94	1.16
Urea fertilizer sources (N)	3	69.62^{**}	72^{**}	6.23^{ns}	3.95^{ns}	142.44^{**}	128.56^{**}
S×N	3	0.74^{ns}	1.46^{ns}	0.04^{ns}	0.01^{ns}	1.03^{ns}	1.21^{ns}
B×N	9	6.33^{ns}	1.97^{ns}	1.35^{ns}	0.96^{ns}	5.69^{**}	3.7^{*}
S×B×N	9	0.34^{ns}	0.34^{ns}	0.04^{ns}	2.03^{ns}	0.66^{ns}	2.67^{ns}
Sub-error	48	3.25	1.84	2.45	2.08	1.28	1.76
Coefficient of variation (%)		6.8	5	14.4	12.4	6.4	8

ns, *, and ** were non-significant and significant at levels of 5% and 1%, respectively

Table 4. *Comparison of the effect of drought stress on some physiological traits of Calendula officinalis L. in two years of experimentation of 2015-2016 and 2016-2017*

Experimental treatments	Total phenol (mg/g dry extract)		Total flavonoid (mg/g dry extract)		Di-hydroxy Guanuzyn enzyme (nano mole per mg protein)	
	First year	Second year	First year	Second year	First year	Second year
Tension levels						
Lack of water stress	25.24 b	25.81 b	16.47 b	15.79 b	10.35 b	11.04 b
Irrigation cut in the budding stage	27.52 a	28.04 a	18.6 a	17.47 a	11.45 a	12.17 a

In each column, the same letters indicate that there is no significant difference between the mean of the treatments

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Table 5. *Comparison of the effect of biological fertilizer on some flowers in the two years of experimentation of 2015-2016 and 2016-2017*

Experimental treatments	Total phenol (mg/g fresh extract)		Biomarker Di-tyrosine (nano mole per mg of protein)	
	First year	Second year	First year	Second year
Bio-fertilizer				
Lack of use (control)	24.42 c	25.24 d	97.92 a	106.47 a
Azotobacter	25.54 bc	25.95 c	97.03 a	104.79 a
Azospirillum	26.48 b	26.89 b	84.52 a	90.31 b
Combination of Azotobacter and Azospirillum (Nitroxin)	29.1 a	29.63 a	87.9 a	90.3 b

In each column, the same letters indicate that there is no significant difference between the mean of the treatments

Table 6. *Comparison of the effects of urea fertilizer on some flowering traits in Calendula officinalis L in two years of experiment in 2015-2016 and 2016-2017*

Experimental treatments	Total phenol (mg/g dry extract)		Biomarker malondialdehyde (Nano mol per mg of protein)	
	First year	Second year	First year	Second year
Lack of use (control)	24 c	24.4 c	3.04 c	2.95 d
75% recommended urea fertilizer with sulphur coating	26.35 b	27.19 b	3.98 b	4.15 c
Recommended amount of urea fertilizer with sulphur coating	27.75 a	28.06 a	4.9 a	4.72 a
Recommended amount of common form of urea fertilizer	27.45 a	28.05 a	4.52 a	4.41 b

In each column, the same letters indicate that there is no significant difference between the mean of the treatments

Total flavonoid

In both years of experiment, the effect of drought stress (S), biological fertilizer (B) and urea fertilizer (N) sources on 1% was significant on Total Flavonoid. Also, in the first year of effect (B×N) at 1% level and in the second year at 5% level, this significant trait was significant (*Table 3*). Drought stress in both years of the experiment led to a significant increase in this trait. The total flavonoid in stress conditions increased 11.4% and 9.6%, respectively, in non-stress during the first and second years (*Table 4*).

Comparing the average interactions of biological fertilizers and urea, the results showed that in the first year, the highest total flavonoid in the treatment of Azotobacter and Azospirillum (Nitroxin) and the recommended amount of urea fertilizer (21.91 mg/g dry extract) and combined treatment Azotobacter and Azospirillum (Nitroxin) and application of recommended amount of urea fertilizer with sulphur coating (22.57 mg/g dry extract). In the second year under Nitroxin application, the use of all three sources of urea fertilizer resulted in an increase in total flavonoid (*Table 7*). Flavonoid antioxidants have a protective effect during dry stress. Many Flavonoids are active ingredients in medicinal plants and have medicinal properties. As active physiological compounds, stress-protecting agents and as absorbents play an important role in plant resistance (Tattini et al., 2004).

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***Table 7.** Comparison of the intermediate effects of biological fertilizers and different quantities of urea sources on the Calendula officinalis L. plant in two trial years of 2015-2016 and 2016-2017*

Experimental treatments		**Total Phenol (mg/g dry extract)**		**Glutathione Peroxidase enzyme (mg of protein per minute)**	
		First year	**Second year**	**First year**	**Second year**
Bio-fertilizer	Urea fertilizer sources				
Lack of use (control)	Lack of use (control)	11.85 i	12 h	21.69 fg	22.24 gh
Lack of use (control)	75% recommended urea fertilizer with sulphur coating	14.02 h	13.81 g	23.36 efg	23.28 gh
Lack of use (control)	Recommended amount of urea fertilizer with sulphur coating	17.77 efg	f16.14	24.22 defg	24.16 efg
Lack of use (control)	Recommended amount of common form of urea fertilizer	15.07 h	16.16 f	23.64 defg	24.06 fg
Azotobacter	Lack of use (control)	12.58 i	gh12.82	22.01 fg	22.58 gh
Azotobacter	75% recommended urea fertilizer with sulphur coating	16.83 fg	13.57 gh	24.1 defg	25.06 defg
Azotobacter	Recommended amount of urea fertilizer with sulphur coating	19.74 ba	17.19 ef	27.54 bcd	27.1 bf
Azotobacter	Recommended amount of common form of urea fertilizer	19.24 bcd	17.65 def	25.54 cdef	27.43 bcde
Azospirillum	Lack of use (control)	16.42 g	13.16 gh	21.81 fg	22.38 gh
Azospirillum	75% recommended urea fertilizer with sulphur coating	18.35 cde	17.43 ef	26.19 bcde	26.64 def
Azospirillum	Recommended amount of urea fertilizer with sulphur coating	20.29 b	18.79 cde	27.61 bcd	28.06 bcd
Azospirillum	Recommended amount of common form of urea fertilizer	18.16 def	19.26 bcd	26.46 bcde	26.9 cdef
The combination of Azotobacter and Azospirillum	Lack of use (control)	16.69 g	16.12 f	21.01 g	20.56 h
The combination of Azotobacter and Azospirillum	75% recommended urea fertilizer with sulphur coating	19.08 b-e	20.02 abc	29.6 b	30.08 bc
The combination of Azotobacter and Azospirillum	Recommended amount of urea fertilizer with sulphur coating	22.57 a	21.25 a	33.47 a	34.28 a
The combination of Azotobacter and Azospirillum	Recommended amount of common form of urea fertilizer	21.91 a	20.73 ab	28.99 bc	30.28 b

In each column, the same letters indicate that there is no significant difference between the mean of the treatments

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Di-hydroxy guanuzyn enzyme

In both years of experiment, the effect of drought stress (S) on the level of 1% was significant on the amount of Di-hydroxy Guanuzyn enzyme (*Table 3*). Drought stress in both years of the experiment led to a significant increase in this trait. The di-hydroxy Guanzine enzyme in stress conditions increased 10.6% and 10.2% in non-stress during the first and second years respectively (*Table 4*). Increasing the amount of di-hydroxy Guanuzyn increased production of superoxide radicals as a result of the occurrence of oxidative stress in the plant. Increasing of di-hydroxy Guanosine under drought stress indicates damage to plant synthetic structures and destruction of nucleic acids (Wenho and Russel, 2000). The results of Davoudi Fard et al. (2012) indicate a significant increase in di-hydroxy Guanuzyn due to drought stress.

Glutathione peroxidase enzyme

In both years of experiment, the effect of drought stress (S), biological fertilizer (B) and urea fertilizer (N) sources on Glutathione Peroxidase enzyme activity was significant at 1% level. Also in the first year of effect (B×N) at 5% level and in the second year at 1% level this trait was significant (*Table 8*). Drought stress in both years of the experiment led to a significant increase in this trait so that the activity of Glutathione Peroxidase enzyme in stress conditions increased 56.6% and 56.2%, respectively, compared to non-stress conditions in the first and second year (*Table 9*). Results showed that in both years, the effect of Glutathione Peroxidase in the treatment of Azotobacter and Azospirillum (Nitroxin) and application of recommended amount of urea fertilizer with sulphur content was highest (*Table 7*).

One of the mechanisms can increase the tolerance of the plant to stress, by activation of antioxidant systems. A large number of physiological disorders in plants are due to the increased production of active oxygen species due to environmental stresses such as drought (Sgherri and Navari-Lzzo, 1995; Zhang and Kirkham, 1994; Miller et al., 2010). Since one of the common effects of drought stress, like other environmental stresses, is primarily oxidative damage (Chen, 2000), plants to cope with oxidative stress and overcome these active species, antioxidant defense system with enzymatic and non-enzymatic mechanisms such as superoxide dismutase, catalaze..., along with a number of non-enzyme antioxidants such as Ascorbic acid, Glutathione, Alpha-tocopherol, and Cartenoids constitute the bulk of this defense system (Nasibi, 2005; Prasad, 2002). The results of Sedghi et al. (2012) indicate that antioxidant enzymes increase the activity of drought stress in an evergreen plant.

Enzyme activity of superoxide dismutase

The results of analysis of variance showed that in both years the effect of drought stress (S) on 1% level was significant on the activity of the enzyme superoxide dismutase (*Table 8*). Drought stress in both years of the experiment led to a significant increase in this trait. The level of enzyme activity of superoxide dismutase in stress conditions increased to 85.7% and 100.6% in the first and second year respectively (*Table 9*). Kawakami et al. (2010) reported that superoxide dismutase enzyme activity increased significantly under drought stress. In this regard, drought stress has been reported to increase the activity of superoxide dismutase in wheat (Tian and Lei, 2007) and rice (Sharma and Dubey, 2005). The results of Sedghi et al. (2012) indicate increased activity of the enzyme superoxide dismutase due to drought stress.

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Table 8. *Analysis of variance of the effect of biological fertilizers and different amounts of urea sources under dehydrating conditions of Calendula officinalis L plant during two years of experiment, 2015-2016 and 2016-2017*

Sources of changes	Degrees of freedom	Average of squares: Biomarker malondialdehyde, First year	Biomarker malondialdehyde, Second year	Glutathione peroxidase enzyme, First year	Glutathione peroxidase enzyme, Second year	Superoxide dismutase enzyme, First year	Superoxide dismutase enzyme, Second year	Biomarker di-tyrosine, First year	Biomarker di-tyrosine, Second year
Repeat	2	0.04 ns	0.2 ns	5.04**	15.02**	3.3**	3.33**	182.3**	92.05**
Drought stress (S)	1	34.62**	50.13**	3043.13**	3109.35**	3029.85**	3573.87**	2146.76**	2227.42**
Main error	2	0.04	0.39	0.19	1.43	0.11	0.11	10.06	8.89
Biological fertilizer (B)	3	118.05**	7.27**	106.22**	117**	8.51 ns	5.14 ns	1062.97**	1889.49**
S×B	3	1.15*	0.31 ns	1.18 ns	2.19 ns	0.62 ns	0.37 ns	69.25 ns	19.86 ns
Sub-error	12	0.26	0.24	18.94	14.91	4.47	4.22	216.86	99.13
Urea fertilizer sources (N)	3	15.49**	14.37**	182.72 ns	189.35**	14.07 ns	9.64 ns	105.15 ns	229.81 ns
S×N	3	0.58 ns	0.6**	10.9 ns	9.4 ns	0.31 ns	0.96 ns	0.97 ns	1.11 ns
B×N	9	0.74 ns	0.38**	20 ns	28.71**	2.82 ns	2.47 ns	185.06 ns	164.25 ns
S×B×N	9	0.27 ns	0.1 ns	1.48 ns	2.53 ns	0.9 ns	0.42 ns	3.79 ns	2.5 ns
Sub-error	48	0.47	0.15	8.79	6.35	5.7	5.52	173.84	152.96
Coefficient of variation (%)		16.6	9.4	11.6	9.7	12.8	12.9	14.4	12.6

ns, *, and ** were non-significant and significant at levels of 5% and 1%

Table 9. *Comparison of the effect of drought stress on some physiological traits of Calendula officinalis L in two years of experimentation 2015- 2016 and 2016-2017*

Experimental treatments	Glutathione peroxidase enzyme		Superoxide dismutase enzyme		Biomarker di-tyrosine (nano mole per mg of protein)	
	First year	Second year	First year	Second year	First year	Second year
Stress levels						
Lack of water stress	19.82 b	20.25 b	13.11 b	12.14 b	87.11 b	93.15 b
Irrigation cut in the budding stage	31.08 a	31.63 a	24.35 a	24.35 a	96.57 a	102.79 a

In each column, the same letters indicate that there is no significant difference between the mean of the treatments

Biomarker malondialdehyde

In both years of experiment, the effect of drought stress (S), biological fertilizer (B) and urea (N) fertilizer sources on 1% level was significant on malondialdehyde

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biomarker. Also in the first year of interaction (B×N) at 5% level and in the second year of effect (B×N) and (S×N) at 1% level, this significant trait was significant (*Table 8*). Application of recommended amount of urea fertilizer with sulphur coating in both years of experiment resulted in significant increase of this trait. So, the amount of biomarker of carotene Dialledium in application of recommended amount of urea fertilizer with sulphur coating increased 61.2% and 60%, respectively, compared to control treatment in the first and second year. In the first year, there was no significant difference between the application of the recommended amount of urea fertilizer with sulphur coating and the application of the recommended amount of urea fertilizer in this regard (*Table 6*).

Comparison of the mean interactions of drought stress and biological fertilizer showed that in the first year of experiment, the highest trait in terms of stress and the combination of Azotobacter and Azospirillium (11.99 Nmol/mg protein) and stress and application of Azospirillum (59.11 nmol/mg^{-1} protein) was obtained (*Fig. 1*). Comparing the mean interactions between drought stress and urea fertilizer sources, the highest biomarker of calcium diphtheria was obtained in the second year under stress conditions and application of recommended amount of urea fertilizer with sulphur coating (5.43 nmol/mg protein) and the lowest in terms non-stress and non-use of urea fertilizers were obtained (*Fig. 2*). Results showed that in the second year, the combination of Azotobacter and Azospirillum (Nitroxin) with three sources of urea fertilizer resulted in a significant increase in this trait (*Fig. 3*). Many plants, when placed in a dry environment, are injured seriously and increase their malondialdehyde content (Grmaetxe, 1998; Saneoka, 2004).

There are reports of reduced malondialdehyde due to the maintenance of membrane lipids from damage induced by free oxygen radicals (Ozdamir et al., 2004). The results of Davoudi Fard et al. (2012), (Pan et al., 2006), (Zhang et al., 2006), (Habibi et al., 2009) and (Li Wen et al., 2006) indicate a significant increase in malondialdehyde due to drought stress.

Biomarker di-tyrosine

In both years of experiment, the effect of drought stress (S) and biological fertilizer (B) on the amount of di-tyrosine biomarker was significant at 1% level (*Table 8*). Drought stress in both years of the experiment led to a significant increase in this trait. So, the amount of biomarker di-tyrosine in stress conditions increased 10.9 and 15.7% in non-stress during the first and second years respectively (*Table 9*).

The application of Nitroxin biological fertilizer in both years led to a significant reduction in the amount of di-tyrosine biomarker. The highest amount of this trait was obtained every two years in non-use of biological fertilizers. In the second year, there was no significant difference between the absence of Bio-fertilizer application and Azotobacter application (*Table 5*). The results of Davoudi Fard et al. (2012) indicate a significant increase in di-prozine due to drought stress. In this study, the use of Bio-fertilizer resulted in the reduction of this trait.

This is consistent with the findings of Davoudi Fard et al. (2012). This conclusion is likely to indicate that bacteria reduce the effects of drought stress through another defense mechanism, which has been prevented from rising levels of Di-tyrosine and antioxidant enzymes. Because antioxidant enzymes activity is reported to be the only defense mechanism against free radical oxidants produced under stress conditions increasing proline can also reduce the free radicals produced under drought stress

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conditions (Aktas et al., 2007). As a result, due to the reduction of the production of free radicals, the amount of protein degradation and, consequently, the production of biomarker of di-tyrosine is reduced (Jose et al., 1999).

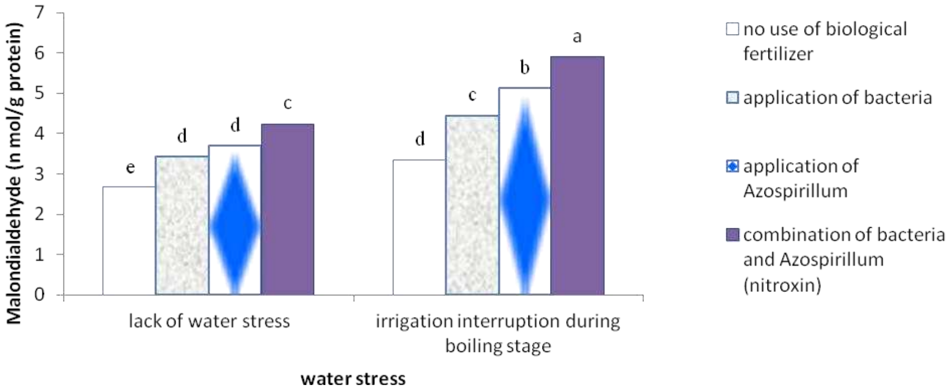

***Figure 1.** Effect of drought stress and biological fertilizer on malondialdehyde biomarker in the first year*

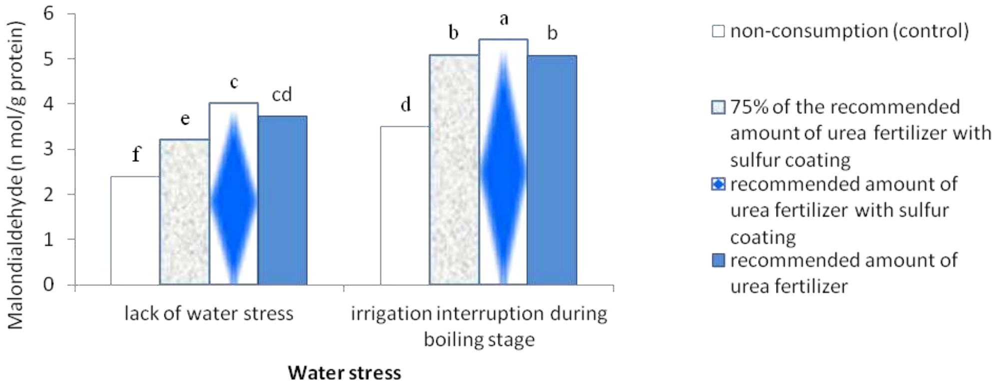

***Figure 2.** Effect of drought stress and biological fertilizer on malondialdehyde biomarker in the second year*

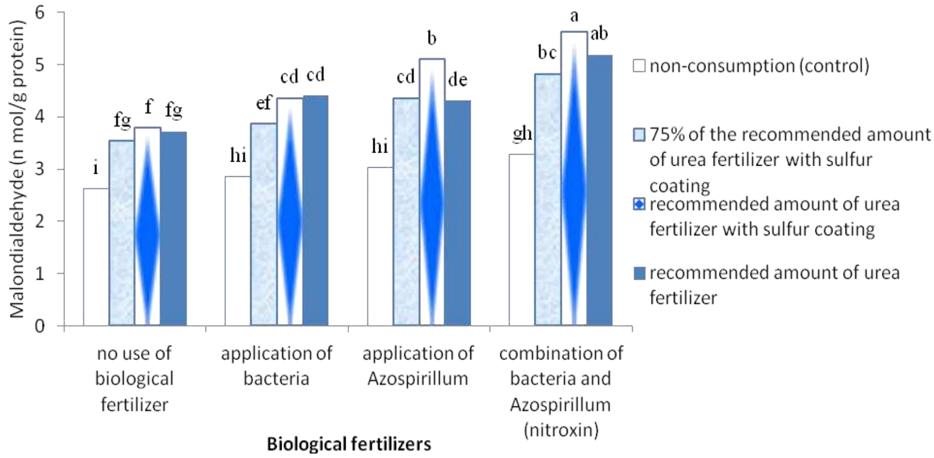

***Figure 3.** Effect of biological fertilizer and urea fertilizer source on malondialdehyde biomarker in the second year*

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Overall conclusion

The results showed that irrigation cut in the boiling stage led to increases in the enzyme di-hydroxy Guanuzyn, glutathione peroxidase, superoxide dismutase, total phenol, total flavonoid and biomarker di-tyrosine. In general, the use of Nitroxin led to an increase in all of these traits except di-tyrosine.

The application of the recommended amount of urea fertilizer with sulphur coating resulted in an increase in total phenol and biomarker malonaldehyde. There was no significant difference between in malonaldehyde, application of recommended amount of urea fertilizer with sulphur coating and the usual form of recommended urea fertilizer. Whilst, results showed that in both years, the effect of Glutathione Peroxidase in the treatment of Azotobacter and Azospirillum (Nitroxin) and application of recommended amount of urea fertilizer with sulphur content was highest. Also, application of recommended amount of urea fertilizer with sulphur coating and recommended amount of urea fertilizer in both years of experiment resulted in significant increase of Total Phenol. In this study, drought stress and application of Nitroxin fertilizer resulted in increased malondialdehyde in the first year. In the second year, malondialdehyde was affected by the interaction of drought stress and urea fertilizer resources. Drought stress and application of recommended amount of urea fertilizer with sulphur coating resulted in increased malondialdehyde. In both years, the application of Nitroxin with the recommended amount of urea fertilizer with sulphur coating resulted in an increase in total flavonoid and glutathione peroxidase enzymes. Comparing the average interactions of biological fertilizers and urea, the results showed that in the first year, the highest total flavonoid in the treatment of Azotobacter and Azospirillum (Nitroxin) and the recommended amount of urea fertilizer and combined treatment Azotobacter and Azospirillum (Nitroxin) and application of recommended amount of urea fertilizer with sulphur coating. In the second year under Nitroxin application, the use of all three sources of urea fertilizer resulted in an increase in Total Flavonoid. There was no significant difference between the recommended amount of urea fertilizer with sulphur coating and urea fertilizer with sulphur coating for Total Flavonoids. Future debate should promote the promotion of information and culture in the field of medicinal plants, as well as the expansion of international cooperation in medicinal plant science and technology, the protection of monolithic plant species, planning for the development of cultivation and domestication of species Important medicinal plants with economical value, the transfer and absorption of the advanced sciences and technologies of other countries in the field of medicinal plants.

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PHYSICOCHEMICAL PROPERTIES AND DISTRIBUTION OF NUTRIENTS ON THE INNER CONTINENTAL SHELF ADJACENT TO THE GULF OF MARANHÃO (BRAZIL) IN THE EQUATORIAL ATLANTIC

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Abstract. The dynamics of the physical and chemical factors that regulate oceanographic processes on the continental shelf off the state of Maranhão (northeastern Brazil) was evaluated using a transect along São Marcos Bay (01°41'S-02°28'S and 43°47'W-44°13'W) in January, March, May, July and September 2014, with a total of seven sampling stations. Water samples were collected from the surface using a Van Dorn water sampler. The following hydrochemical variables were analyzed: water transparency (m), temperature (°C), salinity, conductivity (mS cm^{-1}), total dissolved solids (TDS, g L^{-1}), pH, dissolved oxygen (mg L^{-1}), turbidity (NTU), total suspended solids (TSS) and dissolved nutrients (phosphate, nitrite and silicate). The relationship between these variables and seasonality in the region [rainy season (January to June) and dry season (July to December)] were also evaluated. For data with normality and equal variances, a one-way analysis of variance (ANOVA) was used for the spatial and temporal comparisons of the physicochemical variables. Results showed that the spatial and temporal variability of the physicochemical variables analyzed in this study is associated with local dynamics governed by river discharge, tidal movements, currents and meteorological events. The fluvial transport from the rivers of the state of Maranhão and other freshwater sources in the Amazon region are apparently the major contributors responsible for the maintenance of nutrient availability on the Maranhão continental shelf.

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The present paper aims to broaden the knowledge of the spatial and temporal variability in the physicochemical variables in continental shelves and adjacent waters the coast of Maranhão (North Brazil).
Keywords: *surface water masses, hydrochemical variables, spatial distribution, dissolved nutrients, biogeochemical activity, influence of the discharge freshwater*

Introduction

The continental shelf is an environment with strong biogeochemical activity due to matter transported from land combined with water-sediment interactions and processes of biological absorption, respiration and re-mineralization. Coastal waters receive sediments and discharge from freshwater and underground sources and are therefore more affected by human activities than the open ocean (Lefèvre et al., 2017).

Physicochemical variables are often used to characterize masses of water along continental shelves and adjacent coastal waters and are also important to the determination of biogeochemical processes in response to land and oceanic inputs (Braga et al., 2008). The dynamics of the physical and chemical factors that regulate oceanographic processes on the continental shelf off the state of Maranhão (northeastern Brazil) are not yet fully understood, especially those related to small-scale and medium-scale events governed by freshwater discharge, wave energy, trade winds, macro-tide patterns and the Intertropical Convergence Zone (ITCZ).

The seasonal cycle of the migration of the ITCZ in the tropical Atlantic reflects the behavior and position of surface currents (Stramma and Scott, 1999). The mesoscale circulation in the region of the continental shelf off the state of Maranhão is strongly influenced by the North Brazil Current, directly affecting the spread of the plume of the Amazon River (Nikiema et al., 2007). This cyclic phenomenon alters physicochemical properties and the concentration of nutrients in coastal waters of the São Marcos Estuarine Complex and controls the distribution and abundance of migrant fishing resources in the Gulf of Maranhão.

It is presumed that freshwater discharge from the São Marcos Estuarine Complex serves as an obstacle to the advance of lower temperature marine waters, while also exerting a strong influence on the input and redistribution of nutrients on the inner continental shelf. Thus, the determination of these variables would enable an understanding of the variability in the structure of surface water masses and contribute knowledge on the complex hydrodynamics of the Gulf of Maranhão.

The present paper aim to broaden the knowledge of the spatial and temporal variability in the physicochemical variables in continental shelf and adjacent waters the coast of Maranhão (North Brazil), associated with local dynamics governed by river discharge, tidal movements, currents and weather events.

Material and methods

Study area

The coastal region of the state of Maranhão is classified as being on the northern coast of Brazil, based mainly on the morphology of the coast, climate, oceanographic variables, sediment coverage and width of the continental shelf. This stretch of the coast has been subdivided into three sectors form corresponding to the coast of Maranhão on the eastern Amazonian coast, with a large number of small estuaries bordered by hills

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formed by tertiary sediments of the Barreiras Formation, currently in complete regression (El Robrini et al., 2006). Mangroves occur in protected areas, accentuating the irregularities of the coastline and generating broad tidal plains (Mochel and Ponzoni, 2007).

The coast of Maranhão extends 640 km, with a wide continental shelf and relatively shallow waters under the influence of the discharge of a large number of rivers. The physical characteristics of the coastline enable a division into two distinct parts. The first extends from the border of the state of Pará to Tubarão Bay and is characterized by a low coast of mangroves and deep indentations forming a series of bays and estuaries (denominated the *Reentrâncias Maranhenses*). The second area extends from Tubarão Bay eastward to the mouth of the Parnaíba River. In this area, the coastline is regular and part is covered by a vast area of sand dunes, denominated the *Lençóis Maranhenses*.

The Gulf of Maranhão is located in the center of the coast and is where São Luís Island is located, which separates the gulf into two large bays. São Marcos Bay lies to the west, which is an active estuary where the Mearim and Pindaré Rivers converge. The most intense tides are recorded in the mouth of this bay. São José Bay to the east of the island is a region with shallow depths that receives waters from the Itapecuru and Munim rivers (Silva et al., 2018). Off the Gulf of Maranhão, the continental shelf has a mean width of 150 km and depths less than 45 m, reaching a depth of 75 m near the break of the shelf and dropping abruptly to 2.000 m at the base of the continental slope (Silva and Alvarenga, 1994).

The Maranhão continental shelf can be classified as a high-energy region due to the combined effects of the coastal currents generated by different hydrodynamic forces, such as tides, waves, trade winds and the discharge of the Itapecuru/Munim rivers and Pindaré/Mearim rivers that respectively form the São José Estuarine Complex and São Marcos Estuarine Complex. The volume transported from the Pindaré/Mearim Rivers to the São Marcos Estuarine Complex corresponds to 10 km^3 per year (Jennerjahn et al., 2010), with maximum discharge occurring in March/April, which is the peak of the rainy season.

Data collection

Data were obtained from a transect determined in São Marcos Bay (01°41’S-02°28’S and 43°47’W-44°13’W) in January, March, May, July and September 2014, involving seven sampling stations (*Fig. 1*). Station 1 was located near the city of São Luís and Station 7 was located on the continental shelf before the 50-meter isobath at approximately 100 km from Station 1.

Water samples were collected from the surface using a van Dorn hydrographic bottle. The following hydrochemical variables were determined in the field: water transparency, measured by the depth of the disappearance of the Secchi disk (m); temperature (°C), salinity (g kg^{-1}), conductivity (mS cm^{-1}), total dissolved solids (TDS, g L^{-1}) and pH, using a HANNA HI-9828 multiparameter probe; dissolved oxygen (mg L^{-1}), using a HANNA HI-9146 oximeter together with the Winkler analytical method, as cited in Strickland and Parsons (1972); and turbidity (NTU), using a Tecnopon TB1000® turbidity meter. For the determination of total suspended solids (TSS), water samples were kept refrigerated at -4 °C until filtered and analyzed in the laboratory. TSS (mg L^{-1}) was measured using the gravimetric method, as described in APHA

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(2001). Dissolved nutrients (phosphate, nitrite and silicate) were determined using the method described by Grasshoff et al. (1999).

Climatologic data were acquired from the databank available by INMET (Brazilian Meteorological Institute) recorded by the São Luis meteorological station.

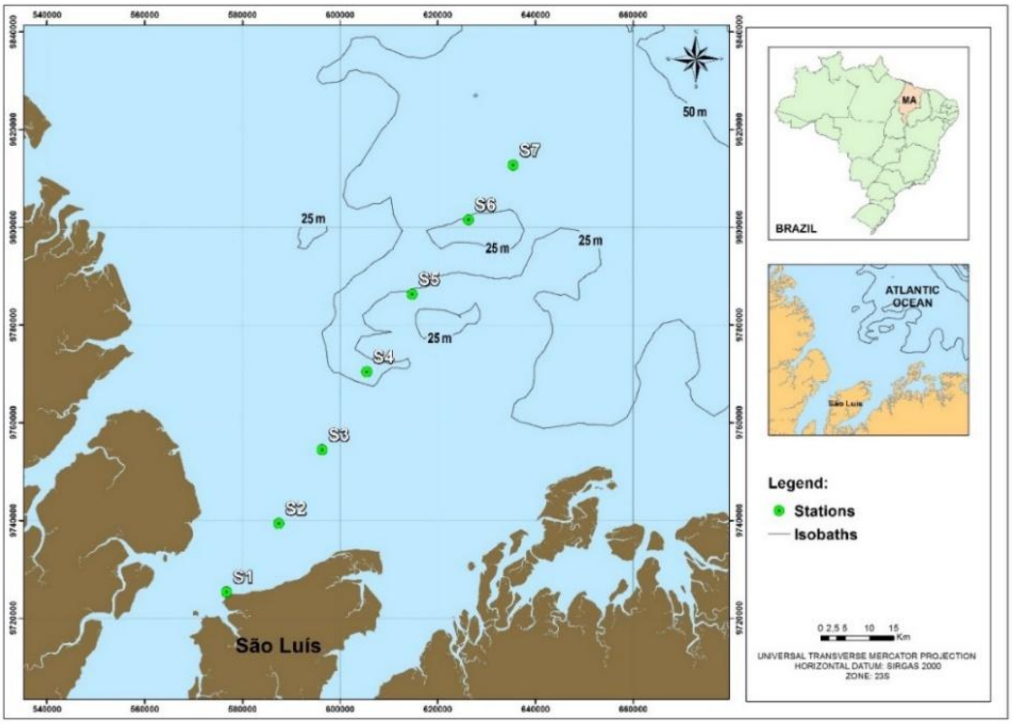

Figure 1. *Location of sampling stations along transect on continental shelf off state of Maranhão*

Data analysis

The physical and chemical variables were expressed as mean, standard deviation, minimum and maximum values determined for each sampling period. The relationship between these variables and seasonality in the region [rainy season (January to June) and dry season (July to December)] were also evaluated. The Shapiro-Wilk test and Levene's test were used to determine the normality of the data distribution and equal variance, respectively. For data with equal variances, one-way analysis of variance (ANOVA) was used for the spatial and temporal comparisons of the physicochemical variables. In cases for which the null hypothesis of variance was rejected, a multi-comparison test (Tukey's test) was used to identify which pairs of means differed significantly. For data with unequal variances, the spatiotemporal comparisons were made using the Kruskal-Wallis (H) test, followed by the Mann-Whitney U test when the null hypothesis was rejected. Principal component analysis was used to identify the main components responsible for the variations in the data on the Maranhão continental shelf, using the correlation matrix as the basis. The significance of Pearson's correlation table was calculated using a two-tailed *t*-test with two degrees of freedom. Excel 2010 and PAST 3.14 were used for the data analysis as well as the creation of the graphs and tables (Hammer et al., 2001). The statistical analyses were evaluated for a critical significance level of $\alpha = 0.05$ (Zar, 2010).

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Results

Hydrological conditions

The accumulated monthly rainfall for 2014 at Station 1 showed values always higher than 100 mm in the months of January to June (*Fig. 2*). In the oceanic region, the precipitation followed the same seasonal cycle compared to the coastal region, with usually slightly lower accumulated rainfall values. The INMET data gave higher rainfall during the wet season. The wet season was associated with the presence of the ITCZ located at its southernmost position in March-April.

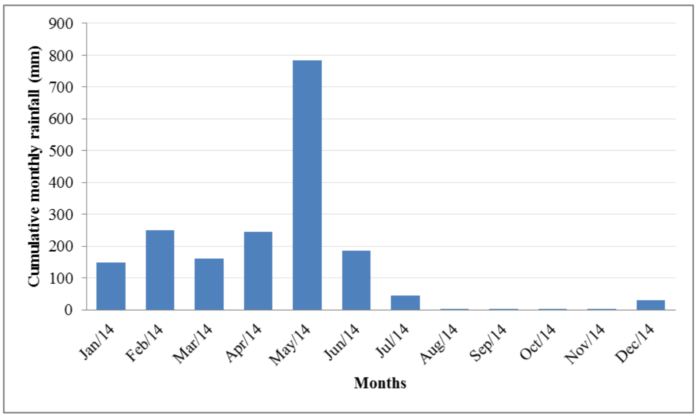

Figure 2. *Accumulated monthly rainfall for 2014 at Station 1 (São Luís, Maranhão). (Source: BDMEP, INMET)*

Abiotic variables

Table 1 displays the mean, standard deviation, maximum and minimum values for the physical and chemical variables of the surface waters measured on the Maranhão continental shelf. Water surface temperature ranged from 27.37 °C to 30.33 °C, corresponding to the wet and dry seasons, respectively (*Fig. 3a*). The mean for all months analyzed was 28.7 °C, which reflects the characteristic of the equatorial region, with small horizontal variations, mean amplitude of approximately 1 °C in the rainy season and 2 °C in the dry season. In statistical terms, temperatures were significantly lower in September 2014 ($H = 14.59$; $p < 0.0001$), but with no significant differences among the different sampling stations ($F = 1.61$; $p > 0.05$).

The spatial distribution of salinity (*Fig. 3b*) ranged from 25.95 g kg^{-1} to 38.08 g kg^{-1} throughout the study. The means per station were 30.98, 32.11, 34.64, 35.39, 35.91, 36.28 and 36.14 g kg^{-1} for S1, S2, S3, S4, S5, S6 and S7, respectively.

A progressive increase in salinity was found with the increase in distance from the coast, with significantly higher values at Stations 5, 6 and 7 in comparison to Station 1 ($F = 4.22$; $p < 0.05$. Mean salinity was 35.04 g kg^{-1} in the rainy season and 33.68 g kg^{-1} in the dry season, with significantly higher values in January 2014 ($Hc = 16.33$; $p < 0.0001$). In May 2014, the lowest salinity was found at Station 1 (25.95 g kg^{-1}), which contributed to the mean value in the rainy season (32.2 g kg^{-1}) and coincides with the period of greater rainfall intensity in the region, with an accumulated rainfall of 784.3 mm in May 2014. The highest mean salinity was found in January 2014 (36.72 g kg^{-1}), which was likely due to the low rainfall in the final trimester of the previous year.

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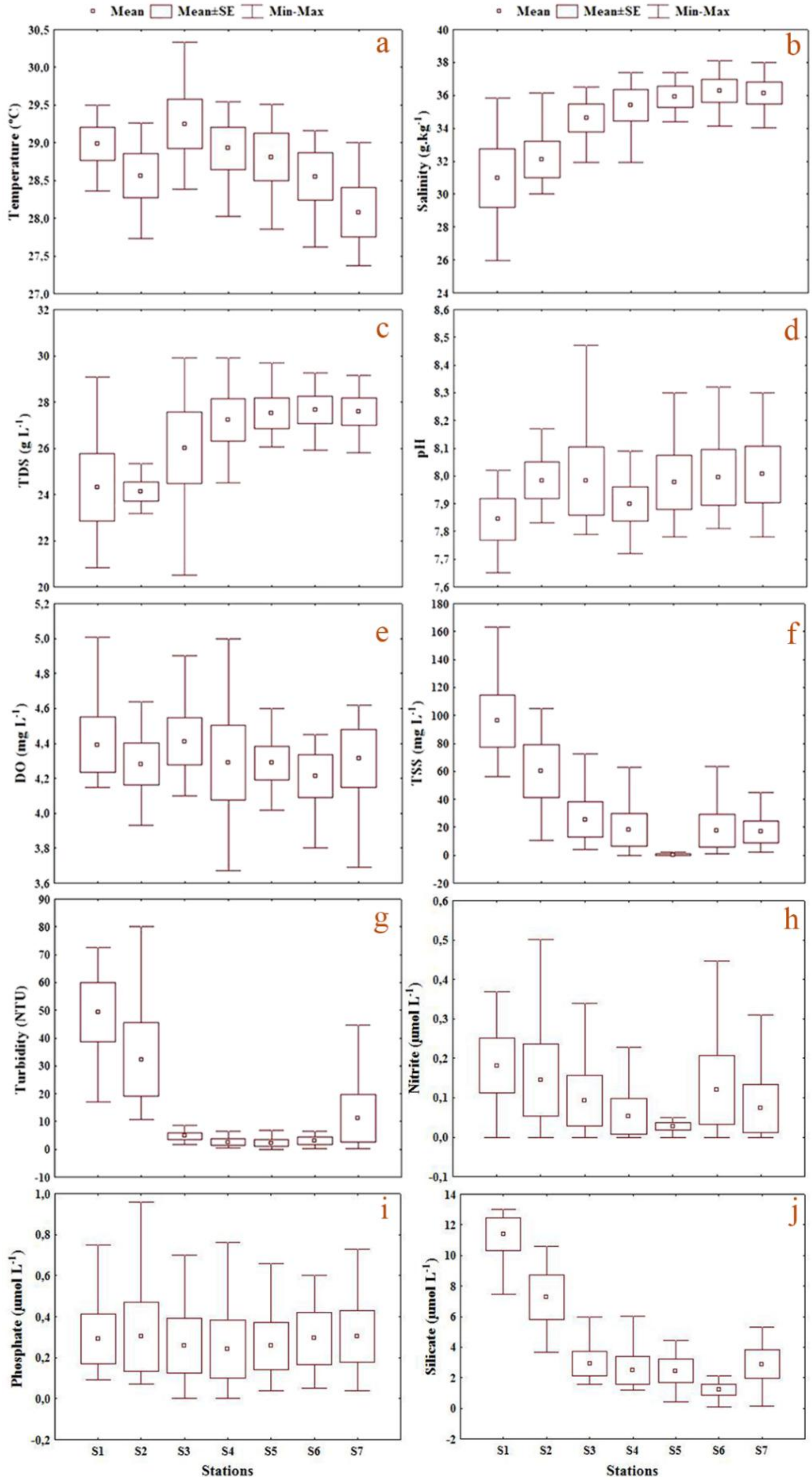

Figure 3. *Spatial distribution of physical and chemical variables of the surface waters along Maranhão continental shelf in each sampling station. a – temperature, b - salinity, c - TDS, d - pH, e - DO, f - TSS, g - turbidity, h - nitrite, i - phosphate, j - silicate*

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***Table 1.** Mean, standard deviation, maximum and minimum values of physical and chemical variables collected on Maranhão continental shelf throughout study period*

Period	Descriptive statistic	Temp (°C)	Sal (g kg^{-1})	TDS (g L^{-1})	Cond (mS cm^{-1})	pH	DO (mg L^{-1})	Secchi (m)	Turb (NTU)	TSS (mg L^{-1})	SiO_2 (µmol L^{-1})	PO_4^{3-} (µmol L^{-1})	NO_{2-} (µmol L^{-1})
Jan/14	Mean	28.84	36.72	28.82	57.40	8.11	4.12	4.26	17.80	21.43	3.75	0.05	0.05
	Deviation	0.72	0.61	1.83	4.20	0.19	0.12	3.50	24.45	31.78	4.06	0.02	0.05
	Maximum	29.50	37.39	29.90	59.80	8.32	4.30	10.37	72.50	91.00	12.78	0.09	0.15
	Minimum	27.73	35.84	24.75	48.01	7.92	3.93	0.13	6.05	1.00	1.51	0.03	0.00
Mar/14	Mean	28.90	36.20	27.48	54.62	7.81	4.16	5.07	19.57	29.14	4.73	0.11	0.07
	Deviation	0.36	2.22	1.57	3.57	0.07	0.21	3.52	33.13	45.77	4.98	0.03	0.10
	Maximum	29.50	38.08	28.68	57.36	7.87	4.27	8.20	80.00	100.00	12.99	0.15	0.27
	Minimum	28.57	32.78	25.35	48.37	7.72	3.69	0.26	0.13	0.00	0.92	0.07	0.00
May/14	Mean	29.28	32.19	24.19	48.56	8.11	4.51	7.57	9.16	19.53	5.76	0.52	0.06
	Deviation	0.20	3.44	2.77	5.55	0.24	0.39	5.48	11.00	27.09	3.41	0.28	0.09
	Maximum	29.54	35.60	27.03	53.84	8.47	5.00	15.26	33.00	70.00	12.85	0.76	0.23
	Minimum	29.00	25.95	20.51	40.61	7.65	3.80	0.41	2.40	0.00	2.14	0.09	0.00
Jul/14	Mean	28.66	33.75	25.74	51.49	7.89	4.54	6.31	19.53	65.17	4.77	0.16	0.32
	Deviation	0.95	3.30	2.25	4.50	0.15	0.24	4.94	27.09	51.34	3.70	0.13	0.15
	Maximum	30.33	36.26	27.42	54.85	8.17	5.01	12.00	70.00	163.00	10.84	0.33	0.50
	Minimum	27.37	28.14	21.90	43.80	7.78	4.30	0.22	0.00	0.00	1.43	0.00	0.05
Sep/14	Mean	28.00	33.61	25.51	51.02	7.86	4.24	3.46	9.27	32.23	2.87	0.55	0.00
	Deviation	0.40	1.43	0.95	1.90	0.03	0.28	2.86	15.43	36.93	3.79	0.23	0.00
	Maximum	28.38	34.98	26.35	52.70	7.89	4.49	7.70	41.50	104.81	9.18	0.96	0.00
	Minimum	27.37	31.48	24.08	48.17	7.82	3.67	0.14	0.15	0.00	0.08	0.35	0.00

On the Maranhão continental shelf, total dissolved solids (TDS) ranged from 20.51 g L^{-1} to 29.90 g L^{-1}, with means of 25.62 g L^{-1} and 26.83 g L^{-1} in the dry and rainy seasons, respectively (*Fig. 3c*). In statistical terms, TDS demonstrated similar patterns to salinity, with higher concentrations in January 2014 ($F = 6.05$; $p < 0.00$). The value at Station 2 was significantly lower in comparison to the values found at Stations 4, 5, 6 and 7 ($F = 2.54$; $p < 0.05$).

The pH values remained basic, ranging from 7.65 to 8.47 throughout the study, with a mean of 8.01 in the rainy season and 7.88 in the dry season (*Fig. 3d*). Values were higher in January 2104 compared to March and September 2014 ($F = 5.97$; $p < 0.0001$).

Dissolved oxygen (DO) concentrations ranged from 3.67 mg L^{-1} to 5.01 mg L^{-1}, with a mean of 4.26 mg L^{-1} in the rainy season and 4.39 mg L^{-1} in the dry season (*Fig. 3e*). The seasonal and spatial distribution of DO demonstrated similar concentrations, indicating a homogeneous pattern for this variable throughout the Maranhão continental shelf.

Spatial distribution of total suspended solids (TSS) concentrations ranged from 0.40 mg L^{-1} to 163.00 mg L^{-1}, with mean concentrations from 23.37 mg L^{-1} in the rainy season to 48.70 mg L^{-1} in the dry seasons, with no statistically significant difference (*Fig. 3f*). Higher concentrations of TSS were found near the coast, with a progressive reduction toward oceanic regions, especially during the campaigns performed in the dry season. The highest concentration was 163.00 mg L^{-1} at Station 1, which differed significantly from all other stations ($H = 21.02$; $p < 0.0001$), except Station 2. The finding suggests the influence of inputs from the mainland combined with tide dynamics.

Turbidity ranged from 0.13 NTU to 80.00 NTU, with a mean of 15.51 NTU in the rainy season and 14.40 NTU in the dry season, demonstrating no significant seasonal

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difference (*Fig. 3g*). A spatial gradient was found during all sampling campaigns, with greater turbidity near the coast (Stations 1 and 2) and a progressive reduction toward the oceanic region to values close to 0 ($H = 20.4$; $p < 0.0001$). Such a pattern was expected due to the influence of total suspended solids and organic matter due to river discharge and the influence of the estuarine plume.

The spatial distribution of nitrite in the surface waters of the Maranhão continental shelf showed a maximum of 0.50 μmol L^{-1}. The highest values were found in July 2014 ($H = 17.6$; $p < 0.0001$), probably due to the advance of the estuarine plume toward the continental shelf. This is corroborated by the increase in organic and inorganic mater demonstrated by the variations in TSS and turbidity, which were higher at the end of the rainy season. Nitrite concentrations were lower than 1 μmol L^{-1} at all sampling stations (*Fig. 3h*).

Phosphorus concentrations ranged from 0.03 μmol L^{-1} to 0.96 μmol L^{-1}, with a mean of 0.23 μmol L^{-1} in the rainy season and 0.35 μmol L^{-1} in the dry season (*Fig. 3i*). Small variations in phosphate concentration were found during the sampling campaigns, with significantly higher concentrations in May 2014 (0.52 ± 0.28 μmol L^{-1}) and September 2014 (0.55 ± 0.23 μmol L^{-1}) ($H = 22.5$; $p < 0.0001$), likely due to the contributions of river runoff and rainfall. In spatial terms, no significant differences were found throughout the sampling stations along the Maranhão continental shelf.

Silicate concentrations ranged from 0.08 μmol L^{-1} to 12.99 μmol L^{-1}, with a mean of 4.75 μmol L^{-1} in the rainy season and 3.82 μmol L^{-1} in the dry season (*Fig. 3j*). Although this transport is evidenced on the Maranhão continental shelf by the increase in rainfall, however no statistically significant difference was found ($F = 0.52$; $p = 0.72$), demonstrating a lack of seasonal heterogeneity with regard to the concentration of silicate. On the spatial scale, however, sampling stations closer to the coast (Stations 1 and 2) had higher silicate concentrations throughout all campaigns ($F = 14.83$; $p < 0.0001$), which was likely due to the strong influence of rainfall and river discharge. Data from the Analysis of Variance (*Table 2*) show the significance of the physicochemical variables in relation to the months and sampling sites.

In the principal component analysis of the environmental variables, the first two axes explained 58.3% of the variability in the data (*Fig. 4*). The significance of the axes was tested using the "broken stick" random model with 9999 bootstrap replicates (Jackson, 1993), which indicated that Components 1 and 2 were sufficient to represent the factorial variance.

Component 1 (41.1%) was positively related to turbidity, total suspended solids, silicate, dissolved oxygen and phosphate, with a tendency toward higher values in the rainy months (January, March and May) as well as at sampling sites closer to the coast, especially Stations 1 and 2. Although July and September are considered to be part of the dry season, both months apparently demonstrated the influence of adjacent drainage stemming from rainfall in the previous months, leading to the maintenance of high turbidity, suspended solids, DO, silicate and nitrite.

Component 1 was negatively correlated with TDS, conductivity, salinity and transparency, with an absence of a pattern in seasonal terms and an expected increase in values at sampling sites more distant from the coast (Stations 3, 4, 5, 6 and 7), which are characterized by greater transparency and dissolved minerals.

Component 2 (17.2%) was negatively correlated with phosphate (PO_4^{3-}) and pH. Phosphate was associated more with May and September. Moreover, the similar distribution throughout the sampling stations suggests the absorption of this nutrient,

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making its concentration quite homogeneous throughout the Maranhão continental shelf. pH was associated more with May, when the highest values were found, as well as sampling sites more distant from the coast (Stations 5, 6 and 7), which is an expected pattern due to the alkaline tendency of oceanic water.

Table 2. *Results of ANOVA the physicochemical variables in the spatial temporal scale. The equal letters represent statistical similarity at the level of 5% of significance, considering each one of the parameters in function of the months and the sampling stations*

Variables	Months of sampling					Sampling stations						
	Jan/14	Mar/14	May/14	Jul/14	Sep/14	S1	S2	S3	S4	S5	S6	S7
Temp (°C)	$28,84^{a}$	$28,9^{a}$	$29,28^{a}$	$28,67^{ab}$	28^{b}	$28,98^{a}$	$28,56^{a}$	$29,25^{a}$	$28,93^{a}$	$28,81^{a}$	$28,55^{a}$	$28,08^{a}$
Sal (g kg-^{1})	$36,72^{a}$	$36,19^{ac}$	$32,19^{b}$	$33,75^{bc}$	$33,61^{bc}$	$30,98^{a}$	$32,11^{ab}$	$34,64^{ab}$	$35,39^{ab}$	$35,91^{b}$	$36,28^{b}$	$36,14^{b}$
TDS (g L^{-1})	$28,81^{a}$	$27,48^{ac}$	$24,19^{b}$	$25,73^{bc}$	$25,51^{bc}$	$24,32^{ac}$	$24,13^{a}$	$25,61^{ac}$	$27,23^{bc}$	$27,51^{bc}$	$27,66^{bc}$	$27,59^{bc}$
pH	$8,11^{a}$	$7,81^{b}$	$8,11^{a}$	$7,89^{ab}$	$7,86^{b}$	$7,84^{a}$	$7,98^{a}$	$7,98^{a}$	$7,89^{a}$	$7,98^{a}$	$7,99^{a}$	$8,01^{a}$
OD (mg L^{-1})	$4,11^{ac}$	$4,15^{c}$	$4,51^{c}$	$4,54^{b}$	$4,24^{c}$	$4,39^{a}$	$4,28^{a}$	$4,41^{a}$	$4,29^{a}$	$4,29^{a}$	$4,21^{a}$	$4,31^{a}$
Secchi (m)	$4,26^{a}$	$5,07^{a}$	$7,57^{a}$	$6,31^{a}$	$3,46^{a}$	$0,23^{a}$	$0,704^{b}$	$3,988^{c}$	$5,728^{cd}$	$7,428^{cde}$	$8,56^{de}$	$10,71^{e}$
Turb (NTU)	$17,8^{a}$	$19,56^{a}$	$9,16^{a}$	$19,53^{a}$	$9,27^{a}$	$49,22^{a}$	$32,36^{a}$	$4,76^{b}$	$2,59^{b}$	$2,23^{b}$	$3,16^{b}$	$11,12^{b}$
TSS (mg L^{-1})	$21,43^{a}$	$29,14^{a}$	$19,53^{a}$	$65,17^{a}$	$32,23^{a}$	$96,02^{a}$	$60,14^{b}$	$25,54^{b}$	$18,18^{b}$	$0,40^{c}$	$17,47^{b}$	$16,75^{b}$
SiO_2 (µmol L^{-1})	$3,75^{a}$	$4,73^{a}$	$5,76^{a}$	$4,77^{a}$	$2,87^{a}$	$11,38^{a}$	$7,28^{a}$	$2,91^{b}$	$2,51^{b}$	$2,45^{b}$	$1,22^{b}$	$2,89^{b}$
PO_4^{3-} (µmol L^{-1})	$0,05^{a}$	$0,11^{a}$	$0,52^{b}$	$0,15^{a}$	$0,55^{b}$	$0,29^{a}$	$0,30^{a}$	$0,26^{a}$	$0,24^{a}$	$0,26^{a}$	$0,29^{a}$	$0,31^{a}$
NO_{2}- (µmol L^{-1})	$0,05^{a}$	$0,07^{a}$	$0,06^{a}$	$0,32^{b}$	$0,00^{a}$	$0,18^{a}$	$0,14^{a}$	$0,09^{a}$	$0,05^{a}$	$0,03^{a}$	$0,12^{a}$	$0,07^{a}$

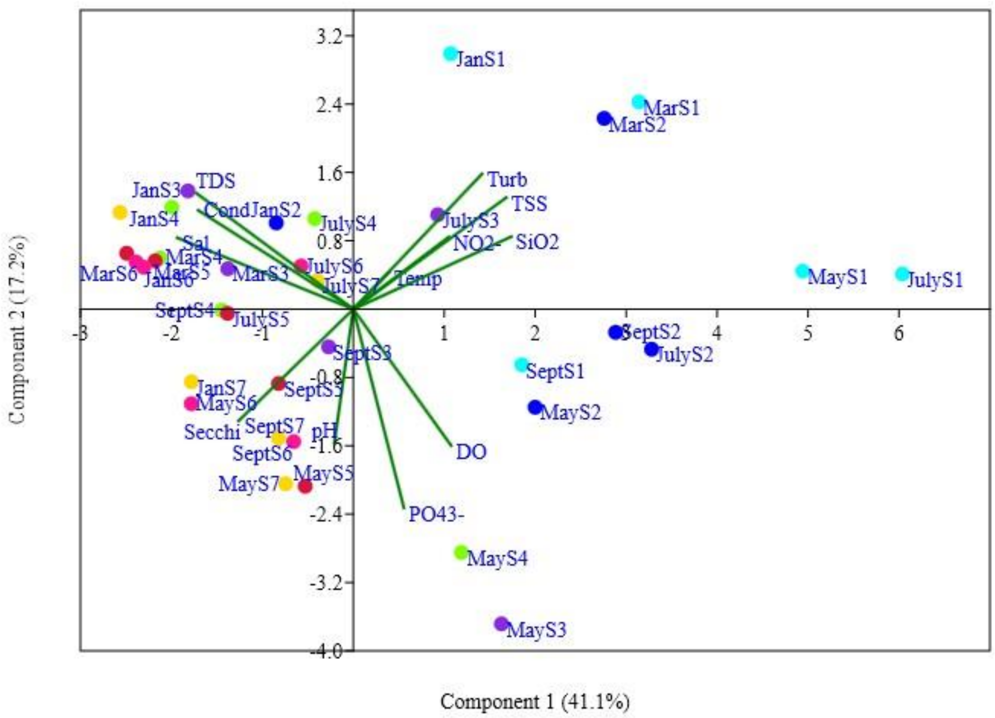

Figure 4. *Principal component analysis of environmental variables sampled on Maranhão continental shelf. Turb = turbidity, TSS = total suspended solids, Temp = temperature, DO = dissolved oxygen, Secchi = transparency, Sal = salinity, Cond = conductivity, TDS = total dissolved solids*

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In the correlation analysis of the environmental variables (*Table 3*), salinity, TDS and conductivity had the largest number of significant correlations with other variables and were positively correlated with transparency and negatively correlated with DO, TSS, silicate, phosphate and nitrate. Silicate was negatively correlated with transparency (r = -0.53) and positively correlated with both turbidity (r = 0.79) and TSS (r = 0.74), confirming the influence of river discharge and rainfall, with higher concentrations of silicate at sites closer to the coast. Nitrite was positively correlated with DO (r = 0.38) and TSS (r = 0.57), which demonstrates its contribution to the increase in biological activity associated with primary productivity and a greater concentration of particulate matter.

Table 3. *Correlation matrix (Pearson's r) for environmental variables sampled on Maranhão continental shelf. Coefficients in bold denote significant correlations ($p < 0.05$, t-test)*

Variables	Temp	Sal	TDS	Cond	pH	DO	Secchi	Turb	TSS	SiO_2	PO_{43-}	NO_{2-}
Temp	1.00											
Sal	-0.16	1.00										
TDS	-0.03	**0.90**	1.00									
Cond	-0.06	**0.90**	**0.83**	1.00								
pH	0.17	0.03	-0.02	0.02	1.00							
DO	0.10	**-0.51**	**-0.52**	**-0.49**	0.19	1.00						
Secchi	-0.14	**0.52**	**0.41**	**0.34**	0.22	0.03	1.00					
Turb	0.07	**-0.41**	-0.27	-0.28	-0.15	0.13	**-0.50**	1.00				
TSS	0.16	**-0.57**	**-0.42**	**-0.44**	-0.24	0.24	**-0.52**	**0.77**	1.00			
SiO_2	0.28	**-0.65**	**-0.52**	**-0.51**	-0.07	0.22	**-0.53**	**0.80**	**0.75**	1.00		
PO_{43-}	-0.14	**-0.37**	**-0.46**	**-0.34**	0.20	0.32	0.12	-0.03	0.06	0.15	1.00	
NO_{2-}	0.07	**-0.36**	-0.28	-0.33	-0.11	**0.38**	-0.13	0.30	**0.57**	0.31	-0.31	1.00

Discussion

The distribution of surface temperature in the ocean is approximately zonal and temperature isolines approximately follow latitude parallels, reaching around 28 °C just north of the equator and diminishing to -2 °C at polar latitudes (Pickard and Emery, 1990). Evidence indicates that inter-annual variability in surface water temperature in the tropical Atlantic occurs more due to local dynamics and interactions with the ocean-atmosphere interface than external perturbations, such as those caused by the El Niño-Southern Oscillation (Hameed et al., 1993; Hastenrath and Greischar, 1993).

Eschrique (2011) found that temperatures demonstrated typical characteristics of the northeastern region of Brazil, with high values throughout the year and stability regarding the period and degree of solar radiation in tropical regions. In studies conducted on the continental shelf off the state of Pará on the border with the state of Maranhão, Nogueira Neto (2013) found surface temperatures around 29.50 °C. Silva et al. (2007b) found a temperature of 28.50 °C in the dry season in an oceanic area adjacent to the Maranhão continental shelf. In studies conducted in the coastal zone of the state of Pará, Martorano et al. (1993) report that high temperatures are typical of equatorial environments, describing a range between 22 °C and 30 °C and monthly means ranging from 24 to 28 °C.

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The salinity range is greater in regions that receive with large volumes of river discharge. The influence of the rivers, together with rainfall, are the main factors responsible for the dynamics of the salinity gradient in the region, which is also strongly affected by the discharge from the Amazon river (Silva et al., 2007a; Araujo et al., 2011). Santos et al. (2008) found a range of 2.49 g kg^{-1} to 36.17 g kg^{-1} along the plume of the Amazon River, with lower values in coastal regions closer to the mouth of the river and higher concentrations in areas more distant from the coast, confirming the enormous influence of the Amazon River on equatorial marine waters. Different behavior is found on the Maranhão continental shelf during the dry season, when rivers contribute very little to the fluvial volume. This explains the increase in salinity at sampling stations closer to the coast due to the oceanic characteristics of tropical water. The increase in the evaporation rate, low river input and high surface water temperatures are directly associated with the intrusion of more saline oceanic water on the Maranhão continental shelf (Pontes and El-Robrini, 2008).

The spatial and seasonal distribution of electrical conductivity demonstrated a similar pattern to that found for salinity, which confirms the correlation between these two variables described by Millero (2006), who states that condumetric ratios have the same proportion as salinity, even if the composition of the salts in surface waters differs. Conductivity depends on concentrations of ions and indicates the amount of salts in water, increasing with the increase in the amount of dissolved solids and demonstrating a close relationship with salinity.

With regard to total dissolved solids (TDS), an understanding of the diverse processes involved enables the identification of interferences in hydrodynamics, affecting tide propagation and the dispersion of suspended nutrients and matter from the mainland basin to the ocean (Vilela, 2011). According to Tundisi and Matsumura Tundisi (2008), all minerals in the water are part of the TDS, including non-ionic components and dissolved organic compounds. Therefore, this variable has a direct and proportional correlation with electrical conductivity due to the concentration of ions, as demonstrated in the present study, in which TDS demonstrated quite similar spatial distribution to that found for salinity and conductivity.

The variable pH is governed by tidal cycles and photosynthesis and/or respiration rates. In aquatic ecosystems, a process of neutralization occurs due to the buffer effect, which maintains pH in a stable balance, with maximum values obtained in environments with greater salinity (Macêdo et al., 2000). Although no significant spatial differences were found, the pH was slightly lower at sampling stations closer to the coast, likely due to the input of freshwater, which has more acidic characteristics stemming, above all, from the large quantity of dissolved organic acids, such as sulfuric, nitric, oxalic, acetic and carbonic acids made available by the metabolic interactions of microorganisms in aquatic environments (Esteves, 2011). The gradual increase in pH toward the open ocean corresponds to carbonate and bicarbonate reactions, which dissociate and increase the alkalinity of surface waters. Santos et al. (2008) found pH values ranging from 7.46 to 8.56 in a transect influenced by the plume of the Amazon River and lower variability in oceanic regions (8.13 to 8.44), which is in agreement with the present study, as pH demonstrated smaller variations at sampling stations located more distant from the coast.

Carvalho et al. (2016) evaluating the phytoplanktonic composition in the continental shelf of Maranhão, also analyzed several physical and chemical variables, however, with a unique seasonal approach (rainy season and dry season). For the present study,

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the approach was taken on a spatial-temporal scale, however, without necessarily grouping the exclusively rainy and dry months, in order to try to show other influencing factors, for the physical and chemical dynamics on the continental shelf of Maranhão.

According to Millero (2006), this relative stability is characteristic of the Atlantic Ocean, unlike records for the Pacific Ocean, for which a range of 7.2 to 8.2 is described.

In tropical coastal regions, the concentration of dissolved oxygen (DO) in the surface layer is subject to rapid fluctuations influenced by external agents, such as river inputs, rainfall, evaporation, winds and tidal patterns, whereas photosynthetic production can contribute to an increase in DO concentrations in the open ocean (Demaster et al., 1996; Santos et al., 2008; Macêdo et al., 2009). In studies conducted in the region of the Saint Peter and Saint Paul Archipelago within the scope of the REVIZEE [Live Resources of the Exclusive Economic Zone] Program, Becker (2001) found minimum and maximum DO values of 2.56 and 4.41 mg L^{-1}, respectively. DO levels ≤ 4 mg L^{-1} in natural waters favors anaerobic processes, which produce a large amount of reducing substances that consume a large quantity of the available oxygen for their oxidation (Esteves, 2011).

With regard to suspended particulate matter, the influence of the tide, geomorphology of the coast and outflow bathymetry in shallow coastal environments are determinants of the re-suspension process. In the present study, Stations 1 and 2 were subject to a greater influence of tidal flow, producing higher TSS values, with lower values at sampling stations more distant from the coast. According to Millero (2006), the decrease in the frequency and intensity of the agitation of bottom layers and the emergence of inorganic and biological reactions in oceanic regions lead to a reduction in suspended particulate matter and more stable TSS values. The values found in the present study were higher than those reported by Souza et al. (2003) in the coastal zone of the continental shelf off eastern Brazil. The authors recorded the distribution of suspended solids in two sampling campaigns conducted off the states of Sergipe, Bahia, Espírito Santo and Rio de Janeiro, with TSS values of 13 mg L^{-1} in the dry season and 90 mg L^{-1} in the rainy season.

On the Maranhão continental shelf, contrary to the expected behavior, the concentration of TSS was greater in the transition between the rainy and dry seasons, with a maximum value in July 2014. This increase may be explained by the periodic dragging of the canals that offer access to the Itaqui port complex, which invariably redistributes the sediment stored on the bottom to the surface layer, thereby contributing to an increase in the concentration of TSS in regions closer to the coast, which are subsequently transported by tidal currents and/or winds to other compartments of the Maranhão continental shelf.

Suspended solids are directly related to turbidity, water transparency and primary productivity, as a greater amount of TSS results in greater turbidity and lower transparency (Macedo, 2003). An increase in the concentration of TSS exerts a direct influence on the reduction in the rate of photosynthesis due to the extinction of light in the photic zone and exerts an negative impact on the availability of food for some species, leading to an imbalance in the marine food chain. Moreover, suspended sediment can be contaminated with pesticides, heavy metals and other toxic substances, which can have a negative impact on the reproduction of fish and other species (Silva, 2006). Da Silva et al. (2009) found higher turbidity in the coastal zone of Maranhão, evidencing the influence of the estuarine sediment plume and the hydrodynamic energy of tidal forces, with the re-suspension of sediments in the water column leading to a

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turbidity range of 14.80 to 39.29 NTU in the dry season and 55 to 200 NTU in the rainy season.

Dissolved inorganic nutrients (nitrite, phosphate and silicate) were recorded in the present study, as the occurrence of these nutrients indicates mainland inputs that affect primary production in marine environments. The allochthonous origin stems from processes such as atmospheric deposition, the infiltration of ground water by lixiviation, the entrance of marine water in estuaries and the flow of rivers, whereas the internal contribution is based mainly on the benthic and pelagic re-mineralization of organic matter. Understanding nutrient distribution patterns and marine biogeochemical cycles remains a considerable challenge (Conkright et al., 2000). Continental shelves are transition zones between the mainland and ocean with a large supply of particulate and dissolved matter stemming from river discharge (Nittrouer et al., 1995), where complex oceanographic processes co-occur on distinct spatial and temporal scales (Arndt et al., 2011).

The increase in biological activity associated with nutrient availability may be attributed to both biogeochemical processes and other oceanographic processes that cause the re-suspension of organic matter (upwelling, small vortices or internal waves), leading to an increase in biological production (Admiraal et al., 1990; Turner and Rabalais, 1994). Along the continental shelf, the spatial distribution of dissolved nutrients is controlled by vertical and horizontal mixtures caused by tides, waves and wind on the water surface (Sánchez-Arcilla and Simpson, 2002). The synergic action of these variables can cause an increase in dissolved nutrients and total suspended solids, thereby increasing primary production (Mann and Lazier, 1996). In coastal zones of tropical regions, estuarine systems serve as a storage site for suspended nutrients, which then become available to the adjacent continental shelf (Ovalle et al., 1999). For instance, Amazon coastal zone is potentiated by the abundance of mangroves and high hydrodynamic energy, creating a rich supply of dissolved nutrients directed to the continental shelf region (Demaster et al., 1996; Kineke et al., 1996; Nittrouer and Demaster, 1996; Pereira et al., 2010).

On the Maranhão continental shelf, nitrite values were low and very similar among all sampling stations ($F = 0.67$; $p > 0.05$), which is in agreement with data described by Demaster and Pope (1996) for various transects determined on the continual shelf of the Amazon. Low nitrite values are typical of tropical waters, except when resulting from re-mineralization, re-suspension of the bottom or input from rivers. Santos (2012) confirms this nitrite instability on the continental shelf off the northeastern region of Brazil, with mean values ranging from 0.02 to 0.05 µmol L^{-1}, which is characteristic of oligotrophic environments. In the period between the rainy and dry seasons (July 2014), an increase was found in the variation of nitrite concentrations, with a mean of 0.32 µMol L^{-1}. This increase can lead to an increase in biological production rates, a greater availability of suspended matter as well as increases in OD, TSS and turbidity, suggesting an increase in the photosynthetic rate and primary production at stations in oceanic regions. All these aspects contribute to an increase in the production of aquatic organisms in this period in regions more distant from the coast. In contrast, lower values were found in the dry season, as occurred in September 2014, when no nitrate was detected. Therefore, nitrate became more unstable in this period, favoring the development of bacterial oxidation, which increases the availability of ammonia. Pereira et al. (2012) report different behavior in a study conducted in the Amazon

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coastal zone; the authors found mean nitrite values of 0.11 µmol L^{-1} at the end of the rainy season and 0.49 µmol L^{-1} in the dry season.

Phosphate concentrations fluctuate little in tropical estuaries. The mainland is the main source of these nutrients in the marine environment, with concentrations tending to diminish with the distance from the source (Eschrique et al., 2010). Monteiro et al. (2015) report values of 0.11 µmol L^{-1} to 1.08 µmol L^{-1} in an Amazonian estuary near the island of Marajó in the state of Pará. However, Lara and Dittmar (1999) found phosphate concentrations between 1.5 and 5.0 µM L^{-1} in a tidal channel in the municipality of Bragança in the same state. Pereira et al. (2012) found low concentrations (mean: 0.11 µmol L^{-1} in the rainy season and 0.92 µmol L^{-1} in the dry season) in a coastal environment in northeastern Brazil, demonstrating the low influence of seasonality on the final concentration of phosphate in this environment.

The rapid absorption of phosphate in oceanic areas by primary producers and bacteria explain its low concentration and availability. In the present study, concentrations were low at the majority of sampling stations. Ramin et al. (2012) and Eschrique et al. (2006) report that phosphate regeneration processes through re-mineralization, excretion by zooplankton and absorption by phytoplankton occur at an accelerated rate, making its residence time in oligotrophic waters extremely short (a matter of minutes) and making its particulate form the main compound found in oceanic waters. The coastal region of the Maranhão continental shelf, which is influenced by a large number of mangroves, is an important vehicle for the transport of nutrients to the oceanic region.

Scientific records describe a reduction in the concentration of dissolved nutrients from the estuary to the coastal region, favoring the development of mixture diagrams as a powerful tool for the characterization of nutrient removal processes from masses of water. According to Noernberg (2001), the transport of sediment through the coastal zone reaches an average of 15 nautical miles in the direction of the open sea, with the coastal environment the main redistribution route of nutrients to oceanic regions. Santos et al. (2008) found high nutrient concentrations in the rainy season at a distance of 120 km from the coast on the continental shelf off the state of Pará, with silicate as the most representative nutrient (range; 14.48 µmol L^{-1} to 108.59 µmol L^{-1}), confirming the considerable capacity and influence of Amazonian rivers in the distribution of this nutrient to the open ocean. In the present study, silicate demonstrated dispersion capacity through the plume of rivers, thereby reflecting the influence of transport from rivers to oceanic regions of the Maranhão continental shelf and giving this nutrient the greatest capacity for concentration and distribution in the area investigated.

The ordination analysis revealed the influence of river discharge and rainfall on the concentration patterns of different physicochemical variables, which is in agreement with data described by Pereira Filho et al. (2009). Pereira Filho et al. (2003) also report the influence of river discharge on the greater presence of nitrogenated nutrients, which are involved in the nutritional process of phytoplankton. Braga et al. (2008) report that the availability of phosphate is closely linked to the mainland input, with the influence of the Prata River and upwelling in the region of Cape Santa Marta in the state of Santa Catarina (southern Brazil). In the present study, the considerable outflow from the mainland, especially from the Mearim and Pindaré Rivers, and the macrotide conditions, with the capacity to transport nutrients from the mangroves, contributed greatly to the availability of phosphate on the Maranhão continental shelf, with means ranging from 0.05 µmol L^{-1} in January 2014 to 0.55 µmol L^{-1} in September 2014. Braga

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et al. (2008) report similar figures for the Santa Catarina continental shelf, whereas Santos et al. (2008) report lower figures for the Amazon continental shelf.

Silicate is a potential tracer of sediment transported from rivers to oceanic regions, enabling the identification of the distribution caused by this flow (Braga et al., 2008). The analyses also indicate an association between silicate and sampling points closer to the coast, demonstrating negative correlations with salinity, TDS and conductivity, for which higher values were found at more external sampling stations along the Maranhão continental shelf. Ciotti et al. (1995) and Weber et al. (1994) also found a greater concentration of silicate in waters closer to the coast. Braga et al. (2008) describe freshwater and terrestrial inputs along the coast as being major contributors to the high concentrations of silicate on the southern/southeastern continental shelf in summer, which is in agreement with the present findings and demonstrates the importance of climate as a regulating agent for the availability of this nutrient. It is therefore important to maintain the levels of river outflow due to the transport of nutrients to the Maranhão continental shelf, which contribute to biological development as well as the maintenance of trophic, reproductive and developmental aspects of species and, consequently, fishing resources, which have dietary, economic and cultural importance to tropical cities in coastal areas.

Conclusion

The spatial and temporal variability in the physicochemical variables analyzed (transparency, temperature, salinity, conductivity, dissolved oxygen and suspended solids) is associated with local dynamics governed by river discharge, tidal movements, currents and climatologic events;

Very low salinity was associated with higher concentrations of nutrients, indicating the influence of terrestrial inputs to the Maranhão continental shelf, especially in areas closer to the coast.

Phosphate concentrations were similar throughout the sampling stations and lower than silicate concentrations. In turn, silicate concentrations were significantly higher in areas closer to the Gulf of Maranhão;

The highest nitrite concentrations were found in July 2014, soon after the period of greater rainfall in the region, demonstrating a direct association with mainland inputs and a greater biological production rate, as nitrite values were positively correlated with dissolved oxygen and total suspended solids;

The fluvial transport from the rivers of the state of Maranhão and other freshwater sources in the Amazon region are apparently the major contributors responsible for the maintenance of nutrient availability on the Maranhão continental shelf, where seasonal influences affect phosphate and nitrite availability, with higher concentrations during and soon after the rainy season. In contrast, silicate values are similar throughout the year.

It is recommended in future studies the increase of transects that would be simultaneously sampled to produce maps of the spatial distribution of physicochemical variables from two-dimensional interpolations (2D).

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EFFECTS OF DIFFERENT CONCENTRATIONS OF CARBOHYDRATE FORMS ON *ORCHIS SANCTA* L. PROPAGATION IN VITRO

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Abstract. In this study, the goal was the germination and propagation of *Orchis sancta* L. which is endangered due to uncontrolled collection and used in obtaining salep, *in vitro* conditions. By the addition of 0 (control group), 20, 40, 60, 80, and 100 g/l concentrations of sucrose, glucose, maltose, galactose, and fructose of carbohydrate forms into Van WaesDebergh culture medium, sugars of effects were determined on germination and development of *Orchis sancta* L. seeds. In the study, germination, formation of protocorm and shoot periods and ratios were examined. The highest average germination ratio was obtained as 77.85% in Maltose40, the lowest average as 44.36% in Galaktose100 mediums. In protocorm formation, when the highest average ratio was detected as 68.53% in Sucrose100, the lowest average as 25.33% in Fruktose100 mediums. Germination period as 12.50-21.33 days, protocorm formation period as 22.50-50.83 days and shoot formation period as 50.66-105.33 days' intervals were determined.
Keywords: *culture medium, germination, orchid, protocorm, shoot*

Introduction

Orchidaceae is one of the largest families of flowering plants in terms of the number of species. While the total of 150 orchid species, of which 13% being endemic, have been specified in Turkey (Erdem, 2004) that there are 117 orchid species among these are used to produce salep (Sezik, 2002). Turkey is one of the richest countries in Europe and Middle East in terms of middle zone orchids, but growing industrialization, rapid urbanization and population growth bring about social, economic and cultural imbalances. The biggest problem faced by the salep orchids, which are among the endangered and damaged plant species in the world, is the excessive uprooting of the plant because of the pharmaceutical importance of the plant drug and its commercial value particularly in the ice-cream and hot drink sector. Hatipoğlu (1981) has listed salep under bulbous-tuberous and rhizomatous ornamental plants, uprooted from natural vegetation without being propagated. According to Sezik (1984), tubers weigh 2 to 7 g when they are fresh. If an average of 4 g is considered, 250 orchids should be removed for 1 kg of fresh tubers. A salep collector normally can collect up to 1 kg of fresh tubers per day. Approximately 1000 to 4000 tubers are required for 1 kg of salep. İşler (2005) stated that the price of 1 kg of mountain salep collected and dried in Van province is 100 Turkish Liras (TL), while meadow salep is sold for 25 TL. It is known that wild collection of orchid tubers for salep production threatens orchids collected in the mountainous areas more compared to the ones collected from the meadows. With each tuber collected, next generation plant and plenty of seeds to be produced by this plant

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are prevented. According to Tekinşen (2006), 15 to 20 tonnes out of 35 to 65 tonnes of salep produced in Turkey is exported in powder from.

Due to its increasing price, powdered tubers of *Muscari* plant, which contains muscarinic substance which can cause harm to human health, and tubers of other plants are used as additives in salep (Hatipoğlu, 1981). The researcher emphasized in those years that heavy wild collection problem due to increasing prices is not at international level like citrus and viticulture, but is greatly a concern of Turkey. It is further mentioned that salep is not consumed in high amounts in the developed countries as in Turkey; even the foreign countries are responsive to destruction of our flora and genetic resources; and they are concerned with this issue. Glicomannan, the active substance of salep, swells with milk or water and generates a fluid solution. Thus, salep has become an essential drink in cold winter days. Additionally, salep is put into the famous Maraş ice-cream to provide late melting and hardness. Among these usages, salep has also a medical importance. It is pointed out that Avicenna (Book of Ibn Sina in C.E. 980-1037) recommends the use of salep as expectorant, appetizer, anti-stroke substance and aphrodisiac (Sezik, 1984). Nonetheless, instead of being used primarily for these purposes, salep is consumed as beverage and gains economic value in ice-cream production. To attain salep, being so useful and having commercial importance, there is an irrepressible and enormous collection from the nature. Among tuberous orchids, salep is rather extracted from orchids with ovoid tuber such as *Anacamptis, Orchis, Ophrys, Serapias, Himantoglossum, Barlia,* and from some species with amorphous tuber such as *Dactylorhiza*. The harvest of every tuber prevents the plant of next year with its many seeds to grow up. Because of this uncontrolled collection, alternative production and propagation methods are aimed and constantly new techniques are developed with seeds in laboratories.

Orchis sancta L. is also an orchid species that has been collected for these purposes and is being studied to be propagated with different methods. This study aims at providing asymbiotic propagation and species continuity of *Orchis sancta* L., a salep orchid, in vitro conditions.

Literature review

According to Stewart and Kane (2010), the role of carbohydrates *in vitro* orchid seed germination and protocorm formation has received some attention, although little in recent years. In the earlier study about asymbiotic orchid seed germination, researchers realized that some carbohydrate sources were better suited to support seed germination than others, and that this response was genus- or species-specific (La Garde, 1929; Smith, 1932; Wynd, 1933). Little attention has been paid by modern researchers to the effects of carbohydrate source on asymbiotic orchid seed germination and protocorm/seedling development, and little information exists on the role of different carbohydrates in the asymbiotic germination of terrestrial orchids (Stewart and Kane, 2010).

Smreciu and Currah (1989) reported that very small orchid seeds have small testas, formed with loose and transparent cell layer and have very small embryos. Germination of orchid seeds which have limited proliferation capacities and do not contain endosperm, an appropriate mycorrhizal relation with a mycorrhizal fungus is required in the environment in addition to appropriate temperature, light, humidity and oxygen conditions (Sezik, 1984). As orchid seeds do not contain nutrient reserves, successful germination do not occur unless a carbohydrate source such as glucose is provided from

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outside (Ingold and Hudson, 1993). Adding carbohydrate to the culture medium as a germination method in addition to symbiotic germination with mycorrhiza might be successful. This way, orchid seeds' utilization of the substances in the culture medium might be changed. These alterative techniques can be tried at different stages such as germination, protocorm and shoot formation, even root and tuber formation. As a result, every study on germination and propagation and every method resulting in yield, first in the laboratory environment and then in the adaptation phase on the land, is treasured for the species continuity. Thanks to this awareness and determination, orchids will be able to seen on the ground, in their living environment.

Material and methods

Seeds

In the study, mature seeds of *Orchis sancta* L. species naturally grown around Aydın province in Turkey but collected and thus destroyed to obtain salep were used and 50 seeds, randomly selected before sowing, were put to Tetrazolium Chloride (TTC) viability test. The seeds soaked with distilled water in Petri dishes were kept for 12 h and the embryo sections were left in 1% tetrazolium chloride solution. While the pink-red coloured ones of the seeds kept at room temperature for 12 h are considered alive, the white-coloured ones are considered inanimate (Grabe and Peters, 1998; AOSA, 2002). According to the test results, 85% of the seeds were alive. The photos of the seeds were captured with OLYMPUS SZ61 binocular microscope dp20 camera and 10X x 1.2 software (*Fig. 1*).

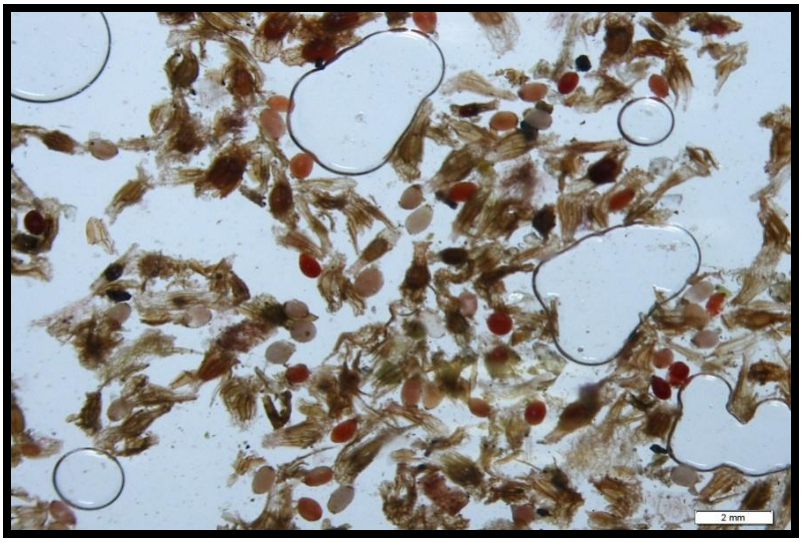

Figure 1. *Aspect of Orchis sancta L. seeds under binocular microscpoe*

The seeds are counted so as to sow 100 units to each Petri dish. The orchid seeds packed in coarse filter paper envelopes were sterilized for 12 min with 1-2 drops of Tween-20 and 10% NaOCl and rinsed 3 times with sterile distilled water after 5 min of shaking with 2% H_2SO_4 (Çığ and Yılmaz, 2016). Sterilized seeds are sown in a total of 15 glass Petri dishes including culture media, in 3 replicates with 5 times for each replicates.

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Preparation of culture medium and seed sowing

Culture mediums were formed by adding 0, 20, 40, 60, 80 and 100 g/l concentrations of the sugar forms of sucrose, glucose, fructose, galactose and maltose into the VWD medium (Van Waes and Debergh, 1986). 0.85 g/l agar was used for all mediums; and were autoclaved at 121 °C and 1.2 atmosphere pressure for 20 min after the pH was adjusted to 5.8 with 1 N HCl and 1 N NaOH. The sterilized Petri dishes with sown seeds were stored in 23 ± 1°C and in dark place and were taken to a light/dark room for 16/8 h with the formation of protocorm. The subculturing process was repeated once a month in aseptic conditions.

Statistical evaluations

In terms of the emphasized specifications, mean and standard error are indicated with descriptive statistics. To determine any possible difference of these specifications between the concentration and culture medium, completely randomized factorial design analysis is implemented (Montgomery, 2001).

The effects model is

$$y_{ijk} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + e_{ijk}$$

where, $i = 1,\dots,6;\ j = 1,\dots,5;\ k = 1,\dots,90$; μ is the overall mean effect, τ_i is the effect of the i^{th} level of concentration, β_j is the effect of the j^{th} level of medium, $(\tau\beta)_{ij}$is the effect of the interaction between τ_i and β_j, and e_{ijk} is a random error component.

An angular transformation is applied to the measured percent variance analysis.

$$Y = arcsine\sqrt{p} = sine^{-1}\sqrt{p}$$

Where p is the proportion and Y is the result of the transformation. The result may be expressed either in degrees or radians. The usual rule of thumb is that they should be used when there are a number of proportions close to 0 and/or close to 1. The transformations will “stretch out” proportions that are close to 0 and 1 and “compress” proportions close to 0.5. The amount of germination, protocorm and shoots was subjected to angular transformation and expressed in terms of their original values in *(Tables 1* and *2).*

Tukey Multiple Comparison test was used in determining significant difference between concentrations and carbohydrates following the variance analysis. The statistical significance level was taken as $p < 0.01$ and the calculations were done with ANOVA procedure by the statistical package JMP 5.0.1a software.

Results and discussions

In the study investigating in vitro germination, protocorm and shoot formation potentials of *Orchis sancta* L., five different carbohydrate (sugar) forms of galactose, glucose, maltose, fructose and sucrose were added into the culture medium with 20, 40, 60, 80 and 100 g/l concentrations (doses) were applied and differences were observed statistically with a significance level of ($p < 0.01$) in the characteristics examined (*Tables 1* and *2*).

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Table 1. *Effect of carbohydrate forms and concentrations on germination, protocorm and shoot formation rates (%)*

Carbohydrate forms	**Carbohydrate concentrations (g/l)**	**Germination rate** (%)**	**Protocorm formation rate** (%)**	**Shoot formation rate** (%)**
Galactose	0	67.53 bcdef	55.31 cdefgh	0.00 h
	20	60.85 fghij	59.79 bcde	0.00 h
	40	59.91 ghij	52.21 efghi	0.00 h
	60	65.93 defgh	45.90 ijk	0.00 h
	80	44.39 n	31.40 l	0.00 h
	100	44.36 n	31.79 l	0.00 h
Glucose	0	67.53 bcdef	55.31 cdefgh	0.00 h
	20	61.62 fghi	68.44 a	9.02 c
	40	66.53 cdefg	59.86 bcde	8.77 cd
	60	52.86 klm	46.87 hijk	0.00 h
	80	47.92 lmn	60.34 abcde	2.80 g
	100	56.39 ijk	41.08 k	0.00 h
Maltose	0	67.53 bcdef	55.31 cdefgh	0.00 h
	20	71.91 abcd	53.74 defghi	0.00 h
	40	77.85 a	64.40 ab	0.00 h
	60	68.87 bcde	49.74 ghij	0.00 h
	80	64.87 efgh	51.22 fghij	0.00 h
	100	59.73 hij	52.66 efghi	0.00 h
Fructose	0	67.53 bcdef	55.31 cdefgh	0.00 h
	20	72.94 abc	57.67 bcdefg	13.16 a
	40	61.18 fghij	55.63 cdefg	6.56 e
	60	47.04 mn	43.03 jk	0.00 h
	80	50.41 klmn	28.94 l	0.00 h
	100	47.91 lmn	25.33 l	0.00 h
Sucrose	0	67.53 bcdef	55.31 cdefgh	0.00 h
	20	68.93 bcde	61.58 abcd	8.01 d
	40	73.88 ab	63.11 abc	13.30 a
	60	63.28 efgh	58.72 bcdef	5.36 f
	80	54.62 jkl	50.52 fghij	3.50 g
	100	66.25 cdefgh	68.53 a	10.90 d

**In the same column, the difference between the averages indicated by the same letter is insignificant at level $p < 0.01$

Germination rate of the seeds (%)

While germination occurred in all mediums and doses, it was observed that the highest germination average is obtained from Maltoz40 with 77.85% (*Table 1*). The lowest average value of germination is obtained from the Galactose100 medium with 44.36%. Taking into consideration all sugar forms in general, it appears that germination rates decrease as the used doses increasing. This suggests that the

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increasing of sugar concentration has a reducing effect on the germination of *O. sancta* seeds (*Figs. 2* and *3*).

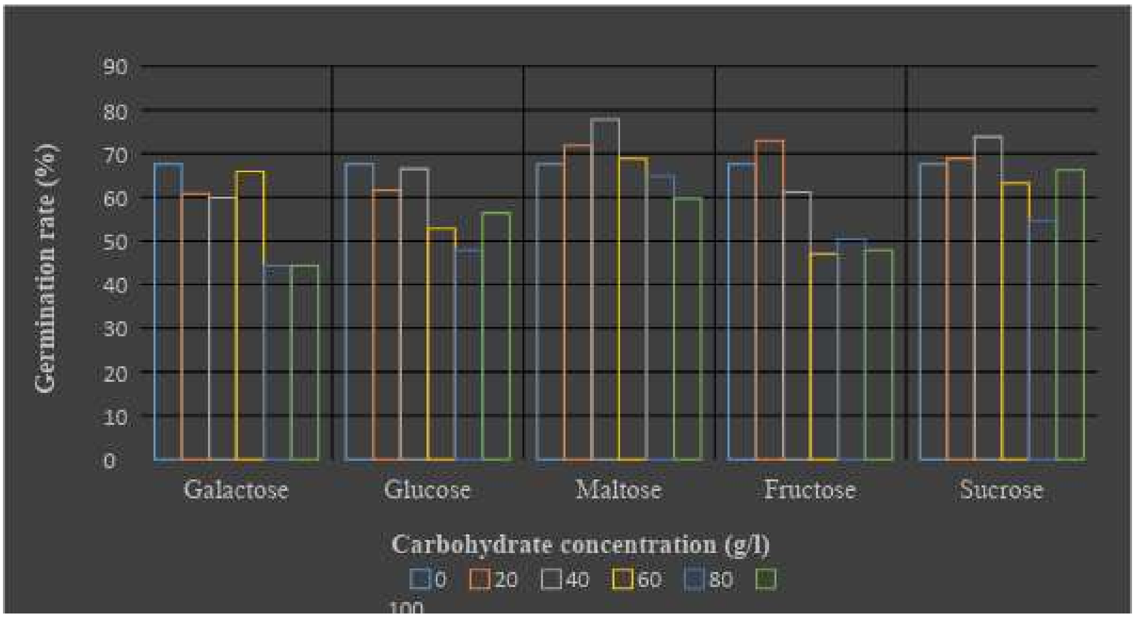

Figure 2. *Effects of carbohydrate forms and concentrations on germination rates (%)*

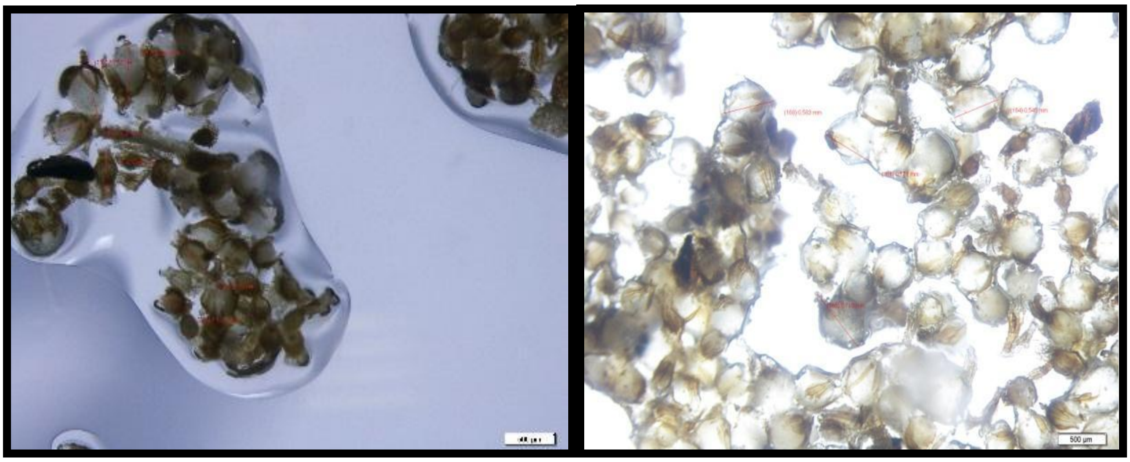

Figure 3. *Germinated O. sancta seeds in culture medium*

In vitro asymbiotic germination of orchid seeds, some inorganic substances and sucrose, glucose and other sugars are useful for the culture medium; dextrose and sucrose can also be used as sugar sources (Harvais, 1973). In our study, sucrose and glucose were sugars that had an effect on germination after maltose. Van Waes and Debergh (1986) reported that sucrose is more effective on germination compared to other sugars, and mentioned a negative effect of galactose. Galactose has the lowest germination rate in our study. Mead and Bulard (1975), succeeded in adding sucrose in their study with species of *Orchis* and *Ophrys* genus. In another study on *Dactylorhiza* species, it is reported that germination was stimulated through glucose and sucrose (Wotavová-Novotná et al., 2007). Considering previous studies, it can be concluded that

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glucose and sucrose induce germination at the most. In our study, these two types of sugar have the highest second and third mean values following maltose (*Fig. 2*). It is presumed that the culture medium and orchid species used by the researchers may be different. Culture mediums used in germination studies on rather different species vary as well. This study of *O. sancta* species has the highest percentage of germination under maltose impact in the VWD medium.

In a study with nine orchid species, in which three of them generated positive results, the germination rate of *O. sancta* was found 100% (Önal, 1999). Yet in another study, 20% sugar was added to 1/10 MS (Murashige & Skoog) medium and ultrasonication was applied to germinate the seeds; and the *Orchis italica* seeds were germination with the rate of 81% (Yararbaş, 2008). 30 g/l of sucrose was added during the study with 6 orchid species executed in full strength MS, VWD and KC (Knudson-C) mediums and ½ MS medium. A germination rate of 7.34%, which is the highest, occurred in VWD medium (Gümüş et al., 2008). In the study with *O. sancta* seeds, KC and VWD mediums with added GA_3, BAP, coconut milk, banana and potato extract was used and the germination rate was found as 20-40% in VWD medium and 20-100% in KC medium Sarı et al. (2011). Bulunuz Palaz et al. (2012) has found the highest germination rate among five orchid species as 30% for the *O. sancta* species. In another study of the researchers, *O. sancta* seeds showed a germination rate of 79.08% in EBP + AC (activated charcoal) medium (Bulunuz Palaz et al., 2014).

Protocorm formation rate (%)

The protocorm phase is described as the formation of a nail-like structure after germination (Sezik, 2002), and the protocorm amount is obtained by calculating percentage ratios to germinated seeds. Again in 60 g/l and above applications of sugar concentrations used in parallel with germination, decreasing mean values were obtained. Protocorm formed in all applied mediums and doses (*Fig. 4*) and the highest protocorm formation rate was obtained from Sucrose100 with 68.53% (*Table 1*). The lowest protocorm mean value was obtained from Fructose100 medium with the rate of 25.33%. Taking into account all sugar forms in general, protocorm ratios decreases with increasing doses. However, the 60 g/l dose seems to be a breaking point and then the protocorm formation increases again in general (*Fig. 5*).

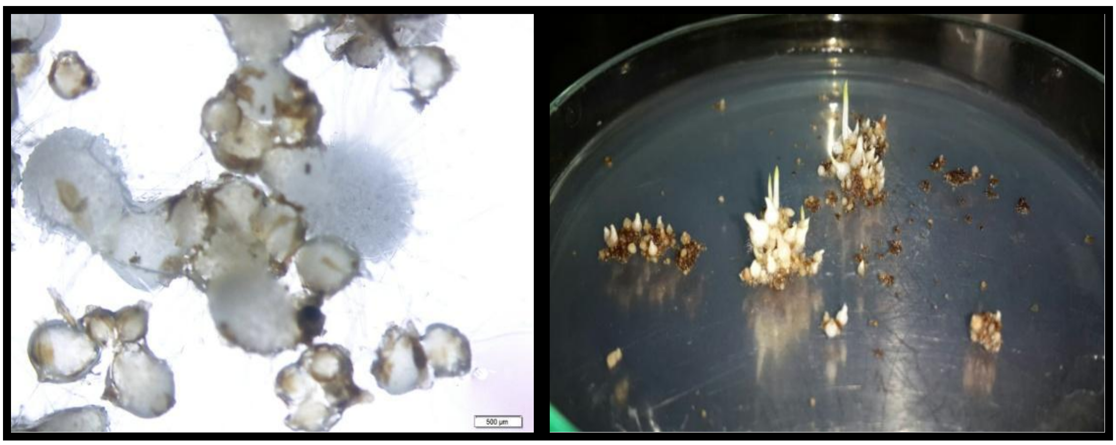

Figure 4. *Protocorm formation in germinated O. sancta seeds in culture medium*

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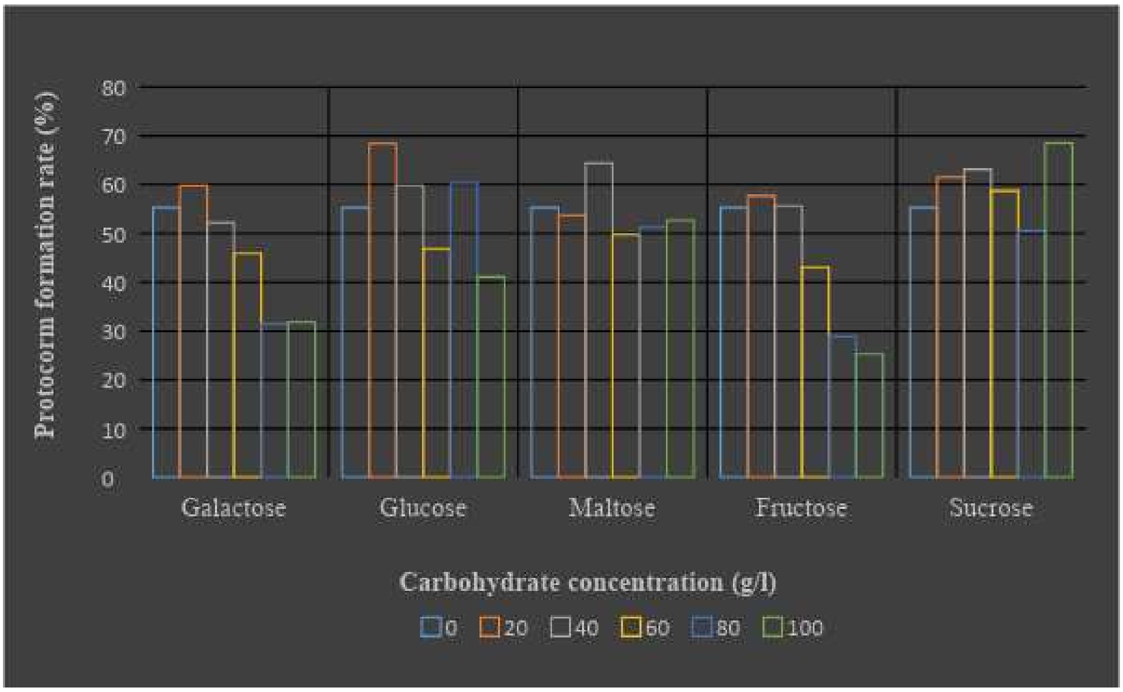

Figure 5. *Effects of carbohydrate forms and concentrations on protocorm formation rates (%)*

The highest rate of protocorm formation for six orchid species was 13.11% (Gümüş et al., 2008). The highest protocorm formation in six orchid species, germinated in five different culture mediums, has been obtained in KC and VWD mediums with a rate of 62.92% (Çığ and Yılmaz, 2016). In the study using KC and KC + 1 mg/l GA_3 mediums for cultivation, protocorm formation occurred in 5.34% of *O. sancta* species which is the highest among the five orchid species (Bulunuz Palaz et al., 2012). In an in vitro study on eight orchid species, *O. sancta* seeds have formed 78.12% protocorm in EBP+AC medium (Bulunuz Palaz et al., 2014). In an in vitro study on five orchid species, the highest rate of protocorm formation occurred in a medium including tripton; recorded 28% for *Serapias vomeracea* species, 48% for *O. sancta* species and 48% for *Orchis coriophora* species (Karakuş, 2015).

As we can conclude from similar studies, protocorm generally showed successful development in VWD mediums. It is thought that especially the rate of 78.12% mean value obtained from *O. sancta* is a result of the EBP+AC medium. A protocorm formation rate of 5.34% was obtained from KC +1 mg/l GA_3 medium, but despite it contained hormones, this was considerably below the mean value occurred in our study. The effect of culture medium on the protocorm formation of *O. sancta* species is obvious.

Shoot development rate (%)

Shoot development did not occur in all mediums and concentrations (*Fig. 6*). In some doses of glucose and fructose; and in all doses of sucrose, shoot development was observed. Shoots were developed with the rate of 0-9.02% in the glucose group; 0-13.16% in the fructose application and 3.50-13.30% in the sucrose application.

The sucrose application was an effective sugar in protocorm formation, while it had the same effect also in shoot development. Likewise, glucose was also a sugar type that

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followed sucrose; nonetheless, while fructose had a lower performance in protocorm formation compared to other sugar types, it showed a more positive effect on shoot development (*Fig. 7*).

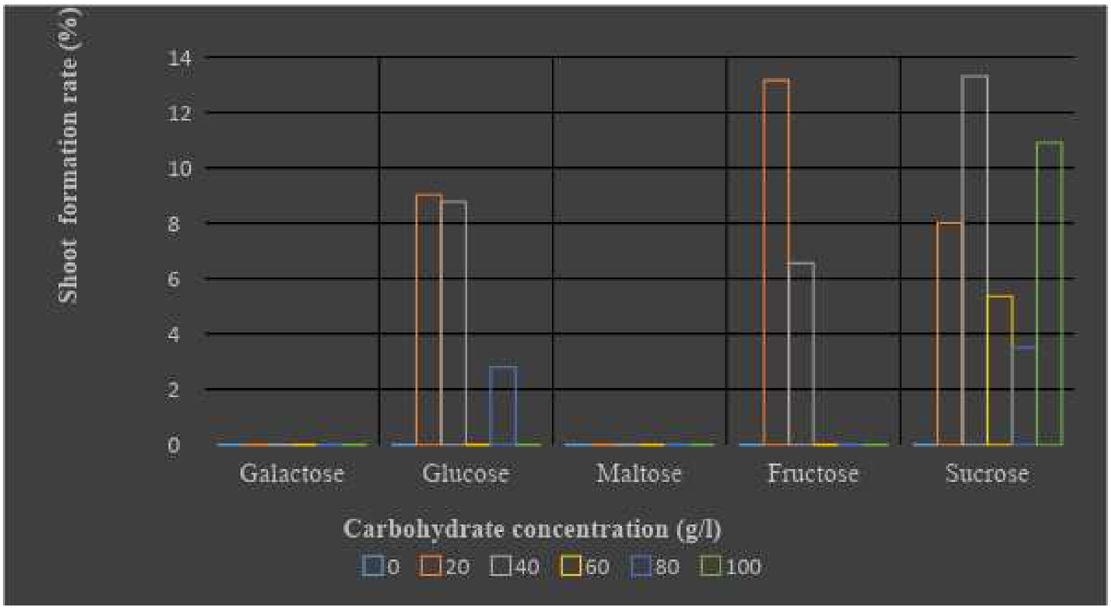

Figure 6. *Effects of carbohydrate forms and concentrations on shoot formation rates (%)*

Figure 7. *Developed O. sancta shoots in culture medium*

On the other hand, maltose and galactose exhibited a negative effect on shoot development, while having positive effects on protocorm formation. It is reported that sucrose has the most positive effect among sugars used for germination and development (Van Waes and Debergh, 1986); and that glucose, glucose, mannose, maltotriose and maltopentaose can be used for seedlings cultivated from in vitro applications (Ernst and Arditti, 1990). In all phases of *O. sancta* seeds, from germination to shoot development, the negative effect of galactose is observed. On the contrary, glucose and sucrose had a stimulating role in all the levels of the study.

In previous research, the amounts of plantlets stemming from protocorm were obtained with rates of 1.86% (Çağlayan et al., 1998) and 80% (Önal, 1999). The highest

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shoot development rates obtained from protocorm was observed in KC medium, MS and 1/10 MS mediums (Çığ and Yılmaz, 2016). In the study with five orchid species applied in two culture mediums, the highest plant development rate determined as 3.5% in *O. sancta* (Bulunuz Palaz et al., 2012). In the study with 8 orchid species, *O. sancta* seeds had a shoot development at the rate of 72.50% in EBP+AC medium (Bulunuz Palaz et al., 2014).

Germination period (day)

The highest germination period was obtained in Galactose80 as 21.33 days; while the lowest period was 12.50 days in Glucose40, Maltose40 and Sucrose20 (*Table 2*). Considering the mediums separately in terms of germination period, the average values were 14.66-21.33 days in the galactose application; 12.50-15.83 days in the glucose application; 12.50-15.33 days in the maltose application; 13.16-20.83 days in the fructose application and 12.50-15.33 days in the sucrose application (*Fig. 8*).

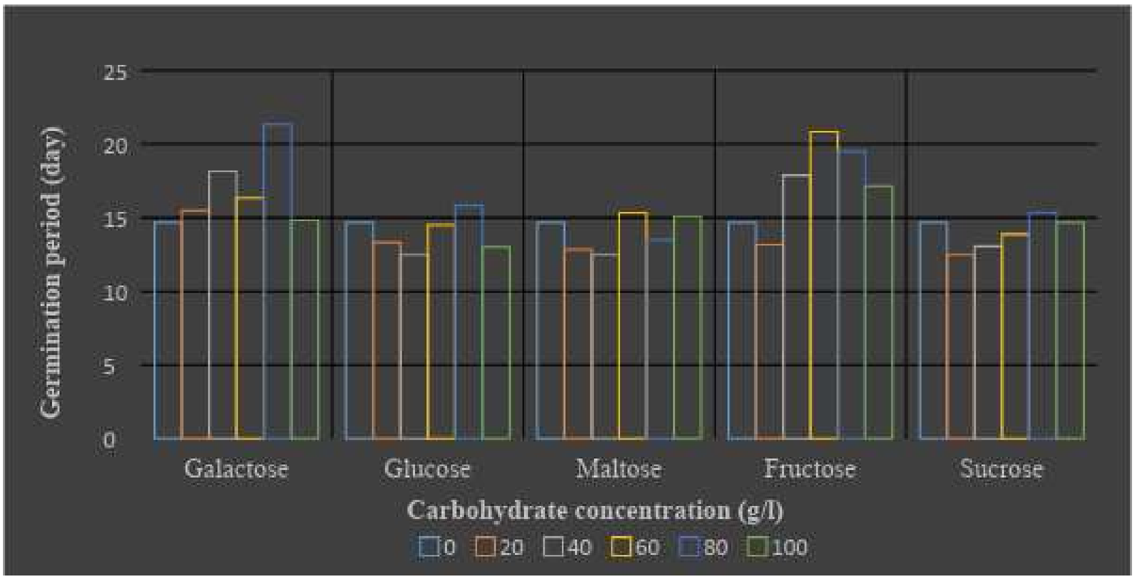

Figure 8. *Effects of carbohydrate forms and concentrations on germination periods (days)*

Germination of seeds cultivated in vitro conditions was observed in 58 days in orchid seeds (Çağlayan et al., 1998), and in15 days in *Orchis italica* seeds (Yurarbaş, 2008). In a study with *O. sancta* and other seed species, the earliest germination was observed in *O. sancta* seeds after a month (Önal, 1999); and after two weeks in another study (Bulunuz Palaz et al., 2012). In a study with Modified Lucke (LM), MS, Lindemann, Vacin & Went, Malmgren Modified (MM) and KC mediums; BAI, Zea, Kin, 2-iP cytokinins and 0/24, 16/8, 24/0 light/dark photoperiods, the *Habenaria macroceratitis* terrestrial orchid seeds showed the highest germination rate of approximately 89% in LM and KC mediums at the 7th week of the study (Stewart and Kane, 2006). In another study, cracking and decolourization were observed in cultivated seeds in 15-20 days (Sarı et al., 2011). The germination period of 4 orchid species cultivated in KC, VWD and Pfeffer nutrient mediums was observed as 3 months (Kısakürek, 2011). In a study to identify the effects of VWD, KC, MS, ½ MS and 1/10 MS culture mediums on the germination period of orchid seeds; the effect of VWD medium on germination

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appeared in the range of 27.67-130.53 days (Çığ and Yılmaz, 2016). Considering the other mediums, this range was 22.00-191 days.

Table 2. *Effects of carbohydrate forms and concentrations on germination, protocorm and shoot development periods (day)*

Carbohydrate forms	Carbohydrate concentrations** (g/l)	Germination period** (day)	Protocorm formation period** (day)	Shoot development period** (day)
Galactose	0	14.66 fghij	23.66 gh	0.00 g
	20	15.50 defghi	22.50 h	0.00 g
	40	18.16 bcd	34.66 cd	0.00 g
	60	16.33 defg	25.83 efgh	0.00 g
	80	21.33 a	51.33 a	0.00 g
	100	14.83 fghij	24.50 gh	0.00 g
Glucose	0	14.66 fghij	23.66 gh	0.00 g
	20	13.33 hij	22.83 h	89.33 c
	40	12.50 j	25.83 efgh	100.66 ab
	60	14.50 fghij	31.83 def	0.00 g
	80	15.83 defgh	32.66 de	75.66 d
	100	13.00 hij	25.00 fgh	0.00 g
Maltose	0	14.66 fghij	23.66 gh	0.00 g
	20	12.83 ij	25.00 fgh	0.00 g
	40	12.50 j	25.66 fgh	0.00 g
	60	15.33 defghij	30.50 defg	0.00 g
	80	13.49 ghij	25.60 fgh	0.00 g
	100	15.05 efghij	27.66 efgh	0.00 g
Fructose	0	14.66 fghij	23.66 gh	0.00 g
	20	13.16 hij	27.16 efgh	99.33 b
	40	17.88 bcde	40.72 bc	105.33 a
	60	20.83 b	50.83 a	0.00 g
	80	19.50 abc	43.66 b	0.00 g
	100	17.16 cdef	39.66 bc	0.00 g
Sucrose	0	14.66 fghij	23.66 gh	0.00 g
	20	12.50 j	25.00 fgh	60.66 e
	40	13.05 hij	24.33 gh	50.66 f
	60	13.88 ghij	28.38 defgh	101.16 ab
	80	15.33 defghij	28.00 defgh	57.99 e
	100	14.66 fghij	28.88 defgh	104.50 a

**In the same column, the difference between the averages indicated by the same is insignificant at $p < 0.01$

According to the studies, the germination period varied in terms of species with diverse mediums and applications (15-191 days). However, in this study, getting close results to those obtained in the previous in vitro study, specifying the germination periods of 1 month and 2 weeks especially with *O. sancta* species, reveals the early

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germination period of the seeds of *O. sancta* species. It is believed that the added carbohydrate and its concentrations of the culture medium were effective.

Protocorm formation period (day)

While glucose, maltose and sucrose applications showed the earliest effect on germination, the mediums with the earliest results in protocorm formation were identified as maltose, sucrose and galactose (*Table 2*). Despite the highest protocorm formation period was obtained from Galactose80 with 51.33 days, the difference with the value of 50.83 days obtained from Fructose60 was considered statistically insignificant. The lowest protocorm formation period averages were obtained from Galactose20 as 22.50 days and Glucose20 as 22.83 days (*Fig. 9*).

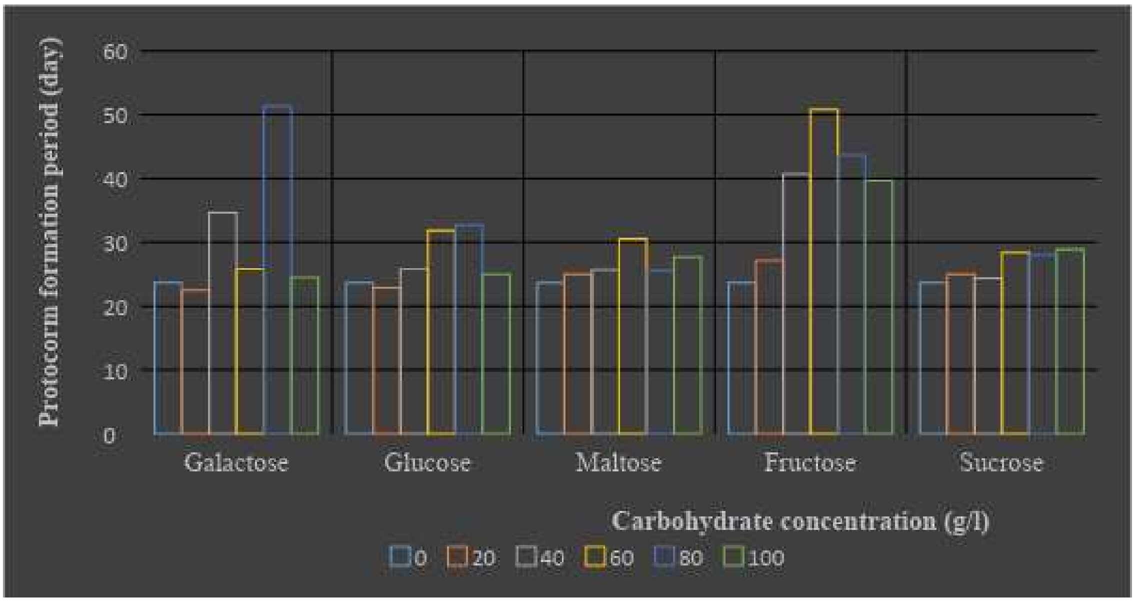

Figure 9. *Effects of carbohydrate forms and concentrations on protocorm formation periods (days)*

Protocorm was obtained in two months in the seeds of *Orchis lutea* and *Orchis fusca*, cultivated in modified Curtis medium (Barroso et al., 1990). Examining the effects of different nutrient mediums, plant growth regulators and light/dark applications on the protocorm formation of *Habenaria macroceratitis* terrestrial orchid, protocorm development was observed in MM medium between 7 and 16 weeks (Stewart and Kane, 2006). In studies utilizing bioreactors, protocorm formation was obtained in 8 weeks (Paek et al., 2001; Young et al., 2000); and in 40 days (Yang et al., 2010) after sowing. In a study, no further development occurred during 8 months after sowing seeds, but protocorm formation was observed after this period by adding 2 mg/l IAA to the nutrient medium (Sarı et al., 2011). As protocorm formation periods ranged between 53.50-309.94 days when all mediums are considered, the periods in VWD medium were 58.50-309.94 days (Çığ and Yılmaz, 2016). In a study to follow the germination and protocorm formation of *Orchis sancta*, which is shown among endangered species, full strength and ½ strength Lindemann (LDM) medium containing 20 g/l sucrose and 7 g/l agar was used and protocorm formation was observed in the 12th week (Acemi et al., 2013).

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Comparing the protocorm formation period of *O. sancta* in this study and the period of the previous one, it is clear that success was achieved in a considerably short time. The protocorm formation range in other studies appears as 40-309.94 days. It is inferred that this period varies in association with the cultivated plant species and medium contents

Shoot development period (day)

The highest shoot development period was obtained from Fructose40 with 105.33 days and Sucrose100 with 104.50 days (*Table 2*). The lowest average shoot development period was obtained from Sucrose40 with 50.66 days (*Fig. 10*). Besides, shoot development was not observed in all the galactose and maltose applications; in 0, 60, 100 g/l doses of glucose; 0, 60, 80 and 100 g/l doses of fructose and 0 g/l dose of sucrose, and therefore, shoot development periods could not be recorded.

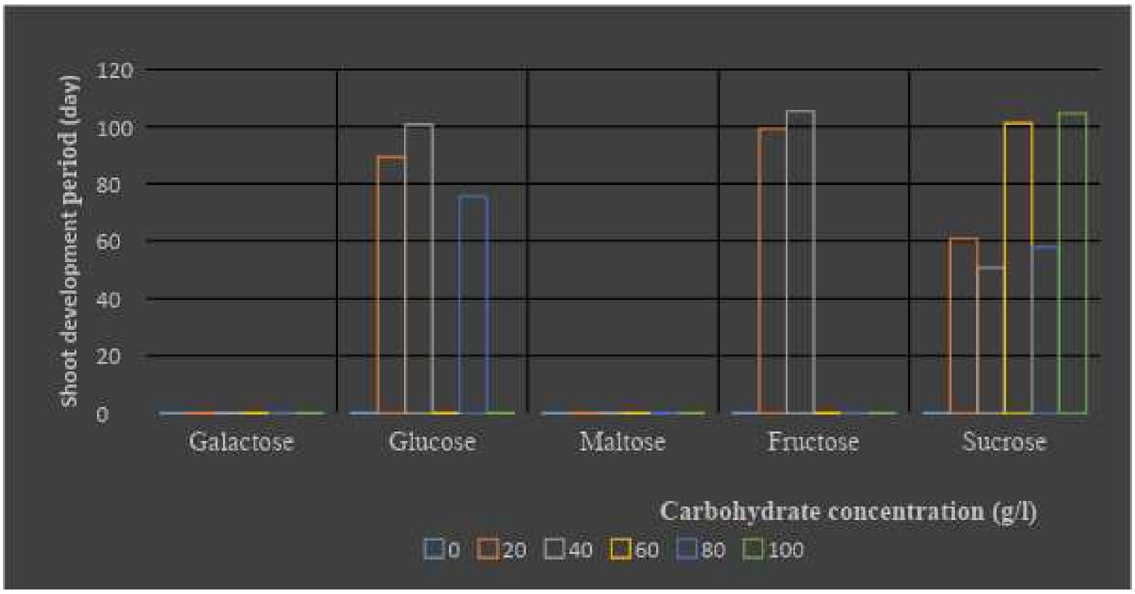

Figure 10. Effects *of carbohydrate forms and concentrations on shoot development periods (day)*

Shoot tips of *Orchis mascula* seeds cultivated in six different nutrient mediums has occurred in 4-5 months (Valetta et al., 2008). The first leaves developed from *Orchis italica* seeds after 2 months (Yararbaş, 2008).

Taking into account the effect of five different culture mediums on shoot development periods, the range of 79.00-406.83 days is noticeable; while the earliest shoot development in VWD medium was 95.33 days, and the latest shoot development emerged in 267.78 days (Çığ and Yılmaz, 2016). In a study testing three orchid species, it was found that sucrose, glucose and fructose were the best energy sources for the *Oeceocladesde caryana* species. It is reported that shoots of *Dactylorhiza majalis* developed slightly weak in fructose, and very weak in glucose (Ponert et al., 2011). These studies indicate that hexoses are not a good carbohydrates source for these species. *Ophrys lojaconoi* exhibited better development in sucrose, weak in glucose, and very week in fructose. Many protocorms have developed better in fructose containing mediums but could not grow further. This proved that fructose has an

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inhibitive effect in the protocorm development. It is reported that sucrose is used more for in vitro orchids but that fructose and glucose are also used (Michl, 1988; Rasmussen, 1995).

Since orchid seeds do not have endosperms, carbohydrate supplement is required to initiate germination. As with the type of carbohydrate, its concentration added in the culture medium is also important. That is because the stimulating role of sugar can be inhibitive in some doses. This study reveals that the sugar types and their concentrations have different effects in germination, protocorm and shoot development phases of *O. sancta* seeds.

Conclusions

As a result, in vitro germination of the *O. sancta* species, the best performances were achieved from maltose, and then sucrose among five sugar types used in the study. Sucrose was the most effective sugar type for protocorm formation. In shoot development, the first sugar with performance was fructose, then sucrose and glucose at last. It is thought that maltose can be used in the germination of *O. sancta* or other orchid species; and sucrose in the protocorm and shoot development. Applications specific for each species as well as general adaptations might be possible. For *O. sancta*, maltose in germination, sucrose in protocorm formation; fructose in shoot development; and as sugar concentrations, doses such as 0, 20 and 40 g/l in germination; 20 and 40 g/l in protocorm; 100 g/l in shoot development can be used. Hence, it is understood that low concentrations for germination and protocorm formation can be used, while high doses should be avoided. The applications in this study to be suggested for the use of sugar and doses together might be: Maltoz40 for germination; Glucose20 and Sucrose20 for protocorm; Furctose20 and Sucrose40 for shoot development.

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EFFECT OF NITROGEN FERTILIZATION LEVELS ON GRAIN YIELD AND YIELD COMPONENTS IN TRITICALE BASED ON AMMI AND GGE BIPLOT ANALYSIS

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Abstract. This study examined the effects of nitrogen doses rate on grain yield (GY) and yield components of spring triticale (×*Tritico secale)* cultivars in dry area, based on cultivars plus cultivar × nitrogen doses interaction GGE biplot and AMMI analysis. The research was designed to evaluate the effects of different nitrogen fertilization levels (0, 40, 80, 120 and 160 kg ha^{-1} of N) on the grain yield and agronomic performance of two cultivars in two growing seasons (2015-16/2016-17). Split plot layout with in randomized complete block design with 3 replications was used in both years. The response to fertilization levels were evaluated through GGE (Genotype main effects and Genotype x Environment interaction) biplot graphic methodologies and regression. Combined analysis of variance of nitrogen applications of two cultivars showed highly significant ($p < 0.01$) difference between the cultivars, nitrogen applications and interaction. The grain yield of triticale was most of all affected by cultivars (70.8%) and then by nitrogen doses (23.5%) and interaction (5.7%). On the other hand, the study showed that plant height, number of spikes per m², number of grain of ears and grain yield increased depending on the increasing nitrogen doses of the cultivars. The highest performance of grain yield of both cultivars were associated with nitrogen N_3 (120 kg/ha) fertilization in regression and AMMI analysis. The results showed that 80 kg/ha N application can be recommended in triticale traits and grain yield with Esin (new) cultivar. Further more, we found that the GGE biplot method generated highly useful results with high visual quality in the study.
Keywords: *triticale, nitrogen doses, yield, yield components*

Introduction

Triticale is a new species in field crops and it is expected to be regarded as a seed in the future as well as being evaluated as forage. It is already produced for seed purposes in many African countries. Especially in dry farming areas, where barley and wheat are not well cultivated, in areas where extreme temperatures are experienced and rainfall is insufficient, triticale can be more economically grown than other cereal crops. In these places, triticale is contribute the several advantages in cropping system because cereal grains can use N, captured in soil profile after lentil or chickpea crops. After lentil and chickpea farming it can use N to accumulate in the soil. In addition, because of the fact that the triticale is abstinent plant, so it is able to get better nitrogen from soil in dry areas compared to wheat and barley. The Syrian border, where the research was carried out, is quite dry and it has become almost impossible to grow wheat without watering. Under these conditions, farmers tend to other cereal crops that may be an alternative to wheat in arid conditions. For this reason, growing triticale is becoming increasingly important (Kendal, 2015; Kendal et al., 2016).

Triticale was designed in order to both biotic and abiotic stressors and thus more suitable for cultivation in marginal areas than wheat (Kendal et al., 2012; Villegas et al., 2010). Triticale is often reported as an interesting product for adverse environmental conditions where environmental factors limit product cultivation (Ugarte et al., 2007;

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Estrada-Campuzano et al., 2008). In such places, each plant does not use the same nitrogen in the same way and the plant cannot make maximum use application amount of nitrogen. Proper N management, are important for obtaining optimum crop yields and positive economic returns while limiting negative environmental impacts of crop production (Gibson et al., 2007). Many authors have reported positive effect of N fertilization on grain yield and the studies showed that N fertilization is one of the most important crop management techniques for spring triticale production (Gülmezoglu, Kinaci, 2004; Lestingi et al., 2010). The weather conditions is affect spring triticale grain yield performance and nitrogen (N) fertilization effect total 35.7% grain yield increase, compared with unfertilized spring triticale (Janušauskaitė, 2013).

Nitrogen is needed for early tiller development of plant to set up the crop for a high yield potential. On the other hand; nitrogen fertilization has an important effect on the final harvest, thus if this element is not take from plant, yield is decreases (Moreno et al., 2003). The amount of nitrogen, barley crop needs to reach maximize yield and quality, will depend on the seasonal conditions, soil type, and rotational history of the soil as well as the potential yield of the cultivars. Nitrogen is the key nutrient input for achieving higher yield of triticale (Alazmani, 2015). The farmers have not enough information about use nitrogen fertilizers and adequate information concerning actual soil requirements. Therefore, the study of use N dozes in triticale cultivars is necessary to recommend optimum nitrogen doses for high yield and yield components in different environment conditions.

The yield of each variety in any environment is a sum of environment (E) main effect, genotype (G) main effect and genotype by environment interaction (GE or GEI) (Farshadfar et al., 2013; Kilic, 2014; Sayar et al., 2013). On the other hand; farmers need varieties that show high performance in terms of yield and other essential agronomic traits by use nitrogen fertilizer. Different statistical analysis, such as correlation, path coefficient and principal component analysis (PCA) can be used to reveal associations between yield and other agronomic traits. The impact of GGE Biplot methods and regression analysis has been clearly showed by different researchers using relationship among factors. This methods; provide the correlative size and interaction (Asfaw et al., 2009; Sayar and Han, 2015; Kendal and Sayar, 2016). So it is very important to identify the use of nitrogen fertilization doses to cultivars for high yield and best quality. The objective of study is to determine the effect of different N rates on yield, yield components, and to identify optimal N deses rate using GGE Biplot analysis to recommend doses for application in Southeast Anatolia Region of Turkey.

Material and methods

The experiment was conducted in the research field of the Kiziltepe Vocational and Training High School, Mardin, Turkey. The experiment was conducted on the basis of split plot layout with completely randomized block design with 3 replications. Main plot was different level of nitrogen fertilizer (0, 40, 80, 120 and 160 kg ha^{-1} of N and sub plot was different two spring triticale cultivars (*Table 1*).

This research was conducted in 2015-2016 and 2016-2017 growing seasons. The seeding rates were 500 seeds m^{-2}. Plot size was 7.2 m² (1.2 × 6 m) consisting of 6 rows spaced 20 cm apart. Sowings were made by using an experimental drill. The fertilization rates for all plots were different N ha^{-1} doses and 60 kg P ha^{-1} with sowing time and different N ha^{-1} doses was applied to plots in double ridge stage. Harvests were

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made using Hege 140 harvester in 6 m². Other normal agronomic practices for barley production were followed. During both of growing seasons, heading time (date), spike per square (m^2), maturity time (date) plant height (cm), length of spike (cm), number of grains per spike, thousand grain weight (g), grain yield (kg/ha^{-1}), hectoliter weight (kg hl), Protein Content (%) were examined (Kendal and Sayar, 2016). The soil analysis results are shown in *Table 2* and the climate data of growing seasons are shown in *Table 3* (Mardin Regional Directorate of Meteorology). Different nitrogen use efficiency (NUE, kg kg^{-1} N) was calculated for each treatment: NUEX Trait = (X trait N – X trait N0) / Nx, where X trait N is X trait from nitrogen (N) fertilized treatments, Xtrait N0 – X trait in unfertilized treatment, and Nx– nitrogen input (N40, N80, N120, N160, kg ha^{-1} N).

Table 1. *Information about cultivars used in the experiment*

Cult. name	Where registration	Regist. time	Type
Tacettinbey	Cukurova University	2000	Spring
Esin	GAP International Agricultural and Training Center	2016	Spring

Table 2. *Soil analysis results of trial*

Texturing (%)			pH	Organic subs. (%)	Total P ppm	Total K ppm	Lime%
Sand	Clay	Mile					
65.6	22.4	12.0	7.5	1.4	22	210	17.15

Table 3. *Information about meteorological data of location of study*

Months	Humidity (%)			Temperature (°C)			Precipitation (mm)		
	2015-16	2016-17	LTA*	2015-16	2016-17	LTA	2015-16	2016-17	LTA
October	49.6	33.3	32.9	19.5	20.5	19.3	58.2	16.5	19.7
November	50.3	35.2	50.2	11.7	11.5	11.2	62.0	27.2	49.1
December	51.7	71.3	47.2	6.4	3.2	4.2	64.7	128.4	58.5
January	74.1	63.6	63.7	2.2	3.1	3.2	146.3	38.3	78.7
February	66.2	51.3	51.0	8.5	3.8	3.9	3.6	23.2	64.4
March	59.1	62.5	62.9	10.0	9.7	9.0	119.8	101.7	99.6
Aprıl	41.3	55.7	55.2	16.8	13.5	15.2	27.1	109.2	98.5
May	42.0	44.0	43.8	19.8	19.7	19.6	20.0	60.3	57.0
June	28.2	26.1	25.8	26.2	26.8	26.0	1.0	0.2	2.2
July	22.4	17.0	16.5	30.6	32.4	32.1	0.1	0.0	0.6
Total							502.8	505.0	528.3
Mean	48.49	46.00	44.9	15.7	14.4	14.3			

LTA: long term average, *Mardin regional directorate of meteorology station (2017)

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The data obtained from the study related to the investigated grain yield and yield components were analyzed respectively for each year and combined with nitrogen doses by using the JMP 5.0.1 statistical software package (SAS Institute, 2002), and the differences between means were compared using a least significant difference (LSD) test at the 0.05 probability level (Steel and Torrie, 1980). Also regression analysis was done by this program. On the other hand; GGE biplot analyses were carried out using GGE biplot software to show the differences among the applied nitrogen doses and crop characteristics and cultivar crop characteristics in two growing seasons (Dogan et al., 2016; Kilic, 2016). GGE biplot analysis also allows comparison amongst nitrogen doses in terms of their discriminating ability and representativeness. These values can be assessed using the discriminating power of the doses' biplot screen of the GGE biplot (Yan and Thinker, 2006). With the AMMI biplot analysis graph in the study: It was aimed at illustrating grain yield performance of N doses and status of triticale cultivars (*Fig. 1b*), grain yield performance of cultivar (*Fig. 1c*), the effect of N doses on grain yield on years (*Fig. 1d*). Also, with the GGE biplot analysis graphs in the study: It was aimed at revealing relation among examined doses and traits (*Fig. 1e*), sector analysis of doses and grouping traits (*Fig. 1f*), ranking of doses on traits (*Fig. 1g*), comparison of doses on traits (*Fig. 1h*)

Results

The combined ANOVA revealed highly significant differences among the years, cultivars and interaction of them for all components ($P < 0.01, 0.05$), the differences among nitrogen doses were highly significant ($P < 0.01, 0.05$) for all components as shown in *Tables 4* and *5*. Generally, breeders interested in the genotypes with high genotypic main effect (average over years and nitrogen doses) and with low fluctuation in yield or yield components (stable). On the other hand, the study showed that plant height, number of spikes per m², number of grain of ears and grain yield increased depending on the increasing nitrogen doses of the cultivars.

The results of the data reviewed

Table 4. *Variance of analysis on grain yield and yield components of triticale*

Sources	DF	HT (date)	SS (m^2)	MT (date)	PH (cm)	SL (cm)	NGS	TGW (g)	GY (kg/ha^{-1})	HL (kg/hl^1)	PC (%)
Year	1	400.4**	1696.0*	589.1**	779.0*	3.3ns	477.7*	0.9ns	522.2ns	123.8*	0.3ns
Error 1	4	0.7	413.9	0.9	36.7	3.5	14.4	9.1	4322.9	4.4	3.6
Cultivar	1	88.8**	380.0ns	26.7**	449.4**	17.1**	492.5**	212.8**	158620.0**	13.1*	1.5*
Year*Cult.	1	4.8**	498.8ns	6.7**	2.5ns	2.4ns	273.5**	28.8**	84.0ns	86.9**	1.3*
Nitr. Doses	4	2.4*	7083.2**	2.5*	2607.4**	58.9**	391.5**	20.4*	52602.6**	33.4*	5.9*
Year*N.Dos	4	1.2ns	5371.2*	1.1*	132.1*	11.6*	69.5*	3.2ns	8718.4**	18.2*	0.5ns
Cul.*N.Dos	4	0.4ns	130.9ns	0.5 ns	57.9ns	10.8ns	47.3ns	40.3**	12757.2**	12.5*	1.1ns
Y*C*ND	4	0.1ns	1247.4ns	0.8ns	120.6*	2.8ns	18.7ns	10.8ns	410.9ns	19.5*	1.2ns
Error 2	36	5.9	6039.5	3.7	267.8	23.1	143.8	49.6	6022.4	40.5	7.5
C. Total	59	504.9	22861.0	632.0	4453.4	133.4	1928.9	376.0	244061.0	352.2	23.0
CV(%)		0.24	9.18	1.03	2.52	7.22	4.37	3.14	3.36	1.37	4.22

HT: Heading Time, SS: Spike ofper Square, MT: Maturity time, PH: Plant Height, LS: Length of Spike, NGS: Number of Grains per Spike, TGW: Thousand Grain Weight, GY: Grain Yield, HL: Hectoliter weight, PC: Protein Content

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***Table 5.** The variance of AMMI analysis on grain yield of nitrogen doses*

Source	Df	SS	MS	F	Explained SS (%)
Total	59	244061	4137		
Treatments	9	223980	24887	70.23	
Cultivar	1	158620	158620	447.65**	70.8
N doses	4	52603	13151	22.26**	23.5
Block	10	5907	591	1.67	
Interactions	4	12757	3189	9.00**	5.7
IPCA1	4	12757	3189	9.00**	
Error	40	14174	354		

Df: degrees of freedom; **: p < 0.01; *: P < 0.05

The mean grain yield (GY) of 2015/16 growing season (3878 kg ha^{-1}) was higher than that of 2016/17 (3819 kg ha^{-1}), the mean yield of growing seasons was changed from 3334 kg ha^{-1} (Esin) to 4362 kg ha^{-1} (Tacettinbey) in *Table 6*.

***Table 6.** The data effect interaction of nitrogen levels and triticale cultivars*

Cultivar		N Doses					Mean		N Doses					Mean
		N_0	N_1	N_2	N_3	N_4			N_0	N_1	N_2	N_3	N_4	
Tacettinbey	HT (date)	163.5	163.8	163.8	163.8	164.2	163.8^{B}	NGS	39.0^{d}	40.9^{cd}	42.6^{c}	46.0^{b}	46.0^{b}	42.8^{B}
Esin		166.0	166.0	166.5	166.3	166.5	166.3^{A}		43.2^{c}	49.5^{a}	50.2^{a}	51.0^{a}	48.8^{a}	48.6^{A}
Mean		164.8^{C}	164.9^{BC}	165.2^{AB}	165.1^{AC}	165.3^{A}			41.1D	45.2^{C}	46.4^{BC}	48.5^{A}	47.4^{AB}	
Tacettinbey	SS (m^2)	125.8	134.0	140.2	137.7	154.8	138.5	TGW	34.9^{cd}	35.9^{cd}	36.0^{c}	35.8^{cd}	34.5^{d}	35.4^{B}
Esin		128.7	136.7	144.0	142.8	165.5	143.5		36.4^{b}	37.2^{b}	37.1^{b}	38.0^{a}	38.2^{a}	39.2^{A}
Mean		127.2^{C}	135.3^{BC}	142.1^{B}	140.3^{B}	160.2^{A}			36.4^{C}	36.9^{BC}	37.3^{A}C	38.0^{A}	37.8^{AB}	
Tacettinbey	MT(date)	30.0	30.2	30.3	30.5	30.7	31.7^{A}	GY(kghl)	2948^{h}	3542^{ef}	3408^{fg}	3485^{f}	3287^{g}	3334^{B}
Esin		31.5	31.5	31.8	31.5	32.0	30.3^{B}		364.8^{e}	4200^{d}	4657^{b}	4847^{a}	4460^{c}	4362^{A}
Mean		30.8^{C}	30.8^{BC}	31.1^{AB}	31.0^{BC}	31.3^{A}			329.8^{D}	3871^{C}	4033^{B}	4166^{A}	3873^{C}	
Tacettinbey	PH (cm)	95.9	100.5	103.5	112.5	114.7	105.4^{B}	HW	74.3^{b}	76.9^{a}	76.9^{a}	77.2^{a}	77.6^{a}	76.6^{B}
Esin		102.1	105.6	112.0	117.5	117.2	110.9^{A}		77.0^{a}	77.6^{a}	77.4^{a}	78.0^{a}	77.7^{a}	77.5^{A}
Mean		99.0^{D}	103.1^{C}	107.7^{B}	115.0^{A}	116.0^{A}			$75.^{6B}$	77.3^{A}	77.2^{A}	77.6^{A}	77.6^{A}	
Tacettinbey	LS (cm)	8.3^{f}	10.2^{e}	10.7^{de}	12.3^{ab}	11.3^{cd}	10.6^{B}	PC (%)	10.5	11.1	10.8	11.5	11.1	11.0^{A}
Esin		10.3^{e}	11.5^{bd}	12.5^{a}	12.2^{bc}	11.7^{bc}	11.6^{A}		10.2	10.3	10.8	11.0	11.0	10.7^{B}
Mean		9.3^{D}	10.8^{C}	11.6^{B}	12.3^{A}	11.5^{B}			10.3^{C}	10.7^{BC}	10.8^{B}	11.3^{A}	11.0^{AB}	

HT: Heading Time, SS: Spike of per Square, MT: Maturity time, PH: Plant Height, LS: Length of Spike,
N Doses – N_0: not applied, N_1: 40 kg/ha, N_2: 80kg/ha, N_3: 120 kg/ha N_4: 160 kg/ha

The yield of application nitrogen doses were ranged from 3298 kg ha^{-1} (N_0) to 4166 kg ha^{-1} (N_3). The yield of cultivar and nitrogen interaction were changed from 2948 kg ha^{-1} to 4847 kg ha^{-1} and the best yield was obtained by N_3 nitrogen doses in Esin, while the low yield obtained from N_0 (without nitrogen) doses and Tacettinbey variety (*Table 7*).

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Table 7. *Influence of different nitrogen levels on yield and yield components of triticale cultivars*

Cultivar		2015-2016					Mean	2016-2017					Mean
		N0	N1	N2	N3	N4		N0	N1	N2	N3	N4	
Tacettinbey	HT (date)	161.0	161.3	161.7	161.7	162.0	161.5^{D}	166	166.3	166	166	166.3	166.1^{B}
Esin		163.0	163.0	163.7	163.7	163.7	163.4^{C}	169	169	169.3	169	169.3	169.1^{A}
N mean		162.0^{c}	162.2^{c}	162.7^{b}	162.7^{b}	162.8^{b}		167.5^{a}	167.7^{a}	167.7^{a}	167.5^{a}	167.8^{a}	
Year mean		162.5^{B}						167.6^{A}					
Tacettinbey	SS (m^2)	121.0	124.7	134.0	139.3	161.3	136.1	130.7	143.3	146.3	136	148.3	140.9
Esin		107.0	110.3	138.7	147.0	173.7	135.3	150.3	163	149.3	138.7	157.3	151.7
N mean		114.0^{d}	117.5^{d}	136.3^{c}	143.2^{bc}	167.5^{a}		140.5^{bc}	153.2^{ab}	147.8^{bc}	137.3^{c}	152.8^{ab}	
Year mean		135.7^{B}						146.3^{A}					
Tacettinbey	MT (date)	27.0	27.0	27.7	28.0	28.0	27.5^{D}	33.0	33.3	33.0	33.0	33.33	33.1^{B}
Esin		28.0	28.0	28.3	28.0	28.7	28.2^{C}	35.0	35.0	35.3	35.0	35.33	35.1^{A}
N mean		27.5^{c}	27.5^{c}	28.0^{b}	28.0^{b}	28.3^{b}		34.0^{a}	34.2^{a}	34.2^{a}	34.0^{a}	34.3^{a}	
Year mean		27.9^{B}						34.1^{A}					
Tacettinbey	PH (cm)	$92.1^{ı}$	$95.3^{hı}$	96.9^{h}	111.6^{df}	112.1^{de}	101.6	99.7^{h}	105.7^{g}	110.0^{eg}	113.3^{ce}	117.3^{bd}	109.2
Esin		96.9^{h}	99.3^{h}	110.9^{ef}	114.3^{be}	116.0^{bd}	107.5	107.3^{fg}	112.0^{de}	113.0^{ce}	120.7^{a}	$118.^{3ab}$	114.27
N mean		94.5^{F}	97.3^{F}	103.9^{E}	112.9^{C}	114.1^{BC}		103.5^{E}	108.8^{D}	111.5^{CD}	$117.^{AB}$	117.8^{A}	
Year mean		104.5^{B}						111.7^{A}					
Tacettinbey	SL (cm)	8.0	9.3	9.7	12.3	11.3	10.1	8.7	11.0	11.7	12.3	11.3	11.0
Esin		9.7	11.0	12.3	13.0	12.0	11.6	11.0	12.0	12.7	11.3	11.3	11.7
N mean		8.8^{F}	10.2^{DE}	11.0^{CD}	12.7^{A}	11.7^{BC}		9.8^{E}	11.5^{BC}	12.2^{AB}	11.8^{AC}	11.3^{BC}	
Year mean		10.9						11.3					
Tacettinbey	NGS	38.6	41.3	41.5	44.6	44.8	42.2^{B}	40.3	44.7	45.0	44.0	43.7	43.5^{B}
Esin		39.4	40.5	43.7	47.3	47.2	43.6^{B}	46.0	54.3	55.3	58.0	54.0	53.5^{A}
N mean		39.5^{e}	43.0^{d}	43.2^{d}	44.3^{d}	44.3^{d}		42.7^{d}	47.4^{c}	49.5^{bc}	52.7^{a}	50.6^{ab}	
Year mean		43.0B						48.5^{A}					
Tacettinbey	TGW (1000)	36.1	36.6	36.9	35.9	34.3	36.0^{C}	33.6	35.1	35.1	35.6	34.7	34.8^{D}
Esin		36.6	36.5	38.3	39.8	40.5	38.3^{B}	39.2	39.3	39.1	40.5	41.8	40.0^{A}
N mean		36.4	36.6	37.6	37.9	37.4		36.4	37.2	37.1	38.0	38.2	
Year mean		37.2						37.4					
Tacettinbey	GY (kgha)	3087	3773	3403	3410	3203	3375^{B}	2810	3310	3413	3560	3370	3293^{B}
Esin		3667	4437	4687	4760	4350	4380^{A}	3630	3963	4627	4933	4570	4345^{A}
N mean		3377^{D}	4105^{AB}	4045^{B}	4085^{B}	3777^{C}		3220^{E}	3637^{C}	4020^{B}	4247^{A}	3970^{B}	
Year mean		3878						3819					
Tacettinbey	HW (kghl)	75.4^{df}	76.7^{ce}	76.8ce	75.2^{ef}	77.6^{be}	76.3^{B}	73.1^{g}	77.1^{cd}	77.1^{cd}	79.3^{cd}	77.5^{ab}	76.8^{B}
Esin		74.3^{fg}	74.5^{fg}	74.3fg	75.5^{df}	75.7^{df}	74.9^{C}	79.6^{a}	80.7^{a}	80.4^{a}	80.5^{a}	79.6^{a}	80.2^{A}
N mean		74.9^{E}	75.6^{CE}	75.6^{CE}	75.3^{DE}	76.7^{C}		76.3^{CD}	78.9^{AB}	78.8^{AB}	79.9^{A}	78.6^{B}	
Year mean		75.6^{B}						78.5^{A}					
Tacettinbey	PC (%)	10.2	10.8	10.6	11.4	10.9	10.8^{B}	10.7	11.3	11.0	11.6	11.4	11.2^{A}
Esin		10.6	10.5	10.6	10.9	11.1	10.7^{B}	9.8	10.1	11.1	11.1	10.8	10.6^{B}
N mean		10.4	10.7	10.6	11.1	11.0		10.3	10.7	11.0	11.4	11.1	
Year mean		10.8						10.9					

NGS: Number of Grains per Spike, TGW: Thousand Grain Weight, GY: Grain Yield, HL: Hectoliter Weight, PC: Protein Content. N Doses – N_0: not applied, N_1: 40 kg/ha, N_2: 80 kg/ha, N_3: 120 kg/ha, N_4: 160 kg/ha

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The results of grain yield showed that first growing season had high yield than the second year and Esin variety was yielding than Tacettinbey during two grooving seasons. The results of grain yield showed that first growing season had higher yield than the second year and Esin variety changed from 4760 kg/hl to 4933 kg/hl (N_3-2015/16-2016/17) had a higher yield Tacettinbey (2810-3087 kg/hl, N_0) during two growing seasons in *Table 7*. Nitrogen use efficiency (NUE) was considerably influenced by cultivars, using N doses and growing seasons. The main factor determining the differences in NUE yield of cultivars was changed from13.1% (Tacettinbey) to 19.6% (Esin) (*Table 7*). The influence of growing season on NUE_{yield} was changed from 21.6(2015-16) to 23.3% (2016-17). The highest NUE_{yield} of interaction was obtained from N_3 fertilization rate (35.9%) in 2016-17 growing season. As expected, depend on increasing basic N fertilization rate increased NUE_{yield}. Each N rate increasing by 40 kg increased NUE_{yield} by 2.5 kg kg^{-1}.

The mean thousand grain weight (TGW) of 2016/17 growing season (37.4 g) was high than 2015/16 (37.2 g), the mean thousand grain weight of cultivars of both years was changed from 34.8 g (Tacettinbey) to 40.0 g (Esin) in *Table 6*. The number of thousand grain weight of nitrogen doses and cultivar interaction of both year means were changed from 36.4 g (N_0) to 38.2 g (N_{4-} 2016/17). The application nitrogen doses of thousand grain weight were ranged from 36.4 g (N_0) to 38.0 g (N_3) in *Table 7*. The thousand grain weight was increased depend on nitrogen rate till N_3 application doses for both varieties.

The mean hectoliter weight (HW) of 2016/17 growing season (78.5 kg/hl) was high than 2015/16 (75.6 kg/hl), the mean hectoliter weight of cultivars of both years was changed from 76.8 kg/hl (Tacettinbey) to 80.2 kg/hl (Esin) in *Table 6*. The number of hectoliter weight of nitrogen doses and cultivar interaction of both year means were changed from 74.9 kg/hl (N_0-2015/16) to 79.9 g (N_3-2016/17). The application nitrogen doses of hectoliter weight were ranged from 75.6 kg/hl (N_0) to 77.6 kg/hl (N_3 and N_4) in *Table 7*. The hectoliter weight was increased depend on nitrogen rate till N_4 doses for both varieties. N3 and N4 doses had the same values, so the N_3 dose application is better for hectoliter weight in tiriticale.

The mean protein content (PC) of 2016/17 growing season (10.9%) was higher than 2015/16 (10.8%), the mean hectoliter weight of cultivars of both years was changed from 11.2% (Tacettinbey) to 10.6% (Esin) in *Table 6*. The number of protein content of nitrogen doses and cultivar interaction of both year means were changed from 10.3% (N_0-2016/17) to 11.4% (N_3-2016/17). The application nitrogen doses of protein content were ranged from 10.3% (N_0) to 11.3% (N_3) in *Table 7*. The protein content was increased depend on nitrogen rate till N_3 doses for both varieties. Nitrogen use efficiency (NUE) of protein content influenced by cultivars, using N doses and growing seasons. The main factor determining the differences in $NUE_{protein}$ of cultivars was changed from 5.3% (Tacettinbey) to 4.2% (Esin) (*Table 7*). The influence of growing season on $NUE_{protein}$ was changed from 4% (2015-16) to 7% (2016-17). The highest $NUE_{protein}$ of interaction was obtained from N3 fertilization rate (10.7%) in 2016-17 growing season. As expected, depend on increasing basic N fertilization rate increased $NUE_{protein}$. Each N rate increasing by 40 kg increased $NUE_{protein}$ by 1.5-2.5 kg kg^{-1} till N3 fertilization rate

The heading time (HT) was calculated from 01 January to heading time as day for both varieties separately in *Table 7*. The maturity time (MT) was calculated from heading time to maturity time as day for both varieties separately. On the other hand,

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ear per square (SS), plant height (PH), long of spike (LE), number of grain per spike (NGS) were recorded and results were showed in *Table 7*. The results showed that there is positive correlation between heading time and maturity time, and depend on N application doses heading time and maturity time are delayed. The cultivars are affected the same on application doses. On the other hand the study indicated the ear per square (SS) and grain per spike (NGS) are contributing the grain yield. Moreover, all traits results (HT, MT, LS, NGS and SS) were increased depend on nitrogen rate till N3 doses for varieties, years and interaction.

AMMI and GGE biplot analysis

Analysis of variance for nitrogen doses (ND) component (C), and the cultivar (C) × component (C) interaction showed significant ($P < 0.01$, $P < 0.01$) effect, and the total sum of squares explained for 96.15%, with PC1 and PC2 accounting 85.03% and 11.12% for nitrogen doses (ND) component (C) (*Fig. 1e-h*), Moreover, the AMMI analysis showed that the grain yield of triticale was most of all affected by cultivars (70.8%) and then by nitrogen doses (23.5%) and interaction (5.7%).

AMMI Analysis was done on grain yield for interaction nitrogen doses, cultivars and years (*Fig. 1b-e*). The results of interaction nitrogen doses and cultivars of grain yield (*Fig. 1b*) showed that grain yield is increased depend on nitrogen doses (*Fig. 1a*) till N_3 application doses, the contribute of N_1 and N_4 to grain yield of triticale is the same, but N_3 application dose is contribute to grain yield in highest level. The interaction of N doses and years showed that the highest grain yield was obtained from the first year (*Fig. 1c*), and the effect of N_3 doses on grain yield was highest than other N doses. The interaction of cultivars and years on grain yield indicated that first year (2015/16) is yielding than the second year (2016/17) and Esin variety is yielding than Tacettinbey variety for both years (*Fig. 1d*).

GGE biplot analysis shows the means over years for nitrogen doses relationships among yield components (*Fig. 1e-h*), the results of relationship between N doses and yield components showed that there is high correlation among components and N application doses (*Fig. 1e*). The figures show that there is high correlation between N_2 application dose and all yield components, while there is high correlation between N_3 doses and GY, LS, NGS, HW TGW and high correlation between N_4 and MT, PC, HT, PH and there is not any correlation among N_0, N_1 and yield components. The sector analysis of N doses and grouping of traits showed in *Fig. 1f,* the results indicated that four sectors occurred on the figure and MT, PC, HT, PH took places in first group in first sector with N_4, and GY, LS, NGS, HW TGW took places in second group in second sector with N_3, while N_2 took places in center of first and second sector, so this dose took places on center of all components. On the other hand N_0 and N_1 located in different sector and opposite of component group, so did not related with any component. The ranking biplot of N doses on components showed in *Fig. 1g,* and the figure indicated that N_2 dose is stable for all components, because N_2 dose located center of stable line with locate above mean of components line, while N_2 doses located side of GY, LS, NGS, HW TGW and desirable for these components, and N_2 desirable for MT, PC, HT, PH. On the other hand, N_0 and N_1 located in under mean of component line, so these doses are undesirable for all components. The comparison biplot of N doses on components showed in *Fig. 1h,* and the figure indicated that N_2 dose is stable for all components, because N2 dose located on ideal enter line, while N_3 doses located side of GY, LS, NGS, HW TGW and desirable for these components, and N_4 desirable

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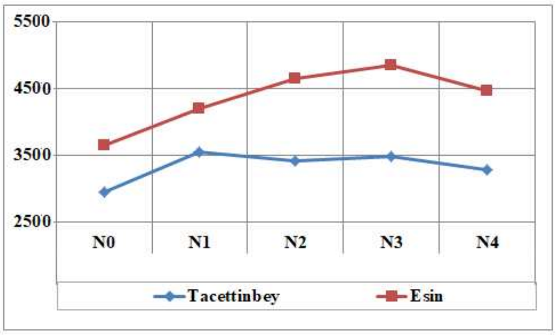
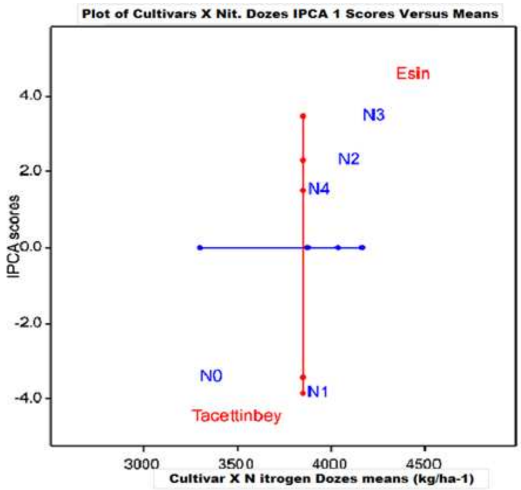
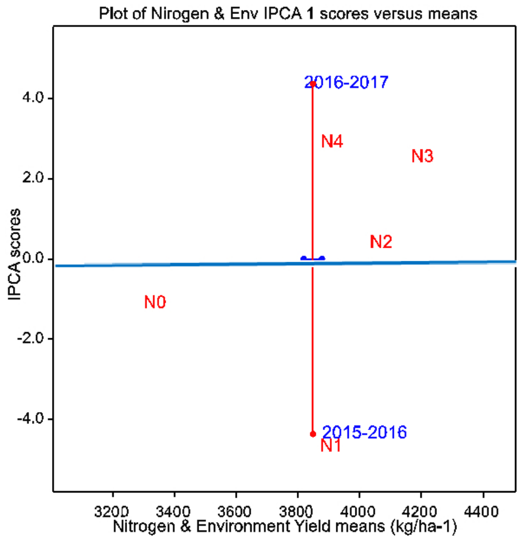
for MT, PC, HT, PH. On the other hand, N_0 and N_1 located in under mean of component line, so these doses are undesirable for all components.



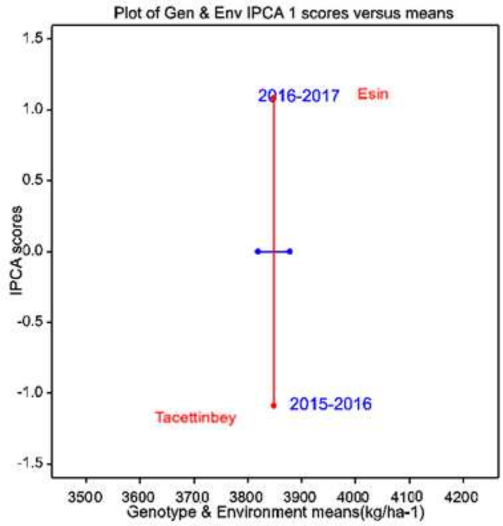
a



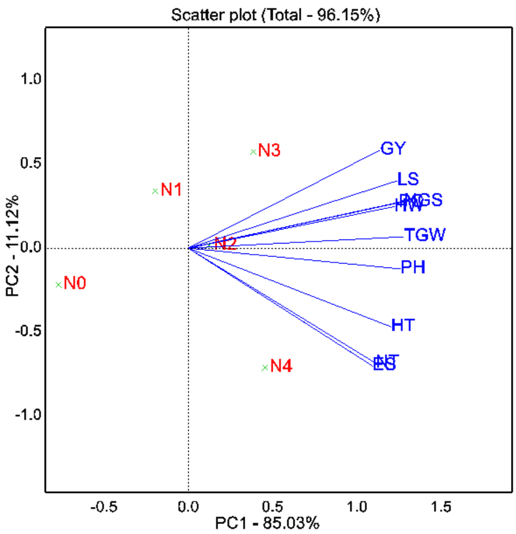
b



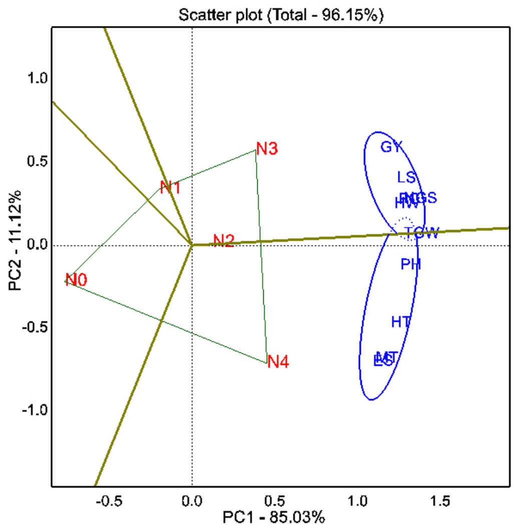
c



d



e



f

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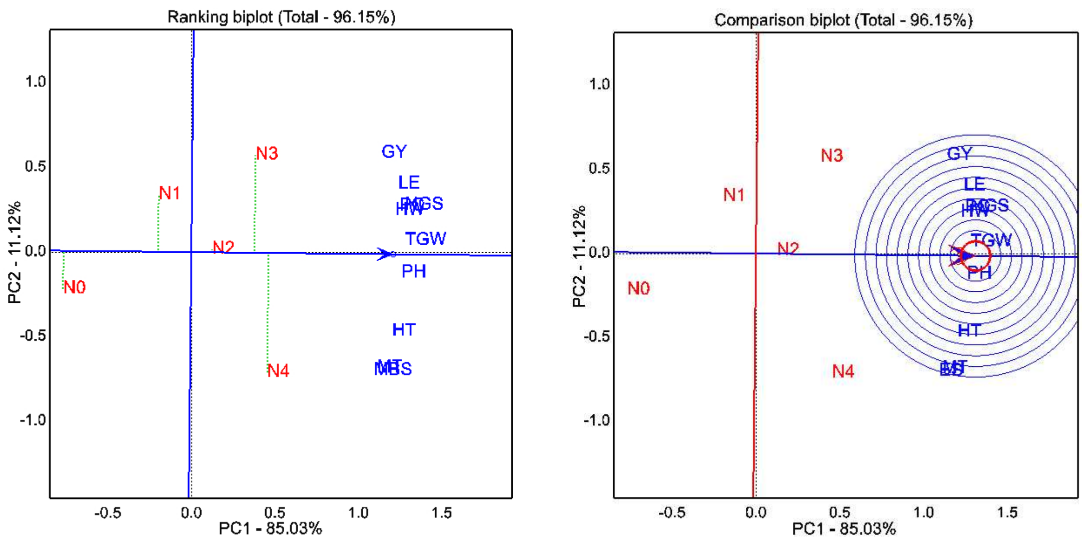

g h

***Figure 1. a.** Regression analysis of grain yield (kg/ha) of cultivar and nitrogen doses. **b.** AMMI analysis of N doses and cultivars on grain yield. **c.** AMMI analysis on grain yield of cultivar. **d.** AMMI analysis of N doses of years. **e.** The relation between N doses and traits. **f.** The sector analysis of N doses and grouping of traits. **g.** Ranking of N doses on traits. **h.** Comparison of N doses on traits*

Discussion

Analysis of variance for grain yield and yield components showed that nitrogen dose, year and cultivar was the main independent factor determining the differences in values of parameters between N doses and results. The significant differences ($P < 0.001$, $P < 0.005$) found of the yield and yield components and the grain yield of triticale was most of all affected by cultivars (70.8%) and then by nitrogen doses (23.5%) and interaction (5.7%) in *Table 5,* therefore the effect of nitrogen doses gives a lead to high variable outputs yield and all its components every year. These results are accepted by Moreno et al. (2003), which proceeds that the N fertilizer highly depends on growing season's variations conditioned by environmental factors. Climatic data of both growing season were indicated that the season of 2015/16 had favorable climate conditions for grain yield, without more cold in winter and good rainfall in planting time (October, November) for early germination and for grain filling time occurs (April, May). On the other hand, the climate conditions for all components without grain yield were good in 2016/17 growing season.

According to results of the study, the application of nitrogen doses had positive effect on yield and yield components. The results increased in parallel with dose increase for both cultivars for all characters. Nitrogen application had positive influence on all the yield components (Fallahi et al., 2008). The best grain yield and majority components results were obtained with N_3 nitrogen dosing. Moreover, N_4 and N_2 nitrogen dosing can be advice for some special components depend on climatic data of seasons. The results of nitrogen use efficiency of the present study agree with the findings of Janušauskaitė (2013) who reported that nitrogen use efficiency for grain yield and protein content as affected by nitrogen rate and its splitting in spring triticale and Yildirim et al. (2007) the findings suggest that advanced breeding lines should be selected at different N levels for better N use efficiency and genetic investigations

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should be conducted in multiple environments and Alaru et al. (2009) who reported that yield values are under the influence of application of N fertilization. There are more N rate application studies on components in different crop as well as triticale which agree in present study; Hadi et al. (2012), increase in number of time to spike by increasing N rate and it might be attributed to the increase in long time filling grain (Gursoy, 2011; Shafi et al., 2011). Hadi et al. (2012), increase SS m-2 by increasing N rate and it might be attributed to increase the time of number spike, Hadi et al. (2012), increase in PH by increasing N rate might be attributed to the increase in vegetative of plant. The increase SL depend on increasing N rate and available environmental in barley and wheat by researchers (Gursoy, 2011; Shafi et al., 2011). Subhan et al. (2004) and Shafi et al. (2011), increase NGS by increasing N rate and it might be attributed to increase the time grain occurs. Fallahi et al. (2008) Alazmani (2015), Yildirim et al. (2016), increasing N rate and it might be attributed to increase the grains per spike, and this is increasing GY.

GGE biplot analysis

The GGE biplot mainly allows the visualization of any crossover GE interaction, which is very important for the breeding program (Sayar, 2017; Kendal, 2015). The GGE biplot method has been widely used to analyze the stability and performance of the genotypes for yield and other components (Goyal et al., 2011; Tekdal and Kendal, 2017). Moreover, GEI and yield stability analyses are important for their consideration of both varietal stability and suitability for cultivation across seasons and ecological circumstances. The GT (genotype-trait) biplot provides an excellent tool for visualizing genotype × trait data (Kendal, 2015).

The AMMI analysis shows good visualization of interaction among nitrogen application doses and cultivars (*Fig. 1b*), among doses and years (*Fig. 1c*), among years and cultivars (*Fig. 1d*). The AMMI method provides considerable flexibility, allowing plant breeders to simultaneously select for yield and stability (Kendal and Sener, 2015).

The GGE biplot could be used to interpret the relationships among nitrogen doses, components, and groups of component (*Fig. 1e*). An understanding of the relationship between doses and components can aid in better understanding doses objectives and in identifying components that are positively or negatively correlated with nitrogen doses. This understanding can also aid in identifying components that can be indirectly selected by selecting for correlated components. It also helps to visualize the strengths and weaknesses of nitrogen doses, which is important for application in barley. If the angle of the vector was less than 90°, there was a positive correlation two observation factors. If the angle was equal to 90°, they were not correlated. There was a negative correlation if the angle was less than 90° (Yan and Thinker, 2006; Dogan et al., 2107). On the other hand, all components took place in two different groups, with N_2, N_3 and N4 doses (*Fig. 1f*). There is high correlation which is took places in same group (Kendal et al., 2016). Moreover, the GGE biplot was accurate in interpreting the ranking and comparing N doses and components (*Fig. 1g*). The doses with both high mean performance and high stability for all of the components were called as table dose (N_2). The dose located side of some components, it means that this dose can be advise for these components (N_3 an N_4). The center of the concentric circles (i.e., ideal dose) was the AEA in the positive direction. In the comparison biplot doses located closer to the ideal dose were more desirable than others (Yan and Tinker, 2006; Benin et al., 2012; Dogan et al., 2016). The result demonstrated that N_2 was ideal application dose in the

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both season for all components, as it was in the center circle for the ideal doses and on the AEA (*Fig. 1h*).

Conclusions

1. In the present study nitrogen application doses had positive effect on grain yield and yield components, and the values of component and grain yield increased in parallel with application nitrogen doses.

2. The nitrogen dose of N3 (120 kg ha^{-1}) are responsible for the maximum productivity of triticale crop in Mardin province environmental conditions. Esin variety showed that it is best cultivar for all components and grain yield except PC.

3. On the other hand; AMMI and GGE biplot analysis revealed that this analysis provided useful results and high image quality to show the correlation among doses, cultivars and components.

4. The results of study showed that the N_3 application of doses in triticale should be recommend for next studies both farm and research area.

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COMPARATIVE SUSCEPTIBILITY OF SOME COMMERCIAL POTATO CULTIVARS TO *FUSARIUM SAMBUCINUM* AND *F. SOLANI* ISOLATES CAUSING TUBER DRY ROT

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Abstract. *Fusarium dry* rot is one of the most important diseases of potato (*Solanum tuberosum* L.), affecting the tubers in storage and the seed pieces after planting. *Fusarium sambucinum* and *F. solani* are common pathogens causing dry rot of stored tubers in temperate areas. In this study, infection of *F. sambucinum* and *F. solani* on tissue discs prepared from tubers of potato varieties that are susceptible or moderately resistant to this disease was studied. Tubers were wounded, inoculated, incubated at 15-20 °C for 5 weeks and the size of the rot was assessed. All isolates were found pathogenic to potato tubers and differed in pathogenicity. Obtained results revealed that *Fusarium* isolates showed variable aggressivity upon the 17 tested potato cultivars. Furthermore, no cultivars were found to be completely resistant to the whole *Fusarium* isolates, and only one cultivar showed a lesser susceptibility to pathogen. This cultivar was Broke®. Moreover, *F. sambucinum* isolates were detected as more aggressive pathogens than *F. solani* in all cultivars. Additionally, one out of three isolates of *F. sambucinum* was more aggressive than the others. All isolates used in this study were identified both based on colony and conodial morphology and also confirmed by molecular methods.
Keywords: *Solanum tuberosum, Fusarium spp., dry rot, assesment, aggressivity, molecular detection*

Introduction

Potato (*Solanum tuberosum* L.) is among the top five crops growing world wide following cereals wheat, rice, corn and barley due to its high carbohydrate content and adaptability. It is an important source of calorie, protein and fat for humans, industrial raw material for starch and alcohol production and also feed for animals. Global annual production of potato in 2016 was about 376.827.000 tonnes (FAOSTAT, 2017). The annual yield loss of potato crop caused by insects, weed and diseases in the developing countries such as Turkey is determined to be 32.4%. Diseases alone are responsible for 21.8% of yield reduction (Eken et al., 2000). Losses associated with dry rot have been estimated to range from 6% to 25%, and occasionally losses as great as 60% have been reported during long-term storage (Estrada et al., 2010; Secor and Salas, 2001). Dry rot is caused by a number of *Fusarium* species affecting sprouting and emergence at the beginning of the season, which results in yield loss and damage to the quality of daughter tubers, especially during storage (Corsini and Pavek., 1986; Hooker, 1981; Al-Mughrabi, 2010; Borca and Carmen., 2013). Furthermore, there may be potential reductions due to other reasons, such as bacterial soft rot. *Fusarium* species are known to cause dry rot, particularly *F. sambucinum* and *F. solani* are the most common pathogens. To establish strategies for the control of this disease, the primary steps would be to make a correct diagnosis and identify the pathogen on potato. In recent

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years, the increasing use of molecular methods in fungal diagnostics enabled a reliable and rapid identification. The most important method of control against this disease is to grow resistant varieties. But potato cultivars vary in their degree of resistance to *F. sambucinum* and other *Fusarium* species

The objectives of this study were: (1) to identify the *F. solani* and *F. sambucinum* isolates obtained from potato dry rot, (2) to identify the virulence of these isolates by comparison with each other, and finally (3) to test some commercial potato varieties for their tolerance to *F. sambicunum* and *F. solani* isolates causing potato dry rot in Turkey.

Review of literature

F. sambucinum Fuckel *(F. sulphureum* Schlecht)- teleomorph *Giberella pulicaris* (Fr.) Sacc.- is the most common pathogen worldwide causing dry rot of stored tubers, but other *Fusarium* species are also known to cause dry rot, particularly *F. solani* (Mart.) Sacc*., F. avenaceum* (Fr.) Sacc. *F. oxysporum* Schltdl., *F. culmorum* (W.G. Sm.) Sacc*.,* and *F. graminearum* Schwabe *(*Boyd, 1972*;* Nelson et al., 1981; Hanson et al., 1996; Eken et al., 2000; Borca and Carmen, 2013; Stefańczyk et al., 2016). Among these fungi, *F. sambucinum* is the most aggressive species both in the world and in Turkey (Boyd, 1972; Hide et al.1992; Hanson et al., 1996; Secor and Salas, 2001; Eken et al., 2000; Du et al., 2012; Aydın et al., 2016). However, some researchers have suggested that other important *Fusarium* species may cause the dry rot. For example, *F. oxysporum* was reported to be the primary agent responsible for potato dry rot among 11 species in Michigan, USA (Gachango et al., 2012). According to Choiseul et al. (2007), *F. avenaceum* was found to dominate pathogens in *Fusarium* population that was examined on potato between 1997 and 2000 in Scotland. Three significant *Fusarium* species, namely *F. coeruleum*, *F. sambucinum* and *F. solani,* have been reported to principally cause the potato dry rot under temperate conditions (Corsini and Pavek, 1986; Daami-Remadi et al., 2006; Daami-Remadi et al., 2012). Isolated outbreaks of disease have been caused by highly pathogenic species and *F. sambucinum* in Scotland was associated with post-storage rotting (Cullen et al., 2005). In addition to destroying tuber tissues, *F. sambucinum* can produce toxins that have been implicated in mycotoxicoses of the human beings and animals (Richardson and Hamilton, 1987; Senter et al., 1991; Schisler et al., 1997; Sveeney and Dobson, 1999).

Potato cultivars vary in their degree of resistance to *F. sambucinum* and other *Fusarium* species (Jellis, 1975; Hooker, 1981; Jellis and Starling, 1983; Wastie et al., 1989). All of the commonly grown potato cultivars in North America are susceptible to the pathogen, but some are less susceptible than the others (Tivoli et al., 1986).

Sources of resistance to pathogens in potato tubers are given in some literature. In resistant tissues lesion enlargement is confined to the infection spot, which might be due to the suberin deposition in the host cells. It involves formation of lignin, suberin, waxes or wound gums in the immediate cells next to the wounded surface. However, further studies imply that formation of lignin and suberin components of periderm may actively be involved in the defense of pathogens. Independent genetic factors are involved in controlling resistance against major *Fusarium* species on potato (Vance et al., 1980; Wastie et al., 1989; Huaman et al., 1989; Valluru et al., 2016). According to Daami-Daami-Remadi et al. (2006) some *Fusarium* species indicated unstable aggressivity upon some of the tested potato cultivars. *F. graminearum* was the most aggressive pathogen on majority of the cultivars, whereas *F. sambucinum*, *F. solani* and *F.*

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oxysporum showed a comparable aggressivity on some cultivars used. All inoculated tubers showed dry rot symptoms with different degrees, which revealed that cultivars' resistance differed against *Fusarium* species (Daami-Remadi et al., 2006). *F. sambucinum* was found to be the most aggressive species in a study conducted by Du et al., 2012 in Chine and according to that study, 56% of the isolates belonged to these species and Sixty-seven clones were identified as susceptible to *F. sambucinum*. Another survey was carried out in Central Europe by Latus-Ziętkiewicz et al. (1987). According to this study, *F. sambucinum* was the main pathogen causing dry rot in Poland between 1985 and 1986.

To establish strategies for the control of this disease, the primary steps would be to make a correct diagnosis and identify the pathogen on potato. *(*Borca and Carmen, 2013; Aydın et al., 2016). It is primarily based on cultural and morphological characters such as the microscopic morphology including shape and size of the macroconidia, the presence or the absence of the microconidia and of the chlamydospores (Gerlach and Nirenberg, 1982; Burgess et al., 1994; Nelson et al., 1983). But in recent years, the increasing use of molecular methods in fungal diagnostics enabled a reliable and rapid identification. These methods are based on the PCR amplification of species-specific DNA fragments using fluorescent oligonucleotide primers, which were designed based on sequence divergence within the internal transcribed spacer region of nuclear ribosomal DNA sequences. This method provided an accurate, reliable and quick diagnosis of *Fusarium* species including *F. sambucinum* (Mishra et al., 2003; Visentin et al., 2009; Powel et al., 1996; Abd-Elsalam et al., 2003; Oechsler et al., 2009; Wang et al., 2011).

Material and methods

Potato cultivars

Potato cultivars tested in this study are listed in *Table 1*. They are provided by the various agricultural companies and they are stored in darkness at 4 °C until the experiments were carried out.

Microorganisms and identification

The strains of *F. sambucinum* (Fs2, Fs3 and Fs4) and *F. solani* (Fs1) are used in this study were taken infected potatoes from Afyon potato production area in the autumn season. They were isolated from the infected potato tubers of cv. Lady Rosetta with typical symptoms of dry rot and it was previously reported to be virulent on potato tubers (Aydın et al., 2016). The strains were maintained on potato dextrose agar (PDA, 38 g and sterile water, made up to 1 litre). Before starting work, pure tubers were infected with isolates and re-isolated. Thus, the isolates were maintained in virulence. The single spore technique was used to obtain a pure culture of *Fusarium* isolates. The color of colonies of strains obtained ranged from white to moderate pink. Isolates were stored in the tube at +4 °C until it starts to work. Synthetic Nutrient Agar (SNA, Difco), Potato Dextrose Agar (PDA, Merck) and 1/10 diluted PDA media were used for morphological and microscopic identification. All *Fusarium* isolates were initially identified according to their morphological and microscopic characters as described by Booth (1977), Gerlach and Nirenberg (1982), Nelson et al. (1983), Hasenekoğlu (1991), Burgess et al. (1994), Leslie and Summmerell (2006), Borca and Carmen (2013). And

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then, identification of the isolates was further confirmed by molecular approach. Processes related to pathogen isolation and identification are given in *Figure 1.*

Molecular identification of Fusarium isolates

DNA isolation, PCR condition and phylogenetic analysis

Fungal Genomic DNA was extracted from mycelium by using the methodology as proposed by Doyle (1987). DNA quality was checked on 1% agarose gel, and then quantification was measured by using the Nanodrop (Thermo Scientific). To achieve the amplification of ITS4-ITS5 region of nuclear genome, PCR was performed in 25 µl volume. This volume occurred from 80 ng of total genomic DNA, 10 pmol both primers, 200 µM dNTP, 2 mM $MgCI_2$, 1X Taq buffer, 1U of Taq Polymerase (5 U/µL, catalog number: EP0402) and ddH_2O for complete the last volume. 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 ITS4-ITS5 region were sequenced by Iontek Company, Istanbul, Turkey.

Alignment and phylogenetic analysis

The obtained sequences were Blasted (basic local alignment search tool) by using the NCBI (National Center for Biotechnology Information) database and percent homology scores were assessed to identify *Fusarium* spp. Phylogenetic trees were made with MEGA version 7 (Tamura et al., 2007). Using a neighbor-joining algorithm, bootstrap analyses for 1000 replicates were performed.

Table 1. *Characteristics of potato cultivars tested for their tolerance to F. sambucinum and F. solani isolates causing potato dry rot in Turkey*

Cultivars*	**Tuber shape**	**Skin color**	**Flesh color**	**Dry matter (%)**	**Usage**
Marabel	Oblong	Yellow	White	19.2	Cooking
Madeleine	Round-oval	Yellow	Yellow	18.2	Cooking
Hermes	Round-oval	Yellow	Ochre	28.4	Crisps
Opal	Round oval	Yellow	Light yellow	Medium	Crisps
Brooke	Round oval	Yellow	Medium yellow	----	Crisps
Lady Claire	Round	Thin yellow	Light yellow	23.8	Crisps
Musica	Long-oval	Light yellow	Yellow	19.7	Cooking
Orchestra	Round oval	Light yellow	Light yellow	17.7	Cooking
Melody	Oval	Yellow	Medium yellow	20.5	Cooking
Vr. 808	Round	Yellow	Light yellow	25.1	Crisps
Lady Rosetta	Round	Red	Light yellow	24.9	Crisps
Desiree	Smooth	Red	Creamy white	21.7	Cooking
Surya	Oblong	White	Pale yellow	----	Crips
Alonso	Round	Yellow	Light yellow	----	-----
Alegria	Oval	Bright-yellow	Light yellow	20.5	Crips
Borwina	Round oval	Bright yellow	Yellow	20.5	Cooking
Soraya	Round	Yellow	Light yellow	----	Cooking

*These potato varieties are grown and registered in Turkey

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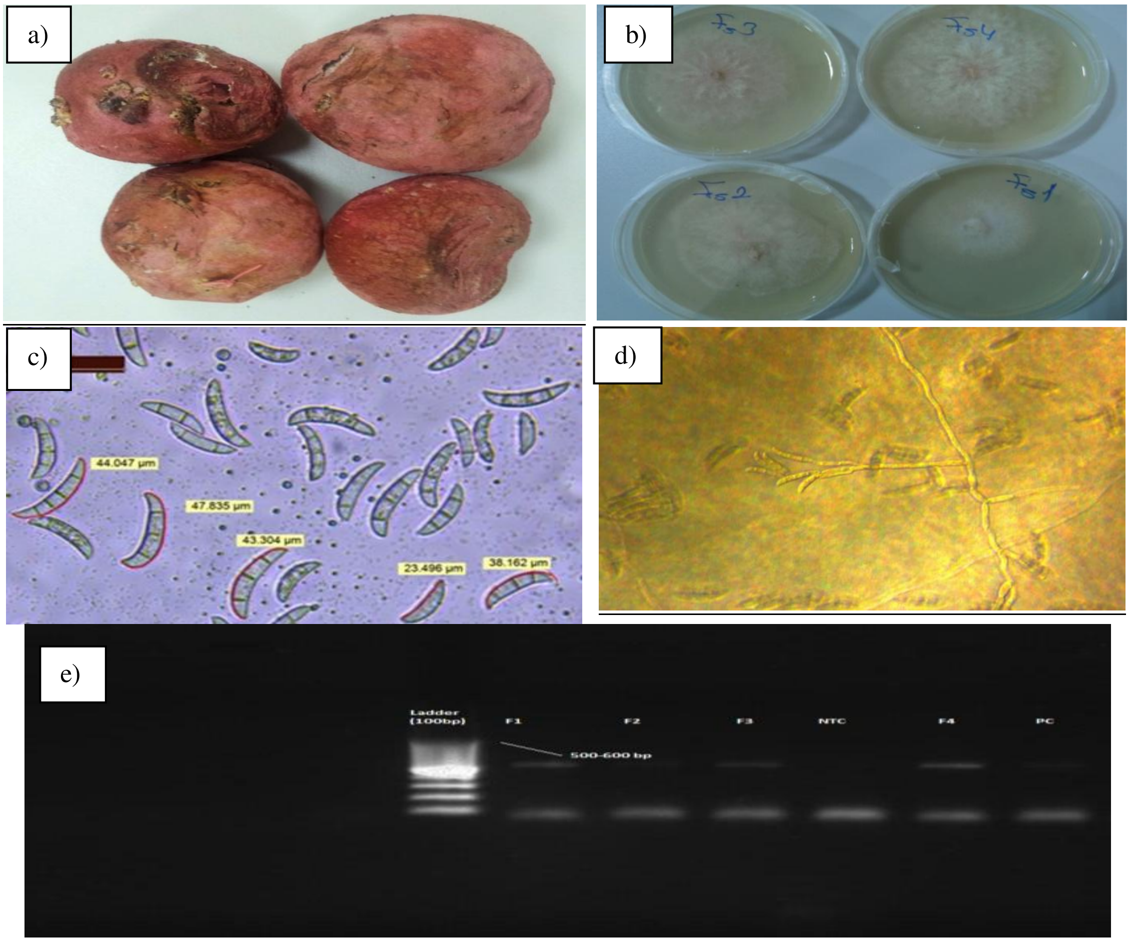

Figure 1. *Isolation and identification of Fusarium isolates. (a) Infected potato tuber by dry rot. (b) Culture purification of Fs isolates. (c) Macrocondia of Fs (d) Microconidia production by Fs. (e) molecular identification of Fs by species specific primer (NTC: No template control, PC: Positive control)*

Evaluation of dry rot susceptibility to F. sambucinum and F. solani

A total of 17 potato cultivars (*Table 1*) were selected to test their resistance to *F. sambucinum* (Fs1, Fs2, Fs3) and *F. solani* (Fs1). Symptom-free tubers of dry rot and other diseases were selected for the experiments and weighed from 80 to 100 g. They were washed in running tap water, dipped in sodium hypochlorite (5%) for 5 min, rinsed twice with double distilled sterile water and air-dried for 24 h. Potato tubers were wounded through inward with a drill 8 mm in diameter and 8 mm in depth. An 8 mm agar plug derived from the edge of a 7-day-old fungi colony on PDA was inserted into the drilled tuber hole. A control treatment consisting of a non-inoculated agar plug was inserted into the wounded tuber. The experiment was set up at room temperature of 15-20 °C in separate plastic boxes with sufficient relative humidity for five weeks. Every treatment was repeated for four times (one tuber x one wound). Treatments applied to seed pieces were: (1) not inoculated, (2) inoculated with *F. sambucinum* (Fs2); (3) inoculated with *F. sambucinum* (Fs3), (4) inoculated with *F. sambucinum* (Fs4) and (5) inoculated with *F. solani* (Fs1). After incubation period, tubers were cut through the inoculation site and the depth and width of the rot area were measured. Parameters of

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dry rot caused maximal width (w), depths (d) were noted, and tubers were calculated by applying the following formula devised by Lapwood et al. (1984):

$$\text{Penetration (mm): } [w/2 + (d\text{-}6)]/2$$

Cultivar's susceptibility to *F. sambucinum* (Fs2, Fs3, Fs4) and *F. solani* (Fs1) was estimated according to this scale: Less or moderately susceptible: mean penetration ≤ 12 mm; Susceptible: 12 mm <mean penetration < 15 mm; Highly susceptible: mean penetration ≥ 15 mm. The data were analysed with SPSS (version 20, SPSS Inc. Chicago, Illinois) and means were separated with Duncan's multiple range test.

Results and discussions

Molecular identification of F. sambucinum (Fs2, Fs3, Fs4) and F. solani (Fs1).

Both *F. sambucinum* (Fs2, Fs3, Fs4) and *F. solani* (Fs1) were identified based on morphological structures as described by Booth (1977), Gerlach and Nirenberg (1982), Nelson et al. (1983), Hasenekoğlu (1991), Leslie and Summerrell (2006) and Borca and Carmen (2013) respectively. In addition for molecular analysis, sequences of ITS4 and ITS5 regions were compared by using the BLAST (basic local alignment search tool) in NCBI (National Center for Biotechnology Information) database in GenBank. Phylogenetic tree generated from the ITS sequence data was found to have a quite consistent resolution with our estimations. The resulting dendrogram (*Fig. 2*) showed that the analyzed ITS gene region represented the variability to differentiate from the isolate of *Fusarium* spp obtained from NCBI.

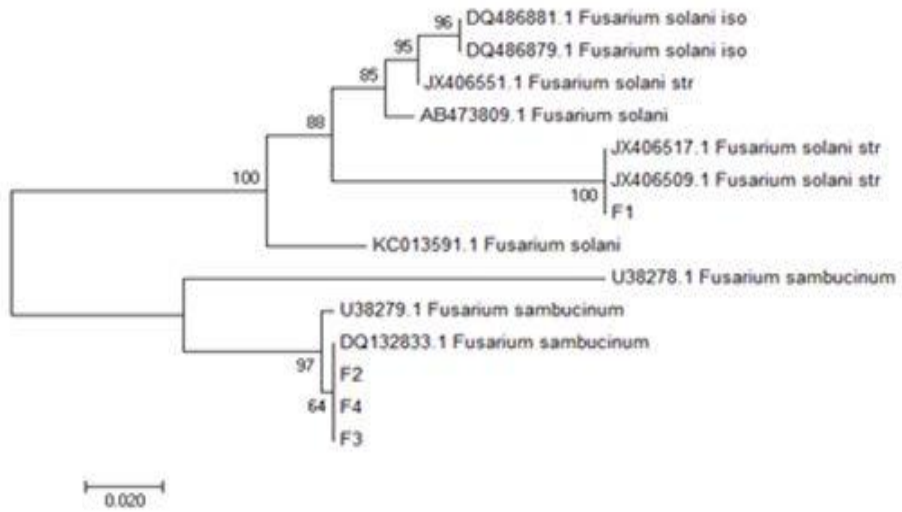

Figure 2. *Phylogenetic tree based on neighbor-joining analysis of ITS sequences from NCBI and 4 isolated Fusarium spp. Bootstrap values are given when above 64% (1,000 replicates)*

Analyzed isolates formed two main branches. First main branch was consisted of investigated F1 and *F. solani* isolates obtained from NCBI. So it can be argued that F1 isolates showed a kinship with *F. solani*. The second main branch was built up by the investigated F2, F3 and F4 and *F. sambucinum* isolates obtained from NCBI. So it can

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be estimated that F2, F3 and F4 isolates showed a genetic kinship with *F. sambucinum.* We reach the conclusion that ITS region was found to be as a diagnostic tool to discriminate the investigated isolates in this study. Informative sequences of ITS region also help the close related *Fusarium* species such as *F. verticillioides* and *F. proliferatum* (Visentin et al., 2009). The least informative or low nucleotide sequence variation of the ITS region to clearly identify many complex species was also previously achieved (Oechsler et al., 2009; Wang et al., 2011). Determination of *Fusarium* spp. using thermal cycler amplification of ITS region of the rDNA using the right primer pairs are reliable, accurate and quick. Duggal et al. (1997) reported that the ITS region shows polymorphisms with in *Fusarium* spp. This is consistent with the previous study (White et al., 1990) and (O'Donnell, 1992) which under line the applicability of using the ITS region as a molecular marker to identify the *Fusarium* spp. It was also defined in these works that the rDNA region of *F. sambucinum* showed a highly conserved. Furthermore, small sequence divergence was found by using the ITS region that can determinate species from the same clan (Turner et al., 1998).

Evaluation of dry rot susceptibility to F. sambucinum (Fs2, Fs3, Fs4) and F. solani (Fs1)

Commercial variety susceptibility to *Fusarium* dry rot decay can affect the market value. Therefore, it is important to grow potatoes that are not susceptible to this disease. The mean dry rot scores are shown in *Table 2,* and analysis of variance is indicated in *Table 3*.

Table 2. *Dry rot severity caused by F. solani (Fs1) and F. sambucinum (Fs2, Fs3, Fs4) in potato tubers in vivo test*

Varieties	The average severity diseases of isolates				
	Severity of Fs1	Severity of Fs2	Severity of Fs3	Severity of Fs4	Mean
Marabel	7.63	13.06	13.88	14.26	12.20 h*
Madeleine	7.25	13.44	18.00	11.94	12.65 gh
Hermes	7.94	18.75	14.38	13.81	13.71 fg
Opal	7.19	18.38	19.62	10.75	13.98 ef
Broke	7.75	13.81	11.25	10.13	10.73 ı
Claire	9.31	10.38	18.19	14.25	13.03 fgh
Musica	8.25	24.50	19.38	16.56	17.17 c
Orchestra	10.44	17.81	13.38	13.06	13.67 fg
Melody	7.09	16.81	24.94	13.88	15.70 d
Vr.808	12.69	16.31	18.94	13.88	15.46 d
Rossetta	12.81	12.13	16.88	8.00	14.95 de
Desire	9.56	14.56	15.13	14.31	13.39 fgh
Surya	8.81	21.13	28.06	6.25	21.06 a
Alonso	12.38	17.93	16.38	6.13	18.20 bc
Alegra	10.25	21.63	23.18	20.00	18.76 bc
Borvira	7.50	19.27	22.50	22.00	17.81 bc
Soraya	13.50	13.25	14.56	19.75	15.26 d

*Values in the same column followed by the same letter are not significantly different at p = 0.05. CV: 9.05%

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Table 3. *Mean squares from the analysis of variance for dry rot inoculation tests*

Variation source (VS)	Degree of freedom (DF)	Sum of squares (SS)	Mean of square (MS)	F
Replication	3	17.088	5.696	2.09
Variety	16	1864.549	116.534	48.82**
Isolate	3	3103.692	1034.564	380.21**
Variety*Isolate	48	2213.389	46.112	16.94**
Error	201	546.939	2.721	
Total	271	7828.586		

Coefficient of variation (CV) = 9.05%

According to the variance analysis results, the disease reactions of the varieties against *Fusarium* isolates appear to be statistically significant at the level of 1%. Susceptibility of Potato varieties to *F. sambucinum* (Fs2, Fs3, and Fs4) and *F. solani* (Fs1) isolates had different grades with average lesion sizes ranging from 10.73 to 21.06 mm (*Table 2*). Cultivars were evaluated in laboratory for 5 weeks at 15-20 °C. Obtained results revealed that Broke had a less or moderately susceptible dry rot potential classification, whereas Musica, Melody, Vr.808, Surya, Alonso, Alegra, Borvira, Soraya had a high dry rot potential. The cultivars of Marabel, Madeleine, Hermes, Opal, Claire, Orchestra, Rossetta and Desire were also susceptible in terms of dry rot (*Table 4, Fig. 3*). Disease development of *Fusarium* dry rot can be increased depending on variety, harvest and handling conditions, tuber characteristics and storage temperatures. Previous studies have shown that the majority of varieties on the market were susceptible to this disease (Tivoli et al., 1986; Wastie et al., 1989; Kumar and Knowles, 2003; Burkhart et al., 2007; Lynch et al., 2003; Daami-Remadi et al., 2006; Du et al., 2012; Baturo-Cieśniewska et al., 2014; Stefańczyk et al., 2016). So, it is essential to incorporate good management practices in order to reduce *Fusarium* dry rot (Leach and Nielsen, 1975; Al-Mughrabi, 2010).

Table 4. *Potato variety susceptibility to Fusarium dry rot*

Less or moderately susceptible	Susceptible	Highly susceptible
Broke	Hermes	Musica
	Opal	Melody
	Claire	Vr.808
	Orchestra	Surya
	Rosset	Alonso
	Desire	Alegra
	Marabel	Borvira
	Madeleine	Soraya

Different rates in terms of disease severity have occurred among the varieties against the same isolate. For example, *F. solani* (Fs1) caused disease severity on Soraya, Alonso, Vr.808, Rossetta, 13.50, 12.38, 12.68 and 12.81 respectively. On the other hand, some isolate again caused on Marabel, Madeleine, Hermes, Opal and Broke 7.63, 7.25, 7.94, 7. 19, 7.75 mm, respectively (*Table 2, Fig. 3*). Similar results were obtained

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in some studies conducted in different countries (Hooker, 1981; Jellis and Starling, 1983; Tivoli et al., 1986; Wastie et al., 1989). According to Daami-Remadi et al. (2006), all inoculated tubers showed dry rot symptoms with different degrees, which reveal that cultivars' resistance differed against *Fusarium* species.

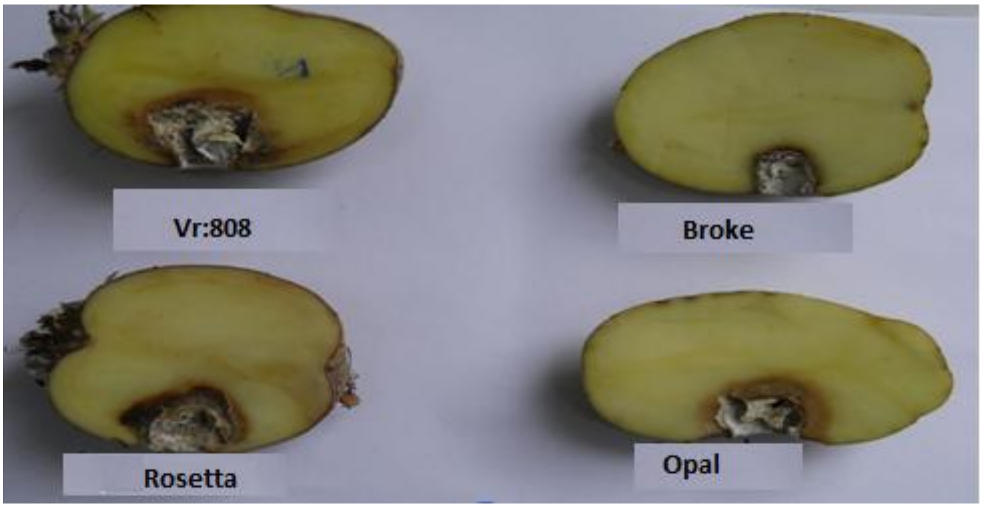

Figure 3. *Dry rot symptom caused by F. solani (Fs1) isolates on tubers of the cultivar Vr.808, Rosetta (left) and Broke, Opal (right) after 5 weeks of incubation at 15-20 °C*

The isolates of whole *F. sambucinum* were more aggressive pathogens than those of the *F. solani* in all cultivars (*Table 2*). As given in *Table 2,* the mean diseases Severity of *F. solani* (Fs1) is 9.43, but the isolates of *F. sambucinum* (Fs2, Fs3, Fs4) were between 16.40-18.15 mm. For example, all isolates (Fs4, Fs3, Fs2, Fs1) caused disease severity on Surya cultivar at different rates, 26.25, 28.06, 21.13, 8.81 respectively (*Table 2, Fig. 4*).

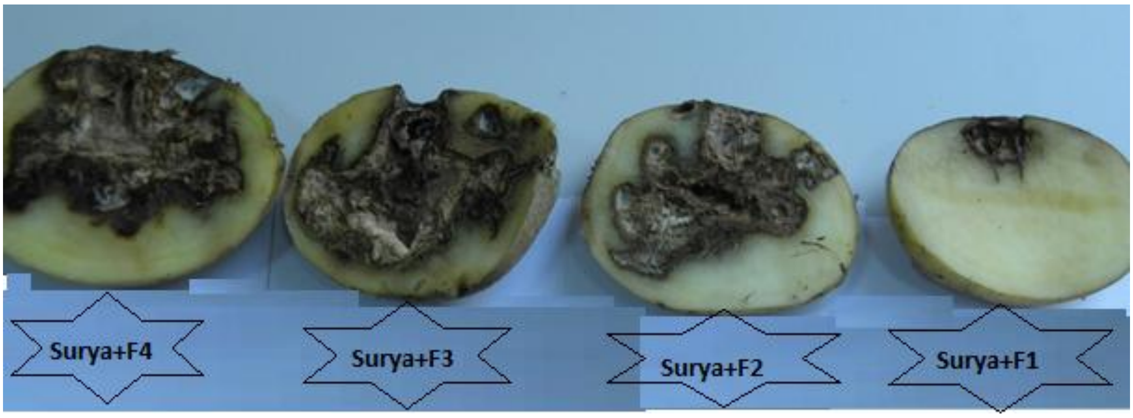

Figure 4. *Dry rot symptoms caused by F. sambucinum (Fs4, Fs3, Fs2) and F. solani (Fs1) isolates on tubers of the cultivar Surya after 5 weeks of incubation at 15-20 °C*

According to this study, *F. solani* (Fs1) was a weaker pathogen. This result is not supported by some researchers. For example, Lenc et al. (2008) and Peters et al. (2008) have reported *F. sambucinum* and *F. solani* are more frequently associated with dry rot

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of tubers but *F. solani is* considered to be a more aggressive pathogen in most parts of Europe. However, this finding was supported by some other researchers who stated that *F. sambucinum* was more aggressive pathogen than the other *Fusarium* species which caused dry rot on potato (Corsini and Pavek, 1986; Wastie et al., 1988; Du et al., 2012; Aydın et al., 2016). In conclusion, this study also supports that several *Fusarium* spp. have been associated with potato dry rot. But depending on the geographic location and the season, the most frequent and devastating of these species is *F. sambucinum.* The isolates are used in this study obtained in Afyon potato production area in the autumn season. The severity of dry rot in this area was usually found to be high (Aydın et al., 2016).

As a result, susceptibility of varieties was found to be distinct to *F. sambucinum* isolates and *F. solani.* While Broke was less or moderately susceptible, other varieties such as Marabel and Madeleine were detected to be susceptible or highly susceptible to *Fusarium* dry rot according to the total disease severity of the four isolates on the varieties (*Table 4.*)

After *Fusarium* isolates grew on the PDA for seven days, they were classified into four different color groups based on mycelium shapes; white (Fs1), Light pink (Fs2), moderately pink (Fs3) and (Fs4). According to phylogenetic analysis also, Fs1 isolate was detected to be close to *F. solani* and the other three isolates are found to be close to *F. sambucinum* (*Fig. 2*). Regarding the color of colonies of isolates on PDA, Fs2 and Fs4 developed close to pink but Fs3 as moderately pink (*Fig. 5*).

Figure 5. *Regarding the color of colonies of isolates on PDA, F. solani Fs1 and F. sambucinum Fs3 (left); F. sambucinum Fs2 and F. sambucinum Fs4 (right)*

All inoculated tubers indicated dry rot symptoms with different degrees against *F. sambucinum* (Fs2, Fs3 and Fs4). Fs2 and Fs4 isolates shared similar levels of pathogenicity, whereas Fs3 caused more severe diseases (*Fig. 6*). Maybe we can make hypothesis that the dark pink of *F. sambucinum* isolates that developed on the medium had more virulence on the potato. Aydın et al. (2016) studied with a dark pink of *F.*

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sambucinum isolate on potato and assumed that the sprouts of tubers were infected with systemic symptoms and severely rot. Thus, it has been suggested that this isolate may be different from other in terms of both the symptom and the severity of the disease.

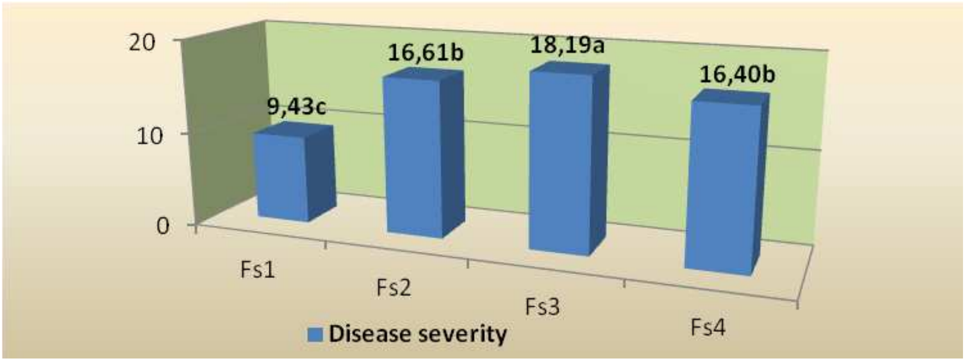

Figure 6. *The mean severity of dry rot caused by F. solani (Fs1) and F. sambucinum (Fs2, Fs3 and Fs4) isolates on potato*

This result shows that the isolates of *F. sambucinum* may be different from each other in terms of virulence. As already noted other researchers have also reported that the majority of the *F. sambucinum* isolates were pathogenic to the potato and caused an average lesion of 21.6 mm. But only one *F. sambucinum* isolate caused an average lesion size that was considerably lower, at 12.1 mm (Stefańczyk et al., 2016).

Conclusion

Briefly, dry rot disease caused by *F. sambucinum* is an economically important field and a postharvest disease throughout the world. This study also reached the conclusion that some strain of *F. sambucinum* investigated were aggressive than the others and caused more severe tuber rots compared to *F. solani.* Many potato cultivars used in this study were susceptible hosts to the pathogen *F. sambucinum.* Only one cultivar showed less or moderate susceptible to pathogen. Phylogenetic tree generated from the ITS sequence data is found to have a quite consistent resolution with our estimations.

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CHANGES IN SOIL HEALTH AND CROPS YIELD IN RESPONSE TO THE SHORT-TERM APPLICATION OF SEWAGE SLUDGE TO TYPIC XEROFLUVENT SOIL IN TURKEY

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Abstract. The aim of this study was to determine the possible effects on soil health and crop yield of the application of anaerobically digested sewage sludge (SS) at doses of 10 (SS_1), 20 (SS_2) and 30 (SS_3) t ha^{-1} yr^{-1} to degraded soils under maize (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) in the semi-arid Mediterranean ecosystem in Turkey. Application of SS at different doses did not show any disruptive effect on microbial biomass and activity in the soil. In the microbiochemical parameters analyzed in the soils under the two plant covers treated with SS, increases were seen of 29-30% compared with control, and of 28-30% in comparison with chemical fertilizer application. Yield increases secured by chemical fertilizers were statistically significantly higher than those from the SS_1. In maize grain yield, statistically significant increases were shown in comparison with the control of 90% with SS_3 and of 86% with SS_2. Similarly, seed cotton showed a statistically significant increase in yield of 72% with SS_3. Results obtained from the study show that for degraded soils in Mediterranean biodegradability conditions, application of SS at a rate of 30 t ha^{-1} yr^{-1} can be used both as a soil improver to maintain the soil and as an organic fertilizer to increase crop yield.
Keywords: *anaerobically digested sludge, soil enzymes, Mediterranean biodegradation condition, maize, cotton*

List of abbreviations: ALKPA – alkaline phosphatase enzyme activity; ArSA – aryl sulphatase enzyme activity; BSR – basal soil respiration; CF – chemical fertilizer treatment; DHG – dehydrogenase enzyme activity; GLU – ß-glucosidase enzyme activity; ha – hectare; MBC – soil microbial biomass carbon; N_{min} – N-mineralization; PRO – protease enzyme activity; SP_1 – first soil sampling period; SP_2 – second soil sampling period; SS – anaerobically digested sewage sludge; SS_0 – 0 t SS ha^{-1} yr^{-1} (control treatment); SS_1 – 10 t SS ha^{-1} yr^{-1} treatment; SS_2 – 20 t SS ha^{-1} yr^{-1} treatment; SS_3 – 30 t SS ha^{-1} yr^{-1} treatment; t – tonnes; UA – urease enzyme activity; yr – year

Introduction

The most important element necessary in ensuring sustainable soil fertility and productivity is soil organic matter. In conditions where soil organic carbon pool has not been well managed, long-term intensive agricultural activity inevitably results in a degradation of soil health. Examining soils affected by the Mediterranean climate, it is seen that the characteristics hindering fertility and crop development are high lime content and pH, and low organic material content. The soils of this region are also characterized by a progressive loss of fertility as a result of high degradation (Pascual et al., 1998). In order to increase soil fertility in a short time, it is necessary not only to add the plant nutrients which may be deficient in the soil by means of chemical fertilizers but also to rectify its characteristics in order to bring it into a sustainable condition. For this reason, in agricultural soils under the effects of the Mediterranean climate whose

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organic material content is low, it is of vital importance to apply organic wastes which have high potential for use.

The sludge varies in the amount and quality of the organic materials remaining according to the stabilization method used, and this determines the direction and strength of its effect on the soil-plant ecosystem. This is because sewage sludge of better quality and with a greater quantity of organic matter will have a greater positive effect on microbial activity and thus on crop yield than the same amount of sludge containing heavy metals. Thus it has been shown not only by researchers working with sewage sludges stabilized by different methods such as thermal drying, aerobic composting, anaerobic decomposition or calcification (Selivanovskaya et al., 2001; Fernández et al., 2009; Jamal et al., 2011), but also by researcher studying with sewage sludge from different wastewater treatment plants of the same municipality (Wong et al., 2001) that the effect of all of them on the soil and crop ecosystem will vary.

The nutrients and organic materials remaining in the sewage sludge at the end of the treatment processes provide for one of the most important methods of disposal of the sludge, that is its use on the soil as a fertilizer or soil improver. The application of sewage sludge to agricultural land is an economical and sustainable method both of recovering nutrients and of disposing of sewage sludge (Lundina et al., 2004; Laturnus et al., 2007; Ghazy et al., 2011). Enriching the soil in organic materials and providing nutrient or fertilizing materials such as nitrogen and phosphorus are the major advantages of this treatment, which can result in an increase in the productivity of the soil (Walia and Goyal, 2010; Saviozzi and Cardelli, 2014). Detailed studies have been conducted on this topic by many researchers in various countries (Gascó and Lobo, 2007; Laturnus et al., 2007).

It has been established in previous studies that the application of sewage sludge improved the physical (Griffiths et al., 2005) and chemical (Speir et al., 2004) characteristics of the soil, and generally supported microbial growth and activity (Debosz et al., 2002; García-Gil et al., 2004). In various other studies, it has been shown that the application of urban sewage sludge has developed the physical characteristics of the soil, such as bulk density, aggregate stability, water retention capacity, total porosity and saturated hydraulic permeability (Kahapanagiotis et al., 1991; Silveira et al., 2003; Rezig et al., 2013), increased the content of organic material, and also prevented erosion (Singh and Agrawal, 2008; Alcantara et al., 2009; Franco et al., 2010; Annabi et al., 2011). However, it has also been shown that the addition of sludge can cause unwanted changes such as a fall in pH values or a rise in salinity, or the concentration of heavy metals (Navas et al., 1998; Veeresh et al., 2003; Singh and Agrawal, 2008). Besides, the long-term repeated addition of sludge can potentially have harmful effects such as the accumulation of toxic metals or damage to microbial communities and their functions, thereby threatening the long-term vitality of the soil. However, observations by different researchers have shown variations in the long-term effects of sludge on the microbial biomass of the soil (Flieβbach et al., 1994; Defra, 2005). The ability to establish the biological characteristics of the soil which give the fastest and most accurate response to all soil management practices in the short term will form an important basis for creating a long-term perspective. Also, there has as yet been little research into the short term effects of the use of dried organic wastes left over after the extraction of energy by anaerobic decomposition on the chemical and biochemical characteristics of the soil and on the yield of annual plants.

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The aim of this study, designed from this starting point, was to research and compare the effect on cotton and maize yield of various doses of sewage sludge (SS) from the Çiğli Wastewater Treatment Plant of İzmir Metropolitan Municipality, stabilized in anaerobic conditions and converted to granules of 90% dryness, with the biochemical characteristics of a degraded soil in a semi-arid Mediterranean agro-ecosystem. In addition, variations in the heavy metal content of the soil were examined. Enzymes considered in the study were those involved in cycling in the soil: protease and urease of N, β-glucosidase of C, alkaline phosphatase of P, and aryl sulfatase of S. Different from the others, the enzyme dehydrogenase is an enzyme which functions in the mechanisms of intracellular respiration and shows the total oxidative capacity of the microbial biomass.

Materials and methods

Anaerobically digested sewage sludge (SS)

The sewage sludge used in the experiment was from the Çiğli Wastewater Treatment Plant of İzmir Metropolitan Municipality of Turkey, stabilized in anaerobic conditions and converted to granules of 90% dryness. Various characteristics of the SS are given in *Table 1*.

Table 1. *Mean values of the some physicochemical properties of anaerobically digested sewage sludge (SS) from Çiğli Wastewater Treatment Plant used in the experiment*

Parameter	Mean[a]	Turkish directive	European directive	USA (Part 503)
pH[c]	**7.18**			
EC[d] ($dS\ m^{-1}$)	**1.95**			
Carbonates ($g\ kg^{-1}$)	**53.50**			
$C_{(Org)}$ ($g\ kg^{-1}$)	**296.98**			
C/N	**9.93**			
N[e] $_{(Kjeldahl)}$ ($g\ kg^{-1}$)	**29.90**			
P[e] ($g\ kg^{-1}$)	**2.28**			
K[e] ($g\ kg^{-1}$)	**3.40**			
Ca[e] ($g\ kg^{-1}$)	**63.60**			
Mg[e] ($g\ kg^{-1}$)	**20.40**			
Na[e] ($g\ kg^{-1}$)	**1.39**			
Fe[e] ($g\ kg^{-1}$)	**12.76**			
Cu[e] ($mg\ kg^{-1}$)	**176.50**	1000	1000-1750	4300
Zn[e] ($mg\ kg^{-1}$)	**1376.59**	2500	2500-4000	7500
Mn[e] ($mg\ kg^{-1}$)	**350.00**			
Cd[e] ($mg\ kg^{-1}$)	**2.83**	10	20-40	85
Cr[e] ($mg\ kg^{-1}$)	**112.53**	1000	-	3000
Pb[e] ($mg\ kg^{-1}$)	**17.44**	750	750-1200	840
Ni[e] ($mg\ kg^{-1}$)	**69.73**	300	300-400	420
Co[e] ($mg\ kg^{-1}$)	**6.71**			

[a]Each value is the mean of three replicates and on an oven-dry (105 °C) basis; [b]Standard deviation; [c]pH of 1:2.5 water extract; [d]Electrical conductivity of 1:5 water extract; [e]Total

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The sewage treatment process was designed according to an advanced biological purification method, biologically removing phosphorus and nitrogen and able to produce water of a better quality, with a capacity of approximately 605 t day^{-1}. The facility has four digestion tanks, two biogas collection tanks and four dryers, each with the capacity to process 200 t day^{-1} of sludge cake. In 2015, the sludge digestion and drying unit produced a total of 11550 t of dried sewage sludge and 11755000 m^3 of biogas (İZSU, 2018). The heavy metal content of the SS used in the study was below the values allowed by Europe Directive 86/278/CEE (CEC, 1986), US (EPA, 1993) and Turkish regulations (RG, 2010) (*Table 1*).

Field experiments

Field experiments were conducted in 2015 at the Research, Application and Production Farm of Ege University Agriculture Faculty (longitude: 27°01'20"-27°01'22" E; latitude: 38°34'47"-38°34'45" N; average altitude 5 m), on sandy loamy soil (sand, silt and clay 558.4, 314.4 and 127.2 g kg^{-1} respectively). *Figure 1* shows the experimental area location and some photos of the experimental period. The soil is classified as Typic xerofluvent (Soil Survey Staff, 2010). The physicochemical properties of the experimental field at the beginning of the experiment are given in *Table 2*. The Menemen Plain, where the research area is located, has a Mediterranean climate, with hot dry summers and cool rainy winters. According to long-term (55 year) climate data, mean total annual precipitation is 525.3 mm. Approximately 50% of this precipitation occurs in winter, 25% in spring, 23% in autumn and 2% in summer. The mean temperature is 16.9 °C, mean relative humidity is 57.5%, and mean annual evaporation is 1532.1 mm (Anonymous, 2009).

Table 2. *Physicochemical characteristics of experimental soil at the beginning of the experiment*

Parameter	Mean[a]		Parameter	Mean[a]	
$pH_{(H_2O)}$	7.66	(0.1)[b]	Na[c] (mg kg^{-1})	30.31	(4.2)
Salinity (mg kg^{-1})	286.7	(69.9)	Fe[d] (mg kg^{-1})	2.21	(0.6)
Carbonates (g kg^{-1})	50.49	(3.5)	Cu[d] (mg kg^{-1})	0.65	(0.2)
Sand (g kg^{-1})	558.4	(33.2)	Zn[d] (mg kg^{-1})	1.54	(0.7)
Silt (g kg^{-1})	314.4	(21.2)	Mn[d] (mg kg^{-1})	1.64	(0.4)
Clay (g kg^{-1})	127.2	(9.0)	Zn[e] (mg kg^{-1})	58.29	(1.3)
Texture	Sandy loam		Cu[e] (mg kg^{-1})	15.49	(0.6)
$C_{(Org)}$ (g kg^{-1})	7.73	(1.6)	Cr[e] (mg kg^{-1})	23.22	(3.1)
C/N	9.72	(1.8)	Cd[e] (mg kg^{-1})	0.82	(0.1)
$N_{(Kjeldahl)}$ (g kg^{-1})	0.79	(0.1)	Pb[e] (mg kg^{-1})	11.66	(2.1)
$P_{(Olsen)}$ (mg kg^{-1})	18.88	(4.6)	Ni[e] (mg kg^{-1})	55.49	(2.6)
K[c] (mg kg^{-1})	231.3	(26.9)	Hg[e] (mg kg^{-1})	61.52	(10.0)
Ca[c] (mg kg^{-1})	2200	(119)	As[e] (mg kg^{-1})	15.93	(2.8)
Mg[c] (mg kg^{-1})	273.8	(23.2)	B[f] (mg kg^{-1})	1.27	(0.4)

[a]Each value is the mean of four replicates of control soils; [b]Standard deviation; [c]NH_4OAc extract; [d]DTPA extract; [e]Total, HCl+ HNO_3 extract; [f]Total, hot water extract

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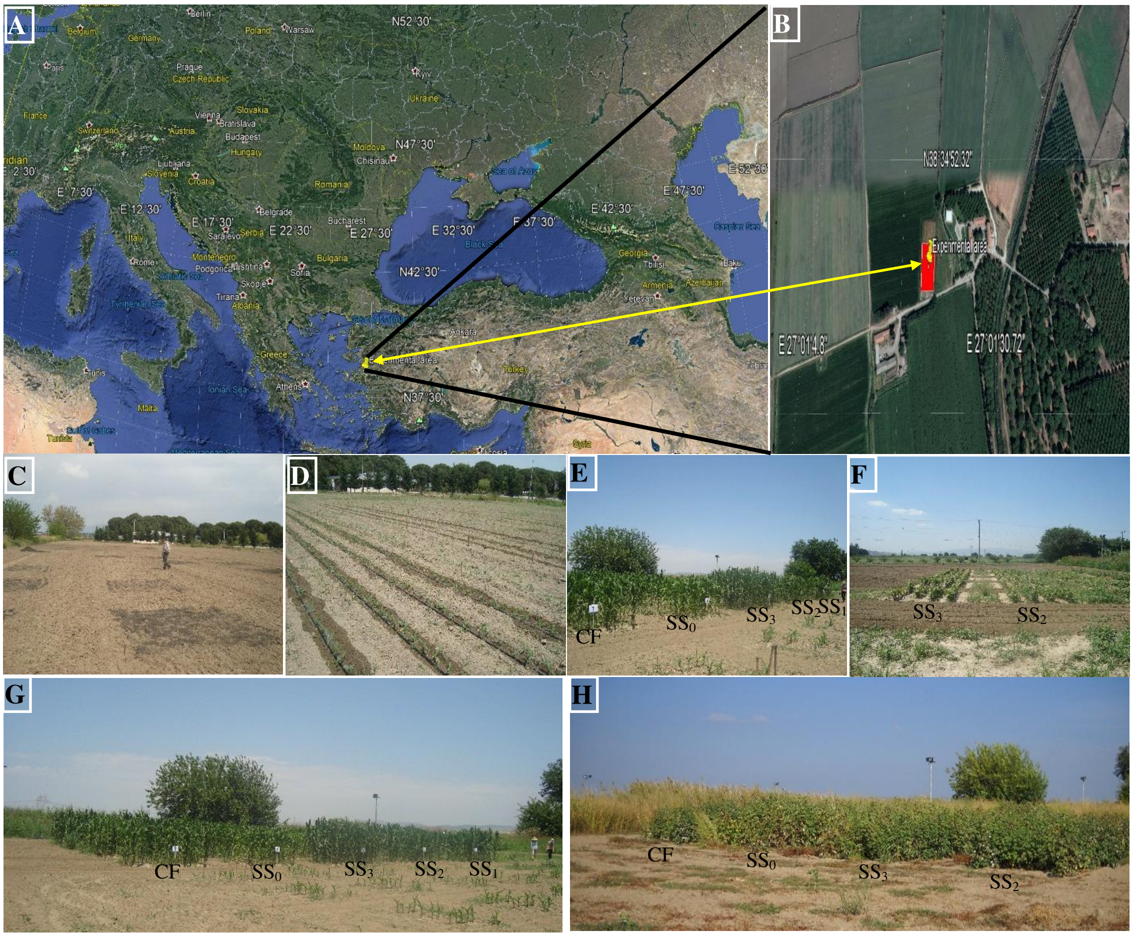

Figure 1. *Map showing (A) the location of the Research, Application and Production Farm of Ege University Agriculture Faculty experimental area in the İzmir Province, Turkey and (B) close-up view of the experimental field, (C) photo of sewage sludge applications, (D) photo of drip irrigation activity, (E) and (G) photos of the maize experiment, (F) and (H) photos of the cotton experiment*

The experiment was performed in four randomized blocks of soil plots (3 m × 3 m) with four replications cropped with maize (*Zea mays* L. var. ZP 737) and cotton (*Gossypium hirsutum* L. var. GSN 12), at the same time. Soil plots were either unamended (SS_0) or amended with SS at rates of 10, 20 and 30 t ha^{-1} on a dry weight basis (SS_1, SS_2 and SS_3, respectively). After application of the SS to the surface of the soil in the experiments, the soil was mixed to a depth of 15 cm using a rotary tiller (21 April 2015). 1 t ha^{-1} 15-15-15 composite fertilizer was used for the mineral fertilizer application in the maize experiment (CF). Maize sowing took place on the same day, 21.4.2015, on all experimental plots after the application of sewage sludge. Seeds were sown in rows 70 cm apart, at 18.3 cm intervals, using a seed drill. As mineral fertilizer, 150 kg ha^{-1} urea fertilizer (46% N) was applied as a top dressing on 4 June 2015. Cotton sowing took place on 29 April 2015. In the cotton experiment, 500 kg ha^{-1} of 15-15-15 composite fertilizer was applied as basic fertilizer to the mineral fertilizer plots (CF) on 21 April 2015. As a mineral fertilizer application in the cotton experiment, 150 kg ha^{-1} of urea fertilizer was applied to the plots as a top dressing on 4 June 2015. The second top dressing in the cotton experiment was applied during the flowering period (8 July

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2015), with only mineral fertilizer at a dose of 270 kg ha^{-1} of CAN (calcium ammonium nitrate). In both the maize and the cotton experiments, drip systems were set up for irrigation purposes. In order to meet the water requirement of the test plants, irrigation activities were carried out every 15 days from the sowing of seeds, taking into consideration the regional climatic conditions and producer practices. Irrigation water was given for maize plant in 6 times (total 600 mm) and for cotton plant in 8 times (800 mm in total) with 100 mm in each irrigation period.

The first soil samling period (SP_1) was taken fifteen days after the application of the SS. The second soil sampling period (SP_2) was taken 120 days later (19 August 2015) for maize and 189 days later (27 October 2015) for cotton. Soon after the plants were harvested individually, surface soil samples were collected randomly from the arable layer (Ap horizon, 0–15 cm depth) of each plot. Each soil sample consisted of a mixture of 10 soil cores, each 3 cm in diameter. Soil samples for biological and biochemical analyses were stored at 4 °C at field moisture. *Table 3* shows the results of the analysis of the soil samples (First period n = 40, second period n = 40) of the microbiological parameters microbial biomass carbon (MBC), basal soil respiration (BSR), N-mineralization (N_{min}), alkaline phosphatase enzyme activity (ALKPA), dehydrogenase enzyme activity (DHG), protease enzyme activity (PRO), urease enzyme activity (UA), ß-glucosidase enzyme activity (GLU) and aryl sulphatase enzyme activity (ArSA), while *Tables 4* and *5* show the Pearson correlation matrix for the chemical parameters analyzed with these parameters for maize and cotton vegetation respectively. When the maize plants came to harvesting maturity, the middle two rows of the four rows on each plot were used (19 August 2015). The cotton bolls were also harvested by hand (27 October 2015). At the end of harvest, plot yields were calculated and converted to t ha^{-1}.

Chemical analysis of soil and sewage sludge

Prior to analysis, soil samples were air-dried and passed through a 2 mm sieve. The principal chemical properties of soil and SS samples were determined by standard methods (Sparks et al., 1996). In particular, the pH was measured on satured soil and on mixtures of sludge:water = 1:2.5; the EC was measured on a 1:5 sample:water extract; and the TOC content was determined by dichromate oxidation of the sample and subsequent titration with ferrous sulfate heptahydrate. The total N content was obtained by the Kjeldahl method (Keeney and Nelson, 1982). Available P content was determined according to the Olsen method (Olsen and Sommers, 1982); and available K content was measured by Flame Photometer in 0.5 M ammonium acetate soil extracts using a ratio of soil to extractant of 1:10 (Sparks et al., 1996). For determination of heavy metals, the soils were extracted with 3 parts HCl + 1 part HNO_3. The concentrations of Pb, Ni, Cr, Co, Hg, As and Cd in the extracts were determined by atomic absorption spectrometry (AAS) (ISO, 1995, 1998). For determination of boron, soils were extracted with hot water for 5 min, centrifuged and filtered. Boron was determined using azomethine-H. Reagents formed using ammonium acetate and disodium ethylenediamine-tetra-acetate for this study are those used by Gupta (1979). The SS samples were dried at 70 °C for 72 h, and their dry weights were recorded. The samples were then ground to pass through a 2 mm sieve for subsequent analysis. After nitric and perchloric acid digestion (4:1), total Mg, Fe, Cu, Mn, Zn, Cd, Pb, Ni, Hg and Cr concentrations in the SS were determined using AAS, and Ca, K and Na concentrations were determined by flame photometry (Kacar and İnal, 2008). Total P content in the acid digest was determined using a spectrophotometer after developing

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the vanadomolybdophosphoric yellow color complex in a nitric acid medium (Kacar and İnal, 2008).

Soil biological and biochemical analyses

Basal soil respiration (BSR) was found using a 0.1 N NaOH solution after a 24 h incubation period at 25 °C (Isermeyer, 1952; Jäggy, 1976). In order to determine microbial biomass carbon (MBC), the moisture content of soil samples was determined, and after fumigation according to Jenkinson (1976), they were agitated with 0.5 M K_2SO_4 (Vance et al., 1987). After that, the amount of C in the filtrate was determined by wet digestion in the presence of strong acid (a mixture of H_2SO_4 and H_3PO_4) and 0.4 N $K_2Cr_2O_7$, after which the excess dichromate was titrated with a 25 mM 1.10-fenantrolin iron sulfate complex indicator solution (Kalembasa and Jenkinson, 1973; Vance et al., 1987). A kEC factor of 0.45 was used in the calculations (Jenkinson and Ladd, 1981). N-mineralization (N_{min}) was assayed according to the method of Keeney (1982). This method involves the incubation of a soil sample under waterlogged conditions at 50 °C. After seven days, NH_4-N released from the soil water mixture was determined with a modified Bertholet reaction. Dehydrogenase enzyme activity (DHG: EC 1.1) was determined after adding TTC (triphenyl tetrazolium chloride) solution at different concentrations according to the soil texture and the amount of organic matter to the soil samples and incubating for 16 h at 25 °C, by photometric measurement at a wavelength of 546 nm of the resulting TPF (triphenyl formazan) (Thalmann, 1968). The activity of urease, an enzyme of the hydrolase group (UA: EC 3.5.1.5), was determined by incubating the soil where urea was used as a substrate at 37 °C for 90 min, and determining the resulting ammonia with a modified Bertholet reaction after extracting it with 2 M KCl (Kandeler and Gerber, 1988). To determine alkaline phosphatase enzyme activity in the soil samples (ALKPA: EC 3.1.3.1), buffered p-nitrophenyl phosphate solution was added and p-nitrophenol, incubated for 1 h at 37 °C, and resulting by phosphomonoesterase activity, was colored with sodium hydroxide and measured photometrically at 400 nm (Eivazi and Tabatabai, 1977; Tabatabai and Bremner, 1970). For protease enzyme activity (PRO: EC 3.4), soils where casein was used as a substrate were incubated at 50 °C for two hours, and the resulting aromatic amino-acids were colored in an alkaline environment with folin-ciocalteu, and detected colorimetrically at 700 nm (Ladd and Butler, 1972). The soil samples were incubated for three hours at 37 °C with salicin, after which the resulting saligen was determined spectrophotometrically at 578 nm in order to determine β-glucosidase activity (GLU: EC 3.2.1.21) (Hoffman and Dedeken, 1965). In order to determine the activity of aryl sulfatase, which has a role in the sulfur cycle (ArSA: EC 3.1.6.1), a solution of p-nitrophenylsulfate was added to the soil samples as a substrate, and the resulting nitrophenol was measured photometrically at a wavelength of 430 nm (Tabatabai and Bremner, 1970).

Statistical analysis

All data were tested for normality and homogeneity of distribution, and were log-transformed if required prior to analyses. Multivariate analysis of variance (MANOVA) test was used to study the effects of treatment on soil chemical, biochemical and microbiological properties. Comparison of average values was performed with the Duncan multiple comparison test and with a significance level of $\alpha = 0.05$. Pearson

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correlation analyses were performed on all the chemical, microbiological and biochemical data. All statistical analyses were performed using the IBM SPSS 20.0. Standard deviation values showing distribution according to the average of data obtained in each period were also calculated by means of the same program.

Results and discussion

Maize and cotton yield

Figure 2 shows the effects on the yield of maize and cotton of SS applied at different doses. It was observed that SS applied in the experiment had no toxic effect on either of the crops. In fact, statistically significant increases in yield parameters of the crops were seen with increasing doses of SS in comparison with the SS_0. A yield increase of 90% was determined in maize grain yield with SS_3 compared to the control. Similarly, seed cotton yield increased by 72% with the same treatment. In a study conducted with maize in a hot, moist and semi-arid eco-subregion soils of India which climate is dominated by monsoons and strongly influenced by the Himalayas and the Thar Desert, a determination was made of the effect of the application of compost, stabilized sewage sludge and nitrogen fertilizer to the crop. According to the results of the field experiments, the two highest doses of stabilized sludge (80 and 160 t ha^{-1}) gave significant increases in yield, but at the same time increases were determined in the heavy metal contents of the crop. The researchers found that treatment at the level of 40-60 t ha^{-1} yr^{-1} of sewage sludge was the most suitable to achieve acceptable crop growth and a minimum of adverse effects on crop and soil quality (Begum, 2011). In a study, laboratory incubation and pot experiment were carried out to determine the effect of heavy metal concentration, adverse effects were shown in the growth of Indian mustard of applications of 40 and 80 t ha^{-1} of sewage sludge which had a heavy metal concentration of twice the limit set by the European Union (Walia and Goyal, 2010).

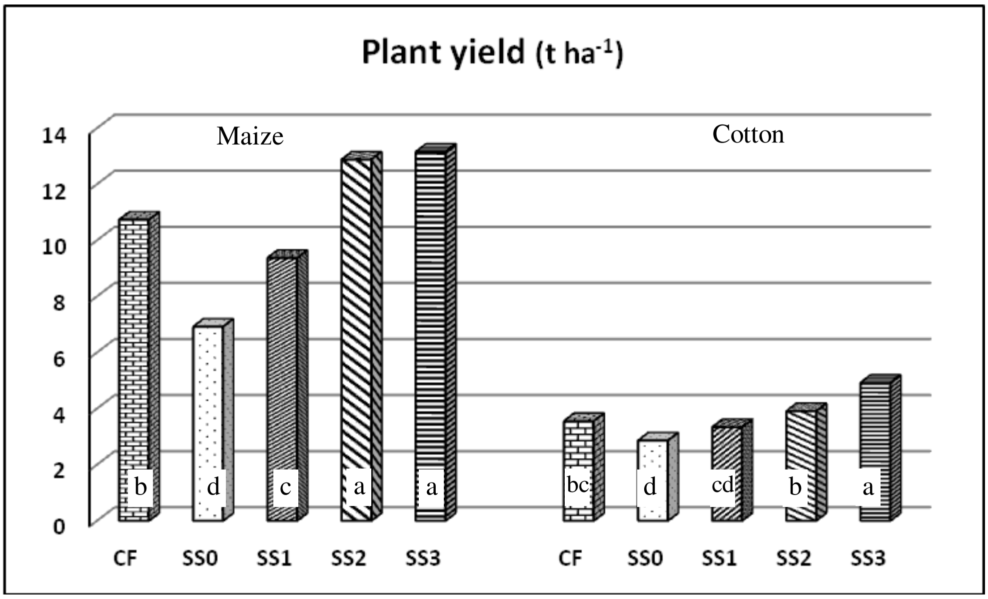

***Figure 2.** The effect of sewage sludge on grain yield of maize and seed cotton yield. Each value is the mean of four replicates. Results within treatmentsof each plant capped with different letters are significantly different (Duncan's test: P < 0.05)*

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The response of the yield parameters of maize to the application of sewage sludge was clearer than that of cotton. In a study by Al Zoubi et al. (2008) on Syrian soils under the effect of a Mediterranean climate, an increase was shown in maize yield with increasing doses of sewage sludge compared with the application of chemical fertilizer. On the other hand, the same researchers found no difference between wheat yields with the application of sludge or inorganic fertilizer. Thus, the effects of the application of sewage sludge vary according to the type of crop. Examining the results obtained in the present study, because not only the increase of 86% in maize grain yield was obtained with the SS_2 but also there was statistically no difference between the SS_2 and SS_3 doses, application at 20 t SS ha^{-1} (SS_2) to Mediterranean soils where maize is grown may be recommended. Additionally, less heavy metal will be added to the soil with the SS_2 than with the SS_3 dose. However, a similar effect was not seen as per seed cotton yield. The statistically greatest yield was obtained with the highest sewage sludge application (SS_3), and so a dose of 30 t SS ha^{-1} may be recommended for soils where cotton is grown in Mediterranean climatic conditions. Of course, even when concentrations of nutrient elements in the sewage sludge are the same for the two crops, the differences in the morphological characteristics of the plants can cause differences to emerge in results in the same field. Similar results in terms of varieties of plant species have been obtained in studies of the effects of the application of sewage sludge on crop yield in field conditions. In a study by Tamrabet et al. (2009) examining the effect of the application of sewage sludge to the soil on the yield of durum wheat (*Triticum durum* Desf.), doses of 33 kg ha^{-1} of mineral fertilizer (urea) and doses of 20, 30 and 40 t SS ha^{-1} were applied to the soil under semi arid cropping conditions of Algeria. It was determined that the most effective application of sludge for wheat yield was 30 t ha^{-1}, and that in semi-moist conditions sewage sludge had a positive effect on the yield of that wheat variety, and could be used safely.

Another result emerging from our study was that the yield increase obtained with the application of chemical fertilizer was higher than that obtained with the 10 t SS ha^{-1} given with the SS_1, but lower than the yield increase provided by SS_2 and SS_3. It was seen that much more effective plant nutrition was achieved by the application of plant nutrient elements provided to the soil by sewage sludge together with the addition of organic materials, especially those which are not present in chemical fertilizers. Therefore, increasing the low level of organic carbon of approximately 7.73 g kg^{-1} in the soil may allow plants to benefit more effectively from plant nutrient elements either in the soil or mineralized in connection with SS applications. In addition to this, it must not be forgotten that organic soil improvers applied to sandy loamy soils can also support an increase in yield by improving the physico-chemical characteristics of the soil. Similar to our findings, a statistically significant values similar to that of a mineral fertilizer treatment were obtained in grain yield of maize at the end of 10 years due to increasing sewage sludge doses which accumulated in each treatment 50, 100 and 147.5 t ha^{-1} in total at the end of the experimental period in agricultural Oxisol under a tropical climate of Brazil (Melo et al., 2018). Researchers have suggested that purification sludge applications can meet all of the benefits of chemical phosphorus and microelement fertilization, while chemical nitrogen fertilization can be partially replaced without loss of yield in the maize plant.

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Soil microbiological and biochemical activities

A brief overview on soil health

All of the doses of SS applied to the soils affected the microbiological parameters analyzed significantly at a rate of 1%. The level of effect varied in connection with the type of crop, and it is thought that the greatest effect was the difference in the crop vegetation periods. Additionally, it can be said that when the root morphology and root secretion of the two test crops are considered, the differences in the rhizosphere zone also have an effect on managing microbial activity. It was observed that biological activity in maize plants with regard to the parameters examined was approximately 6.5% higher than that of cotton (*Table 3*). In maize soils, SS_3 more stimulated microbiological and biochemical parameters at a statistically significant level. The situation was somewhat different with soils under cotton, and it can be said that the effect of sewage sludge was almost the same on a dose basis. Thus, it can be seen from *Table 3* that an effect was shown at the same level of statistical significance on seven of the nine microbiological and biochemical parameters analyzed by both SS_3 and SS_2, and on three by SS_1. This is one of the important conclusions emerging from the results of the experiment: that is, that in soils under cotton, which has a longer growing period than maize, the differences between the microbiological and biochemical parameter values analyzed resulting from the treatments are reduced. The reason for this may be said to be that after the easily available carbon in SS is applied to the soil, it is used in a short time by heterotrophic microorganisms as a source of energy and carbon, leaving in the soil a valuable and stable humus which is resistant to breakdown. Looking at the overall picture, what emerges is that with crops with a short growing period, sewage sludge can be used as an organic fertilizer because of its capacity for mineralization.

Table 3. *Effects of stabilized sludge (SS) applications on some microbiological and biochemical properties of the soil under maize and cotton vegetation*

Parameters		**1st SP [a]**	**2nd SP**	**Mean**	**1st SP**	**2nd SP**	**Mean**
		Maize			**Cotton**		
BSR [h]	**SS_0 [b]**	0.100 *b* [g]	0.091 *bc*	**0.096 *b***	0.097 *b* [f]	0.112	**0.104 *b***
	CF [c]	0.113 *b*	0.111 *a*	**0.112 *ab***	0.129 *ab*	0.119	**0.124 *ab***
	SS_1 [d]	0.157 *a*	0.073 *c*	**0.115 *ab***	0.141 *a*	0.112	**0.127 *a***
	SS_2 [e]	0.130 *ab*	0.102 *ab*	**0.116 *ab***	0.164 *a*	0.124	**0.144 *a***
	SS_3 [f]	0.158 *a*	0.084 *bc*	**0.121 *a***	0.150 *a*	0.122	**0.136 *a***
MBC [i]	**SS_0**	221.7 *b*	160.1	**190.9 *b***	179.3 *b*	120.4	**149.9 *b***
	CF	209.6 *b*	186.7	**198.2 *b***	195.0 *ab*	160.5	**177.7 *ab***
	SS_1	223.0 *b*	199.0	**211.0 *b***	185.6 *b*	159.9	**172.7 *ab***
	SS_2	337.5 *a*	215.7	**276.6 *a***	246.6 *a*	202.4	**224.5 *a***
	SS_3	194.6 *b*	214.8	**204.7 *b***	220.2 *ab*	142.0	**181.1 a*b***
DHG [j]	**SS_0**	147.6 *c*	124.8 *b*	**136.2 *c***	158.2 *b*	81.11 *b*	**119.67 *b***
	CF	130.8 *c*	113.1 *b*	**122.0 *c***	157.0 *b*	81.83 *b*	**119.44 *b***
	SS_1	228.9 *b*	161.5 *ab*	**195.2 *b***	230.8 *a*	110.6 *ab*	**170.7 *a***
	SS_2	327.6 *a*	193.8 *a*	**260.7 *a***	272.6 *a*	143.2 *a*	**207.9 *a***
	SS_3	307.7 *a*	207.0 *a*	**257.3 *a***	271.9 *a*	143.7 *a*	**207.8 *a***

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ALKPA[k]	**SS_0**	583.8 *d*	556.6 *b*	**570.2 *d***	518.0 *c*	529.4 *c*	**523.7 *c***
	CF	533.6 *d*	560.6 *b*	**547.1 *d***	523.1 *c*	547.8 *bc*	**535.4 *c***
	SS_1	728.9 *c*	727.5 *a*	**728.2 *c***	747.9 *b*	685.2 *ab*	**716.6 *b***
	SS_2	848.9 *b*	768.4 *a*	**808.6 *b***	758.3 *b*	787.4 *a*	**772.9 *b***
	SS_3	957.8 *a*	864.3 *a*	**911.0 *a***	1009.6 *a*	780.4 *a*	**895.0 *a***
PRO[l]	**SS_0**	151.1 *b*	108.3 *c*	**129.7 *c***	117.7 *d*	93.3 *c*	**105.5 *c***
	CF	128.6 *b*	90.3 *c*	**109.4 *c***	103.8 *d*	91.1 *c*	**97.45 *c***
	SS_1	267.2 *a*	226.1 *b*	**246.7 *b***	185.8 *c*	185.5 *b*	**185.6 *b***
	SS_2	270.9 *a*	256.7 *b*	**263.8 *b***	284.0 *b*	270.1 *a*	**277.0 *a***
	SS_3	360.9 *a*	385.1 *a*	**373.0 *a***	352.7 *a*	260.4 *a*	**306.5 *a***
UA[m]	**SS_0**	60.19	54.33	**57.26 *ab***	52.31 *c*	61.31	**56.81 *b***
	CF	57.84	53.25	**55.55 *b***	52.84 *bc*	55.41	**54.13 *b***
	SS_1	72.00	54.37	**63.19 *ab***	64.0 *abc*	55.22	**59.63 *ab***
	SS_2	68.23	61.46	**64.84 *a***	65.97 *ab*	69.08	**67.52 *a***
	SS_3	71.49	60.53	**66.01 *a***	74.15 *a*	61.41	**67.78 *a***
ArSA[n]	**SS_0**	197.3 *b*	174.9 *b*	**186.1 *cd***	222.2 *ab*	173.5 b	**197.9 *b***
	CF	187.6 *b*	162.2 *b*	**174.9 *d***	204.7 *b*	168.8 *b*	**186.7 *b***
	SS_1	235.6 *ab*	193.6 *ab*	**214.6 *bc***	239.6 *ab*	222.9 *a*	**231.2 *a***
	SS_2	262.3 *a*	206.1 *ab*	**234.2 *ab***	248.0 *a*	244.9 *a*	**246.5 *a***
	SS_3	273.4 *a*	223.1 *a*	**248.3 *a***	241.5 ab	223.8 *a*	**232.7 *a***
GLU[o]	**SS_0**	59.81 *b*	58.35 *c*	**59.08 *c***	58.07 *c*	60.71 *b*	**59.39 *c***
	CF	61.24 *b*	58.98 *c*	**60.11 *c***	63.87 *bc*	69.70 *ab*	**66.78 *bc***
	SS_1	80.11 *a*	65.73 *b*	**72.92 *b***	76.29 *ab*	73.59 *ab*	**74.94 *ab***
	SS_2	84.52 *a*	81.86 *a*	**83.19 *a***	84.29 *a*	85.86 *a*	**85.08 *a***
	SS_3	90.12 *a*	83.10 *a*	**86.61 *a***	77.86 *ab*	80.14 *ab*	**79.00 *b***
N_{min}[p]	**SS_0**	3.01 *bc*	2.97 *c*	**2.99 *c***	3.32 *d*	2.75 *ab*	**3.03 *c***
	CF	2.74 *c*	3.20 *c*	**2.97 *c***	3.43 *d*	1.62 *b*	**2.52 *c***
	SS_1	4.78 *ab*	3.83 *bc*	**4.31 *b***	5.20 *c*	3.35 *ab*	**4.28 *b***
	SS_2	5.93 *a*	4.61 *b*	**5.27 *ab***	6.62 *b*	3.29 *ab*	**4.95 *b***
	SS_3	6.18 *a*	5.66 *a*	**5.92 *a***	7.97 *a*	4.88 *a*	**6.42 *a***

[a]SP, sampling period; [b]SS_0, Unamended soil; [c]CF, Chemical fertilizer; [d]SS_1, 10 ton ha^{-1} SS; [e]SS_2, 20 ton ha^{-1} SS; [f]SS_3, 30 ton ha^{-1} SS; [g]same small letters in the same column (different treatments) do not differ by adjusted Duncan test ($P < 0.05$); [h]Basal soil respiration (mg CO_2-C g^{-1} 24 h^{-1}); [i]Microbial biomass carbon (µg C_{mic} g^{-1}); [j]Dehydrogenase enzyme activity (µg TPF g^{-1}); [k]Alkaline phosphatase enzyme activity (µg p-NP g^{-1} h^{-1}); [l]Protease enzyme activity (µg Tyrosin g^{-1} 2 h^{-1}); [m]Urease enzyme activity (µg N g^{-1} 2 h^{-1}); [n]Aryl sulphatase enzyme activity (µg p-NP g^{-1} h^{-1}); [o]β-glucosidase enzyme activity (µg Saligenin g^{-1} 3 h^{-1}); [p]Nitrogen mineralization (µg NH_4-N g^{-1} 24 h^{-1}). *Each value was given on a dry matter basis as the average of four replicates

Even the heavy metal content of the 10, 20 and 30 t ha^{-1} doses of sewage sludge applied to the experimental soils in treatments SS_1, SS_2 and SS_3 respectively was not shown to have any statistically significant adverse biological activity on the soil. As can be seen from *Tables 4* and *5*, there was not even a negative correlation between the values of heavy metal in the sludge and microbiological activity in the soil. Many of the

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enzymes which are catalyzers of the microorganisms playing an active role in changes and cycles occurring in the soil contain various heavy metals. Examining the correlations of the analysis results, it was seen that DHG activity showed a greater number of positive correlations with the chemical parameter total Cu than with other parameters (*Tables 4* and *5*). A positive correlation of between 1% and 5% was found between all microbiological parameters and Hg and B content in cotton soils, while As content showed a significantly negative correlation with most parameters. In contrast to maize soils, these correlations found between B and As concentrations and microbiological parameters are thought to arise from the larger amounts of irrigation water supplied to the area where cotton is grown: the irrigation periods and thus the amount of irrigation water are greater than for maize.

Also, increases were seen of on average 29-30% compared with the SS_0 and 28-30% against the CF in the microbiological and biochemical parameters of the soils under each plant. Considering the average values obtained from soils under maize and cotton, all increases occurring with SS applications compared to SS_0 and CF treatments were found to be statistically significant ($\alpha = 0.05$). The greatest increase in the experimental area under each crop was related to PRO activity. According to the variance analysis of the maize experiment, sampling periods were statistically significant apart from ALKPA, PRO and Nmin, while treatments had a significant effect on all parameters except BSR. Period x treatment interaction was only significant on DHG and BSR. However, according to the variance analysis of the cotton experiment, periods were statistically significant apart from parameters other than ALKPA, GLU and UA, while treatments had a significant effect on all parameters other than MBC. Period x treatment interaction was found to be significant only on ALKPA. Examining the results obtained regarding the times of sampling of the soils in the experimental area, in relation to the soil samples taken 15 days after the application of sewage sludge (SP_1), the sampling in the harvest period (SP_2) was 120 days after the application of sewage sludge (19 August 2015) for maize, and 189 days after it (29 October 2015) for cotton growth, and it was seen that the values of the parameters analyzed in the soil samples were determined to be on average 13.5% lower. However, this reduction did not obstruct the difference in the effect on microbial activity which could occur in connection with the applications. It is thought that the slight reduction which occurred between sampling periods arose from the harvest times not providing suitable conditions for microbial activity in terms of the soil moisture in the soils under maize and the climatic conditions in the soils under cotton. In fact, this decline was expected to be more pronounced for the reasons given, but unexpectedly, only a slight decrease was determined in our study. The high enzyme activity determined in the soil samples taken in the first period after the application of SS shows the existence of large amounts of substrates which can be broken down biologically in these soils, which is consistent with high unstable-C content, stimulating microbial activity. However, the process of root development in the cotton rhizosphere especially can support biological activity in the soil up to the period of harvest sampling. Root secretion, which mostly stimulates microbial growth, is a side product which represents a part of growth and development, and apart from allowing the development of symbiotic relationships, in a simple way becomes the substrate of microorganisms (Uren, 2007). In addition, plant roots are accepted to be a source of extracellular enzymes in the soil (Egamberdieva et al., 2011). The low levels of difference in activity between the sampling times may also be an indicator of the continuation, even though slight, of the mineralization process of the SS, and this is of

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great importance from the point of view of sustainable management of organic matter in soils under the effect of a Mediterranean climate, especially those degraded by intensive agriculture.

Table 4. *Correlation between average values of heavy metals and microbiological parameters of maize soils at the end of the experiment*

Parameters[a]	BSR	MBC	ALKPA	GLU	DHG	ArSA	PRO	UA	N_{min}
BSR[b]	1			0.333*	0.540**	0.405**		0.597**	0.371*
MBC[c]		1		0.334*	0.458**	0.409**		0.331*	0.404**
ALKPA[d]			1	0.824**	0.862**	0.711**	0.866**	0.524**	0.830**
GLU[e]	0.333*	0.334*	0.824**	1	0.812**	0.850**	0.718**	0.727**	0.796**
DHG[f]	0.540**	0.458**	0.862**	0.812**	1	0.810**	0.744**	0.712**	0.771**
ArSA[g]	0.405**	0.409**	0.711**	0.850**	0.810**	1	0.602**	0.677**	0.657**
PRO[h]			0.866**	0.718**	0.744**	0.602**	1	0.488**	0.712**
UA[i]	0.597**	0.331*	0.524**	0.727**	0.712**	0.677**	0.488**	1	0.578**
N_{min}[j]	0.371*	0.404**	0.830**	0.796**	0.771**	0.657**	0.712**	0.578**	1
Total Zn		0.443**	0.758**	0.676**	0.742**	0.674**	0.700**	0.370*	0.528**
Total Cu	0.400*	0.336*	0.733**	0.764**	0.789**	0.742**	0.745**	0.530**	0.597**
Total Cr			0.625**	0.601**	0.465**	0.419**	0.577**	0.414**	0.616**
Total Cd		0.400*	0.499**	0.471**	0.460**	0.393*	0.532**		
Total Pb									0.355*
Total Ni	0.604**	0.348*			0.528**	0.508**		0.467**	
Total Hg		0.374*	0.590**	0.460**	0.551**	0.482**	0.479**	0.376*	0.451**
Total As									
B[k]					0.313*				

[a]The units of each variables are those given in *Table 2* and *3;* [b]Basal soil respiration; [c]Microbial biomass carbon; [d]Alkaline phosphatase; [e]ß -glucosidase; [f]Dehydrogenase; [g]Aryl sulphatase; [h]Protease; [i]Urease; [j]N-mineralization; [k]Hot water soluble boron. **Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level

Evaluating the experimental results, it is seen that one remarkable finding was the positive correlation between total Hg concentration in the soils and the analyzed microbiological and biochemical parameters. This correlation, found to be significant at a level of 1% to 5%, was determined in all parameters except BSR in the maize experiment (*Table 4*) and in all parameters in the cotton experiment (*Table 5*). Similar to this finding, it was shown in a study by Campos et al. (2018) in Spain that microbial biomass parameters and DHG activity were not adversely affected in the expected way by pollution levels caused by the toxic metal Hg.

Soil microbiological activities

Basal soil respiration (BSR)

Organic materials found in or applied to the soil are used as a carbon and energy sources by heterotrophic microorganisms. As a result of the growth and activities of them heat, CO_2, water vapor and humus are formed (Epstein, 1997) while the amount of CO_2 generated is defined as BSR. Taking into account the mean values of BSR obtained in the results of the experiment, there was a statistically significant increase in maize

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soils with SS_3, and in cotton soils with SS_1, SS_2 and SS_3 in comparison with SS_0 and CF. It is thought in studies conducted with sewage sludge that climatic conditions where the soils to which SS was applied are located were significant in the variation in the effect of the sludge. In a similar study in Russia, where climatic conditions are different, it was found that applications of sewage sludge stabilized by anaerobic decomposition did not have a statistically significant effect on MBC and BSR (Selivanovskaya et al., 2001). In our study, a significant high positive correlation was also found between BSR and the other biological parameters examined independent of crop type. The highest positive correlation with BSR in the experimental soils was with DHG (r = 0.668**) and the highest negative correlation was with total As (r = 0.372*) (*Table 5*).

Table 5. *Correlation between average values of some chemical and microbiological parameters of cotton soils at the end of the experiment*

Parameters[a]	BSR	MBC	ALKPA	GLU	DHG	ArSA	PRO	UA	N_{min}
BSR[b]	1	0.348^{*}	0.475^{**}	0.526^{**}	0.668^{**}	0.406^{**}	0.534^{**}	0.355^{*}	0.577^{**}
MBC[c]	0.348^{*}	1			0.506^{**}	0.377^{*}	0.373^{*}		
ALKPA[d]	0.475^{**}		1	0.678^{**}	0.652^{**}	0.663^{**}	0.849^{**}	0.491^{**}	0.684^{**}
GLU[e]	0.526^{**}		0.678^{**}	1	0.526^{**}	0.650^{**}	0.653^{**}	0.370^{*}	0.514^{**}
DHG[f]	0.668^{**}	0.506^{**}	0.652^{**}	0.526^{**}	1	0.705^{**}	0.670^{**}	0.446^{**}	0.806^{**}
ArSA[g]	0.406^{**}	0.377^{*}	0.663^{**}	0.650^{**}	0.705^{**}	1	0.645^{**}		0.575^{**}
PRO[h]	0.534^{**}	0.373^{*}	0.849^{**}	0.653^{**}	0.670^{**}	0.645^{**}	1	0.589^{**}	0.738^{**}
UA[i]	0.355^{*}		0.491^{**}	0.370^{*}	0.446^{**}		0.589^{**}	1	0.427^{**}
N_{min}[j]	0.577^{**}		0.684^{**}	0.514^{**}	0.806^{**}	0.575^{**}	0.738^{**}	0.427^{**}	1
Total Zn			0.633^{**}	0.513^{**}	0.317^{*}		0.578^{**}	0.601^{**}	0.401^{*}
Total Cu	0.383^{*}		0.707^{**}	0.562^{**}	0.584^{**}	0.558^{**}	0.708^{**}	0.464^{**}	0.576^{**}
Total Cr			0.446^{**}		0.490^{**}	0.413^{**}	0.423^{**}		0.458^{**}
Total Cd			0.369^{*}	0.452^{**}			0.381^{*}	0.343^{*}	
Total Pb	0.373^{*}	0.408^{**}	0.343^{*}		0.763^{**}	0.427^{**}	0.339^{*}		0.623^{**}
Total Ni	0.380^{*}	0.350^{*}			0.724^{**}	0.471^{**}			0.565^{**}
Total Hg	0.384^{*}	0.326^{*}	0.619^{**}	0.346^{*}	0.578^{**}	0.344^{*}	0.686^{**}	0.453^{**}	0.509^{**}
Total As	-0.372^{*}	-0.349^{*}			-0.595^{**}	-0.414^{**}			-0.394^{*}
B[k]	0.394^{*}	0.383^{*}	0.464^{**}	0.384^{*}	0.700^{**}	0.543^{**}	0.448^{**}	0.350^{*}	0.506^{**}

[a]The units of each variables are those given in *Tables 2* and *3;* [b]Basal soil respiration; [c]Microbial biomass carbon; [d]Alkaline phosphatase; [e]ß -glucosidase; [f]Dehydrogenase; [g]Aryl sulphatase; [h]Protease; [i]Urease; [j]N-mineralization; [k]Hot water soluble boron. **Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level

Microbial biomass carbon (MBC)

Regarding the mean MBC values, the application of 20 t ha^{-1} of sewage sludge (SS_2) was found to provide an increase in both maize and cotton soils which was statistically significant (α = 0.05) in comparison with SS_0 and CF (*Table 3*). It was observed that the MBC values of soils under both crops were affected by the SS_3 dose, and showed the greatest activity with SS_2. A decrease was determined in the amount of MBC in connection with sewage sludge applied at 30 t ha^{-1} compared to the dose in SS_2 because of the concentration of heavy metals, of 26% in maize soils and of 19% in cotton soils. In spite of this decrease, the MBC values of soils under both crops were still analyzed to

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be at high levels compared to plots with SS_0 and CF treatments. Furthermore, according to some studies (Flieβbach et al., 1994; Filip and Bieleck, 2002), a decrease in MBC can be the result of metal pollution.

Microbial biomass is an available storage of nutrients in the soil, such as C, N, S and P, and an indicator of the cycle of organic materials in the soil (Jenkinson and Ladd, 1981). Although it represents a small amount of the total soil N, C and P, N and other plant nutrients make an important contribution to plant nutrition because of their rapid mineralization. The higher MBC value is thought to be caused by the organic C content of SS. In plots to which the SS_2 dose was applied it was found to be 47% higher than the control and 33% higher than the plot treated with chemical fertilizer. Alongside the heavy metal load of the sewage sludge, the stabilization method may have an effect on its mineralization levels. In a study conducted in field conditions over three years with two different sewage sludges stabilized by composting and thermal drying, microbial biomass increases at the end of three consecutive years of 20 t ha^{-1} yr^{-1} of SS were 3.5 times greater than the control in the case of the SS stabilized by composting and 1.8 times greater in the case of the SS stabilized by thermal drying (Fernández et al., 2009). As was shown in previous studies, when substrate C is added to soils, it stimulates growth in the autochthonous soil microbiota in relation to an increase in energy sources in the soil (Antolín et al., 2005; Pascual et al., 2007). However, in the same study, even though an increase was observed in MBC value with an application of the highest dose of sewage sludge (80 t ha^{-1} yr^{-1}) similar to that of our study, this increase was lower than that provided by 20 t ha^{-1} yr^{-1}. Therefore, microorganisms can to a certain extent tolerate potential toxic effects with both the C content and mineralization potential of SS, which are effective parameters on MBC. When the dose of SS increased beyond a certain point, toleration levels decrease, but MBC activity was still higher than that of the control or that of the chemical fertilizer treatment. In addition, the effects of two different crops on the MBC were not clearly revealed.

Although a significant correlation was found between MBC content and DHG and ArSA activity at the 1% level and GLU and UA enzyme activity at a level of 5%, no correlation was determined between ALKPA and PRO enzyme activities (*Table 4*). In cotton soils, DHG activity showed the highest correlation, while ArSA and PRO enzyme activities showed a slight correlation with MBC content. These results show that ArSA, GLU, UA and PRO activities, and especially DHG enzyme activity, were a good indicator of general microbiological activity in the soils examined in this study, and in addition to this, they can provide valuable information on plant nutrient cycle processes. In both maize and cotton soils, MBC gave the highest positive correlation with DHG activity, and the only negative correlation with total As.

Nitrogen mineralization (N_{min})

N-mineralization, the conversion of nitrogen in organic form to its inorganic form, is performed by microorganisms in the soil with various physiological characteristics. The realization of this process depends on the suitability of environmental conditions, the presence of the relevant microorganisms in the environment, and a supply of suitable organic material. An increase was observed statistically in N_{min} values in relation to SS_0 and CF soils with all three doses of sewage sludge applied to the experimental soils. Also, from among the three doses, SS_3 had the greatest effect on N_{min}. It is thought that the observed increase in nitrogen mineralization during the growing period of each of the crops is an indicator that this waste has a high potential to increase crop

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productivity. Similarly, in a study in which SS was applied to sandy soil at a rate of 20-320 g kg^{-1} for 100 days, it was shown that N mineralization took place although at a low rate, and for this reason N was added to the soil by mineralization throughout the production season for plants growing in a single year (Alva et al., 2006). Also, the fact that no decline was determined in the levels of N-mineralization with increasing SS doses. This showed that the SS applications up to 30 t ha^{-1} did not have a restrictive effect on ammonification and nitrification bacteria in the same year. The N_{min} value shows a high correlation with the other microbiological and biochemical parameters analyzed in the soils under both crops (*Tables 4* and *5*). N_{min} showed the highest positive correlation in maize soils with ALKPA (r = 0.830**) and on cotton soils with DHG (r = 0.806**).

Soil biological activities

Dehydrogenase enzyme activity (DHG)

Dehydrogenase is an intracellular enzyme which functions inside living cells. Different from the other enzymes examined, which function both inside and outside cells, DHG gives more reliable information on the size and activity of the living microbial population (Bergstrom et al., 1998). Applications of sewage sludge, and especially treatments SS_2 and SS_3, increased DHG by between 42 and 53% in relation to treatments SS_0 and CF, with a 5% level of significance. Similar studies are also to be found in which SS applied to soils increased DHG activity (Fernández et al., 2009; Mondal et al., 2015). On the other hand, some researchers have reported that DHG activity was inhibited by the toxic effects of heavy metals with the addition of organic wastes rich in Pb (Marzadori et al., 1996) and Cu (Chander and Brookes, 1991). Thus, the lack of a statistically significant difference between DHG activity analyzed in relation to SS_2 and SS_3 treatments in maize soils and all three applications in cotton soils is an indicator of the lack of a clear increase in the number of living microbial cells. The observed trend in MBC values supported this situation. However, even though an increase in the amount of organic C added to the soil provided by the increased doses did not seem to support a numerical increase in living cells, positive changes observed in other microbiological and biochemical parameters may be an indication that various species of microorganisms with different functions in the ecosystem are supported. High positive correlations were found between DHG and all the microbiological parameters analyzed (*Tables 4* and *5*). With the result that there is a positive correlation between the heavy metal content of the soil and DHG, it may be concluded that soils to which sewage sludge is applied for a single year are not negatively affected in terms of microbial activity.

Alkaline phosphatase enzyme activity (ALKPA)

Phosphatase enzymes hydrolyze organic phosphorus compounds to ortho-phosphate, a form which plants can uptake (Amador et al., 1997). Because they are only produced by microorganisms, alkaline phosphatases are directly related to microorganism activity in the soil (Cayuela et al., 2008). A statistically significant increase was seen in ALKPA activity at an average of 37-41% compared to SS_0 and CF treatments with SS_3, and this was found to be 911.04 µg p-NP g^{-1} h^{-1} for maize soils and 894.97 µg p-NP g^{-1} h^{-1} for cotton soils. In another study, conducted with soils in a Mediterranean climate, aerobically stabilized sewage sludge was applied to soils in proportions varying

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between 6.2 and 10 g SS kg^{-1} soil (equivalent to 0.5 g P_2O_5 kg^{-1} soil). After incubation periods of 25 and 87 days following the incorporating of sewage sludge to the soil, an increase was found in phosphatase enzyme activities which decreased with a lengthening of the incubation period (Criquet et al., 2007). Other studies have shown similar patterns in soil enzyme activities following the application of SS (Pascual et al., 1998; Antolín et al., 2005; Criquet et al., 2007). Considering all these similar results, it is seen that sewage sludge has a short-term increasing effect on soil phosphatase activities, followed by a rapid decrease. Although we determined a decrease in ALKPA activity, it cannot be said that this decrease was on a large scale. Presumably, just as the quantity of living microorganisms in the sewage sludge and later their activity in the soil to which it was applied may result in an increase in enzyme activity (Dick and Tabatabai, 1984), at the same time the content of the existing substrates in the soil is increased due to SS has various organic substrates, and in this way the activity of microorganisms is supported (Kizilkaya and Bayrakli, 2005). These existing substrates include acid and alkaline phosphomonoesterase substrates (García et al., 1993) and phosphodiesterases (Turner and Haygarth, 2005) in generally large amounts in the organic matter in sewage sludge. Thus, when the amount of available P in the soil is a limiting factor for microbial growth, microorganisms can increase phosphatase production in order to be able to mineralize available P from organic P substrates added to the soil with SS (Criquet et al., 2007). Another result emerging from our study concerned correlations. The highest positive correlation with ALKPA was with PRO for maize and cotton vegetations ($r = 0.866^{**}$and 0.849^{**}, respectively).

Protease enzyme activity (PRO)

Protease hydrolyzes proteins into polypeptides, oligopeptides and amino acids. Because most N compounds are in an organic-related form in mineral soils, organic nitrogen must be converted to an inorganic form in order for N to be taken up by plants. Organic wastes applied to the soil stimulate protease (Rezende et al., 2004), but the breakdown products of these wastes can hinder this enzyme (Dilly and Nannipieri, 2001). In the present study, higher protease activity was determined in the soils to which the three doses of sewage sludge were applied than in SS_0 and CF soils; breakdown products of the sewage sludge did not hinder this enzyme, but on the contrary increased the amount of substrate necessary for the enzymatic reaction. Among the treatment doses, SS_3 resulted in PRO activity at the highest and most significant levels for maize soils, while with cotton soils, SS_2 and SS_3 showed PRO activity at a significant level. The highest correlations with the PRO value were shown by ALKPA both in maize soils ($r = 0.866^{**}$) and in cotton soils ($r = 0.849^{**}$).

Urease enzyme activity (UA)

Urease is an enzyme catalyzing the hydrolysis of urea to ammonia or ammonium, depending on soil pH (Tripathi et al., 2007). According to García et al. (2000), the presence of substrates or the demand of plants and microorganisms for nutrients increases the activity of this enzyme involved in the N cycle. Indeed, as Fernández et al. (2009) determined previously, the highest UA levels in connection with SS applications coincide with the highest crop yield parameters determined (*Fig. 2*). Statistically significant increases of 12-20% were determined with SS_2 and SS_3 compared to treatments SS_0 and CF. Nickel is necessary as a co-factor for the urease enzyme. In

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other words, in order for the urease enzyme to become active, it must join with two nickel ions for each sub-unit. The positive correlation (r = 0.467**) between UA and total Ni determined in maize soils may be an indication of this.

Aryl sulfatase enzyme activity (ArSA)

Aryl sulfatase enzyme which is responsible for the hydrolysis of aryl sulfate esters by the fusion of oxygen-sulfur bonds (Tabatabai, 1994). Ester sulfate, the substrate of aryl sulfate, is only found in fungi, and therefore it can also be a direct indicator of the presence of fungi in the soil (Bandick and Dick, 1999). ArSA was determined at approximately the same levels with all SS treatments, showing that there was a suitable substrate of the enzyme at approximately the same amounts in all sewage sludges. Renella et al. (2005) indicated that the coarse sandy soils from long-term field experiments under maize contaminated with high Cd–Ni-containing sludge reduced arylsulfatase and β-glucosidase activities and were significantly inhibited them by Cd and Ni at concentrations of 13.1 and 52.3 mg kg^{-1}, respectively. On the other hand in our study, the highest ArSA activity analyzed in maize soils was 248.25 μg p-NP g^{-1} h^{-1} with SS_3, and this value was achieved in cotton soils at a level of 246.47 μg p-NP g^{-1} h^{-1} with SS_2. ArSA activity showed an increase of 13-30% with SS applications relative to SS_0 and CF. Positive correlations were determined between ArSA and all microbiological and biochemical parameters. The highest correlation for maize soils was with GLU activity (r = 0.850**), and for cotton soils with DHG activity (r = 0.705**).

β-glucosidase enzyme activity (GLU)

Sugars with low molecular weight, which are hydrolysis products of glucosidases, are an important source of energy for soil microorganisms. One of the most important glucosidases in the soil is β-glucosidase which catalyzes the hydrolysis of cellobiose and contributes to the mineralization of the main organic carbon compound in nature, cellulose (Landgraf et al., 2003). The highest GLU activity in soils was shown with SS_3 in maize soils (86.61 μg Saligenin g^{-1} 3 h^{-1}), and in cotton soils with SS_2 (85.08 μg Saligenin g^{-1} 3 h^{-1}). An average increase of 22-32% was achieved in relation to SS_0 and CF. Hattori (1988) and Dick et al. (1988) determined higher GLU in applications of stable organic material containing cellulose. The highest positive correlation with GLU activity for maize soils was with ArSA (r = 0.850**), and for cotton soils with ALKPA (r = 0.678**).

Conclusions

Increases were seen in all microbiological parameters analyzed in relation to sewage sludge treatments and were found to be 22% for SS_1, 35.5% for SS_2, and 33% for SS_3 in relation to SS_0 and CF. In particular, it was observed that the SS applications of 20 and 30 t ha^{-1} increased microbiological parameters more. Correlation matrices showed that the SS doses of 10, 20 and 30 t ha^{-1} yr^{-1} applied did not result in a negative correlation between heavy metals and microbiological parameters, but rather showed positive correlations. This is because the result of the application to the soil of sewage sludge containing organic matter of a quality and amount to encourage microbial activity in the soil is to mask the negative effect created by the heavy metals it contains either on

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microorganisms or on the plant. Even though there was a decrease of 13.5% in the biological parameters analyzed in the second period compared with the first period, this decrease was not at a level to eliminate the differences between SS applications. Moreover, this is important for the management of sustainable organic matter in agricultural soils, and it brings the sewage sludge stabilization method to the fore. Biological activity in soils under different vegetation was also different. Microbial activity in the rhizosphere area of maize plants was found to be 6.5% greater than that of cotton. The highest dose of SS gave an increase in maize grain yield of 90% and in seed cotton yield of 72% over CF soils, and these increases were both much greater than the increases secured by chemical fertilizer applications and also different at a statistically significant level. The potential of organic matter to improve the physicochemical characteristics of a sandy loamy soil may have made a positive contribution to this. Even with the highest dose of sewage sludge applied in the experiment (SS_3), no toxic effect was observed in either maize or cotton plants. Therefore, in soils with a poor light-textured and low organic matter content in a Mediterranean climate and with one-year applications of sewage sludge, applications of stabilized sewage sludge at a level of 30 t ha^{-1} whether for the purpose of sustainable management of the soil's organic matter or to increase the crop yield can be used without ecotoxicological effects. This study, however, was conducted in a representative area of the Mediterranean Basin where arid and semi-arid climate conditions are dominant, so the results obtained can also be applied to other Mediterranean countries where SS incorporating to agricultural soil at a rate up to 30 t ha-1 yr-1 can be a cheap valuable solution to manage and utilize this waste as both fertilizer and soil conditioner. Our results suggest that attention should be paid to reducing the environmental risks of heavy metals in sludge utilization as soil improvement, not only to control the sewage sludge application dosage, but also to cultivate appropriate plant species. In the future, more field experiment is needed to evaluate the long-term residual effects of sewage sludge in the soil in terms of microbiological and biochemical properties of soils under different climate conditions and crop patterns

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APPENDIX

Table A1. *Multivariate hypothesis tests (MANOVA) for maize experiment*

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.998	962.414[b]	9.000	19.000	.000
	Wilks' Lambda	.002	962.414[b]	9.000	19.000	.000
	Hotelling's Trace	455.880	962.414[b]	9.000	19.000	.000
	Roy's Largest Root	455.880	962.414[b]	9.000	19.000	.000
Sampling Periods	Pillai's Trace	.780	7.467[b]	9.000	19.000	.000
	Wilks' Lambda	.220	7.467[b]	9.000	19.000	.000
	Hotelling's Trace	3.537	7.467[b]	9.000	19.000	.000
	Roy's Largest Root	3.537	7.467[b]	9.000	19.000	.000
Treatments	Pillai's Trace	1.917	2.250	36.000	88.000	.001
	Wilks' Lambda	.019	3.806	36.000	72.939	.000
	Hotelling's Trace	13.314	6.472	36.000	70.000	.000
	Roy's Largest Root	10.861	26.548[c]	9.000	22.000	.000
Replications	Pillai's Trace	1.116	1.383	27.000	63.000	.146
	Wilks' Lambda	.205	1.498	27.000	56.132	.101
	Hotelling's Trace	2.471	1.617	27.000	53.000	.067
	Roy's Largest Root	1.845	4.304[c]	9.000	21.000	.003
Sampling Periods * Treatments	Pillai's Trace	1.668	1.748	36.000	88.000	.018
	Wilks' Lambda	.075	2.019	36.000	72.939	.006
	Hotelling's Trace	4.496	2.185	36.000	70.000	.003
	Roy's Largest Root	2.110	5.158[c]	9.000	22.000	.001

a. Design: Intercept + Sampling Periods + Treatments + Replications + Sampling Periods * Treatments
b. Exact statistic
c. The statistic is an upper bound on F that yields a lower bound on the significance level

Table A2. *Multivariate hypothesis tests (MANOVA) for cotton experiment*

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.997	832.912[b]	9.000	19.000	.000
	Wilks' Lambda	.003	832.912[b]	9.000	19.000	.000
	Hotelling's Trace	394.537	832.912[b]	9.000	19.000	.000
	Roy's Largest Root	394.537	832.912[b]	9.000	19.000	.000
Sampling Periods	Pillai's Trace	.917	23.172[b]	9.000	19.000	.000
	Wilks' Lambda	.083	23.172[b]	9.000	19.000	.000
	Hotelling's Trace	10.976	23.172[b]	9.000	19.000	.000
	Roy's Largest Root	10.976	23.172[b]	9.000	19.000	.000
Treatments S	Pillai's Trace	1.898	2.207	36.000	88.000	.001
	Wilks' Lambda	.017	3.983	36.000	72.939	.000
	Hotelling's Trace	18.014	8.757	36.000	70.000	.000
	Roy's Largest Root	16.405	40.100[c]	9.000	22.000	.000
Replications	Pillai's Trace	.837	.903	27.000	63.000	.605
	Wilks' Lambda	.318	.998	27.000	56.132	.487
	Hotelling's Trace	1.680	1.099	27.000	53.000	.375
	Roy's Largest Root	1.375	3.207[c]	9.000	21.000	.013
Sampling Periods * Treatments	Pillai's Trace	1.504	1.473	36.000	88.000	.073
	Wilks' Lambda	.108	1.643	36.000	72.939	.037
	Hotelling's Trace	3.705	1.801	36.000	70.000	.018
	Roy's Largest Root	2.435	5.951[c]	9.000	22.000	.000

a. Design: Intercept + Sampling Periods + Treatments + Replications + Sampling Periods * Treatments
b. Exact statistic
c. The statistic is an upper bound on F that yields a lower bound on the significance level

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COMPARISON OF METHODS FOR PREDICTING SOIL PROPERTIES IN DONGHAI COUNTY OF CHINA

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Abstract. Different methods have been used to predict soil properties and obtain continuous spatial distributions. However, studies that compare the accuracy of these methods are scarce. In addition, few studies considered human activity variables as well as landscape ecology variables to predict soil properties. The objective of this research was to determine the optimal method for predicting soil properties using human activity and landscape ecology variables in regions with complex topographies. The study results are as follows. (1) Introducing land-use type as human activity variable to the soil-landscape model can improve the accuracy. (2) In the study region, the mean absolute error (MAE) and mean relative error (MRE) of the soil organic matter, pH, and soil particle size predicted by the soil-landscape model were lower than those predicted by ordinary Kriging method. (3) Specifically, the regions in which the soil-landscape prediction model was more accurate were all concentrated in the central and west hilly regions in the study region, whereas the regions in which the geostatistical interpolation was more accurate were mainly in the east plain region. The differences in the results are based on the topography of the study area.
Keywords: *soil-landscape, geostatistical interpolation, Donghai County, hilly region, precision comparison*

Introduction and review of literature

The purpose of soil sampling is to acquire the spatial distribution characteristics of target variables and study the soil characteristics across an entire study region. Soil surveys can achieve optimal land management by exploiting the available information in the study region (Dent and Young, 1981; Carter, 1993; Minasny and McBratney, 2006). Because current land management is becoming more refined and soil sampling is limited by human, material, and financial resources, methods that reduce sampling costs through rational sampling yet can still satisfy soil survey accuracy requirements have received increasing attention. Current scholars have conducted plenty of in-depth research on sampling design optimization methods. Some scholars (Hengl and Rossiter, 2003) have constructed a prediction model of soil properties using auxiliary variables and simultaneously optimized the distribution of sampling points. In addition, some researchers (van Groenigen et al., 2000; Ferreyra et al., 2002; Simbahan and Dobermann, 2006; Brus and Heuvelink, 2007) optimized the number and spatial

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distribution of sampling points using a simulated annealing algorithm and obtained the optimal distribution of soil sampling points. However, optimizing the methods of collecting soil samples may promote a heterogeneous spatial distribution of soil sampling points and soil properties, which can affect the accuracy of soil quality assessments. Under the assumption that additional soil sampling points cannot be added, methods that infer soil property information in unknown regions must be used to obtain a continuous spatial distribution of soil properties. Current soil studies primarily apply soil-landscape models and geostatistical methods for predicting different soil properties.

Soil-landscape models are derived from Jenny's equation (Jenny, 1941). This equation describes soil as a function of climate, potential biota, relief, parent material, and time (Jenny, 1941; Zhou and Wang, 2004). Numerous studies have used this model to predict soil properties. Jones (1973) studied the West African Savannah and found that the soil carbon, nitrogen and soil particle size were related to annual precipitation. Some research (Jones, 1973) found a piecewise linear relationship between the soil organic carbon, nitrogen, pH value and the gradient angle. Using soil-landscape models, Grunwald (2016) predicted the soil organic carbon content under different land-use types using a neural network with climate and topography factors as the input variables. A geostatistical approach was first applied by the South African mining engineer Krige (1951) for mineral exploration calculations used in the mining industry in the early 1950s. In soil property studies, the spatial variability of soil physical properties became a focus starting in the 1970s in North America and certain Western European countries (Krige, 1951; Minasny and McBratney, 2006).

In summary, numerous studies have investigated issues related to soil property predictions performed using soil-landscape models as well as geostatistical methods. However, the study regions in which these two methods were applied to predict soil properties were mostly concentrated in plains regions where the soil properties were relatively homogenous. Studies on regions with large topographic variations (such as mountains and hilly regions) are scarce, and comparative studies on the prediction results and accuracy of the two methods are limited. Therefore, in the present study, (1) the Huanghuaihai hilly region of Donghai County in Jiangsu Province was selected as the study region, and based on the large amount of data available for this study region, a soil-landscape model and a geostatistical model was used to predict the soil properties and then the prediction results and accuracy of the two methods were compared to provide information for selecting methods of predicting soil properties in regions with large topography variations; and (2) based on the frequent human activities in the study region, land-use types were added to represent of the effects of human activities as landscape factors to improve the prediction accuracy of the soil-landscape model and then analysed and verified the prediction results.

Materials and methods

Study area

Donghai County (*Fig. 1*) is located in the northeast of Jiangsu Province, China at 34°11′~34°44′N, 118°23′~119°10′E. Donghai County encompasses a plains hilly region at the southeast margin of the Huanghuaihai region. Its area is 2073 km^2. The elevation is high in the west and low in the east. The east plains region is flat and presents numerous lakes and reservoirs, the west region is a hilly region with an undulating relief, and the centre region presents gentle slopes as the plains transition to hills. The

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Maling Mountains, on the western boundary, extend from north to east. The substrate consists of purple sandshale, and purple soil is distributed as well. The parent material of the purple soil is the weathered remnants of the purple sandshale. In the centre and west, numerous hills and gullies are distributed and cross each other, and the terrain is primarily hilly. The parent material of the brown soil distributed throughout these regions is the weathered remnant of acidic metamorphic granitic gneisses, representing an ancient alluvial deposit covered by slope wash. The elevation of the lacustrine plain in the east is only 2-5 m, and the parent material of the soil is secondary ancient fluvial-lacustrine loessial deposits formed in the Quaternary.

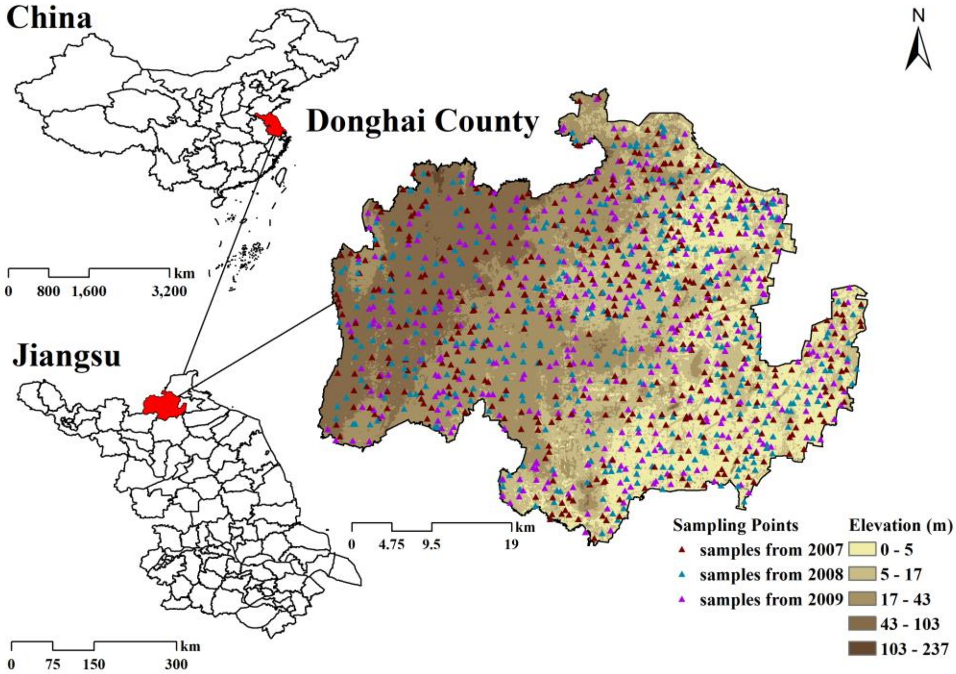

***Figure 1.** Location map of the study region and design of the sampling points*

Soil sampling

The 1268 sample points used in this study were collected in November 2007, November 2008, and November 2009. Each soil sample has been collected only one time in three-year period (detailed design shown in *Fig. 1*). The sampling locations were all on farmlands, and the collection times were all after the rice harvesting period and before the winter wheat sowing period. The sampling locations were determined using geographic coordinates obtained with Garmin-76 GPS receivers to reduce the accuracy errors associated with global positioning systems. The soil samples were all collected using stainless steel soil samplers, which collected soil from the 0-20 cm topsoil layer. At each sampling location, 5 samples were collected along 10-m diagonals and then mixed homogenously. After that, 1 kg of soil was retrieved by quartering, and the samples were sealed in plastic bags and brought back to the laboratory. Plant roots and rocks in the collected soil samples were removed, the soil samples were spread out on plastic disks, and naturally dried in ventilated locations. The dried samples were crushed and ground using wood rods or mortars, passed through 20-mesh, 60-mesh, and 100-mesh nylon sieves, and separately sealed in plastic bags until the physiochemical

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analyses. The physiochemical analyses of the samples were conducted at the Institute of Soil Science, Chinese Academy of Sciences, in Nanjing. Each sampling point included measurements of the pH value, organic matter content, and soil particle size. During the laboratory evaluation, each sample was divided into four parts and tested paralelly to prevent any errors that may occur during test to ensure the accuracy of soil properties. The three soil indices used in this study and their measurement methods were as follows: (1) soil pH was determined using the potential method; (2) soil organic matter was determined using the potassium dichromate volumetric method; and (3) soil particle size was determined using a laser particle size analyser and the Malvern method following the US grade system. 70% of the sample points were randomly selected (918) as the training set to build the soil-landscape prediction model and geostatistical model (ordinary Kriging interpolation). The remaining 30% of the sample points (350) were used as the test dataset for the models.

The statistical features of the three soil properties measured in areas with different land-use types are shown in *Table 1*. The organic matter in the soil from dry lands was significantly less than that from paddy fields. The soil moisture in the paddy fields was high, and organic matter decomposed slowly and accumulated more easily. The soil from the dry land was more acidic than that from the paddy fields. The particles of the soil from the paddy field were significantly larger than those of the soil from dry land, and the paddy field soil was mainly loamy and clayey, whereas the dry land soil was mainly sandy. The three soil properties of the paddy field showed a more significant normal distribution pattern, whereas the soil properties of the dry land showed a skewed distribution, with high kurtosis and concentrated data.

Table 1. *Statistical features of the soil properties of the different land-use types in intensively sampled regions*

		Minimum	Maximum	Mean	Variation coefficient	Skewness	Kurtosis
Paddy field	Organic matter (g/kg)	3.5	41.0	20.7	30.68%	0.42	-0.24
	pH value	5.0	8.2	6.8	8.85%	0.17	-0.31
	Soil particle size (%)	4.0	89.7	41.1	58.35%	0.15	-1.30
Dry land	Organic matter (g/kg)	1.2	28.7	12.5	29.63%	0.70	2.11
	pH value	5.1	9.9	6.4	8.45%	1.29	8.77
	Soil particle size (%)	3.5	78.3	17.8	65.73%	1.68	4.15

The above analysis indicates that the differences in the three soil properties from the paddy field and dry land were significant. The data statistics showed that the land-use types significantly affected the soil properties and that land-use types should be used as a factor in soil-landscape models.

Soil-landscape multivariate stepwise regression method

Soil-landscape models establish quantitative relationships between soil properties and different environmental factors (topography, landscape, hydrology, and human activities) by studying the correlation features between soil and environmental factors, and variation patterns of the spatial and temporal distribution of the soil properties are

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predicted based on quantitative relationships (Gessler et al., 2000; Kerry and Oliver, 2004; Grunwald, 2016).

This study determined the environmental factors that affect the soil in the study region based on the soil-forming factor theory and soil-landscape multivariate stepwise regression method. The soil-forming factor theory determined five categories of soil formation factors: climate, parent material, relief, potential biota, and time. The soil-landscape model theory proposes that different soil environmental conditions can form different soil types. When specific combinations of soil-landscape factors are determined, the soil type that is formed is also uniquely determined (Grunwald, 2016). Therefore, this study selected landscape factors that are relevant to soil forming and predictive for topography, morphology, climate, hydrology, vegetation, and human factors. For the soil factors, the soil organic matter and pH in the study region are important soil properties. Furthermore, because the study region is hilly, the soil particle size varies significantly. Additionally, the organic matter, pH, and soil particle size represent the comprehensive index, chemical index, and physical index of the farmland soil quality, respectively, and can be used to represent farmland soil quality to a great extent (Xia et al., 2000; Yuan et al., 2008; Zhu, 2008). Therefore, this study selected soil organic matter, pH, and soil particle size as the soil property indices. The definitions, equations, and meanings of the landscape factors in this study are provided in *Table 2*. To acquire specific data acquisition, a 30 m × 30 m digital elevation model (DEM) of the study region was used to select the relevant topographic factors in ArcGIS 9.3, including the elevation, gradient angle, plane curvature, profile curvature, topographic wetness index, and stream power index. The 5-year mean normalized difference vegetation index (NDVI) value was calculated in the region based on 30 m Landsat Thematic Mapper (TM) remote sensing data for the five year period 2005-2010. The land-use types were identified based on the current land-use map of Donghai County for 2010 (1:5000 scale). This study used the soil properties of the 918 sample points in the training set and a multivariate stepwise regression method to build the soil-landscape prediction model.

Geostatistical interpolation method

To predict the target variables (soil properties) of many independent and common variables in space, the commonly used geostatistical interpolation method, the ordinary Kriging (OK) method, was used. Based on the 918 sample points, the ordinary Kriging interpolation was used to predict the soil organic matter, pH value, and soil particle size in the study region. The Kriging interpolation is also called the spatial local interpolation method, and it is based on variogram theory and structural analyses and yields the best unbiased estimations of regionalized variables in limited regions. The Kriging method is one of the most important methods in geostatistics and provides the best linear unbiased estimations of elevation based on the spatial location of the points to be interpolated and the measured elevation of neighbouring points. The original topography in the study region is represented by a Kriging interpolation map of the elevation. The overall equation is as follows (*Eq. 1*):

$$\mathrm{Z}(x_0) = \sum_{i=1}^{n} \lambda_i Z(x_i) \quad \text{(Eq.1)}$$

where $\mathrm{Z}(x_0)$ represents the value of the unknown sample points; $Z(x_i)$ represents the value of the known sample points surrounding the unknown sample points; N is the

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number of known sample points; and λ_i is the weight of the i[th] sample point. This equation is determined by a semivariogram analysis, and based on the statistical requirements of unbiased and optimal, the equation between the weights and semivariance can be derived using Lagrange's minimization principle. For a more detailed and comprehensive description of the method, please refer to Cressie (1988), Odeh et al. (1995), and Mishra et al. (2009).

Table 2. *Definitions, equations, and meanings of the landscape factors*

Environmental factor	Definition and equation	Representation
Elevation (m)	Vertical distance from a point to the absolute reference surface	Changes in vegetation and hydrothermal conditions, affects soil development
Gradient angle	Angle between the normal and vertical directions at a ground point $\alpha = \arctan\sqrt{f_x^2 + f_y^2}$ (Gunstensen et al., 1991)	Reflects the degree of tilt of the local surface, which affects the topsoil stability and surface runoff discharge and convergence
Plane curvature (m-1)	At any given surface point, the curvature of the curve defined by the intersection between the horizontal plane passing through this point and the ground surface $H_c = -\frac{f_y^2 f_{xx} - 2f_x f_y f_{xy} + f_x^2 f_{yy}}{(f_x^2 + f_y^2)(1 + f_x^2 + f_y^2)^{3/2}}$ (Roddier, 1988)	Affects the convergence and divergence of the movement of surface material
Profile curvature (m-1)	At any given surface point, the curvature of the curve defined by the intersection of the plane passing through the normal direction and the direction with the maximum elevation gradient at this point and the ground surface $P_c = -\frac{f_y^2 f_{xx} + 2f_x f_y f_{xy} + f_x^2 f_{yy}}{(f_x^2 + f_y^2)(1 + f_x^2 + f_y^2)^{3/2}}$ (Zevenbergen and Thorne, 1987)	Affects the acceleration and deceleration of the movement of surface material
Topographic wetness index	$\mathrm{TWI} = \ln\left(\frac{A}{L \times \tan\alpha}\right)$ (Barling et al., 1994)	Quantitatively reflects the comprehensive condition of soil water storage and discharge
Stream power index	$\mathrm{SPI} = \frac{A \times \alpha}{L}$ (Hack, 1973)	Quantitatively reflects the potential erosion capability of runoff
Normalized difference vegetation index	$\mathrm{NDVI}_i = \frac{band4 - band3}{band4 + band3}$ $\overline{NDVI} = \sum_i^n \mathrm{NDVI}_i$ (Townshend and Justice, 1986)	Quantitatively reflects the vegetation distribution and coverage in the region
Land use type	Paddy field, dry land	Different land use types reflect the degree of human disturbance and change to soil

Note: f_x and f_y are the first derivatives of the elevation surface in the x and y directions, respectively; f_{xx} and f_{yy} are the second partial derivatives of the elevation surface in the x and y directions, respectively; f_{xy} is the second mixed partial derivative of the elevation surface in the x and y directions; A is the confluence area; and L is the length of the contour lines

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Method of analysing accuracy errors

The method of determining the accuracy errors in this study includes the mean absolute error (MAE) and mean relative error (MRE) indices to describe the differences between the model predictions and the measured values. Smaller values indicate a higher accuracy of the model predictions. $\widetilde{Z}_i$ is the predicted value, and Z_i is the measured value (*Eqs. 2* and *3*).

$$\mathrm{MAE} = \frac{1}{n}\sum_{i=1}^{n}\left|\widetilde{Z}_i - Z_i\right| \tag{Eq.2}$$

$$\mathrm{MRE} = \frac{1}{n}\sum_{i=1}^{n}\left|\widetilde{Z}_i - Z_i\right|/Z_i \tag{Eq.3}$$

Results

Prediction results of the two prediction methods

Soil-landscape models

The study object in this study is farmland; thus, all of the sample points were on farmland. Prediction models were built separately for paddy fields and dry land, which are shown in *Table 3*. All of the prediction models passed the F test at the 95% confidence level. The test analysis results showed that the model prediction results were good. The R^2 values of the soil property prediction models showed that the goodness of fit of the prediction model for the three soil properties in the paddy fields was higher than that in the dry land. The landscape factors in the paddy fields could explain 65.9%, 51.7%, and 63.5% of the spatial variance of the soil organic matter, pH value, and soil particle size, respectively. The soil-landscape factors in the dry land could explain 56.1%, 46.4%, and 48.3% of the spatial variance of soil organic matter, pH value, and soil particle size, respectively.

Table 3. *Soil-landscape prediction models for the different soil properties*

Land use	Soil property	Regression equation	R^2	F
Paddy field	Organic matter	$y_1 = 27.647 - 4.489\ln x_1 + 0.245x_2 + 4.832\,x_3 + 2.713x_7$	0.659	183.49
	pH	$y_2 = 6.753 - 0.119\ln x_1 + 0.009x_5 + 0.244\,x_7$	0.517	108.05
	Soil particle size	$y_3 = 0.443 - 0.122\ln x_1 + 0.016\,x_2 + 0.005x_5 + 0.194x_7$	0.635	128.88
Dry land	Organic matter	$\ln y_1 = 3.382 - 0.234\ln x_1 - 0.04331\ \ln x_4$	0.561	77.02
	pH	$y_2 = 6.572 + 0.153\ln x_1 - 0.062x_4 - 0.06221\ \ln x_5$	0.464	44.62
	Soil particle size	$y_3 = 0.371 - 0.043\ln x_1 - 0.00771\ \ln x_6$	0.483	46.84

Note: x_1: elevation; x_2: gradient angle; x_3: plane curvature; x_4: profile curvature; x_5: topographic wetness index; x_6: stream power index; and x_7: NDVI

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For the three soil properties, the goodness of fit of the prediction models was highest for organic matter. The soil-landscape model provided accurate predictions of the condition of the soil organic matter. Among the eight soil-landscape factors in the paddy fields and dry land, elevation was always a factor for the prediction models, indicating that elevation is one of the most important factors that affects soil properties.

Using the above soil property prediction models, The organic matter content, pH value, and soil particle size was separately predicted in the paddy fields and dry land. The prediction results are shown in *Figure 2*.

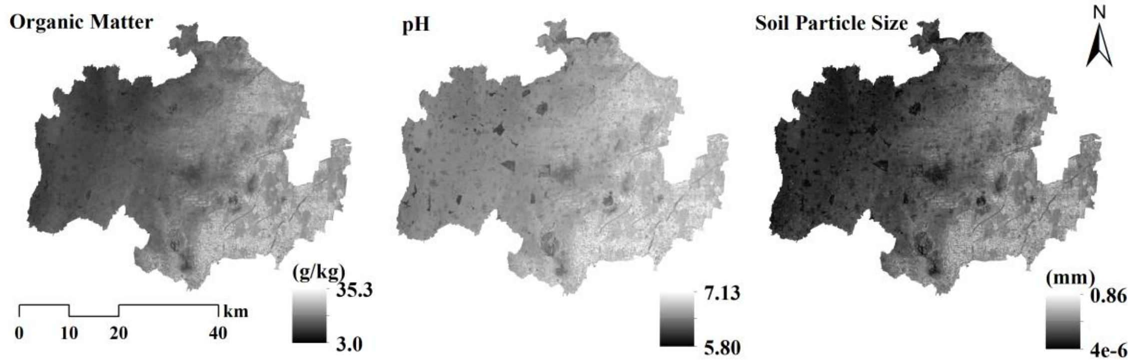

Figure 2. *Soil property prediction results of the soil-landscape model*

The results indicate that the soil organic matter content gradually decreased spatially from east to west. The pH values were high in the east and west of the study region but low in the centre. The soil particle size showed a similar spatial variation as the organic matter. The soil in the east region was made up of large/small particles, whereas the soil in the west region showed more sandy content.

Geostatistical interpolation

Ordinary Kriging interpolation was used to predict the soil organic matter content, pH, and soil particle size for the 918 sampling locations in the study region, and the results are shown in *Figure 3*.

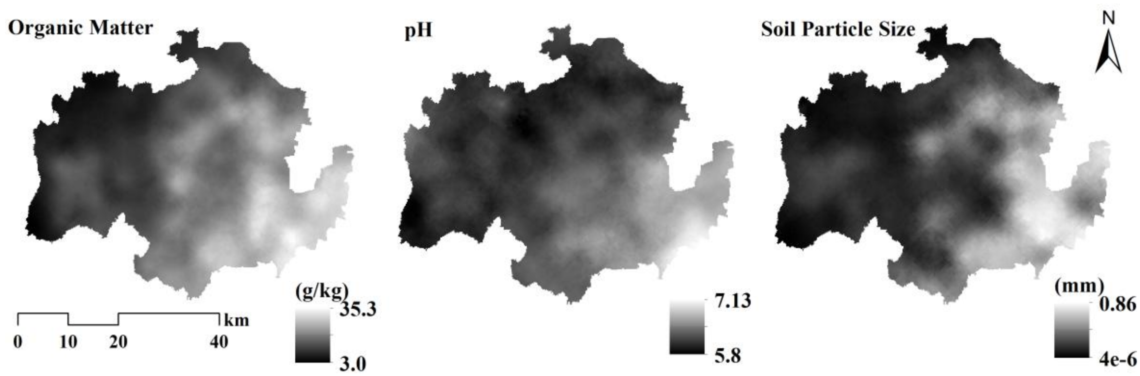

Figure 3. *Soil property prediction results of the Kriging interpolation*

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Based on the spatial distribution of the interpolation results, the overall spatial distribution pattern of the three soil properties were similar between the Kriging interpolation results and soil-landscape model prediction results. However, certain local regions showed significant differences. The Kriging interpolation prediction was based on the characteristics of the data statistics and spatial distribution of the interpolation points. The spatial variation of the soil properties in the interpolation results was smooth, although several regions showed a significant ‘bull’s eye’ effect. Several extremely high or low property data values strongly affected the interpolation results, which is expected when using this geostatistical interpolation method.

Accuracy errors of the two prediction methods

Soil-landscape model

350 sampling points were used to construct a test set and then quantitatively analysed the accuracy of the soil property prediction results in relation to the measured values.

The error analysis of the soil property prediction results is shown in *Table 4*. The analysis results show that the difference between the predicted results and measured values of the three soil properties in the paddy fields and dry land was extremely small; therefore, the three soil properties predicted by the model and interpolation method were accurate based on the prediction results. The mean absolute errors of the soil organic matter content, pH, and particle size in the dry land were smaller than those in the paddy fields by 41.18%, 20.00%, and 121.28%, respectively, although the relative error of the prediction results for the organic matter content in the paddy fields was smaller than that in the dry land by 13.33%, indicating that the model prediction results for the organic matter in the paddy field were more accurate than those in the dry land. The relative errors of the pH, soil particle size, and topsoil thickness in the paddy fields were extremely close to those of the dry land. All of the differences presented a significance of 0.01, which indicated that the prediction accuracy of the three soil properties by the prediction models was similar between the paddy fields and dry land.

Table 4. *Error analysis of the prediction results by the soil-landscape models*

Soil property	Paddy field (n = 220)				Dry land (n = 130)			
	Measured value	Predicted value	MAE	MRE	Measured value	Predicted value	MAE	MRE
Organic matter (g/kg)	20.6	21.2	0.24	0.13	12.1	12.6	0.17	0.15
pH	6.7	6.7	0.24	0.04	6.4	6.4	0.2	0.03
Soil particle size (%)	38.9	41.8	10.71	0.48	16.8	18.9	4.84	0.47

The relative errors of the 350 test points were interpolated to further study the spatial distribution of the prediction errors for the three soil properties predicted by the soil-landscape models in the whole study region. The results are shown in *Figure 4*.

The spatial distribution of the relative errors of the prediction results indicated that the relative error of the predicted soil organic matter content was high in the dry land in the west; therefore, the model showed low accuracy in this region. The relative error of the prediction results for soil pH value was high in the paddy fields in the centre and east of the study region but low in dry land, and the relative error of the prediction

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results for the soil particle size was high in the central study region. Overall, the accuracy of the soil-landscape model predictions for the three soil properties was spatially heterogeneous and showed high values in most regions and low values in certain regions.

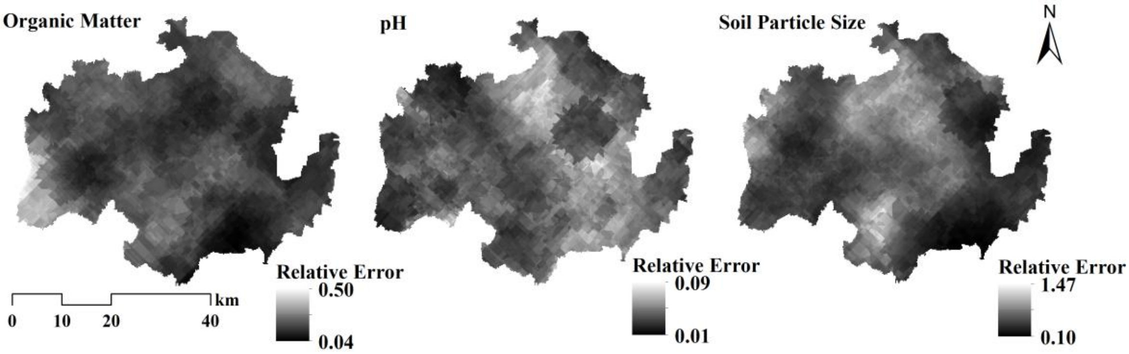

Figure 4. *Spatial distribution map of the relative error of the soil property predictions by the soil-landscape models*

Geostatistical interpolation

The accuracy of the soil properties predicted by the statistical interpolation was calculated, and the error analysis of the interpolation results is shown in *Table 5*. Among the three soil properties, the MAE and MRE of the interpolation results for the organic matter content and pH value in the paddy fields were slightly smaller than those in the dry land, with the MAE’s smaller by 3.57% and 6.90%, respectively, and the MRE’s smaller by 40.00% and 20.00%, respectively. These results indicate that the Kriging interpolation results for the organic matter content and pH value in the paddy fields were more accurate than those in the dry land. The MAE and MRE of the interpolation results for the soil particle size in the paddy fields were larger than those in the dry land by 29.17% and 8.47%, respectively, which indicated that the accuracy of the Kriging interpolation results for this soil property was higher in the dry land area.

Table 5. *Error analysis of the prediction results by the Kriging interpolation*

Soil property	Paddy field (n = 220)				Dry land (n = 130)			
	Measured value	Predicted value	MAE	MRE	Measured value	Predicted value	MAE	MRE
Organic matter (g/kg)	20.6	21.1	0.27	0.15	12.1	13.1	0.28	0.25
pH	6.7	6.7	0.27	0.04	6.4	6.5	0.29	0.05
Soil particle size (%)	38.9	42.2	10.76	0.64	16.8	21.0	8.33	0.59

A kriging interpolation was conducted on the relative errors of the 350 test points to further study the spatial distribution of the prediction errors of the three soil properties predicted by the Kriging interpolation for the entire study region. The results are shown in *Figure 5*.

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Based on the spatial distribution of the relative error of the prediction results, the relative error of the predicted soil organic matter content and pH value was high in the west dry land region. The accuracy of the results predicted by the Kriging interpolation for the organic matter content and pH value in this region was low. The relative error of the interpolation results for the soil particle size was low in the central study region, whereas the accuracy of the predicted soil particle size was high in this region.

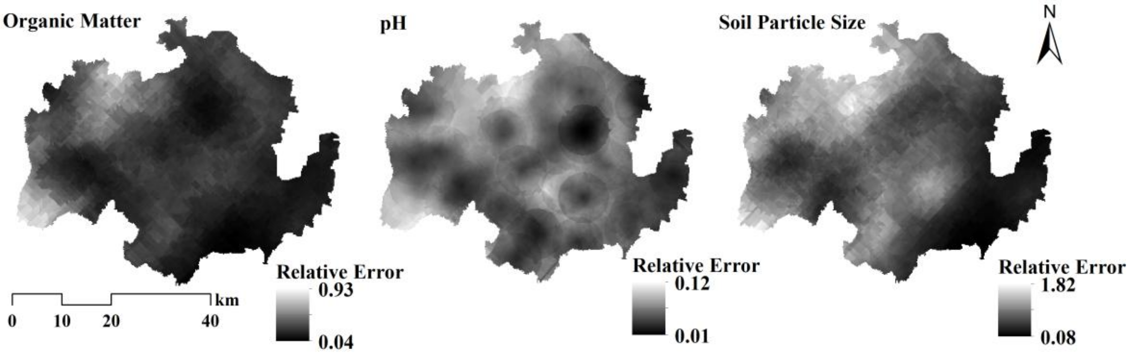

Figure 5. *Spatial distribution map of the relative errors of the soil properties predicted by the Kriging interpolation*

Comparison between the accuracy of the two prediction results

The comparison between the errors of the predicted soil properties by the two prediction methods is shown in *Table 6*. The results indicate that the MAE and MRE of the soil-landscape model predictions for the soil organic matter, pH value, and soil particle size were all smaller than those of the Kriging interpolation, indicating that the overall accuracy of the three soil properties predicted by the soil-landscape model was higher than that of the Kriging interpolation.

Table 6. *Error comparison of the results predicted by the soil-landscape model and Kriging interpolation*

Soil property	Soil-landscape model				Kriging interpolation			
	Measured value	Predicted value	MAE	MRE	Measured value	Predicted value	MAE	MRE
Organic matter (g/kg)	17.0	18.0	0.21	0.14	17.5	18.1	0.27	0.19
pH	6.6	6.6	0.23	0.03	6.6	6.6	0.28	0.04
Soil particle size (%)	30.7	33.3	8.53	0.48	30.7	34.3	9.86	0.63

The spatial distribution of the errors of the two prediction results (the MRE of the soil-landscape model - the MRE of the Kriging interpolation) were subtracted and further compared the spatial distribution of the accuracy of the two prediction results. The regions in which the MRE difference was less than 0 represent regions where the soil-landscape model was more accurate, and regions in which the MRE difference was higher than 0 represent regions where the Kriging interpolation was more accurate. The prediction accuracy of the three soil properties were partitioned based on these results,

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which is shown in *Figure 6*. In the figure, GIP represents geostatistical interpolation optimum and SLP represents soil-landscape optimum.

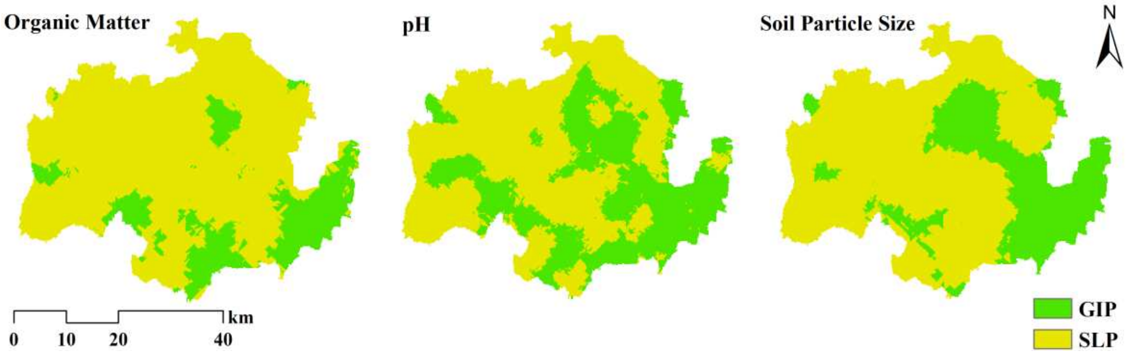

Figure 6. *Partitioned prediction accuracy of the soil-landscape model and geostatistical interpolation (the yellow colour represents areas where the prediction accuracy of the soil-landscape model is better, and the green colour represents areas where the prediction accuracy of the geostatistical interpolation is better)*

Based on the partitioned accuracy map, the prediction accuracy for organic matter, pH value, and soil particle size by the soil-landscape model was higher than that of the geostatistical interpolation method in most regions. For organic matter, the regions in which the soil-landscape prediction model was more accurate were mainly in the centre and west of the study region and constituted 80.59% of the total area. The regions in which the geostatistical interpolation was more accurate were mainly in the southeast region. For the pH value, the regions in which the soil-landscape prediction model was more accurate were mainly in the centre and west of the study region and constituted 60.82% of the total area. The regions in which the geostatistical interpolation was more accurate were distributed in the east and southwest regions. For the soil particle size, the regions in which the soil-landscape prediction model was more accurate were also mainly in the centre and west regions and constituted 70.58% of the total area. The regions in which the geostatistical interpolation was more accurate were mainly in the east region. Therefore, the soil-landscape model was more accurate for the three studied properties in the centre and west of the study region, and the relative error of the prediction results was smaller than that of the geostatistical interpolation by more than 20%. In the east study region, however, the prediction accuracy of the geostatistical interpolation was slightly higher than that of the soil-landscape model, and the relative error of the prediction results was smaller than that of the soil-landscape model by approximately 10%.

Discussion

Comparison with previous study on soil properties prediction

As mentioned before, Donghai County belongs to hilly region in Jiangsu Province, which usually makes it difficult to predict soil properties. To solve this problem, Former researchers took data from terrain attributes to raise the accuracy of predictions. Compared with their work such as results form Florinsky et al. (2002), higher R^2 can be achieved (0.561 vs 0.37) is this research. It is mainly because our soil-landscape model

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use terrain data in addition with landscape data such as TWI and NDVI. In addition. Human activity data is taken consideration in this research. This finding shows that landscape data and human activity data can improve the performance of soil prediction model in hilly regions.

Accuracy comparision between soil properties

In this research, it can be found that no matter what method is used, the prediction error of soil particle size is greater than that of organic matter and pH. It can be seen from *Table 1* that the variation coefficient of soil particle size is large yet variabilitys of soil organic matter and ph are small. At the same time, soil particle size is affected by precipitation, agricultural activities as well as organisms (Tyler and Wheatcraft, 1992), far r exceeding the coverage of the factors constructed in this study. While the physical and chemical processes of soil organic matter and ph are clearer and the changes are more stable. The above reasons explain why the MAE and MRE of soil particle size is always slightly worse than soil organic matter and pH.

Error analysis of two prediction methods

When it comes to the results from soil-landscape model, results indicated that the MAE and MRE of the three soil properties were relatively small. The prediction accuracy of the soil properties by the prediction model was good, which verified and further extended the stepwise linear relationship between the soil organic carbon content and pH value and the gradient angle observed by previous studies (Mishra et al., 2009). The mean absolute errors of the soil pH and particle size in the dry land region were all less than those in the paddy fields, which is mainly because paddy fields and dry land present different types of human activities. The differences among the three soil properties were significant. The dry land region is in the west hilly regions of the study region, and values for the three soil properties were significantly lower in this region compared with that in the paddy fields. Therefore, under the same error conditions, the absolute errors in the paddy fields will be naturally higher than those in the dry land. The relative error of the organic matter content in the paddy field was smaller than that in the dry land, which indicated that the prediction results for the organic matter in the paddy field by the prediction models were more accurate than those in the dry land. External factors (e.g., land management measures, such as fertilizing) reduce the effect of internal factors (e.g., soil-forming factors, such as soil-forming parent material) (Yasrebi et al., 2009). Therefore, the present study introduced land-use types to represent the effect of external factors to improve the model prediction accuracy.

When it comes to the results from kriging, the MAE and MRE of the interpolation results for the organic matter content and pH value in the paddy field were slightly smaller than those in the dry land, which indicated that the Kriging interpolation results for the organic matter and pH value in the paddy field were more accurate than those in the dry land. The MAE and MRE of the interpolation results for the soil particle size in dry land were smaller than those in the paddy field, indicating that the Kriging interpolation results for this soil property were more accurate for the dry land region. The main reason for this result is that the organic matter and pH value in the paddy fields were high and the spatial variance was weak; therefore, the prediction results by the Kriging interpolation were better. The dry land region presented the opposite results, with the organic matter and pH values showing significant spatial variations; thus, the

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accuracy of the interpolation prediction was low. The overall soil particle size in the dry land was low and presented small variations and weak spatial variance; thus, the accuracy of the interpolation prediction was good. These results are consistent with the findings of Yasrebi et al. (2009), who indicated that the prediction accuracy of Kriging interpolations was generally affected by the variation coefficient.

Comparison between two prediction methods

As proposed by previous studies, soil property prediction methods that consider topographic properties predict more smooth values and show a reduced effect of outliers on the prediction performance (Junxiao et al., 2016). The soil-landscape model in this study considered topographic factors. Thus, when comparing the accuracy of the soil property predictions based on the soil-landscape model and geostatistical interpolation, the accuracy of the prediction results by the soil-landscape model was overall higher than that of the geostatistical interpolation results.

Specifically, for the organic matter, pH, and soil particle size, the regions in which the soil-landscape prediction model was more accurate were all concentrated in the centre and west of the study region, whereas the regions in which the geostatistical interpolation was more accurate were mainly in the east region, which is because the centre and west of the study region are hilly regions with large topographic variations. The landscape factors in the soil-landscape model reflected this topographic difference, whereas the geostatistical method only predicted the soil properties based on data similarity. Therefore, the prediction results by the soil-landscape model in the hilly region were more accurate compared with the prediction results by geostatistical interpolation. Inversely, the east study region is a flat plain, and the soil properties were relatively homogenous. Therefore, the prediction accuracy of the geostatistical interpolation was better than that of the soil-landscape model.

Conclusions

The following specific conclusions can be reached.

- The study object was farmland soil in which human activities have strongly affected soil properties. Therefore, when the soil-landscape model was constructed, land-use types were added to represent the effect of human activities on the soil properties. The study results indicated that in the hilly region with large topographic variations, introducing the human activity variable improved the soil property predictions.
- The study region belongs to the Huanghuaihai hilly region. A comparison of the accuracy of the soil property predictions based on the soil-landscape model and geostatistical interpolation in the study region showed that the soil-landscape model was more accurate than the geostatistical interpolation. Specifically, for the organic matter content, pH, and soil particle size, the regions in which the soil-landscape prediction model was more accurate were all concentrated in the central and west hilly regions in the study region, whereas the regions in which the geostatistical interpolation was more accurate were mainly in the east plain region. Therefore, the prediction results by the soil-landscape model in the hilly region were more accurate compared to the prediction results by geostatistical interpolation.

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However, this study attempted to introduce a human activity variable into soil-landscape models to improve prediction accuracy; therefore, the variables in this model must be selected more rigorously, comprehensively, and appropriately. For example, taking into account the geo-environmental factors of the soil itself, the influence of human activities and the similar area with the same topography in the study area, we need to select variables that are suitable to local conditions. These issues require further study.

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PREDICTION OF LAND USE CHANGE AND ECOSYSTEM SERVICES VALUE: A CASE STUDY IN NANPING, CHINA

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Abstract. Changes in land use affect the structure, processes, and functions of ecosystems, and thus influence the ability of ecosystems to serve human beings. The prediction of land use and ecosystem services value can provide reference for the rational planning of land resources and relevant policy-making. Based on the CA-Markov model and the service value per unit area of terrestrial ecosystems in China, the area of land use types and ecosystem services value were predicted for 2030 and the corresponding countermeasures and suggestions were put forward. The study found that from 2015-2030, the area of forest land had the largest increase, while the area of land for construction had the largest decrease. The spatial distribution of land use in 2030 was predicted to be almost the same as that in 2015. The slight increase in the value of ecosystem services in 2030 was mainly due to improvement in the service capacity of waste treatment and gas regulation. The contribution rate of the service value of forest land to the total ecosystem services value was predicted to gradually decrease from 2015-2030.
Keywords: *landsat, LUCC, urbanization, Nanping, CA-Markov*

Introduction

Land use involves the long-term and cyclical management activities undertaken on land through use of biological resources and technology in accordance with the natural characteristics of the land and the purposes of economy and society (Fu et al., 2013, 2015; Lawler et al., 2014; Song et al., 2015). Ecosystem services refer to the environmental conditions and utilities formed by ecosystems necessary for the survival and development of human beings (Tolessa et al., 2016; Gaglio et al., 2017; Inkoom et al., 2017). Land use change affects the composition, function, and processes of ecosystems by altering the type of land cover, thereby affecting the availability of ecosystem services and human well-being (DeFries et al., 2004; Koellner et al., 2013). Prediction of land use change is a topic of interest in current research on land use change (Liu et al., 2009). However, there is less research in this area at present. Research on the prediction of land use change and ecosystem services value is helpful for governments to realize the ecological planning of land with comprehensive consideration of the national economy and social development (Inkoom and Frank and Fürst, 2017), to understand the supply capacity and demand of land in order to develop programs for land use planning according to the optimization of the ecological environment and sustainable development (Simmons et al., 2008; Cetin, 2016), and to carry out eco-friendly land use in order to fully utilize the ecological functions of land to ensure ecological security and to promote sustainable land use (Cetin, 2015; Cetin et al., 2018).

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Land use change assessment examines the land's utility and its limitations. According to the intended use of land, the assessment can be divided into the suitability of land for agriculture and cities. The assessment allows us to clarify whether the regional land is suitable for production in various industries, such as agriculture, fruit, forestry, aquaculture, and urban construction, and can also examine the quantity, quality, and distribution of land resources with unreasonable use. It provides scientific basis for the adjustment of regional land use structure and planning. Therefore, land use change assessment is an important method for studying land use change and developing land use policy (Veldkamp and Verburg, 2004).

The land use change model is a powerful tool for land use assessment, and it can help decision-making authorities to formulate land use policies (Veldkamp and Verburg, 2004; Verburg et al., 2004). Land use models can be divided into three categories according to different combinations. The first is a quantitative prediction model, such as the Markov model (Yang et al., 2007; Subedi et al., 2013). This model is based on probability theory and stochastic processes, which can be used to predict land use changes in future years or decades using the initial state of various types of land use and mathematical algorithms, such as the transfer matrix. It is more commonly used in the prediction of land use change because the conditions for using the model are simple; only the area and transfer matrix of land use categories are needed to predict land use changes in future decades. However, the model is not perfect because it cannot reflect the spatial location information, but instead simulates the changes in area of various types of land (Kim and Lee, 2007).

The second type of model is a spatial prediction model, such as a cellular automaton model. This type of model adds spatial information. The cellular automaton model, or CA model, was invented by von Neumann in 1950 (Zhao et al., 2011). This model is based on cells with discrete properties that are scattered in a grid with a set of rules and are changed according to changing rules. Cells with various properties form a complex system through simple interactions. However, there are deficiencies in these cells. The state of a cell in the next moment depends on the state of itself and its neighboring cells. However, the state of the cell should also be related to socio-economic factors, such as population, policies, and transportation (Li et al., 2010). In addition, the simulation accuracy of the CA model is affected by the subjectivity of land use conversion rules.

In addition to the above-mentioned models, there is a third type of model called the CA-Markov model, which is the coupling of the first two models. This coupled model has the advantages of the first two models in that it has both spatial information and improved accuracy of land use simulation (Ramezani and Jafari, 2014). Therefore, we used the CA-Markov model to predict land use changes and ecosystem services value in this study.

The Western Taiwan Straits Economic Zone was established in 2010. Fujian Province is at the core of the zone, and it also includes areas in Zhejiang Province and Jiangxi Province. It aims to cooperate with Taiwan to establish a new pole of economic growth in the southeast of China, thus driving economic growth in China. Nanping City, the study area, is located in the central position of the Economic Zone because it is situated at the border of Fujian Province, Jiangxi Province, and Zhejiang Province. Therefore, it has an important significance in predicting the change in land use and ecosystem services value in Nanping. In addition, Nanping City is at the headstream of the Minjiang River, which is the drinking water source for Fujian Province. Therefore, research on the prediction of land use change and ecosystem services value in Nanping

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can provide useful information for ecological security, using land resources rationally, alleviating the contradiction between humans and land, and promoting social harmony and sustainable development.

In this study, we aimed to determine the future change in land use and ecosystem services value in Nanping City from 2015 to 2030 based on the CA-Markov model and the equivalent value per unit area of ecosystem services created by Costanza (1997) and Xie et al. (2006, 2010). This research not only can reveal the future change in land use and ecosystem services value in Nanping, but also can provide a reference for scientific and effective land use planning and for research on ecosystem services value in other provinces and regions.

Materials and methods

Study area

Nanping City was chosen as the study area in this research. It is located in the southeast part of China across the sea from Taiwan (*Fig. 1*).

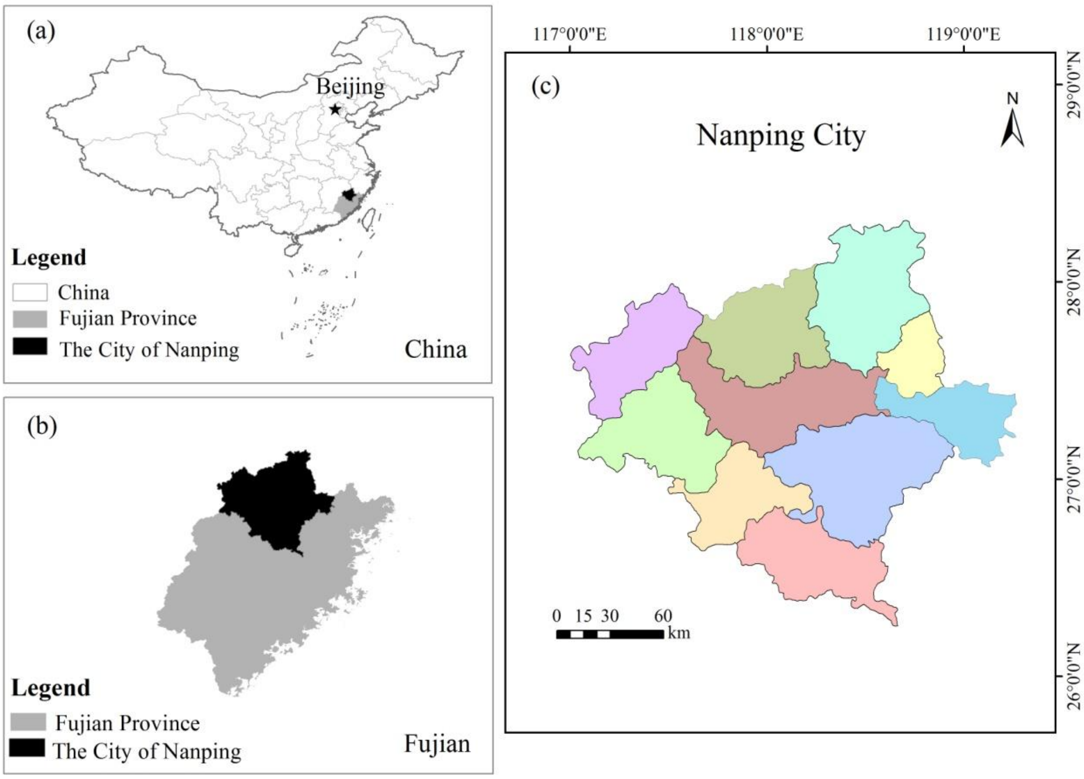

Figure 1. *Map of study area*

The area has a subtropical humid monsoon climate with an annual average temperature of 17-19 °C, an average winter temperature of 6-9 °C, an average summer temperature of 28-29 °C, and an average annual rainfall of 1684-1780 mm. Nanping City has a history of over 4,000 years and is one of the cradles of culture in Fujian Province. Nanping City is the largest city in the province with an area of 26,300 km^2, accounting for about one-fifth of the total area of the province. The area of cultivated land and

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forest land in Nanping accounts for approximately one-quarter of that in the province. The area of bamboo forest land accounts for approximately one-tenth of that in China. By the end of 2015, the forest coverage rate was 74.75%, the total household registration population was 3.2 million, and GDP was 20.933 billion USD. Nanping is also the food production base of Fujian Province; its grain output ranks first among that of other cities in the province. Nanping has one of the best ecological environments compared to other areas at the same latitude. Wuyishan, which is under the jurisdiction of Nanping, is one of the four World Natural and Cultural Heritage Sites in China.

Data source

Acquisition and processing of remote sensing data

We downloaded remote sensing images, including Landsat 5 (2005 and 2010) and Landsat 7 (2015) images, from the Chinese Geospatial Data Cloud (Xu et al., 2016). The images from Landsat 7 were processed using strip processing. The images with cloud coverage of less than 10% were selected. Seven scenes fully covered the total study area, so twenty-one scenes were downloaded for the research. Then, color synthesis, mosaic, clipping, and object-oriented classification were conducted using ENVI 5.2 to process these images.

In this study, bands 5, 4, and 3 (RGB) were used for color synthesis. According to the needs of the study and the geographical features of Nanping City, we divided the land use types into 6 categories, including forest land, grassland, cultivated land, construction land, water area, and unused land. After the object-oriented classification was conducted, post-classification maps for three periods were created (*Fig. 2*). Finally, the areas of all land use types for three periods were extracted using ENVI (*Table 1*).

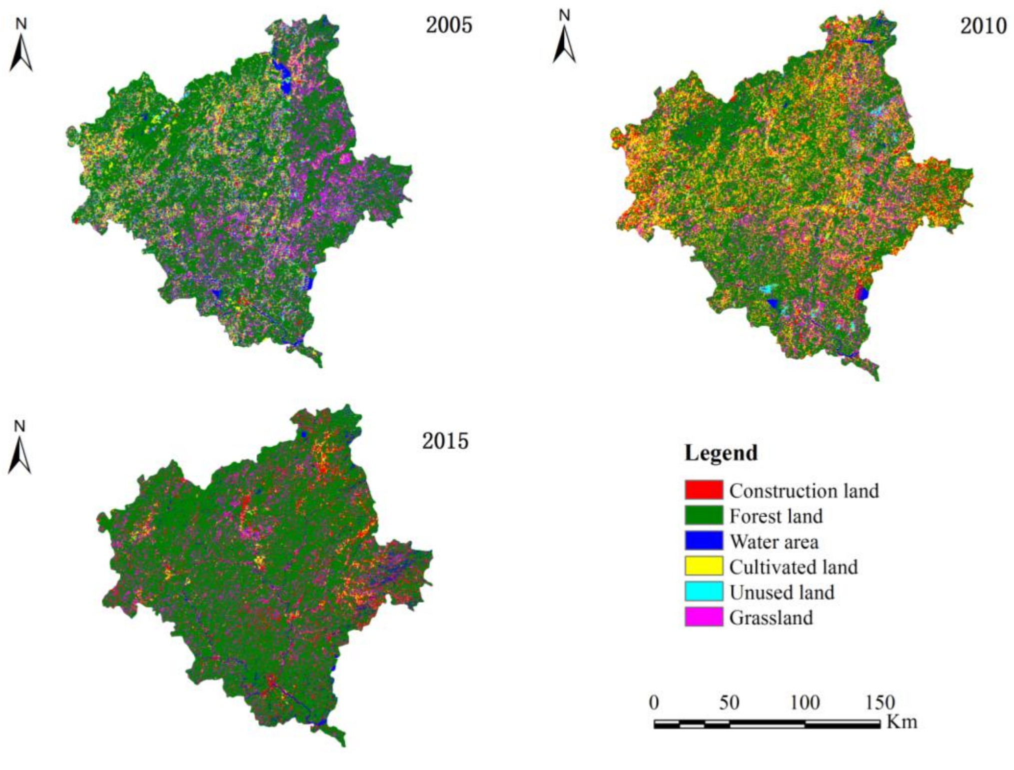

Figure 2. *The distribution of land use types in three periods in Nanping*

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Table 1. *Area of different land use types in three periods (km²)*

Land use types	2005	2010	2015
Construction land	498.94	1350.44	1101.63
Forest land	19495.21	19258.94	21380.69
Water area	712.06	645.76	624.84
Cultivated land	2124.34	2452.60	1106.44
Unused land	1076.98	770.43	410.36
Grassland	2362.45	1796.45	1644.75

Preparation for creation of a suitability atlas

In this study, the CA-Markov module in IDRISI 17.0 was used to predict land use change in Nanping City. A gradient vector map and vector maps of the water system buffer and administrative center buffer were needed before using the CA-Markov model.

Acquisition of the gradient map

Chinese land use planning is a project of the development, utilization, governance and protection of land in a specific area, time and space according to the requirements of Chinese sustainable development as well as local natural and economic conditions (Zhang and Feng, 2017). According to the grading standards adopted in Chinese Land Use Planning, the slope of land use in Nanping City was divided into five grades (*Table 2*). The gradient map was then obtained using digital elevation model (DEM) and ArcGIS 10.1 software (*Fig. 3*). It can be seen from *Table 2* that the area with a gradient >6° accounted for 84.73% of the total study area, while the area with a gradient of 0-6° only accounted for 15.27% of the total area. This also confirms that mountains are the major landform in Nanping.

Table 2. *Gradient classification and area in Nanping City*

Grades	Gradient (°)	Area (km²)	Proportion (%)
1	0-2	1009.52	3.85
2	2-6	2995.24	11.42
3	6-15	9978.90	38.05
4	15-25	8290.77	31.60
5	>25	3954.76	15.08

Acquisition of the buffer maps of the water system and administrative centers

The buffer maps of the water system and administrative centers (*Fig. 4b* and *d*) were obtained by setting up four buffer zones at intervals of 1 km, which was the influence area of the water system and administrative centers, by means of distribution maps of the water system, the county government, and ArcGIS 10.1 (*Fig. 4a* and *c*).

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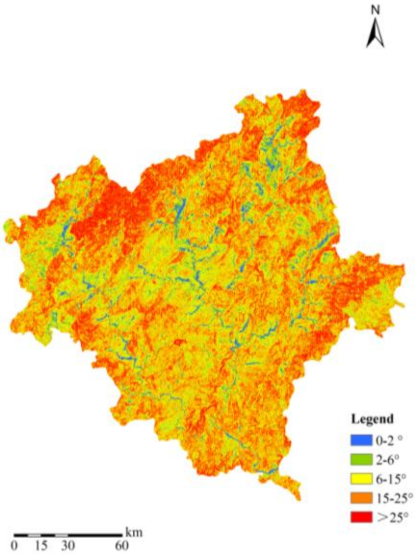

Figure 3. Gradient map of Nanping City

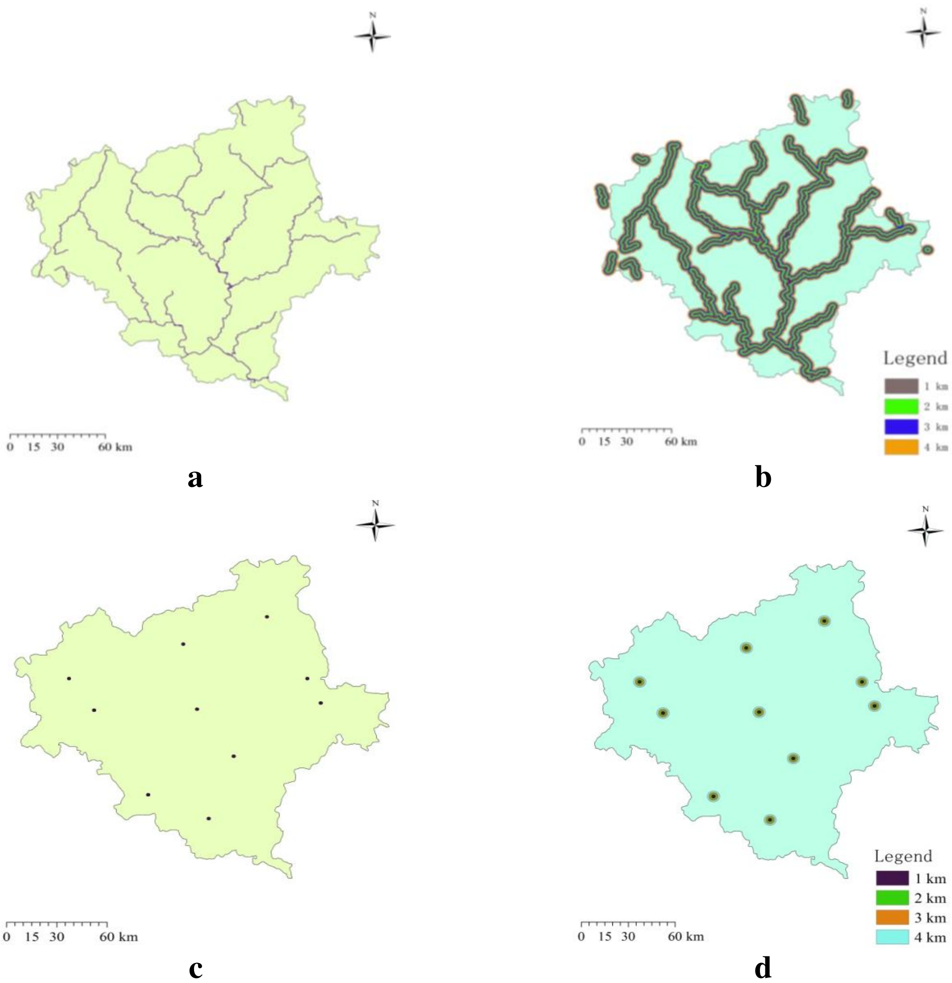

***Figure 4. a** Distribution of the water system in Nanping City. **b** The buffer of the water system in Nanping City. **c** Distribution of the county government administrative centers in Nanping City. **d** The buffer of the county government administrative centers in Nanping City*

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Study methods

CA-Markov model

The CA model was proposed by Stanislaw Ulam in the 1940s. It has been widely used in geography, ecology, information science, military science, social science, and other fields since being improved by a number of scholars.

The CA model formula is as follows (*Eq. 1*):

$$A = (G, U, E, f_x) \tag{Eq.1}$$

where, G represents the cell grid; U refers to the cell neighborhood; E is the initial state of the cell; f_x represents the transformation rules of cell x; and A is the cellular automaton model.

Each cell is in different states at different times, and the states change according to the rules. Cellular automaton can be seen as a model of cellular assembly with rules. It can be used to study land use change because it can change over time and space, which is similar to the process of land use change, and the structure and composition of the cells in cellular automata are similar to those of raster data.

The Markov model can also be used to predict land use change using a transition probability matrix. *Equation 2* describes the transition process from land use type E_t to another land use type E_j. P_0 is the probability of land use type E_t being transformed to another land use type E_j.

$$P(E_t \to E_j) = P(\frac{E_t}{E_j}) = P_0 \tag{Eq.2}$$

Equation 3 is the state transition probability matrix. P_{ij} represents the state transition probability from land use type E_i to another land use type E_j.

$$P_{ij} = \begin{bmatrix} P_{11} & P_{12} & \cdots & P_{1n} \\ P_{21} & P_{22} & \cdots & P_{2n} \\ \vdots & \vdots & \vdots & \vdots \\ P_{n1} & P_{n2} & \cdots & P_{nn} \end{bmatrix} \tag{Eq.3}$$

Before using the Markov model, we must assume that the change in each land use type is only related to the state in the present moment, and not related to the state in any previous moment. At the same time, the probability of transition from one type of land use to any other type of land use is needed (*Eqs. 4* and *5*).

$$\begin{cases} 0 \le P_{ij} \le 1 (i, j = 1, 2, \cdots, n) \\ \sum_{j=1}^{n} P_{ij} = 1 (i = 1, 2, \cdots, n) \end{cases} \tag{Eq.4}$$

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$$\pi_j(k) = \sum_{i=1}^{n} \pi_i(k-1) P_{ij} (j = 1,2,\Lambda\ ,n) \tag{Eq.5}$$

where $\pi_j(k)$ represents the probability that the land use type transitions into the final land use type after the passing of k time from the initial state. P_{ij} is the transition probability from one state of land use to another.

We can achieve the value of P_{ij} from *Equation 6:*

$$P_{ij} = \frac{f_{ij}}{\sum_{j=1}^{n} f_{ij}} \tag{Eq.6}$$

where f_{ij} represents the time of transition from state i to state j in one step.

$$S^{(k)} = S^{(0)} \cdot \pi_j^{(k)} \tag{Eq.7}$$

where $S^{(k)}$ is the state of land use at k moment. $S^{(0)}$ is the initial state of land use and $\pi_j^{(k)}$ represents the transition probability matrix after K steps. From *Equation 7* we can obtain the state of land use at any time by means of the initial state of land use and the transition probability matrix after K steps.

Both the CA model and Markov model are discrete mathematical models in terms of time and state. They are widely used in the fields of geography and ecology. However, they both have drawbacks. The Markov model relies mainly on the transfer matrix of each type of land use to make a prediction. It does not take into account the fact that the driving force of land use change is constantly changing. In addition, the Markov model can provide data on how land use will change, but cannot predict the spatial distribution of land use change. Therefore, the accuracy of predicting land use change using the Markov model is lacking. The CA model can simulate the dynamic spatiotemporal changes of land use in the future. However, the prediction accuracy of the CA model depends on the conversion rules of land use, which are subjective. Additionally, the state of the cells in the CA model are not only affected by spatial location, but are also affected by many other aspects, such as nature, economy, and society. To summarize, the CA-Markov model combines the Markov model and the CA model, and takes into account the impact of the drivers of land use change, thereby more accurately predicting land use change in the study area.

Operation of IDRISI

In this study, the CA-Markov module in IDRISI 17.0 was used to predict the land use change in Nanping City. The grid maps of land use post-classification, Markov transfer probability matrix, and multi-criteria evaluation module (MCE) suitability atlas were prepared before using the model to predict trends in land use change.

Grid maps of land use post-classification

The vector maps of land use post-classification described in 2.2.1 were transformed into grid maps using the conversion tool of ArcGIS 10.1.

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Markov transfer probability matrix

The matrices of the Markov transfer probability from 2005-2010 and from 2010-2015 were obtained using IDRISI: GIS Analysis-Change/Time Series-Markov (*Tables 3* and *4*).

Table 3. *The transfer probability matrix of land use from 2005-2010 (%)*

2005 / 2010	Construction land	Forest land	Water area	Cultivated land	Unused land	Grassland
Construction land	27.04	8.86	14.73	14.51	16.07	9.48
Forest land	27.27	65.62	33.71	31.90	29.17	28.59
Water area	4.76	2.84	17.39	4.02	3.05	2.65
Cultivated land	33.27	13.66	25.94	35.90	36.79	32.92
Unused land	0.85	0.85	0.71	1.38	1.59	1.06
Grassland	6.81	8.17	7.52	12.29	13.33	25.30

Table 4. *The transfer probability matrix of land use from 2010 to 2015 (%)*

2010 / 2015	Construction land	Forest land	Water area	Cultivated land	Unused land	Grassland
Construction land	18.05	1.20	13.53	14.04	9.60	9.67
Forest land	64.39	87.93	51.51	56.66	58.87	60.02
Water area	3.75	1.47	26.67	2.91	4.37	1.92
Cultivated land	3.58	4.52	2.91	2.39	10.44	3.33
Unused land	0.20	1.80	0.17	0.39	1.98	0.28
Grassland	10.03	3.08	5.21	18.52	14.74	24.78

Creation of a suitability atlas

A suitability atlas is a set of images containing rules. Using the atlas can better simulate the influence of factors on land use change. The creation of the atlas is a key and difficult component in predicting land use change. We used the MCE module in IDRISI to create the suitability atlas. In the MCE module, we set up the images according to Boolean rules, which were that 1 indicated suitability and 0 indicated unsuitability, and conversion rules. These conversion rules were as follows:

(1)The conversion rules of cultivated land

a) The area with a slope >25° was chosen as an unsuitable area for cultivated land.
b) The zones within 2 km of administrative centers were set as inappropriate areas for cultivated land.

(2)The conversion rules of construction land

a) The conversion of construction land to any other type of land use was forbidden.
b) The conversion of an area with a slope >15° to construction land was forbidden.

(3)The conversion rules of forest land, grassland, and water area

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The restrictions of forest land, grassland, and water area were simpler compared to other types of land because of their functions of environmental protection. Hence, their rules were as follows:

a) The conversion of forest land and grassland to cultivated land was prohibited.
b) The conversion of water area within 1 km of a water system to construction land was forbidden.

(4)The conversion rules of unused land

The conversion of unused land to other types of land use was prohibited, and unused land could only be allocated to areas with a larger gradient.

Suitability images for each land use type were created by combining each image, obtained using the rules above, using Boolean intersection in the MCE module. We then created the suitability atlas using the Collection Editor tool. Finally, the future change in land use in Nanping was predicted using the CA-Markov module in IDRISI.

Accuracy of the prediction of land use change

In order to ensure the feasibility of the CA-Markov model in predicting land use change, we tested the accuracy of the model. There were two ways to test the accuracy: the quantitative accuracy test, which tested the accuracy of the structure and quantity of land use types; and the spatial accuracy test.

The quantitative accuracy test

The equation of this method is as follows (*Eq. 8*):

$$a = \frac{x_{ib} - x_{ia}}{x_{ib}} \times 100 \quad \text{(Eq.8)}$$

where a is the error precision and i indicates a specific land use type. x_{ib} and x_{ia} represent the values of the predicted and actual areas, respectively. When $a > 0$, it indicates that the predicted value is greater than the actual value.

The spatial accuracy test

$$y = \frac{|(x_{ib} - x_{ic}) + (x_{ia} - x_{ic})|}{x_{ia}} \times 100 \quad \text{(Eq.9)}$$

where y represents the spatial error of the land use prediction and i indicates a specific land use type; x_{ia} and x_{ib} represent the number of cells in the reference image and the predicted image, respectively; and x_{ic} represents the cell number of the overlapping parts of x_{ia} and x_{ib}.

Value per unit area of ecosystem services

According to the research results of Costanza et al. (Costanza, 1997) and Xie et al. (Xie et al., 2006, 2010) combined with the actual situation in China, a table of equivalent value per unit area of terrestrial ecosystem services in China was created,

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which provides a reference for the calculation of ecosystem services value in China (*Table 5*). The table is widely used by Chinese scholars (Liu et al., 2009; Xu et al., 2016; Li et al., 2010; Peng et al., 2016; Wang and Sun, 2016; Xu et al., 2016). According to the study by Xie (Xie and Xiao and Lu, 2006), we chose 9 types of ecosystem services, including gas regulation, climate regulation, water conservation, soil formation and protection, waste treatment, biodiversity conservation, food production, supply of raw materials, and entertainment and culture.

Table 5. *Equivalent value per unit area of terrestrial ecosystem services in China*

Ecosystem services	Forest land	Grassland	Cultivated land	Wetland	Water area	Unused land
Gas regulation	3.50	0.80	0.50	1.80	0.00	0.00
Climate regulation	2.70	0.90	0.89	17.10	0.46	0.00
Water conservation	3.20	0.80	0.60	15.50	20.38	0.03
Soil formation and protection	3.90	1.95	1.46	1.71	0.01	0.02
Waste treatment	1.31	1.31	1.64	18.18	18.18	0.01
Biodiversity conservation	3.26	1.09	0.71	2.50	2.49	0.34
Food production	0.10	0.30	1.00	0.30	0.10	0.01
Supply of raw materials	2.60	0.05	0.10	0.07	0.01	0.00
Entertainment and culture	1.28	0.04	0.01	5.55	4.34	0.01

In this study, the land use types were divided into 6 categories, including forest land, cultivated land, water area, unused land, grassland, and construction land. Therefore, *Table 5* was slightly modified to form a table of equivalent value per unit area of terrestrial ecosystem services in Nanping City (*Table 6*). Construction land was not included in *Table 6* because ecosystem services provide a positive utility that meets the needs of human beings. However, construction land, such as rural residential land and industrial and mining land, constantly releases sewage, waste gas, and solid waste to the environment, which have a devastating effect on the natural ecological environment. Consequently, we did not account for the value of ecosystem services of construction land.

Table 6. *Equivalent value per unit area of terrestrial ecosystem services in Nanping City*

Ecosystem services	Forest land	Cultivated land	Water area	Unused land	Grassland
Gas regulation	3.50	0.50	0.00	0.00	0.80
Climate regulation	2.70	0.89	0.46	0.00	0.90
Water conservation	3.20	0.60	20.38	0.03	0.80
Soil formation and protection	3.90	1.46	0.01	0.02	1.31
Waste treatment	1.31	1.64	18.18	0.01	1.95
Biodiversity conservation	3.26	0.71	2.49	0.34	1.09
Food production	0.10	1.00	0.10	0.01	0.30
Supply of raw materials	2.60	0.10	0.01	0.00	0.05
Entertainment and culture	1.28	0.01	4.34	0.01	0.04

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We can see from *Table 6* that the equivalent value per unit area of ecosystem services of these land use types is based on the value per unit area of food production of cultivated land in Nanping, which can be calculated by *Equation 10*:

$$E_a = \frac{1}{7} \cdot T_a \cdot T_b \qquad \text{(Eq.10)}$$

where E_a represents the value per unit area of food production of cultivated land (CNY/hm^2); T_a refers to the average grain yield per unit area (kg/hm^2) in the study area; and T_b is the average grain price in China (CNY/kg). By consulting the Nanping Statistical Yearbook, we found that the average grain yield per capita in Nanping City in 2015 was 5,700 kg/hm^2, and that China's average grain price in 2015 was 5.24 CNY/kg. Hence, we obtained the value per unit area of food production of cultivated land in Nanping, which was 4266.85 CNY/hm^2, by means of *Equation 10*.

The values per unit area of ecosystem services for land use types, except for cultivated land, were obtained using *Equation 11* and *Table 6*.

$$CV_{ij} = E_a \cdot f_{ij} \qquad \text{(Eq.11)}$$

where i is a specific land use type. j represents a specific ecosystem service. CV_{ij} represents the value per unit area of specific ecosystem services of a specific land use type (CNY/(hm^2·year)). E_a represents the value per unit area of food production of cultivated land (CNY/hm^2). f_{ij} refers to the equivalent value per unit area of specific ecosystem services of specific land use types.

Table 7. *The value per unit area of ecosystem services of land use types in Nanping City (ten thousand CNY/hm^2)*

Ecosystem services	**Forest land**	**Cultivated land**	**Water area**	**Unused land**	**Grassland**
Gas regulation	1.4933	0.2133	0.0000	0.0000	0.3413
Climate regulation	1.1520	0.3797	0.1962	0.0000	0.3840
Water conservation	1.3670	0.2560	8.6953	0.0128	0.3413
Soil formation and protection	1.6640	0.6229	0.0042	0.0085	0.5589
Waste treatment	0.5589	0.7012	7.7567	0.0042	0.8320
Biodiversity conservation	1.3909	0.3029	1.0624	0.1450	0.4651
Food production	0.0427	0.4267	0.0427	0.0042	0.1280
Supply of raw materials	1.1093	0.0427	0.0042	0.0000	0.0213
Entertainment and culture	0.5461	0.0042	1.8517	0.0042	0.0171
Total	9.3243	2.9497	19.6136	0.1791	3.0891

Prediction of ecosystem services value

The predicted value of ecosystem services of all land use types can be calculated by *Equation 12:*

$$ESV = \sum_{i=1}^{n} \sum_{j}^{m} A_i \times CV_{ij} \qquad \text{(Eq.12)}$$

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where ESV is the predicted value of ecosystem services, A_i is the predicted area of a specific land use, and CV_{ij} represents the value per unit area of ecosystem services of specific land use types. n and m refer to the number of land use types and ecosystem services, respectively.

Results

Prediction accuracy of land use change

The area of land use types in Nanping City in 2015 was predicted using the CA-Markov model and the area of land use types in Nanping City in 2005 and 2010. According to *Equations 8* and *9,* quantitative and spatial errors were present (*Table 8*). We found from *Table 8* and *Figure 5* that the quantitative and spatial prediction accuracy were greater than 90% and 89.85%, respectively. Therefore, it is feasible to predict future changes in land use with the CA-Markov model.

Table 8. *Accuracy of prediction of land use in 2015*

2015	Construction land	Forest land	Water area	Cultivated land	Unused land	Grassland
Actual value (km^2)	1101.63	21380.69	624.84	1106.44	410.36	1644.75
Validated value (km^2)	1154.26	21057.99	645.50	1212.29	429.36	1775.21
Quantitative error (%)	4.78	-1.51	3.30	9.56	4.63	7.93
Spatial error (%)	13.09	4.28	12.32	12.76	7.41	11.05

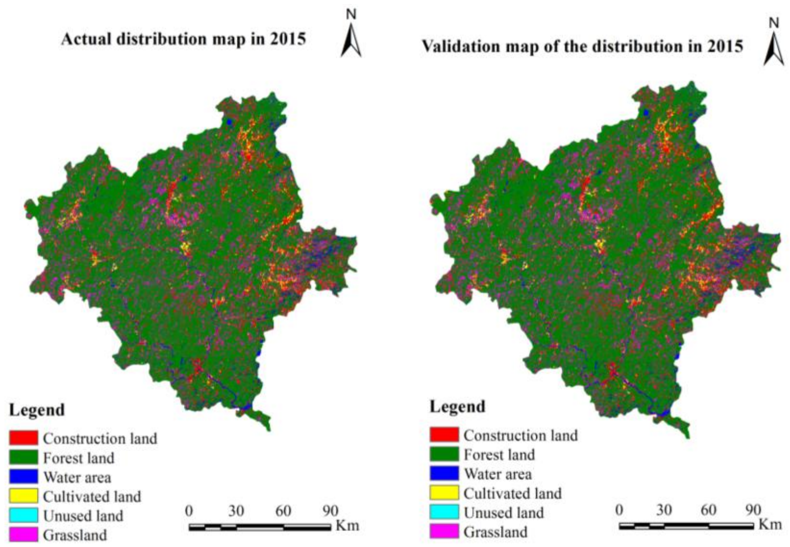

Figure 5. *Comparison of the actual distribution and validated distribution of land use in 2015*

Prediction of land use change

After completing the accuracy test, we predicted the change in land use types in 2020, 2025, and 2030 in Nanping by changing the number of simulated cycles in IDRISI to 5, 10, and 15, respectively. However, the change in land use was insignificant from 2015-2025, so we only demonstrated the results of land use change in 2030. As

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shown in *Table 9* and *Figure 6,* there were observable characteristics in the future changes in land use in Nanping.

Table 9. *Comparison of the change in area of land use in 2015 and 2030*

Land use types	2015		2030		2015-2030
	Area (km^2)	Proportion (%)	Area (km^2)	Proportion (%)	Area change (km^2)
Forest land	21380.69	81.39	21780.14	82.89	399.45
Grassland	1644.75	6.26	1430.55	5.44	-214.20
Cultivated land	1106.44	4.21	1189.61	4.53	83.17
Construction land	1101.63	4.19	846.36	3.22	-255.27
Water area	624.84	2.38	592.86	2.26	-31.98
Unused land	410.36	1.56	435.10	1.66	24.74
Total	26268.71	100.00	26274.62	100.00	5.91

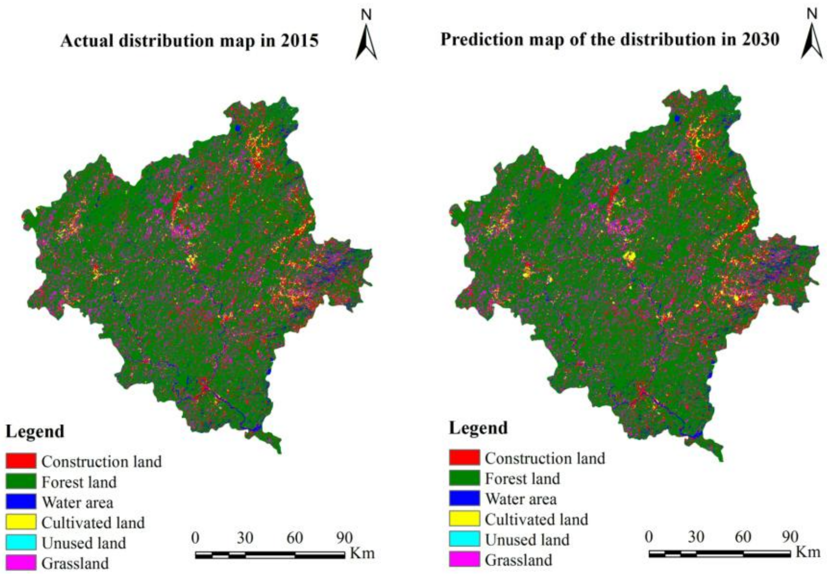

Figure 6. *Comparison of the distribution of land use in 2015 and 2030*

In the 2030 prediction, the total area of forest land reached 21780.14 km^2 and the proportion of forest land to the study area increased from 81.39% in 2015 to 82.89% in 2030, which is an increase of 1.50%. This shows that over the 15 years, the ecological environment continues to be strengthened and the forest cover rate is sustained.

Compared to the area of grassland in 2015, the area in 2030 showed a significant reduction with a decrease of 214.20 km^2. The decrease was closely linked with the development of the regional economy and the constant adjustment of the industrial structure. Consequently, more attention should be given to pasture degradation caused by overgrazing and natural disasters (Wang, 2013).

The area of cultivated land was predicted to increase by 83.17 km^2 during the 15 years, indicating that the government's policy of protecting basic farmland and stabilizing the food supply will continue to play a role in this stage.

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Water area would decrease slightly in 2030 compared to 2015. Due to the continuous increase in population, the change in life style, and the increase in water utilization for agriculture and industry, the water area could continue to decrease, which should be given adequate attention.

By 2030, the area of construction land would be reduced from 1101.63 km^2 in 2015 to 846.36 km^2 in 2030, which is a decrease of 255.27 km^2. This means that the intensive development of land would increase in the process of urban construction and economic development in Nanping during those 15 years.

The change in area of unused land during the 15 years would not be significant. This is due to the fact that most of the unused land is situated in regions with harsh natural conditions and steep slopes, which is not easily accessible to humans.

Prediction of ecosystem services value

The services value and rate of contribution of ecosystems in 2015 and 2030 were calculated using *Tables 7* and *9* and *Equation 12*.

It can be seen from *Table 10* that the service value of the ecosystems from 2015-2030 would continue to increase slightly. By 2030, the total value of the services in Nanping would reach 227.72 billion CNY, which is an increase of 2.69 billion CNY. From the services value of different ecosystems, the value of forest land showed a slow growth but it remained at a relatively high value during the 15 years. The contribution rate of forest land in 2030 was 0.58% higher than that in 2015. The services value and contribution rate of cultivated land and unused land also increased slightly. However, during the 15 years, the services value of water area and grassland continued to decrease, with a total decrease of 0.63 billion CNY and 0.66 billion CNY, respectively. The contribution rate of ecosystem value of water area and grassland was predicted to decrease by 0.35% and 0.33%, respectively. Consequently, more attention should be given to the reduction in the services value of water area and grassland during these 15 years.

Table 10. *The services value and contribution rate of different ecosystems in 2015 and 2030*

Ecosystem types	Ecosystem services value (billion CNY) and contribution rate (%)				Changing services value (billion CNY)	Changing contribution rate (%)
	2015		2030		2015-2030	2015-2030
Forest land	199.36	90.61	203.08	91.18	3.72	0.58
Cultivated land	3.26	1.48	3.51	1.58	0.25	0.09
Water area	12.26	5.57	11.63	5.22	-0.63	-0.35
Unused land	0.07	0.03	0.08	0.04	0.01	0.01
Grassland	5.08	2.31	4.42	1.98	-0.66	-0.33
Total	220.03	100.00	222.72	100.00	2.69	0.00

As shown in *Table 11*, excluding the service of waste treatment, the value of the remaining service types showed a slight increase over the 15 years. Most notably, the service values of soil formation and protection, gas regulation, and supply of raw materials in 2030 were over 0.40 billon CNY higher than those in 2015. The order of various service values in 2030 were as follows: soil formation and protection > water conservation > gas regulation > biodiversity conservation > climate regulation > raw

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materials > waste treatment > entertainment and culture > food production. In short, the total value of ecosystem services in Nanping was predicted to steadily increase over the 15 years.

Table 11. *The value of ecosystem services in Nanping City in 2015 and 2030 (billion CNY)*

Ecosystem services	2015	2030	Changing value over 15 years
Gas regulation	32.72	33.26	0.54
Climate regulation	25.80	26.20	0.40
Water conservation	35.51	35.72	0.21
Soil formation and protection	37.19	37.78	0.59
Waste treatment	18.94	18.79	-0.15
Biodiversity conservation	31.56	32.01	0.45
Food production	1.41	1.64	0.23
Supply of raw materials	23.80	24.24	0.44
Entertainment and culture	12.86	13.02	0.16
Total	219.82	222.71	2.89

Discussion and suggestions

Land is a precious natural resource that is necessary for the survival of human beings and for urban development. Sustainable land use and rational planning of land are basic requirements for the development of the city, and are also the issues of most concern for land use decision-making departments (Bateman et al., 2013). Therefore, we provide the following countermeasures and suggestions according to the study results, which are aimed at providing useful information for land administration departments.

(1) Cultivated land

From the above prediction results, it was found that the area of cultivated land would decrease slightly from 2015 to 2030. However, the population in Nanping City will continue to increase during these 15 years. It would be particularly important to guarantee the basic supply of grain. Therefore, the protection of basic farmland as a basic land use strategy is of great significance for maintaining regional grain production and regional ecological security. The specific countermeasures and suggestions are as follows:

a) High-quality areas of cultivated land are defined as a basic farmland protection area, ensuring that the area and the quality of basic farmland do not decrease.

b) It is forbidden for other land uses to occupy basic farmland areas.

c) For ordinary cultivated land, construction projects should use as little cultivated land area as possible. If it is necessary to occupy cultivated land, only farmland with poor quality should be occupied.

d) Due to the abandonment of rural areas in Nanping City, it is forbidden to abandon cultivated land. Check the farmland that has been abandoned, and take corresponding measures to resume farming.

e) Developing unused land with a moderate slope as a reserve resource for cultivated land. It is forbidden to develop areas with slopes greater than 25°.

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f) Developing a responsibility system for farmland protection; designating the relevant leaders of the government as the first responsible person, and ensuring that the tasks of farmland protection are completed.

(2) Grassland

This study found that the grassland area would be significantly reduced by 2030. Therefore, appropriate measures should be taken to protect the grassland area.

a) Implementation grassland development plans to increase grassland utilization so as to ensure adequate forage supply for livestock raised by farmers.

b) Planting grass according to scientific methods and actively introduce suitable pasture varieties.

c) Protecting the grassland and forbidding the burning of grassland. When weeds are eliminated and pests and diseases are removed, scientific measures must be taken to prevent damage to the grasslands.

d) It is forbidden to overgraze so as to prevent degradation of grassland. For degraded grasslands, measures should be taken to increase grass production and livestock husbandry.

e) Destruction of grassland should be investigated.

For forest land, construction land, and unused land, the prediction results performed well, but it is still necessary to make recommendations to ensure that this favorable situation is maintained. The specific recommendations are as follows:

(3) Forest land

It is forbidden to develop and use natural forests. Practice the closing of mountains and forests, and encourage tree planting on barren slopes.

(4) Construction land

a) Construction land should be used intensively. Develop and utilize ground and underground space to support the construction of multi-level industrial plants in order to improve the utility rate of space.

b) Encouraging farmers to build houses in a centralized manner and prohibiting scattered distribution of rural buildings.

(5) Unused land

On the premise of protecting the ecological environment, developing the gentle slope zone will provide backup support for other land resources.

Conclusions

Based on CA-Markov model and equivalent value per unit area of terrestrial ecosystem services in China, we predicted the area of the land use types and ecosystem services value in 2030. The following main conclusions are as follows.

From 2015 to 2030, the area of the forest land and cultivated land increase obviously. The area of the grassland and construction land show a significant decrease, while the water area and unused land area do not change significantly. Compared with 2015, the change of the spatial distribution pattern of land use types in 2030 is not significant.

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It has been found that the total value of ecosystem services will continue to rise from 2015 to 2030. It has been estimated that the ecosystem services value in Nanping City will reach 222.72 billion CNY in 2030. The increasing speed of the service value of forest land will slow down, but the contribution rate of forest land in 2030 is still 0.58% higher than 2015. The improvement of the ecosystem services value in the study area is mainly due to the increasing value of service types such as gas regulation, soil formation and protection, biodiversity conservation and supply of raw materials. More significantly, the services value of the gas regulation and soil formation and protection increase by over 0.50 billion CNY during the 15 years.

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RESPONSES OF THE MORPHOLOGICAL TRAITS OF ELM (*ULMUS MINOR* 'UMBRACULIFERA') LEAVES TO AIR POLLUTION IN URBAN AREAS (A CASE STUDY OF TEHRAN METROPOLITAN CITY, IRAN)

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Abstract. In this study, changes in the morphological traits of the leaves of elm tree (Ulmus minor 'Umbraculifera') were investigated against the air pollution in Tehran City, capital of Iran. For this purpose, the healthy and fully developed leaves were sampled from even-aged trees of the same diameter class from 3 districts of Tehran City with different traffic volume and air pollution load. Then, the micro-morphological traits of the stoma including length, width, density, size, stomatal shape, trichome density, and theoretical stomatal resistance were measured using Scanning electron microscopy (SEM) images. The macro-morphological traits, including length, width, shape, and area of the leaf, as well as the number of teeth on the blade, length of petiole, and asymmetry of the blade were measured. Determining the wet and dry weights and leaf turgor, the specific leaf area and relative water content were estimated. The results showed that the macro-morphological traits of the leaves were resistant and did not change significantly in the presence of air pollutants. In the contaminated areas, unlike the stomatal density that showed an increase, the stomatal size decreased. With increasing leaf thickness in the polluted areas, the stomatal density also showed an increase. Due to the inherent resistance of the leaves of elm tree to the reduction of leaf water potential, the failure of the photosynthesis rate, caused by the presence of pollutants, is compensated. In the species of elm, the traits of stomatal density and size are better descriptors for reflecting leaf changes in different environmental conditions.
Keywords: *air pollutants, urban areas, stomatal density, stomatal size, green space development, resistance plant*

Introduction

Air pollution in urban and industrial areas affects the survival of plant species. Although plants in industrial environments can be effective in controlling and reducing air pollution, they are affected by air pollutants and damaged (Dineva, 2004). Different plants show different sensitivity and performance in the face of air pollution (Dineva, 2004; Verma and Singh, 2006; Stevovi et al., 2010). The response of plants to environmental conditions and changes is strongly associated with their structural and functional characteristics (Gostin, 2009; Wang et al., 2011). Environmental changes lead to anatomical and morphological changes in the organs of plants (Verma and Singh, 2006; Wuytack et al., 2010; Stevovi et al., 2010).

Several studies have been done on the structure and anatomy of vegetative organs under conditions of environmental pollution (Arriaga et al., 2014; Chaturvedi et al., 2013; Lima et al., 2000; Kardel et al., 2010; Balasooriya et al., 2009). Plant species that are resistant to air pollution can show adaptations and continue to grow. The resistance of different tree species to air pollution is different, and some species are able to withstand pollutants and can remain normal and healthy (Magtoto et al., 2013). Among plant organs, leaves are more sensitive to environmental tension because most of the physiological processes, such as photosynthesis, are carried out through leaves. However, leaves have greater flexibility to adapt to the environmental conditions (Dineva, 2004; Rani et al., 2006). Hence, studying the reactions and adaptations of leaves in the face of air pollutants can be a good indicator of the performance of plant species in polluted environments. In cities and industrial areas, wooden species and trees, with their high leaf area, are effective in controlling and reducing air pollutants (Saebo et al., 2012; Petrova et al., 2013).

Having informed on the capability and performance of species and determining the ability of different species to deal with air pollution can be the first step to maximize the benefits of trees in urban and industrial areas (Escobedo et al., 2011; Saebo et al., 2012). It also provides a suitable basis for the selection of plant species and improving air quality (Wang et al., 2011).

Tehran, as the largest city in Iran, is considered as one of the world's populous cities (Naddafi et al., 2012). The city faces problems such as high population density and heavy traffic jam. It is the most industrialized city of Iran. Many industries such as power plants, refineries, furnaces, and metal processing and smelting plants are located on the outskirts of the city (Kermani et al., 2016). In Tehran, air pollution is a serious problem, and pollutants from vehicle traffic are a major contributor to this pollution (Kermani et al., 2016, 2003; Naddafi et al., 2012; Salmanzadeh et al., 2015; Kord et al., 2010; Leili et al., 2008; Sowlat et al., 2011). The present study was carried out to study the capabilities and resistance of elm (Ulmus minor 'Umbraculifera') tree against air pollution. The study seeks to answer the question that is it advisable to plant elm tree with the purpose of development of green spaces in urban and industrial areas with high rate of air pollution. Accordingly, changes in the morphological traits of the leaf of street-side trees of Tehran City were investigated.

Materials and methods

Study area

Tehran is the capital city of Iran, located in 35° 36' northern latitude and 50° 53' eastern longitude. The average altitude of the city is 1191 m above sea level. The maximum and minimum levels of temperature are, respectively, 22.5 °C and 11.2 °C. Majority of industries are located on the western part of the city and its suburbs where the prevailing wind blows towards the east-west direction (Alijani and Safavi, 2005). Tehran's topographic conditions are such that the mountains surround the city from the north and east and these elevated barriers prevent the evacuation of pollutants from the city. Moreover, on the west side, due to being openness, suburban pollutants also enter the city with the help of winds (Alijani and Safavi, 2005; Golbaz et al., 2010).

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Research procedure

Three districts of Tehran City were selected to take samples from the leaves of street-side elm trees. These three districts in terms of the traffic volume and air pollution load were divided in two categories of low emission (Site 1: Chitgar Station) and high emission (Site 2: Azadi Street and Site 3: Gisha Bridge) (*Fig. 1*).

The first site, Chitgar Station, was located in a forest park on the western outskirt of the city with a dense green space, where the traffic volume was very low. The distance of Site 1 from the nearest highway (Tehran-Karaj Highway, as most crowded highway of Iran) was more than 1000 m. Despite the low traffic of this station, under the influence of the prevailing wind direction, numerous small and large industrial sites in the region, and the discussed topographic conditions, this site deals with the consequences of air pollution.

Since majority of the particles released from the vehicles are sedimentated at the shortest distance from the emission source, attempts were made to collect the samples from the leaves of road-side trees.

Samples in Site 2 were collected from street-side trees in Azadi Square, the west and southwest of Tehran. Traffic volume in Azadi Square is very heavy. However, due to wide streets and pedestrian walkways, as well as lower density of residential and commercial buildings, air conditioning is better in this site, compared to the Site 3.

Site 3 is located in the central part of Tehran. A vehicle overpass, the intersection of two highways, proximity to residential and commercial structures, such as higher education institutions and offices have caused massive traffic jams and reduced air conditioning in this part of the city.

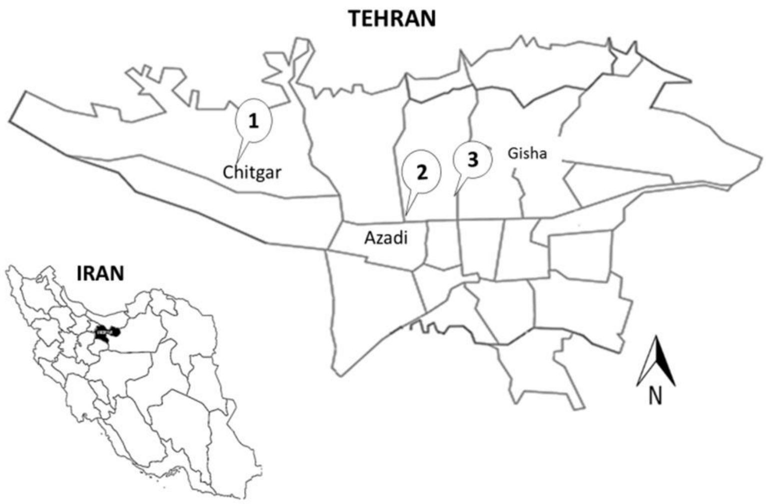

Figure 1. *Situation of the sampling sites in Tehran City and in Iran*

Figure 2 shows air quality indicators of Tehran in 2014. The data were obtained from the annual report on air quality of Tehran in 2014 published by Tehran Air Quality Control Company. The Site 1 was located in a forest park in the outskirt of the city, where there was no active air monitoring station nearby. The nearest air monitoring stations to the Sites 2 and 3 were placed at a distance of about 500 m away from the sampling area (*Fig. 2*).

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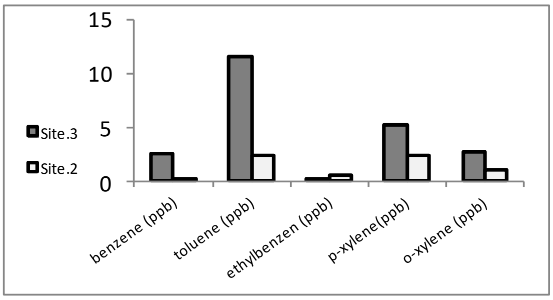

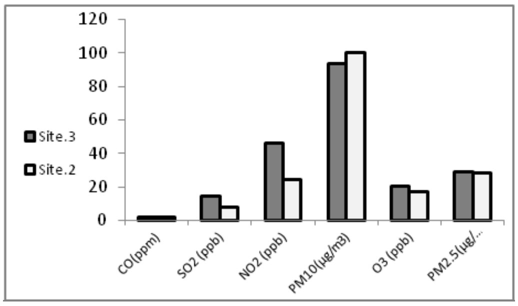

Figure 2. *Air quality indicators of Tehran City in 2014 obtained from Tehran Air Quality Control Company*

Sampling and analysis

Traits of elm tree

Elm (Ulmus SPP.) includes 20 to 45 species, scattered in the northern hemisphere and mountainous parts of tropical regions (Wheeler and Manchester, 2007). Iran is one of the countries wherein some elm species have been expanded (Oladi et al., 2013). Because some species of elm, due to their vault-like crown, are widely used in development of green spaces in urban areas.

Leaf sampling

In each sampling site, 5 healthy and disease-free trees with the least distance from the street were selected randomly. The orientation of the trees towards the street and the direction of sunlight and wind were considered to be the same. The trees were even aged and selected from similar diameter classes. The sampling was done at the end of the growing season in the second half of September, 2014.

The perfectly healthy leaves were collected from the street side outermost part of the canopy. To ensure the opening of stomata, the leaves were collected at 10 to 11 am. They were placed in a wrapped nylon in an ice tank with minimal hand contact and transferred to the laboratory in the shortest time. To take images with Scanning Electron Microscopy (SEM), the leaves were dried in a dry place away from sunlight at normal room temperature.

Using the images taken from the lower leaf surface, micro-morphology traits of the leaves, including Stomatal pore length (µm) (SL), Stomatal pore width (µm) (SW), Stomatal density (SD), Stomatal pore shape (SPS) (length to width ratio of stomatal), Stomatal Pore Surface (SPS), and Theoretical minimal stomatal resistance (Rs) were measured. Assuming the oval shape of the pores, the SPS was measured for other pores using *Equation 1* (Balasooriya et al., 2009). The Rs was also calculated using *Equation 2* (Balasooriya et al., 2009).

$$SPS = \frac{(SL \times Sw \times \pi)}{4} \quad \text{(Eq.1)}$$

$$Rs = \left(4\frac{l}{n} \times \pi \times Sl \times Sw \times D\right) + \left(Sl + \frac{Sw}{4n \times Sl \times Sw \times D}\right) \quad \text{(Eq.2)}$$

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where Rs is the stomatal resistance (sm^{-1}), l is the stomatal pore depth (m), D is the water vapor diffusion coefficient in air (24.2×10^{-6} m^2s^{-1} at 20 °C), and n is the stomatal density (number of stomata m^{-2}). Stomatal depth was considered to be 10 µm (Samson et al., 2000; Olyslaegers et al., 2002). To study the macro-morphological parameters of the leaves, from each tree base, the images of 50 leaves were prepared in a JPEG format with the help of a scanner. According to which, the parameters of Leaf length (LL), Leaf Width (LW), LS (length to width ration), Leaf area, teeth density, petiole length, and fluctuating asymmetry blade (FA) (*Eq. 3*) were calculated using Image J software (Kovacic and Nikolic, 2005).

$$Fa = \frac{|Wl - Wr|}{(Wl + Wr)} \quad \text{(Eq.3)}$$

in which Wl is the left half area of leaf blade and Wr refers to the right half area.

To measure SLA, 30 leaves were collected from each tree. From each leaf, 4 cm^2 pieces were punched and dried for 24 h at 60 °C. The weight of the dried parts was measured by a digital scale with a precision of 0.001 g. Then, the SLA was calculated using *Equation 4*. SLA = area of the punched parts/weight of the punched parts (cm^2 g^{-1}).

To measure Relative Water Content (RWC), 20 leaves were sampled from each tree and punched into the 4 cm^2 pieces. The wet weight of the punched pieces was measured. These pieces were placed in distilled water in low light intensity for 4 h. After measuring their turgor weight, they were dried at 60 °C for 24 h and weighed (Ritchie and Nguyen, 1990) (*Eq. 4*).

$$RWC = \lfloor (\text{wet weight} - \text{wet weight}) | (\text{turgor weight} - \text{dry weight}) \rfloor \times 100 \quad \text{(Eq. 4)}$$

Statistical analysis

The normality of the data was tested by Kolmogorov–Smirnov test in SPSS 16.0 software. Homogeneity of variance was tested using Levene's test. To compare the data, ANOVA was used. For multiple comparisons of the average values, Duncan, Kruskal-Wallis, and Mann-Whitney U tests were used.

Results and discussion

As *Figure 2* suggests, the level of most of the pollutants in Gisha Bridge is higher than Azadi Square. This is mainly due to the lower wind speed, high density of buildings, heavy traffic, and transport of worn out vehicles at the central parts of Tehran City (Shamsipour et al., 2013; Naddafi et al., 2012; Leili et al., 2008). *Figure 3* shows a SEM image taken from LSS of elm leaf.

Micro-morphological traits of elm leaves

The results of statistical testes showed a significant difference between the average values of the leaf micro-morphological traits in tree sampling stations, including SL (Sig = 0/000), SSH (Sig = 0/000), stomatal density (Sig = 0/000), stomatal pore surface (Sig = 0/001), and RS (Sig = 0/000) (*Fig. 4*). No significant difference was found between the measured values of SW in the three sampling sites (Sig = 0/289) (*Table 1*).

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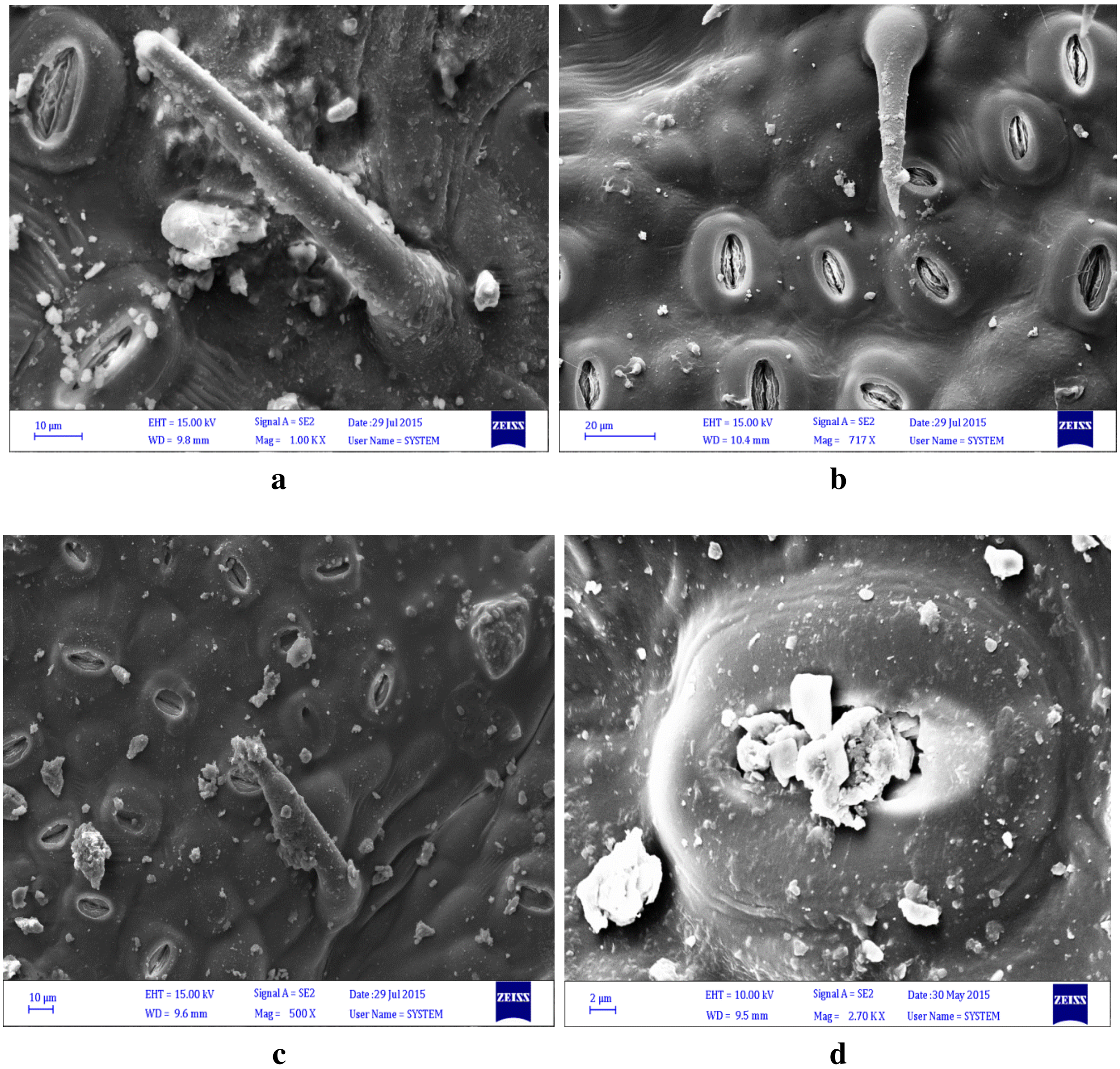

Figure 3. *SEM images taken the lower surface pores of elm leaf.* ***a*** *Chitgar Station,* ***b*** *Gisha Bridge,* ***c*** *Azadi Square,* ***d*** *complete blockage of stomata by airborne particulates*

According to the table, the SL value, compared to Chitgar Station, showed a decline of 27% in Azadi Square and 19% in Gisha Bridge (*Fig. 4a*). In addition, the values of stomatal length-to-width ratio in Azadi Square and Gisha Bridge were respectively 27% and 17% lower than that in Chitgar Station. (*Fig. 4b*). Comparison of the average stomatal density in the studied areas showed that in Azadi and Gisha, 31% and 144% increase in density was observed in comparison with Chitger (*Fig. 4c*). Stomatal pore surface was reduced in the polluted sites so that its value at Azadi and Gisha was 31% and 26% lower than Chitgar, respectively (*Fig. 4d*). The Rs increased in Gisha Bridge compared to Chitgar and was 30% less than Azadi (*Fig. 4e*).

The response of stomata to tension and mechanisms for reducing and controlling the input of pollutants into leaves are one of the most important traits of plants under stress conditions (Andersen, 2003). Balasooriya et al., 2009). Stomatal density is an important eco-physiologic parameter, which affects gas exchange between the atmosphere and plant (Lake et al., 2001; Uprety et al., 2002; Pompelli et al., 2010).

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***Table 1.** A comparison on the average values of the micro-morphological traits of Elm leaves in the three sampling sites*

Parameter	Location	mean ± SE	Sig.	Significant difference
Stomatal pore length (µm)	Chitgar	16.84 ± 0.37	0.000	**
	Azadi Square	12.29 ± 0.25		
	Gisha Bridge	13.56 ± 0.42		
Stomatal pore width (µm)	Chitgar	6.92 ± 0.33	0.289	ns
	Azadi Square	6.53 ± 0.18		
	Gisha Bridge	6.31 ± 0.24		
Stomatal pore shape	Chitgar	2.62 ± 0.15	0.000	**
	Azadi Square	1.91 ± 0.05		
	Gisha Bridge	2.17 ± 0.05		
Stomatal density (mm2)	Chitgar	322.43 ± 29.01	0.000	**
	Azadi Square	420.87 ± 17.30		
	Gisha Br.	780.03 ± 82.01		
Stomatal pore surface (µm2)	Chitgar	92.47 ± 0.55	0.001	**
	Azadi Square	63.54 ± 0.26		
	Gisha Bridge	68.33 ± 0.45		
Theoretical minimal stomatal resistance (sm-1)	Chitgar	90.2 ± 10.3	0.000	**
	Azadi Square	63.4 ± 4.33		
	Gisha Bridge	100.13 ± 18.2		

Ns = no statistically significant difference, * = a statistically significant difference at the probability of 95%, ** = a statistically significant difference at the probability of 99%

Researchers have found that traits such as stomatal density, epidermal cell density, and stomatal pore length are not hereditary and influenced by environmental conditions. Stomatal pore shape, increased stomatal density, and reduced stomatal pore surface in polluted areas are mechanisms for adapting species to contaminated environments (Wagoner, 1975; Kardel et al., 2010; Wuytack et al., 2010; Rani et al., 2006).

Increasing the stomatal density and reducing the size of the stomach can control and reduce gas exchanges and the entry of pollutants through the stomata (Wuytack et al., 2010; Alves et al., 2008). The entry of pollutants into plants through stomata has an inhibitory effect on plant physiological activities, such as photosynthesis (Younis et al., 2013). Therefore, plants reduce the size of stomata to minimize the entry of pollutants (Verma and Singh, 2006). Plants, which have good resistance to air pollution, can offset photosynthetic deficiency, caused by reduced gas exchange, by increasing pore density.

In the present study, decreasing changes in stomatal pore length, stomatal pore surface, and stomatal pore shape as well as increasing stomatal density from clean areas towards contaminated areas indicates the strong and effective correlation of these parameters with each other in order to adapt the species to air pollution. Many studies highlighted the importance of measurement of stomatal resistance under air pollution conditions (Alessio et al., 2002; Wuytack et al., 2010). Alessio et al. (2002) believed that stomatal resistance increases in areas with heavy traffic to reduce gas exchanges with the surrounding environment under tension conditions (Verma and Singh, 2006; Wuytack et al., 2010).

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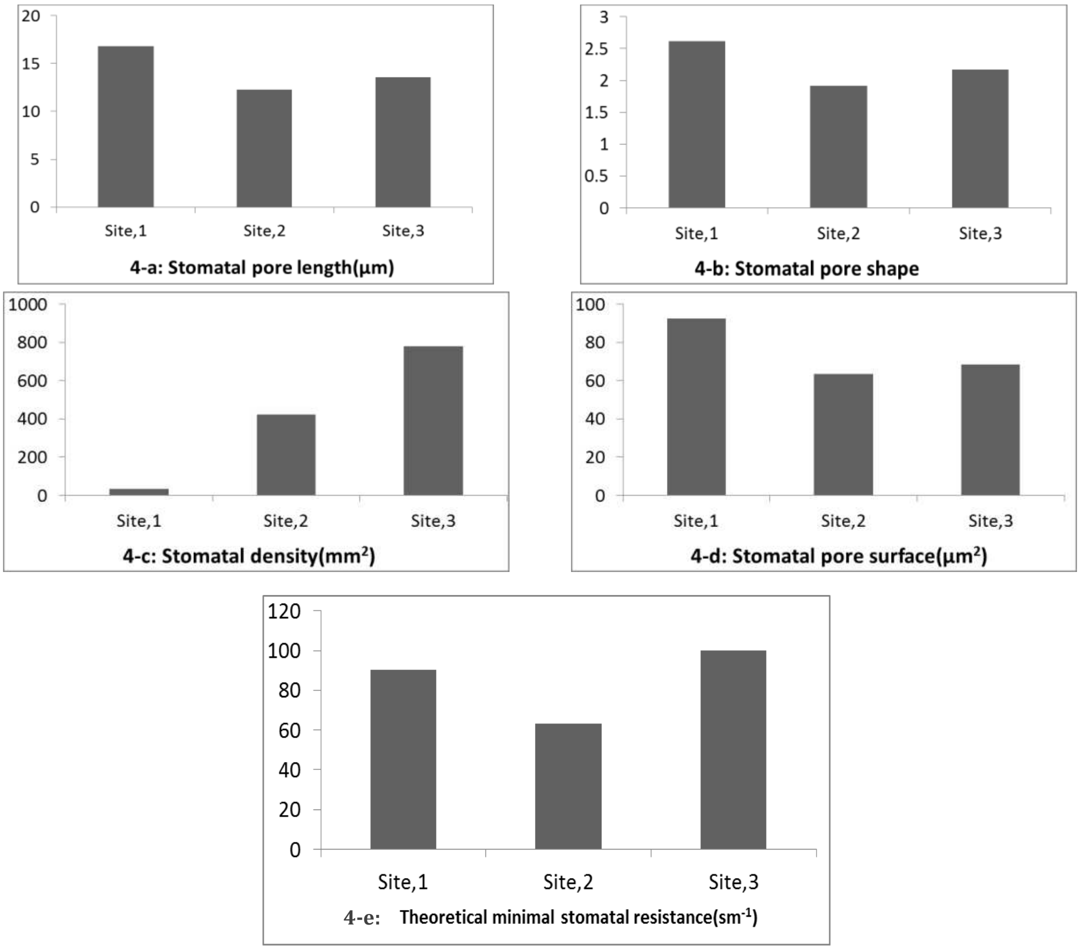

Figure 4. *Micro-morphological traits of the lower surface stomata of elm leaf in the sampling sites*

In the present study, in the site of Gisha Bridge, in addition to increasing the stomatal density and decreasing the SPS, the Rs showed an increase. This finding is in line with that reported by Wuytack et al. (2010). Mahecha et al. (2013) found that stomatal resistance increases with increasing age. As time passes, stomatal resistance decreases with the activation of tension adaptation mechanisms such as changes in stomatal and enzyme activities. In the present study, Rs reduced as a response to air pollution. The reduction of stomatal resistance in leaves may be due to premature aging caused by environmental tension. In Azadi Site, as the tension increased in leaves, the mechanisms of adaptation were quickly applied; these adaptations caused premature aging, replacement of new leaves, changes in stomatal density and stomatal pore surface, reduced Rs, and increased mesophyll conductance of the leaves. It is also necessary to note that in the present study, stomatal resistance was not measured in the environment; hence the trend of observed stomatal resistance could be due to the effect of local climates and local temperature changes in the sampling areas.

Macro-morphological traits of the leaves of elm tree

As *Table 1* shows, there was found no significant difference between the average values of leaf length (Sig. = 0.73), leaf width (Sig. = 0.64), leaf area (Sig. = 0.64), leaf

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shape (length-to-width ratio of leaf blade) (Sig. = 0.40), and teeth density (Sig. = 0.11) in the sampling stations. No significant difference was observed between the average values of fluctuating asymmetry blade (Sig. = 0.00) and petiole length (Sig. = 0.02) in the three sampling sites (*Table 2*).

Table 2. *Statistical analysis of the leaf macro-morphological traits of elm leaf in the sampling sites*

Parameter	Sampling site	mean ± SE	Sig.	
Leaf length	Chitgar	76.79 ± 4.67	0.732	ns
	Azadi Square	76.26 ± 6.66		
	Gisha Bridge	82.33 ± 6.62		
Leaf width	Chitgar	45.97 ± 3.57	0.645	ns
	Azadi Square	50.171 ± 5.87		
	Gisha Bridge	52.21 ± 4.89		
Leaf area	Chitgar	23.46 ± 2.05	0.645	ns
	Azadi Square	26.94 ± 5.64		
	Gisha Bridge	29.43 ± 4.61		
Length to width ratio of leaf blade	Chitgar	1.687 ± 0.05	0.404	ns
	Azadi Square	1.55 ± 0.07		
	Gisha Bridge	1.60 ± 0.06		
Fluctuating asymmetry blade	Chitgar	0.07 ± 0.01	0.000	**
	Azadi Square	0.08 ± 0.010		
	Gisha Bridge	0.17 ± 0.016		
Petiole length	Chitgar	6.66 ± 1.38	0.026	*
	Azadi Square	10.72 ± 1.51		
	Gisha Bridge	11.76 ± 0.99		
Teeth density	Chitgar	2.39 ± 0.246	0.115	ns
	Azadi Square	1.37 ± 0.35		
	Gisha Bridge	1.60 ± 0.378		
Specific leaf area	Chitgar	123.89 ± 24.4	0.470	ns
	Azadi Square	101.92 ± 11.4		
	Gisha Bridge	91.83 ± 17.9		
Relative water content	Chitgar	81.4 ± 2.55	0/000	**
	Azadi Square	45.4 ± 4.22		
	Gisha Bridge	83.1 ± 1.00		

Ns = no statistically significant difference, * = statistically significant difference at the probability of 95%, ** = statistically significant difference at the probability of 99%

Despite the insignificant difference found in the average values of leaf area in three sites, this parameter in the sites of Azadi Square and Gisha Bridge increased respectively by 15% and 25% compared to the clean environment in Chitgar Site.

Although the difference between the average values of fluctuating asymmetry blade of the three sites was not confirmed statistically, however the parameter in the sites of Azadi and Gisha Bridge increased respectively by 21% and 136% compared to Chitgar. Petiole length in Azadi and Gisha Bridge increased respectively by 61% and 77% compared to Chitgar. Although there was found no significant difference in the average

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values of number of teeth per unit area, it reduced respectively by 43% and 31% in Azadi and Gisha Bridge, compared to Chitgar. The comparison of the average values of specific leaf area in the sampling sites showed no significant difference among them (Sig. = 0.47) comparing the average values of relative water content in the sampling sites showed a significant difference (Sig = 0/00) and demonstrated a reduction of 44% in Azadi Site compared to Chitgar, and it was relatively equal in Gisha Bridge and Chitgar.

Under tension conditions, plants make adaptations that decline gas exchange to the environment in order to reduce contact surface with the polluted environment (Kardel et al., 2010; Arriaga et al., 2014; Verma and Singh, 2006; Balasooriya et al., 2009). The alteration of plant traits in air pollution conditions has been emphasized by many researchers, such as Vujic' et al. (2015) and Glukhov et al. (2015).

Various studies have referred to the reduction of leaf length, leaf width, and leaf area in different plant species under polluted conditions (Kardel et al., 2010; Arriaga et al., 2014; Verma and Singh, 2006; Lima et al., 2000; Balasooriya et al., 2009). Balasooriya et al. (2009) and Verma and Singh (2006) reported that the leaf area in contaminated sites is one of the most important biological markers for contamination monitoring In the present study, the lack of significant difference in mean leaf length, leaf width, leaf area, teeth density, and leaf shape indicated that the macro-morphological variations of elm leaf in polluted sites are not noticeable. The elongation of the leaf and the petiole represents its tolerance to stress (Xu et al., 2009). The findings of the present study confirm those reported by Xu et al. (2009). Incremental changes in petiole length, leaf area, and dry weight from the clean site to the polluted areas showed a positive relationship between these parameters and adaptation of the species to environmental pollutants (Meziane and Shipley, 2001; Chaturvedi et al., 2013).

By reducing the specific leaf area, the amount of chloroplast per unit area of plants increases. Thick leaf has a higher photosynthetic potential. In better words, the concentration of chlorophyll of chloroplast increases in thick leaves and the loss of light or the light passing through the leaves decreases. Consequently, leaf photosynthetic capacity increases.

Those species maintain their photosynthesis rate under tension conditions will be more responsive to stress Insignificant statistical differences in the present study indicated that elm, with the preservation of the photosynthesis potential in the leaves, can resist contamination and continue its growth.

In the polluted sites of Azadi and Gisha Bridge, a reduction in specific leaf area was observed. This is a strategy to improve stress tolerance (Xu et al., 2009). The leaf area variation reflects the growth rate of plants (Meziane and Shipley, 1999). Its reduction reduces water loss and transpiration. It also slows leaf growth and increases resistance of species to tension. Plants that are resistant to tension have higher relative water content (Mahecha et al., 2013). By increasing the thickness of leaves, the water loss is reduced and the relative water content is maintained at an optimum level.

In the present study, there was found no statistical difference between the relative humidity content of clean and contaminated sites, indicating the resistance of the species against the reduction of water content and the increase of transpiration in the presence of air pollutants. Changes in relative water content and specific leaf area revealed a negative relationship between these variables in order to adapt to environmental tension conditions.

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Based on the macro-morphological traits of the elm leaf in this study, it can be argued that the elm leaf is resistant to air pollutants. The elm, in conditions of increased stress, can increase its resistance to airborne contaminants, with adaptations.

Conclusion

The results of the study suggested that the macro-morphological traits of elm leaf, such as its shape, area, width, and length in the presence of air pollutants do not change significantly and are relatively resistant. The stomatal traits at the lower leaf surface of elm showed that the tree controls the entry of air pollutants by reducing the size of stomata, and stomatal density increases to compensate for the shortage of gas exchanges.

The elm tree can maintain the water contents of leaf tissue to the optimum level, and by increasing the leaf thickness, preserve the photosynthetic potential of its leaves.

In the polluted sites, with increasing leaf thickness, stomatal density also increased. Regarding the inherent resistance of this species to the reduction of leaf water potential, the loss of photosynthetic rate caused by the presence of pollutants was compensated. Reducing the relative water content at Azadi Site can lead to the closure of stomata and eventually reduced photosynthesis. In this site, the species also reduced the size of stomatal pore to cope with this tension. According to the findings of this study, elm can resist against air pollution by applying cost-saving mechanisms. The findings showed that the traits of the elm leaf on polluted sites vary hardly. Hence, based on morphological studies of elm leaf, it can be admitted that the elm species is resistant to air pollution stress in industrialized cities and industrial areas, as well as the sites with high traffic volume. Anatomical studies of leaf can supplement this idea.

In many studies, stomatal density and stomatal pore surface are introduced as a good indicator for well differentiation of the sites with different air quality. Similarly, in elm species these two parameters were obviously better descriptors of leaf changes in different environmental conditions. The findings of the present study indicated that in urban and industrial areas that need green spaces resistant to air pollution, the planting of elm tree can play an effective role in achieving this goal.

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ENVIRONMENTAL RISK DETERMINATION OF FLOOD IN PORSUK RIVER BASIN VIA ONE-DIMENSIONAL MODELLING

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Abstract. Floods, which are frequently occurring natural disasters, may interrupt the social and economic activities, and cause serious environmental and health problems in the region under effect. Floods may cause environmental problems such as soil loss, pollution of fresh water resources and epidemic diseases transported by floodwater. In order to prevent these adverse effects, flood risk maps, flood prevention and sustainable flood management plans must be established at the settlements which are vulnerable to floods. In this study the flood frequency analysis of Porsuk River basin located between the Porsuk Dam (which is the only source of domestic water of the city) and Eskisehir city center was conducted. The return period and probability distribution of flood magnitude were identified by flood frequency analysis. The effects of floods on the region located between the Porsuk Dam and Eskisehir city center were examined, the probable maximum flood for 25, 50, 100, and 500 years of flood return periods were determined by Log Pearson III method using the maximum flow data of years 1963-2009. MIKE 11 was presented in this study as one-dimensional flood model, which was conducted to determine the flood risk with efficient, easy and quick decision making approaches in Turkey.
Keywords: *flood risk, flood frequency analysis, flood modelling, one-dimensional modelling, MIKE 11, flood mapping*

Introduction

Floods are listed between natural disasters with huge detriment forces because of their adverse effects on environment, health and economy in vulnerable areas (Düzgün, n.d.; Uşkay and Aksu, 2002). In environmental aspect, floods affect biodiversity of flora and fauna (Tingsanchali and Karim, 2005), cause soil depletion (Uşkay and Aksu, 2002), pollute surface water sources (Haltaş, 2013), create epidemic disasters by infectious microorganisms and death (Çetin, 2013). Also, floods have social impact, such as, physiologic health problems caused by natural disaster, and the damaging of historical buildings and economic effects (Morss et al., 2005).

Recently, focus has shifted towards developing most appropriate strategies, including the promotion of vulnerability assessment and development of skills, methods and technologies to cope with flood, the assessment of costs and benefits and flood management methods (Calder and Aylward, 2006; Zhang et al., 2008; Marchi et al., 2010; Merz et al., 2010). Floods Action Program and Directive on The Assessment and Management of Flood Risk (2007/60/EC) was published by European Parliament in October 2007 to create general frame politics of water bodies', develop flood management plans for every basin areas, and aim reducing the devastating effect of floods (Griffiths, 2002). The major goals of the directive are both decreasing food risks

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and determining possible effects caused by climate changes (Brown and Damery, 2002). In line with the Flood Action Programme, Ministry of Forestry and Water Affairs in Turkey organized National Basin Management Framework (NBMF) with aim to protect water basin, sustainable and profitable usage of water resources, and providing guidance to investment programs engendered as a result of long-term discussions with different institutes with different views (Çetin, 2013; Hopur, 2013; Ün, 2013). Under the scope of NBMF, the Ministry planned to create twenty five basin management committees including local authorities, universities, civil organizations, and other foundations and these committees provide the implementation of the principles of NBMF in order to prevent the adverse effects caused by the floods on the environment, generate flood risk maps, and establish flood prevention and sustainable flood management plans (Düzgün, n.d.; Akpınar, 2013; Aras, 2013).

The evaluation of increased flood risk has been investigated among engineers and economists. The areas under the flood risk are determined with the return period, the water depth and the flowrate of flood (Düzgün, n.d.; Uşkay and Aksu, 2002: Tiryaki, 2013). Different hydraulic models such as one-dimensional hydraulic models (1-D), two-dimensional hydraulic models (2-D), and integrated hydraulic models (1D-2D) can used to investigate flood risk. HEC-RAS (Özdemir, 2013), HEC-GEORAS (Özdemir, 2013) and MIKE 11 (Thompsona, 2004; Patro et al., 2009) 1-D hydraulic models are performed to determination the water depth of flood with the flowrate and the return period along the rivers and canals. 2-D hydraulic models can be listed MIKE 21, FLO2D, and CCHE2D providing flood depth with cross-section area along the rivers and canals. The integrated models like MIKE FLOOD (Liu et al., 2007; Patro et al., 2009) combine both 1-D hydraulic models (MIKE 11) and 1-D hydraulic models (MİKE 21) to determine flooded area settled around the rivers and canals.

MIKE 11 is a 1-D modelling system for rivers, channels, reservoirs and structures. The model is a world standard in 1-D river modelling for simulating flow and water level, water quality and sediment transport in rivers, floodplains, irrigation canals and other inland water bodies. The model application range is large enough to include simple design investigation and huge forecasting projects requiring complex hydraulic structure operation policies as well (Thompsona, 2004; Patro et al., 2009). This model is a versatile modelling platform enabling hydrodynamic modelling of rivers, irrigation systems, flood control, advection dispersion modelling, water quality modelling and sediment transport modelling (Tingsanchali and Karim, 2005; Marchi et al., 2010; Wen, 2013; Wolfs, 2013).

The structure of the model includes data and modules of the basin such as, rainfall-runoff data, river hydrodynamics, advection-dispersion data, water quality, topographical data and time series data of the river. The topographical data are the channel cross-section, the floodplain topography, the roughness and the structure geometry, Time series data are the boundary conditions of the river, the water levels, the discharges and the Q-h boundary data of the river. Modelling of unsteady flow via MIKE 11 is based on three fundamental elements, which are a differential relationship expressing the physical laws, a finite difference scheme producing a system of algebraic equation, and a mathematical algorithm to solve these equations. MIKE 11 general assumptions are listed below:

- Incompressible and homogeneous fluid,
- The flow is one-dimensional (uniform velocity and water level in cross-section),

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- The bottom slope is small,
- Small longitudinal variation in geometry, and
- Hydrostatic pressure distribution.

This paper presents an approach that can be used to identify flood risk and creating flood risk map in Porsuk River basin. According to flood risk assessment framework introduced to our country by EU Flood Risk Directive, this study can demonstrate an integrated approach used to determine flood impact assessment, flood modelling, economic tool, and risk assessment. The method, MIKE 11 was presented in this study, can be considered as an important, easy and quick decision support tool that can develop existing decision making approaches like HEC-RAS, which are generally used for flood risk in Turkey. Also, this study will be useful in design of dams, culverts and flood control structures in the mentioned area in relation to urban drainage, river basin and canals.

Materials and methods

In this study the flood frequency analysis, the return period and probability distribution of flood magnitude were identified by flood frequency analysis for Porsuk River basin. The one-dimension flood modelling was performed on the region located between the Porsuk Dam and Eskisehir city center. The probable maximum flood of flood return periods were determined using the maximum flow data.

Working area

The project area selected in this study is located in Porsuk River basin. The Porsuk River is the sub basin of Sakarya River basin, and covers an area of 11325 km² in the northwest Anatolia Basin. The basin is located between 29°38'-31°59' east longitude and 38°44'-39°99' north latitude and has 202 km in length to the East-West direction and 135 km in length to the North-South direction. The Basin contains Eskisehir and Kutahya centers, and the 7 district of these two cities and some parts of it can be bordered by Ankara, Usak and Afyon borders. More than 60% of the basin is mountainous. The project area has been identified between area of Eskisehir and Porsuk Dam and it covers an area of 4253 km². The project area is shown in *Figure 1* and its properties between the town and dam are listed in *Table 1*.

Table 1. *The project area properties in the Porsuk River basin*

Property	Value
X (top left)	107951.11978
Y (top right)	143651.70000
X (bottom left)	111378.88022
Y (bottom right)	141963.30000
West longitude	29° 59' 11.1198" E
North longitude	39° 54' 11.7000" N
East longitude	30° 56' 18.8802" E
South longitude	39° 26' 3.3000" N
Scale	1:156700
Area	4253.1 km^2

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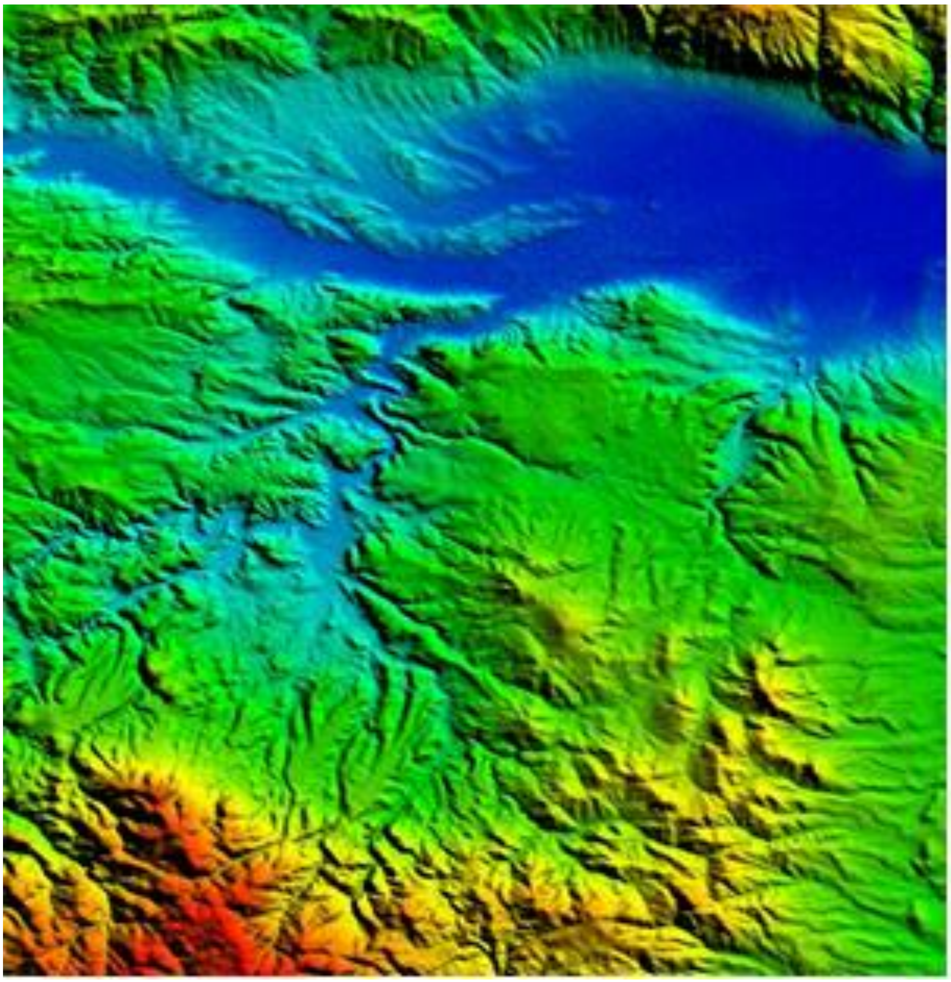

Figure 1. *The project area in the Porsuk River basin*

Determination of flood return period in Porsuk River basin

The return period and probability distribution of flood magnitude were identified by flood frequency analysis. The effects of floods on the region located between the Porsuk Dam and Eskisehir city center were examined, the probable maximum flood for 25, 50, 100, and 500 years of flood return periods were determined by Log Pearson III method using the maximum flow data of years 1963-2009 (47 years period) provided by The State Hydraulic Works in Turkey.

MIKE 11-one dimension flood modelling

MIKE 11 (MIKE, DHA 2012 SOFTWARE) 1-dimensional simulation software is composed of four subfiles associated with the data files. These files are the network data for the river line, the river cross-section file, and the boundary data file of the study area boundary conditions, and the files of river hydrodynamic parameters. The file of river hydrodynamic parameters has been identified as high-order full dynamic. After entering the files associated with the sub-model to simulation model, 1D simulation models was performed with prepared data by the return period of flood. MIKE 11 model setup is shown in *Figure 2*.

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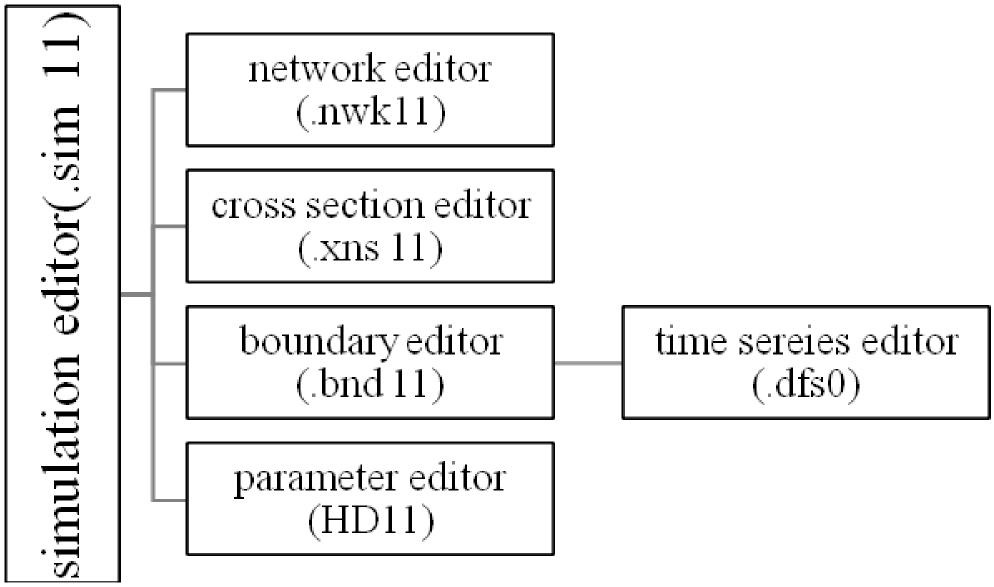

Figure 2. *MIKE 11 model setup*

To perform, MIKE 11 with data of Log Pearson III methods, a background map of the study area, the shape files map contains the river line, the field measurement data including the river cross-section, the data with the topographic heights of the study area, the boundary conditions for the workspace to create a terrain mesh and the flood periods files of the water level and the height of the flood flow were prepared. A background map (bacground.gif) for the work area to create the model grid, the shape file map (network.shp) containing the Porsuk River line, the site measurement data (surveyed_cross-section.txt) containing river sections, the topographic heights of the study area (dem.xyz), the boundary conditions for the study area and the water level heights of the floods and the floods were prepared as sub-file entrance data to run simulation model for MIKE 11.
Firstly, a background map of the working area was digitized by the manual marking method in the file containing the river line data in network editor (. nwk 11), the representation on the associated background file was shown in *Figure 3* after digitizing. The section data obtained by field measurements previously was transferred to the file containing the data for the river section line (. xns 11), the graphs of the processed and unprocessed section data are shown in *Figure 4*. Manning's n value for the river line was chosen as 0.025.

The existing water level was used as the downstream limit condition in the file containing the boundary conditions of the river lines (. bnd 11), triangular hydrographs of 65, 124, 166, 220, 261, 309, 408, 450 m^3/s and the peak condition with one-hour interval calculated by respectively 2, 5, 10, 25, 50, 100, 200, 500 and 1000 years flood frequency period, was used as the upstream limit condition. The sample hydrograph constructed for a 2-year periodic renewal was shown in *Figure 5*. In the file containing the hydrodynamic parameters, the wave approximation is defined as "high order full dynamics". After the sub files created by using the prepared model bases were associated with the simulation file, 1-dimensional models were created by entering the simulation period of the flood flowrates.

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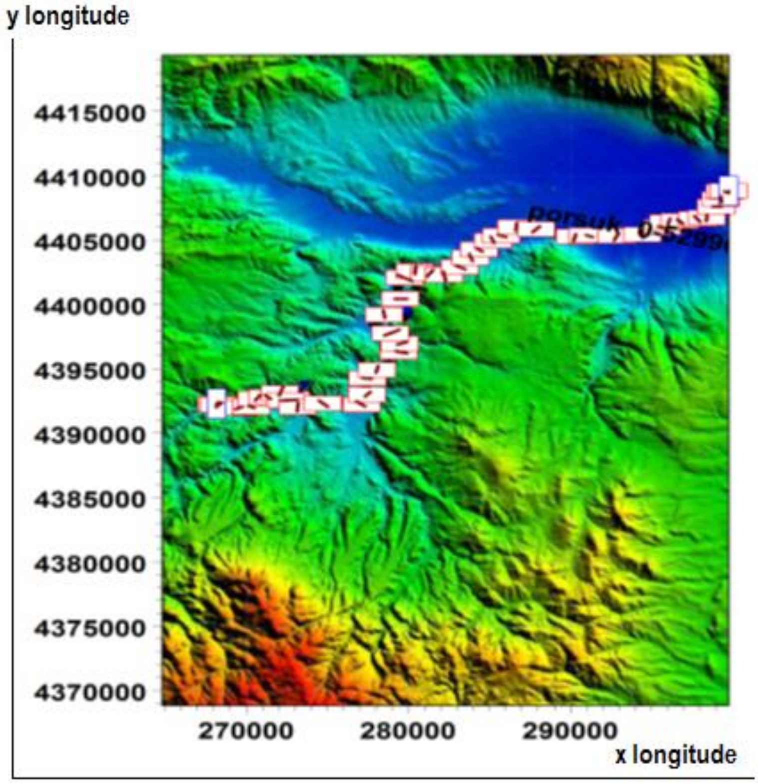
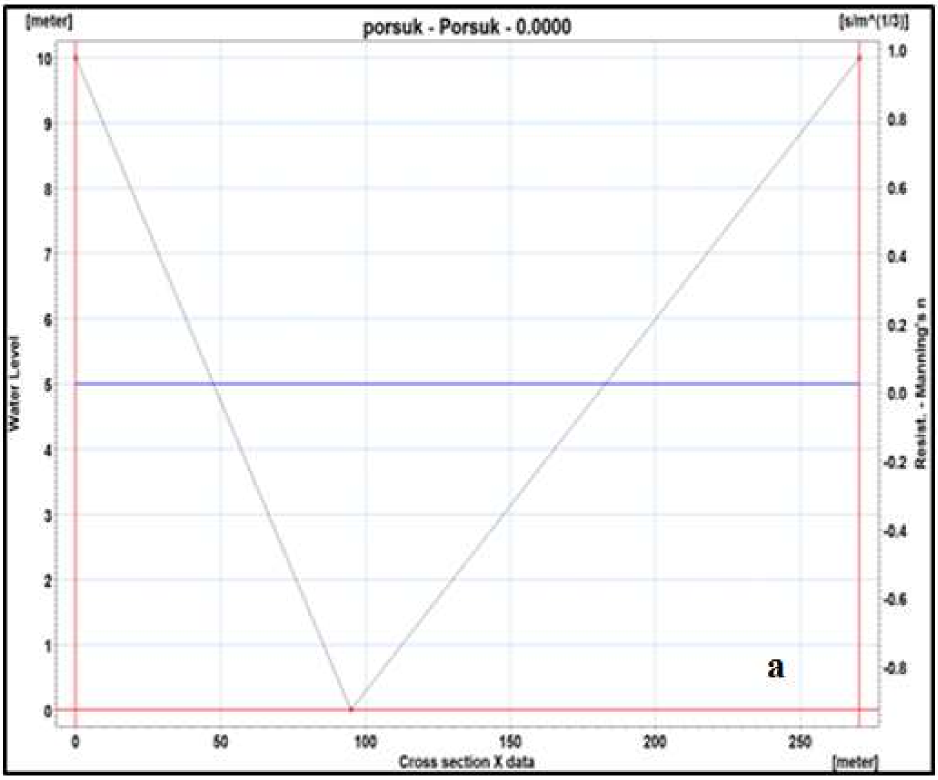


Figure 3. *Project area in the Porsuk River basin with digitized cross section in x and y longitudes with dimensionless along the river (boxes refer processed cross section with n: 0.025)*



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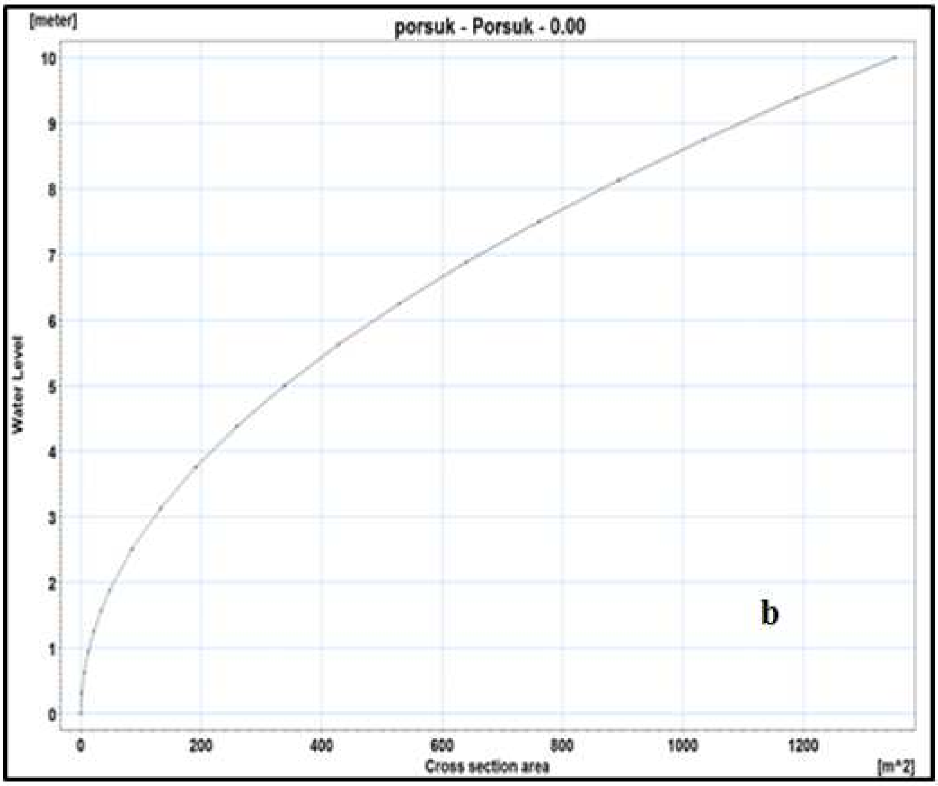

Figure 4. ***a*** *Unprocessed section data.* ***b*** *Processed section data*

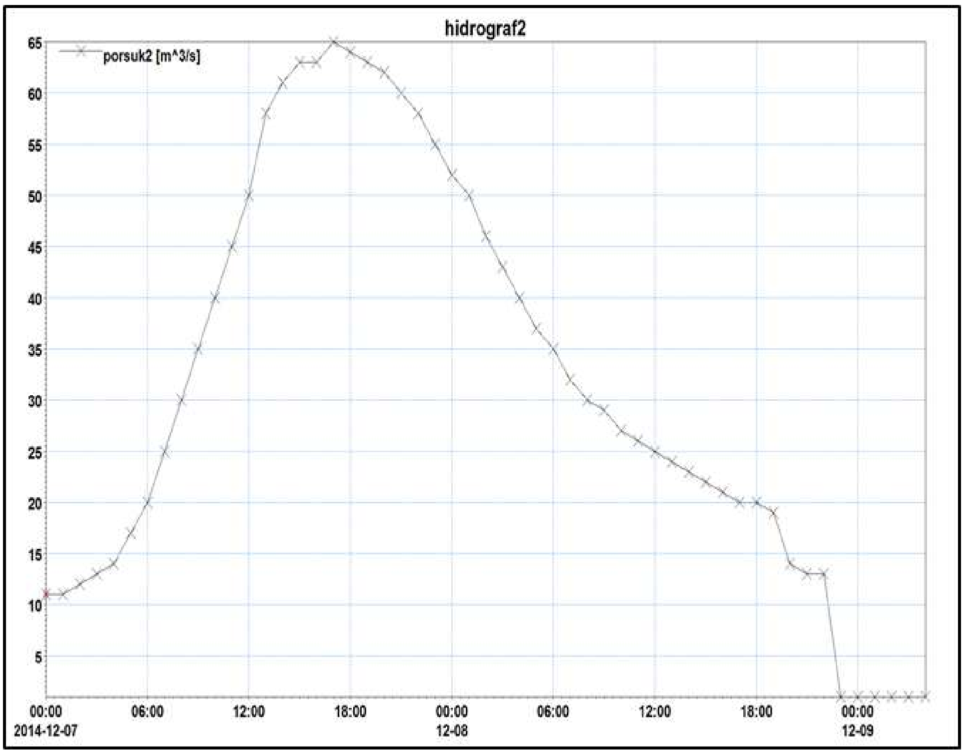

Figure 5. *The sample hydrograph constructed for a 2-year periodic renewal*

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Results

Results of flood return period analysis

The return period and probability distribution of flood magnitude were identified by flood frequency analysis in the region located between the Porsuk Dam and Eskisehir city. The probable maximum flood return periods were determined by Log Pearson III method using the maximum flow data of years 1963-2009 (47 years period) provided by State Hydraulic Works in Turkey. The results of the flood return period analysis are given in *Table 2*.

Table 2. *Results of flood return period analysis*

i	Return period (years)	Possibility *P* (%)	Frequency coefficient (*K*)	$Y = \log(Q)$	Flood flow rate Q (m^3/s)
1	2	50	0.296	2.232	480
2	5	20	0.786	2.921	834
3	10	10	0.916	2.985	966
4	25	4	0.991	3.022	1051
5	50	2	1.018	3.035	1083
6	100	1	1.031	3.041	1100
7	200	0.5	1.038	3.045	1108
8	500	0.2	1.044	3.047	1115
9	1000	0.1	1.045	3.048	1117

As a result of flood return period analysis, the lowest flood flowrate is 480 m^3/s and the return period is 2 years with 50% possibility. The biggest flood flowrate is 1117 m^3/s and the return period is 1000 years with 0.1% possibility. 52-h flow rate based on the flood return period and their flowrates was used for MIKE 11 simulation. The 52-h calculated flowrates used in simulations were given in *Table 3*.

Table 3. *52-h flow rate based on the flood return period and their flow rates*

t (hour)	Return period (years)/flow rates Q (m^3/s)							
	2/480	**5/834**	**10/966**	**25/1051**	**50/1083**	**100/1108**	**500/1115**	**1000/1117**
0	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00
1	11.13	11.30	11.41	11.56	11.68	11.81	12.08	12.20
2	11.67	12.49	13.06	13.80	14.38	15.03	16.39	16.98
3	12.60	14.58	15.96	17.73	19.11	20.69	23.96	25.37
4	14.38	18.53	21.44	25.17	28.08	31.40	38.28	41.25
5	16.88	24.10	29.16	35.65	40.71	46.49	58.46	63.63
6	20.43	32.01	40.12	50.53	58.65	67.91	87.12	95.41
7	24.77	41.68	53.53	68.73	80.58	94.11	122.16	134.27
8	29.78	52.84	68.99	89.73	105.89	124.33	162.58	179.09
9	35.13	64.77	85.53	112.18	132.94	156.65	205.80	227.02
10	40.46	76.63	101.98	134.51	159.86	188.79	248.79	274.69

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t (hour)	Return period (years)/flow rates Q (m^3/s)							
	2/480	**5/834**	**10/966**	**25/1051**	**50/1083**	**100/1108**	**500/1115**	**1000/1117**
11	45.49	87.83	117.51	155.58	185.25	219.13	289.36	319.68
12	49.92	97.68	131.16	174.10	207.58	245.79	325.03	359.22
13	57.91	110.21	146.86	193.88	230.53	272.37	359.12	396.56
14	60.65	116.31	155.32	205.38	244.39	288.92	381.27	421.12
15	62.55	120.57	161.22	213.39	254.04	300.45	396.68	438.22
16	63.49	122.64	164.10	217.29	258.75	306.07	404.21	446.56
17	64.68	124.23	165.96	219.51	261.24	308.88	407.67	450.28
18	64.20	123.16	164.49	217.51	258.79	305.90	403.59	445.75
19	63.36	121.30	161.88	213.86	254.39	300.65	396.46	437.80
20	61.86	117.94	157.14	207.44	246.61	291.26	383.87	423.83
21	60.15	114.02	151.71	200.00	237.59	280.50	373.92	423.41
22	57.85	108.82	144.51	190.12	225.69	266.26	378.94	428.97
23	55.40	103.31	136.81	179.67	213.09	262.55	378.35	428.29
24	52.50	96.79	127.71	167.34	200.82	255.48	368.87	439.47
25	49.50	90.12	118.42	154.74	190.64	243.32	362.44	439.17
26	46.30	82.98	108.50	141.26	176.01	225.85	350.03	417.78
27	43.20	76.09	98.96	128.29	158.56	205.00	326.63	384.77
28	40.14	69.25	89.49	115.42	138.71	181.30	295.54	342.73
29	37.35	63.05	80.90	103.75	121.48	157.00	259.75	300.19
30	34.75	57.25	72.86	92.84	108.33	131.60	221.57	256.58
31	32.48	52.21	65.87	83.35	96.90	112.36	185.33	215.58
32	30.42	47.63	59.53	74.74	86.51	99.96	149.44	175.23
33	28.68	43.76	54.16	67.45	77.73	89.47	122.22	139.34
34	27.14	40.35	49.44	61.03	70.00	80.24	107.53	117.65
35	25.82	37.41	45.36	55.51	63.34	72.29	95.15	103.46
36	24.66	34.84	41.80	50.68	57.53	65.34	83.97	92.13
37	23.65	32.60	38.70	46.47	52.45	59.28	75.07	81.35
38	22.55	30.18	35.35	41.92	46.98	52.74	65.48	70.97
39	21.78	28.47	32.97	38.72	43.10	48.12	58.37	63.15
40	20.86	26.42	30.14	34.87	38.47	42.59	50.80	54.31
41	20.28	25.17	28.41	32.52	35.65	39.21	46.27	49.24
42	19.59	23.64	26.28	29.65	32.18	35.07	40.73	43.10
43	19.19	22.78	25.09	28.03	30.23	32.75	37.63	39.65
44	13.69	15.88	17.41	19.35	20.87	22.60	26.20	27.75
45	13.43	15.30	16.60	18.26	19.55	21.03	24.09	25.41
46	13.05	14.46	15.44	16.68	17.65	18.75	21.05	22.04
47	0.94	0.99	1.02	1.04	1.06	1.08	1.12	1.13
48	0.94	0.99	1.02	1.04	1.06	1.08	1.12	1.13
49	0.94	0.99	1.02	1.04	1.06	1.08	1.12	1.14
50	0.94	0.99	1.02	1.04	1.06	1.08	1.12	1.14
51	0.94	0.99	1.02	1.04	1.06	1.08	1.12	1.14
52	0.95	0.99	1.02	1.04	1.06	1.08	1.12	1.14
Volume	5.98	10.30	13.31	17.17	20.28	24.18	33.31	37.49

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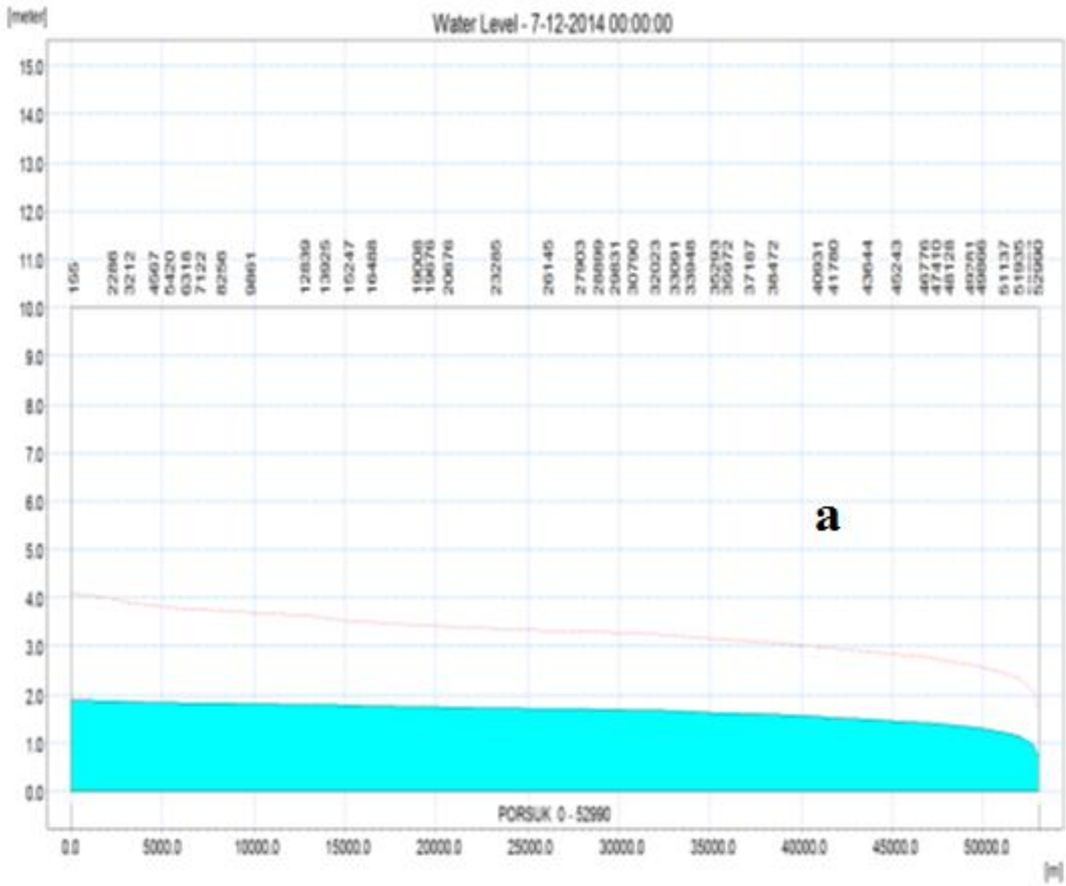
Results of MIKE 11-one dimension flood modelling

Determining the areas risked by flood hazard in the area, the modelling methods were chosen by using the flood return period and the flood water level. With the aim of using 1D hydraulic modelling, MIKE 11 software powered by DHI was used in this study. In this model, the flood water level is obtained with the flood flow rates depending the flood return periods. The return period for simulation was specified with 52 h, depending on the calculated flood flowrate data.

The flood level of area between Porsuk River and Eskisehir city was simulated with MIKE 11 according to 25, 50, 100, and 500 years-return period and possible flood flowrates. The simulation results obtained for specified periods are given in the *Figure 6* by the result of MIKE View Program. The relation between return period-flood flow and the relation between the return period- flood water level are illustrated in the *Figure 7*.

When the flood return period is 25 years with 4% possibility, the flood flow rates are 1051 m^3/s and the flood water will be observed in the river is 4.1 m. In case of the return period is 50 years with 2% probability, flood flow rates equal to 1083 m^3/s and the flood water levels are 4.3 m. In case of the return period is 100 years with 1% probability, flood flow rates equal to 1100 m^3/s and the flood water levels are 5.2 m. If the flood return period is 500 years with 0.1% possibility flood flow rate danger from 1115 m^3/s and the flood level will be observed in the river is 5.15 m. As a result of one-dimensional flood model of project area, it was stated that there is no linearity both between the return period and flood flow (R^2:0.5826) and also between return period and flood water level (R^2:0.4256).



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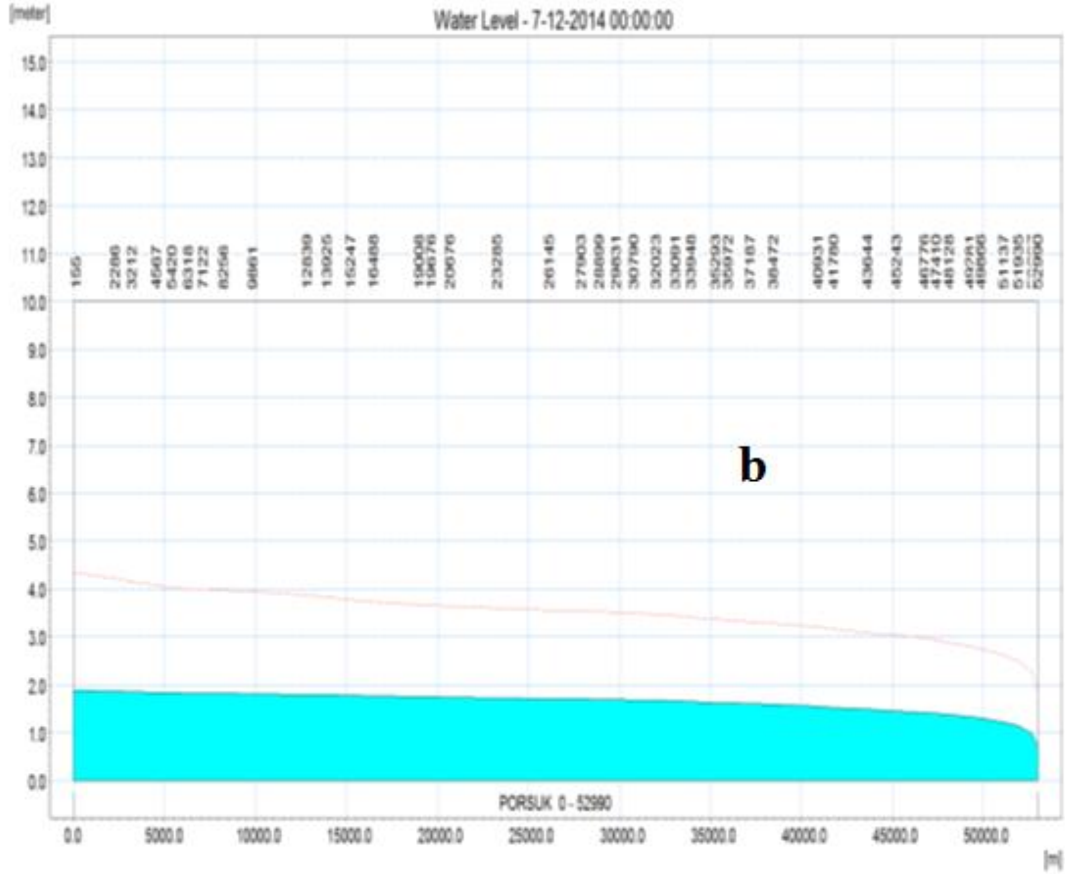

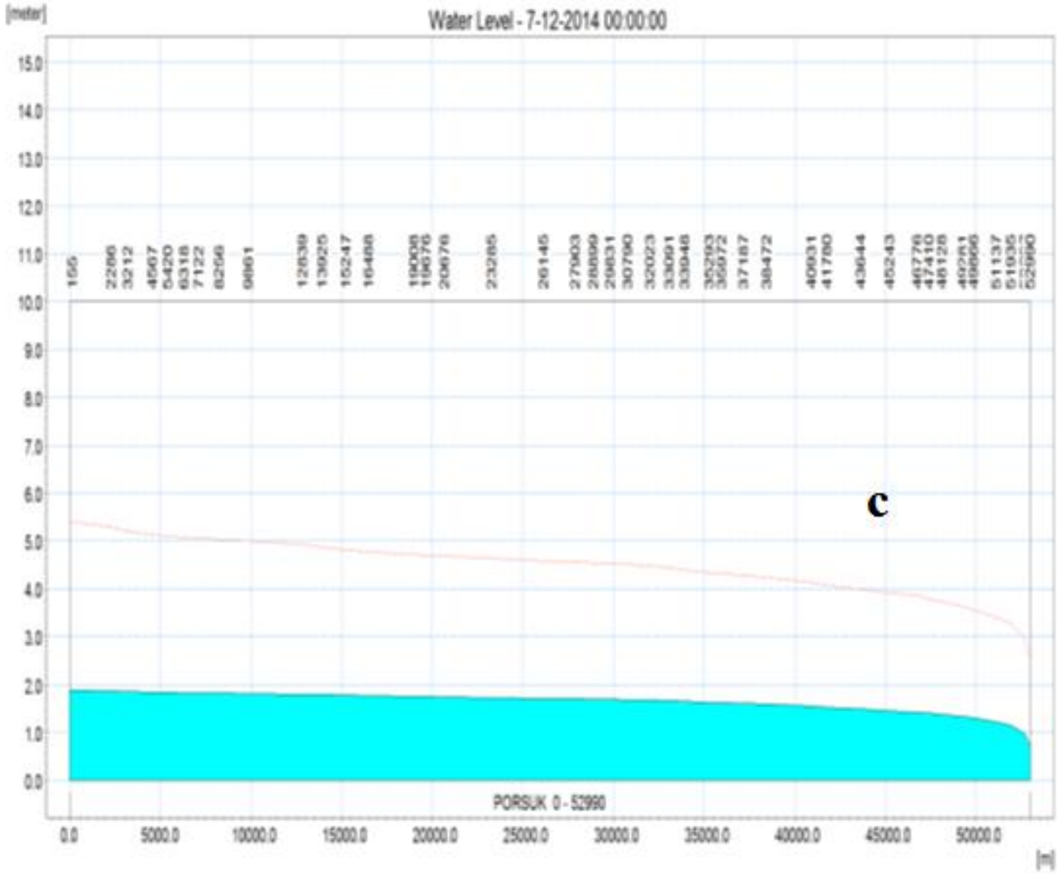

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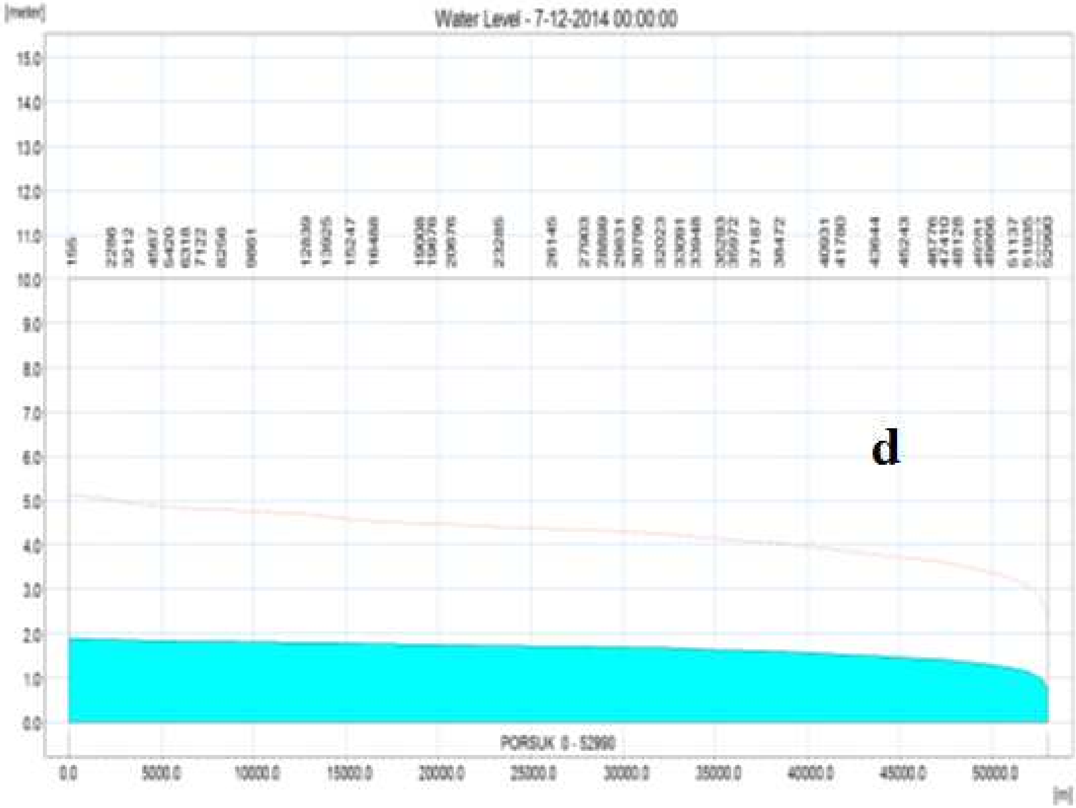

Figure 6. *Results of MIKE 11-one dimension flood modelling for 25 (a), 50 (b), 100 (c) and 500 (d) years return periods*

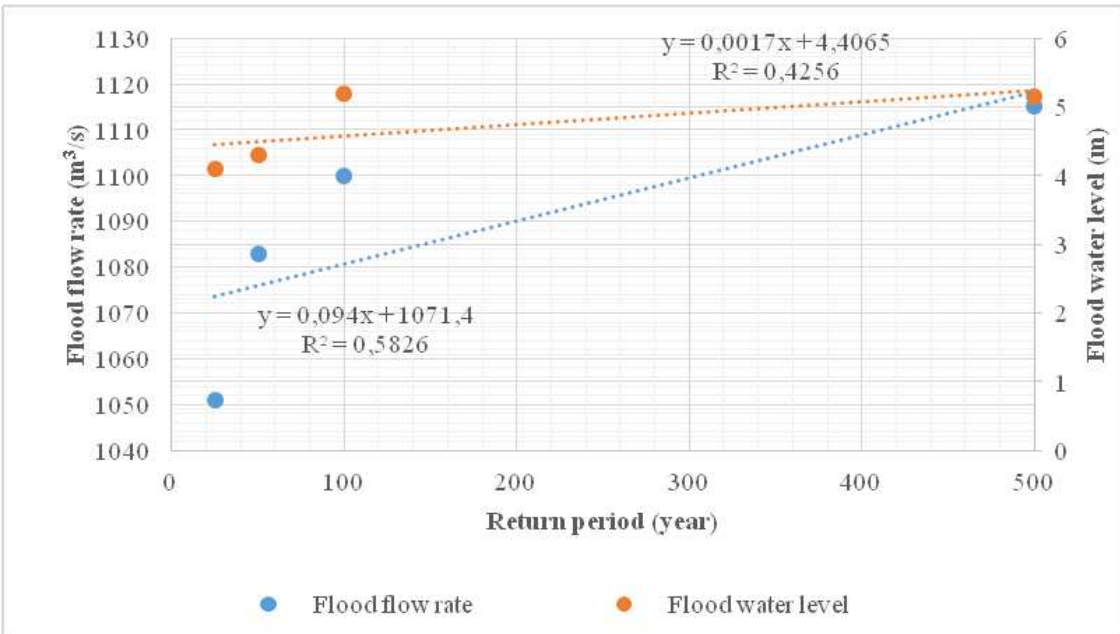

Figure 7. *The relation between return period-flood flow and the relation between the return period-flood water level*

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Discussion

Within the scope of the study, the necessary return period and flood size probability distribution were determined using flood frequency analysis for project area located in Porsuk Dam - Eskisehir City. Result of the flood frequency analysis, maximum possible flood flow rates were determined and these flow rates were used for one-dimensional model to determine flood water level. Validation and calibration studies were not performed in project area due to lack of real data of flood flow rate and flood water level. However, previous researchers performed validation and calibration studies of MIKE 11. Panda et al. (2010) calibrated MIKE 11 model for the monsoon periods of the years 2006 and 2001 and they stated that simulated flood levels by MIKE 11 one dimensional model fit real data in validation studies (R^2: 0.921) and calibration studies (R^2:0.912). Also they determined that observed peak water levels in MIKE 11 fit better than other artificial neural network models because of their hidden neurons in the hidden layer (Panda et al., 2010). Haldar and Khosa (2015) stated that MIKE 11 hydrodynamic model was useful to diminish the flood risk level for river zone and they validated the model for the year 2000 with short available data.

Our results can be evaluated within the scope of the objectives of the National Flood Management Strategy for other settlement which have flood risk. There are similar researches in literature especially in Europe. Booij (2003) argued that decision support systems could be used to select the plan to be used for flood control and ecosystem rehabilitation. In his study, he created the model environment for the Red River basin aiming decision support system usage. Meteorological, hydrological, hydraulic, social and economic data of Red River were used to the PCRaster system. Also Birkland et al. (2003) emphasized that the environmental damage caused by floods in river ecology has increased due to the inadequate flood hazard control policies, and the environmental, social and environmental impacts of flood risk areas have been increasing in order to improve ecosystem functions, protect sensitive environmental resources, they argued that local governments should take an active role in creating an optimized flood management policy from economic approaches.

Akadiri et al. (2008) aimed to improve the resilience of the buildings located on the coast of England against flood in his study. He listed the factors affecting flood risk as soil type, basic geological structure and humidity conditions of existing soil, density of precipitation, average annual precipitation, rainfall transport capacity of the channel and precipitation regime. He stressed that the most serious effects created by climate change are accelerated sea level rise and greater coastal overflow due to rising winds. DEFRA cited increased flood numbers with climate change by referring to 2004 data. Again based on this data; the preliminary views on the total number of domestic, industrial and commercial buildings under flood risk, the human population to be affected by flood risk, the total value of vulnerable agriculture areas against floods, annual average flood protection and costs to avoid losses. The homeowners ranked flood-resistant buildings with proposals increase the defense strength of their buildings against floods. The one dimensional model created in the study can be used as a base and 2D and integrated models can be created for Porsuk Dam - Eskisehir City. Flood frequency analysis performed in this study; Porsuk Dam - Eskişehir City can be used for engineering purposes in the design of bridges, dams, culverts and flood control structures. It also provides the necessary data to determine the economic value of flood control projects, clarify the flood deposits and determine the area on the flood bed.

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In this study, the area between Porsuk Dam, which is source drinking water source of project area, and Eskisehir was simulated to investigate flood risk and flood frequency analysis and flood water level was determined by MIKE 11 model. Flood frequency analysis performed in this study for area between Porsuk Dam- Eskisehir. The results of one-dimension flood model can be used for engineering purposes to design urban basins, dams, culverts and flood control of these structures. In addition to determining the economic value of flood control projects, the results of MIKE 11 model provide efficient, easy and quick response in the case of emergency action against flood risk to reduce flood detrimental effects.

Formation of flood risk maps and flood protection schemes of our country, which is taking important steps in the process of accession to the European Union, is ultimately required in accordance with the Council of Europe and the European Parliament Directive 2007/60/EC of October 23, 2007 on Flood Risk Assessment and Management. For all these reasons; In areas under flood risk; appropriate flood prevention structures should be determined by investigating the environmental, social and economic effects that floods can create, creating flood risk maps, flood intervention and prevention plans of risky areas in water and river basins.

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ADJUSTMENT FOR THE OPTIMUM DISTRIBUTION OF DUST AND GAS IN FULLY MECHANIZED HEADING FACE

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Abstract. The forcing type ventilation at the current fully mechanized heading face is inadequate to meet the requirement of underground coal mine, which could lead to serious disaster of dust and gas explosion and environmental pollution in the roadway. In this paper, the airflow field, dust field and gas field at different distances from the duct outlet to the heading face were numerically simulated, the formation mechanism of each field and the reasons of unreasonable distribution were analyzed, an airflow adaptation adjustment method was proposed for realizing the optimal distribution of airflow, dust and gas by comprehensively changing the position, caliber and direction angle of the duct outlet. The method was applied to Ningtiaota coal mine in Northern Shaanxi of China. The adjustment schemes obtained with airflow adaptability adjustment method provided better results when prepared to before optimization. The dust concentration at the driver position decreased by 35% and the gas concentration reduced by 54%, which provides a theoretical basis for the scientific management of ventilation in the fully mechanized heading face.
Keywords: *duct outlet, airflow field, dust field, gas field, optimal adjustment of airflow*

Introduction

The fully mechanized heading face is vulnerable to the severe disaster of dust and gas explosion. Currently, the expansion of cross-section size and overlong distance excavation contribute to the dust and gas accumulation and severely pollute the underground working environment (Zhou et al., 2017; Wang and Ren, 2013). The caliber and the distance between the outlet and heading face of traditional forcing type ventilation cannot be changed. Under the premise of the specific total capacity of ventilation air, with the continuous extension section of drivage roadway, the airflow cannot efficiently dilute gas and dust within effective range due to the wind energy losses caused by the increasing air resistance along the roadway (Whang, 1999). If the air volume increases in the process of ventilation, the energy wasting, secondary dust (Geng et al., 2018) and discomfort feelings of workers will occur. Besides, the direction angle of the outlet cannot be changed, causing the limitation of airflow path. Thus, even increasing in air volume, the gas and dust still cannot be efficiently diluted because they are accumulated in the blind ventilation zone at the heading face. Therefore, the current forcing type ventilation cannot meet the requirements for underground coal mine. In order to satisfy the actual needs under various conditions, the dynamic adaptability adjustment of airflow in the fully mechanized heading face is essential. Domestic and foreign scholars have carried out a lot of researches on distribution laws and optimization analysis in the single field, including airflow field, dust field and gas field (Li, 2016; Wang et al., 2015; Zhou et al., 2014; Luo et al., 2015; Zhang et al., 2016). However, the study on the comprehensive consideration of airflow, dust and gas field

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under the different parameters of duct outlet, including caliber, direction angle and back-forth distance, has not been proposed yet. In light of the current situation of underground coal heading face and based on analysis of the migration mechanism and distribution law of airflow, dust and gas field, the optimum migration of airflow, dust and gas field is realized by changing the parameters of outlet, which provides a theoretical basis for the safety, energy-saving and green ventilation environment in fully mechanized heading face.

Materials and methods

Material

We propose a method of the optimal adjustment for distribution of dust and gas in fully mechanized heading face. This method is applied in the Ningtiaota coal mine, which is located in Ningtiaota industrial park in Northern Shaanxi province of China in *Figure 1*. It is one of the four mining fields in the state's planning for the southern district of Shenfu mining, and the total output is 12.00 mt/a. The forcing type ventilation is uesd in Ningtiaota mine. The air duct outlet cannot be adjusted, the path of airflow is limited, the problems of gas accumulation, dust accumulation and ventilation of dead corner in heading face should be solved.

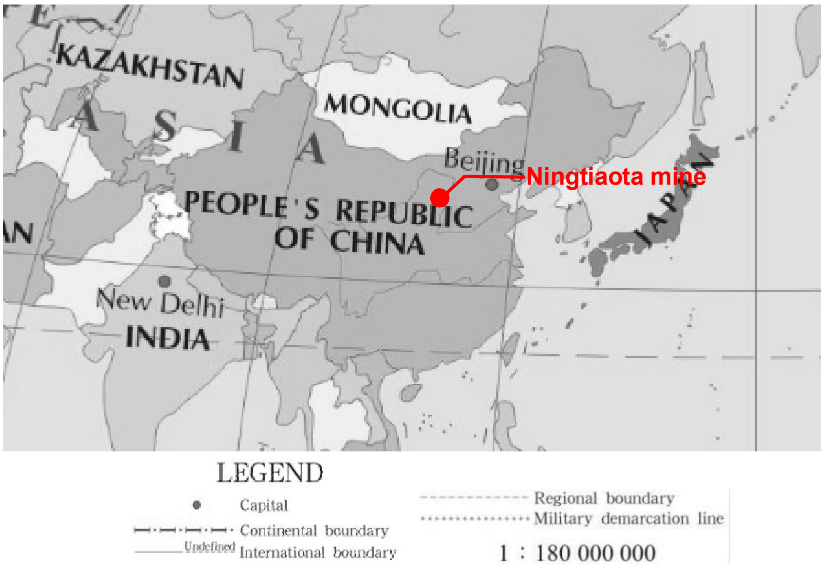

Figure 1. *Location of study area in China*

Theoretical analysis

(1) Airflow

Assuming no heat source in the roadway and neglecting the thermal radiation from the wall, the air flowing meets the continuity equation and the momentum conservation equation. For the flowing mode is a restricted-wall-attached jet, the airflow is turbulent, incompressible fluid, whose diffusion is a random orbit model, thus the SIMPLEC (Tang et al., 2015) algorithm and Realizable k-ε turbulence model are used to control and calculate the flow field.

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k equation (*Eq. 1*):

$$\frac{\partial}{\partial t}(\rho\varepsilon)+\frac{\partial}{\partial x_i}(\rho\varepsilon u_i)=\frac{\partial}{\partial x_i}\left((u+\frac{u_i}{\sigma_k})\frac{\partial k}{\partial x_j}\right)+G_k+G_b-\rho\varepsilon-Y_M+S_k \qquad \text{(Eq.1)}$$

ε equation (*Eq. 2*):

$$\frac{\partial}{\partial t}(\rho\varepsilon)+\frac{\partial}{\partial x_i}(\rho\varepsilon u_i)=\frac{\partial}{\partial x_j}\left[\left(\mu+\frac{\mu_t}{\sigma_\varepsilon}\right)\frac{\partial\varepsilon}{\partial x_j}\right]+\rho C_1 S_\varepsilon-\rho C_2\frac{\varepsilon^2}{k+\sqrt{v\varepsilon}}+C_{1\varepsilon}\frac{\varepsilon}{k}C_{3\varepsilon}G_b+S_\varepsilon \qquad \text{(Eq.2)}$$

where ρ (kg/m^3) is the density of airflow, x_i, x_j coordinate element, k (J) is the turbulence kinetic energy, σ_k, σ_ε are the prandtl number, it indicates the relationship between temperature boundary layer and flow boundary layer, ε is the dissipation rating of k, S_ε (kg/m^3 s) is the coupling for additional equations, G_k (J) is turbulent kinetic energy formed by average air speed, the G_b (J) is the turbulent kinetic energy formed by buoyancy, Y_M is the affect from the expansion of turbulent motion on ε, μ is the viscosity coefficient of molecular, μ_t is the viscosity coefficient of turbulent, $C_2 = 1.9$, $C_{1\varepsilon} = 1.44$, S_k, S_ε (kg/m^3 s) are the source item defined by customer.

(2) Gas field

The assumptions of gas field are as follows: the same wall roughness along the roadway and the gas migration in roadway meets the species mass conservation equation, which is shown as (*Eq. 3*):

$$\frac{\partial(\rho c_s)}{\partial t}+\frac{\partial(\rho c_s u)}{\partial x}+\frac{\partial(\rho c_s v)}{\partial y}+\frac{\partial(\rho c_s w)}{\partial z}=\frac{\partial}{\partial x}\left(D_s\frac{\partial(\rho c_s)}{\partial x}\right)+\frac{\partial}{\partial y}\left(D_s\frac{\partial(\rho c_s)}{\partial y}\right)+\frac{\partial}{\partial z}\left(D_s\frac{\partial(\rho c_s)}{\partial z}\right)+S_s \qquad \text{(Eq.3)}$$

where ρc_s (kg/m^3) are the species mass conservation equation, D_s is diffusion coefficient of components, u, v w (m/s) is the component of velocity vector in the three directions of x, y, z, S_s (kg/m^3 s) is the quality source item of gas component.

(3) Dust field

The dust in the heading face is a sparse phase, dust particles are a discrete phase, and air flows continuously. And the discrete phase model (DPM) of Euler-Lagrange method is used to simulate the motion of dust. The force between dust particles is neglected because the quality of particles is light, dust particle should be loaded with gravity, buoyancy pressure gradient force, etc. (Liu, 2010). Two-phase gas-solid flow mathematical model is used to analyze the migration law of dust. The mechanical model is as follows (*Eqs. 4* and *5*):

$$\frac{du_p}{dt}=F_D(u-u_p)+\frac{g_x(\rho_p-\rho)}{\rho_p} \qquad \text{(Eq.4)}$$

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$$F_D = 0.75 \frac{C_D \rho \left| \mu_p - \mu \right|}{\rho_p d_p} \tag{Eq.5}$$

where $F_D(u\text{-}u_p)$ (N/kg) is the mass per unit mass drag force, C_D is the drag coefficient, u (m/s) is the air velocity of fluid phase, u_p (m/s) is the particle velocity, μ (m^2/s) is the fluid viscosity, ρ (kg/m^3) is the fluid density, ρ_p (kg/m^3) is the particle density, d_p (m) is the particle diameter.

Finite element analysis method and verification

The air duct is installed at the upper of the side of roadway wall. For ensuring the precision of model calculation, the roadheader is simplified into two parts, one is a machine part, and another is a cutting part. The end of the outlet is set as an inlet, the tail section of the roadway is set as an outlet, the outlet pressure is 0, and there is no slip on the wall surface. In the gas field, the exposed coal wall at heading face is set as the gas emission source. In dust field, the dust source is set at the heading face. Tetrahedron method is used to mesh the geometric model, and the surface mesh unit is set as a triangle. The adjustment schematic diagram of duct outlet on the fully mechanized heading face is shown in *Figure 2*, and the formulas are as follows.

Hydraulic caliber (*Eq. 6*):

$$d_H = \frac{4A}{S} \tag{Eq.6}$$

where d_H (m) is the hydraulic caliber, A (m^2) is the fluid cross-sectional area, S (m) is the wetted perimeter.

Reynolds number (*Eq. 7*):

$$\mathrm{Re}_H = \frac{\rho v d_H}{\mu} \tag{Eq.7}$$

where ρ (kg/m^3) is the air density, v (m/s) is the speed of airflow, μ (Pa·S) is the coefficient of viscosity.

Formula turbulence intensity can be derived from *Equations 8* and *9*:

$$I = 0.16(\mathrm{Re}_H)^{-\frac{1}{8}} \tag{Eq.8}$$

where I is turbulence intensity, R_{eH} is Reynolds number.

Gas emission source term:

$$s_i = \frac{CQ\rho\varphi}{60V} \tag{Eq.9}$$

where s_i ($kg{\cdot}m^{-1}{\cdot}s^{-1}$) is the gas emission source term, C (g/m^3) is the concentration of emission gas, Q (m^3/min) is the air volume in roadway, ρ (kg/m^3) is the gas density, V (m^3) is the volume of emission gas, φ is the proportion of each gas component.

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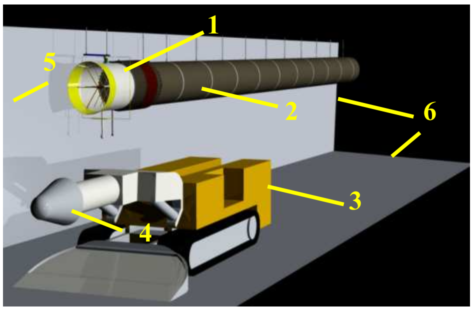
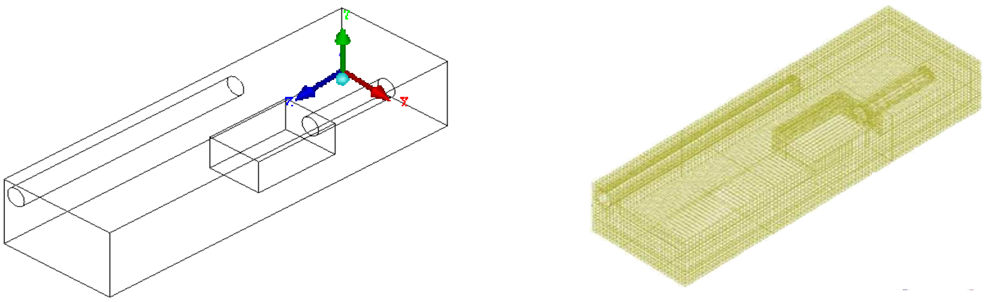


1-Adjustment device of duct outlet; 2-air duct；3-Mian body of Roadheader; 4-Cutting part of roadheader; 5-Tunnelling heading face; 6-Roadway

Figure 2. *Schematic diagram of air outlet adjustment in the fully mechanized mining face*

Taking Ningtiaota coal mine as the research object, the numerical simulation scheme is established, and the feasibility of the numerical simulation scheme is verified. The cross-section of roadway is rectangular (6.25 m × 3.75 m), the airspeed at the duct outlet is 8.089 m/s, and the parameters of other boundary conditions of airflow field and gas field are set as shown in *Tables 1*, *2* and *3*. The geometric model and finite element model of the fully mechanized heading face are shown in *Figure 3*.

The air velocity at the height of pedestrian breathing in the air return side at the distance of 6 m from outlet to heading face and the dust concentration at the distance of 8 m from the duct outlet to heading face are selected as measured objects, the measured points 1 as shown in *Figure 4*. The results of comparing the measured data and simulation data are shown in *Figure 5* that indicates the distribution of airflow and dust is consistent with the actual situation, which proves the numerical simulation scheme of dust field is feasible.



a **b**

Figure 3. *The fully mechanized heading face model.* ***a*** *Geometric model.* ***b*** *Finite element model*

Table 1. *Boundary condition setting of airflow field*

Condition	Define
Air Density (kg/m^3)	1.199
Air Viscosity (P•s)	1.7894e-0.5
Operating Pressure (Pa)	101325
Hydraulic caliber (m)	1
Turbulence Intensity (%)	2.526

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Table 2. *Boundary condition setting of the gas field*

Name	Material	Define
CH_4	Density (kg/m^3)	0.6679
	Viscosity	1.087e-05
Coal	Density (kg/m^3)	1530
	Cp (Specific Heat) (j/kg-k)	2700
	Thermal Conductivity (w/m-k)	0.17
	Porosity of porous media	0.2
	Quality source term ($kg/(m^3 \cdot s)$)	0.001

Table 3. *Boundary condition setting of dust field*

Material	Define
Model	Discrete Random Walk Model
Minimum particle (m)	5e-07
Maximum particle (m)	2e-04
Medium particle size (m)	3.25e-05
Mass flow rate ($kg \cdot s^{-1}$)	0.016
Particle size distribution	Rosin-Rammler
Particle size distribution exponential	1.42

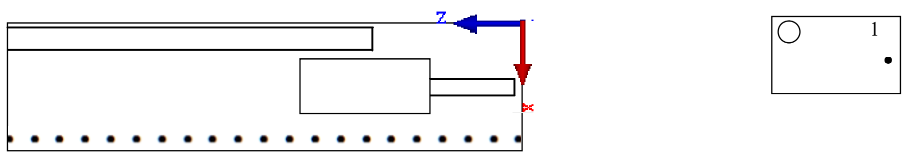
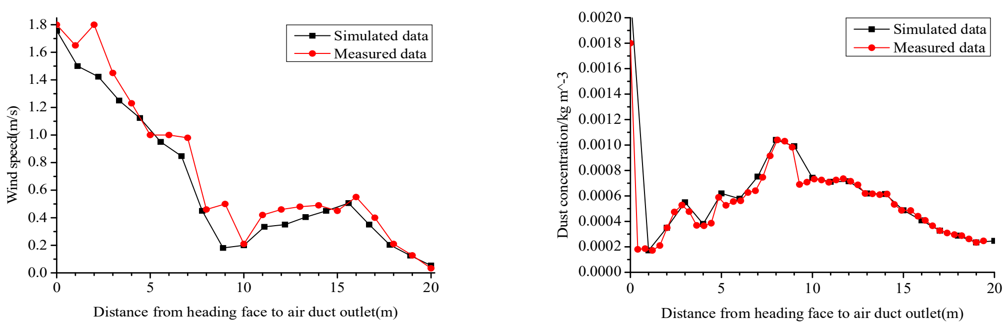


Figure 4. *Distribution of measured points*



a **b**

Figure 5. *Comparing the measured data and simulation data of airflow-dust field.* ***a*** *Air velocity distribution at air return side.* ***b*** *Dust concentration distribution at air return side*

According to the contour of the gas concentration of different cross-section at the distance of 6 m from the duct outlet to heading face (see *Figure 6*), Z = 1.0 represents

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X-Y plans, which is 1.0 m away from heading face, and the direction of Z axis is the reverse direction of roadheader working. It is known that the gas concentration at the side of duct outlet is apparently lower than that at the air return side. According to the average values of simulation, the gas concentration at the cross section of Z = 0.2 m and Z = 1 m are 0.186% and 0.147%, which is consistent with the measured values of 0.187% and 0.146%, respectively, verifying the numerical simulation scheme of gas field is feasible.

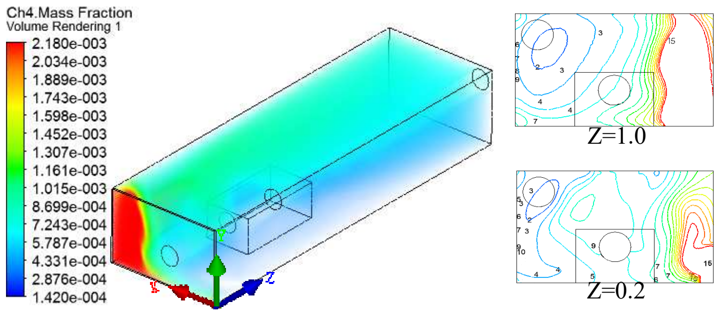

Figure 6. *Contour map of gas concentration at roadway cross section*

Migration mechanism and existing problems in airflow dust and gas field

Migration mechanism and existing problems in airflow field

According to the definition of hydrodynamic, it is known that the airflow at the duct outlet is restricted attachment jet-flow under the forcing type ventilation (Wang et al., 2004), and the jet-flow zone is in front of duct outlet (see *Figure 7*).

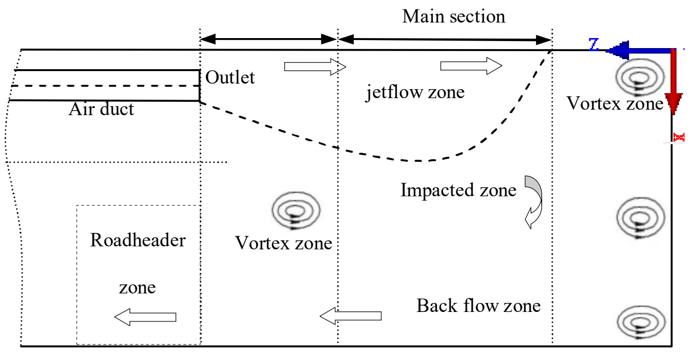

Figure 7. *Schematic diagram of air flow structure in the fully mechanized heading face*

In the process, the airflow will continuously absorb air surrounding, resulting in energy loss. Then with the jet expanding continually, the airspeed at the axis of outlet is equal to the initial velocity at the outlet and keeps constant, which is the start section of jet-flow. Then the speed begins to decrease to 0, which stage is the main section, the effective range of the jet-flow includes the start section and the main section. With the increasing distance from the outlet to the heading face, the effective range is reduced by the resistant force along the roadway. The air-flowing is attached to the wall and limited by roadway boundary, leading to the backflow formed at the bottom of roadway. The dust and gas will be brought out utilizing the backflow. However, the airspeed at return

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side is low, which has a slight influence on the air disturbance at the upper and lower corners of the air-return side where the original air keeps relatively stable state. When the boundary layer of original air is loaded by tangential friction force or disturbed by air, the vortex is formed. Similarly, the jet-flow boundary layer disturbs air at the rear of roadheader where the vortex is formed, and the airflow of normal migration is affected and the vortex formed in front of the heading machine for the large volume of roadheader.

According to the literature (Zan et al., 2010), the motion parameters of restricted attachment jet-flow are related to restriction degree *n*, the calculation formula of jet restriction degree is as follows (*Eq. 10*):

$$n = \frac{A_0}{A} \tag{Eq.10}$$

where A_0 (m^2) is the section area of the outlet, A (m^2) is the section area of roadway.

From the formula above, the smaller restriction degree n is, the stronger the characteristics of the free-wall-attached jet will be, whereas the restricted-wall-attached jet characteristics become weaker.

The distribution regularity of airflow field, gas field and dust field are simulated at distances of 5, 6, 7, 8, 9 and 10 m, respectively, from the duct outlet to the heading face, then the existing problems of original field are obtained.

Turbulence is the main factor for gas dust diffusion, and vortex should be emphasized (Gong et al., 2017a). To analyze clearly the airflow distribution law at workers' activity district and ensure the position of turbulence, the airflow distribution under different distances from the outlet to heading face is shown in *Figure 8*, the specific results of air distribution shown in *Table 4*.

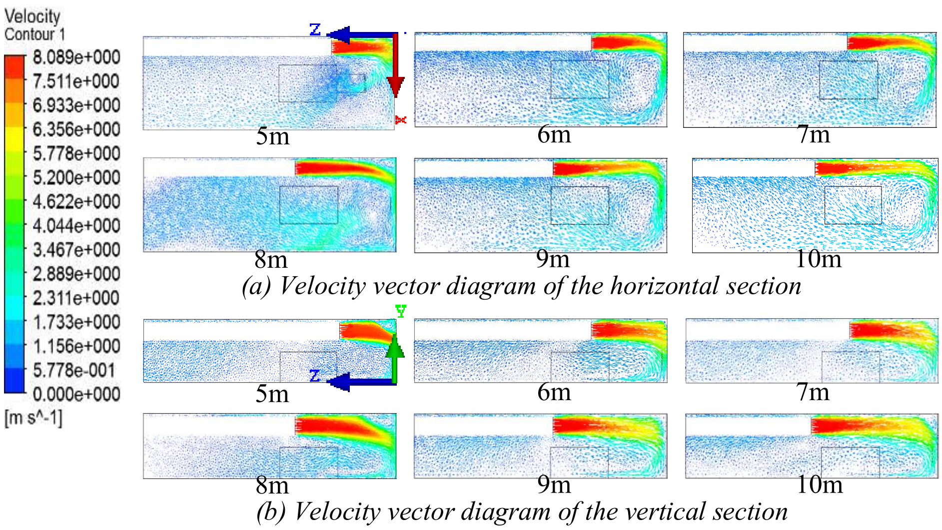

Figure 8. *Airflow distribution under different distance from outlet to heading face*

The simulation results show that when the duct outlet is close to the heading face (5 m), the driver will feel extremely uncomfortable because of overhigh velocity of

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airflow. The vortex formed above the roadheader, when the airflow impacts the heading face and destroys air return zone, which causes dust accumulation and environment pollution. Expanding outlet can decrease airspeed and shorten the effective range of jet-flow to make sure the back-flow district formed at the heading face, which is conducive to bringing gas and dust out effectively. Whereas, when the duct outlet is far away from the heading face (9-10 m), the irregularly circulated motion of gas and dust occurs for the vortex formed in the front of roadheader. At the same time, the jet zone becomes longer, but the effective range will be reduced by resistance along the roadway, leading to the low air velocity when it arrives in the heading face, and resulting in the dust and gas at the producing source are difficultly diluted. Consequently, the caliber and the distance from outlet to heading face should be in a reasonable range.

Table 4. *Airflow field distribution*

Distance from heading face (m)	Air velocity at diver place (m/s)	Air velocity at walking place (m/s)	Turbulence formed position
5	0.3~1.4	0.2~4.7	The end of the jet-flow
6	0.28~3.0	0.27~2.9	In front of roadheader
7	0.3~2.8	0.25~3.0	The right side of roadheader
8	0.25~0.8	0.22~0.75	Above the jet flow zone and the right side of roadheader
9	0.1~0.8	0.23~0.8	The right side and in front of roadheader
10	0.1~0.9	0.2~1.7	In front of roadheader

Migration mechanism and existing problems in gas field

The wall is a porous medium in roadway, and gas flows out from heading face, gas molecules are irregularly moved from high to low concentration. The gas is mainly produced from heading face, its density is lighter than that of air and easily accumulated on the roof. The gas at the upper corner will be disturbed by airflow, then vortex formed. However, the wind intensity at the lower corner is weak, so that its disturbance to the gas is light, resulting in the gas concentration at lower corner is higher than that at upper corner. The gas in the air-return area is brought out, because vortex formed near the roadheader.

The method of gas diluting is to blow fresh air to the heading face. We proposes the gas in the ventilation dead corner of roadway should be diluted by changing the caliber, direction angle and distances from the heading face, then the air volume be controlled at heading face.

The explosion would most likely occur due to the high gas concentration. The gas concentration distribution at different distances from the duct outlet to the heading face are numerically simulated, and the reasons of gas accumulation are investigated. Aimed at revealing the gas field distribution, we obtained the gas concentration distribution at the closest distance (5 m), middle distance (7 m) and the farthest distance (10 m) as shown in *Figure 9*.

The simulation results show that: with the increase of distance from the heading face, the gas concentration increases, but the average gas concentration gets lower. It is mainly accumulated in the upper and lower corner of the air return side, and the gas is difficult to be brought out because the airflow is weak in the lower position. The gas

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concentration tends to be stable as the distance further away from the heading face. When the duct outlet is 5 m away from the heading face, the airflow impacts the heading face, and the ventilation return path cannot be formed, resulting in the gas diffussion thus it is difficult for gas to be efficiently moved out. When duct outlet is 6 m from the heading face, compared with the gas concentration at the backflow side, it is lower in the jet flow zone. When the distance from duct outlet to heading face is 7-8 m, the jet flow disturbs the air, and gas is well diluted. When the duct outlet is 9-10 m from the heading face, the jet flow will be affected by resistance in the roadway, causing the effective range becomes shorter and severe gas accumulation takes place at the heading face. Based on the analysis above, we should focus on optimizing the gas field at specific distances of 5-10 m between duct outlet and heading face.

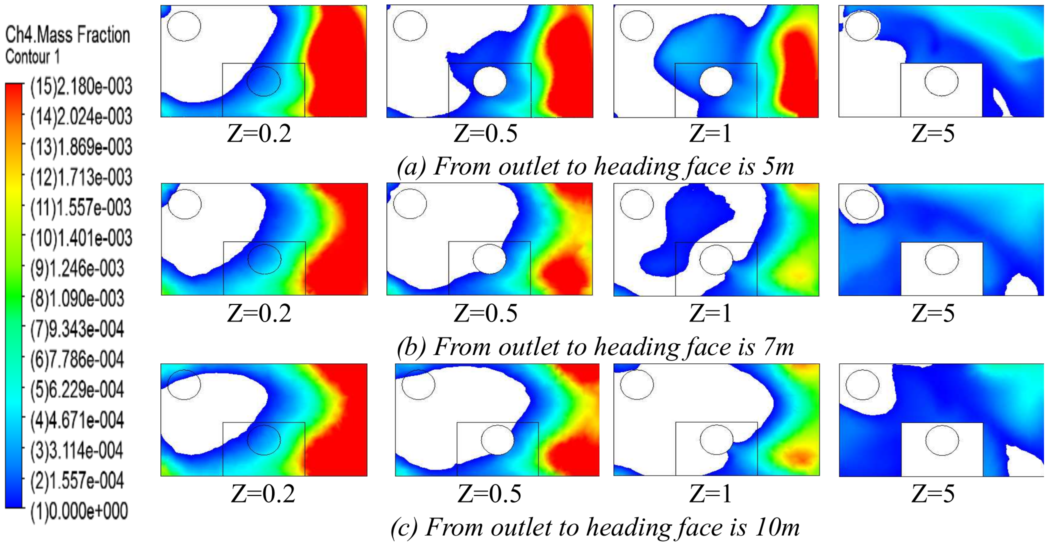

Figure 9. *Contour of gas concentration in different cross-section of roadway*

Migration mechanism and existing problems in dust field

The jet flow zone is generated by the directly impact of airflow from the outlet, which can effectively disperse dust. The dust concentration is lower than that in the vortex zone. At the same time, the jet absorbs the dust in the surrounding air, the absorbed dust will be taken to the heading face, resulting in high dust concentration. The vortex formed at the front of roadheader, causing the dust diffusion in the roadway and difficultly settle down. Under the forced type ventilation, the air velocity is low at the back of roadheader. The respirable dust tends to move toward the backflow side, where the small particle dust accumulates mainly at the upper corner (Alam, 2006; Candra et al., 2014), thus the dust concentration in the backflow zone is higher than that in the jet zone (Gong et al., 2017b).

To better describe the distribution of dust concentration in each zone, calculation models in each zone were established (Liu et al., 2002), and the equations are as follows (*Eq. 11, 12* and *13*):

$$\overline{C}_a = C_0 + \frac{GQ'}{Q_0(Q_0 + Q')} \qquad \text{(Eq.11)}$$

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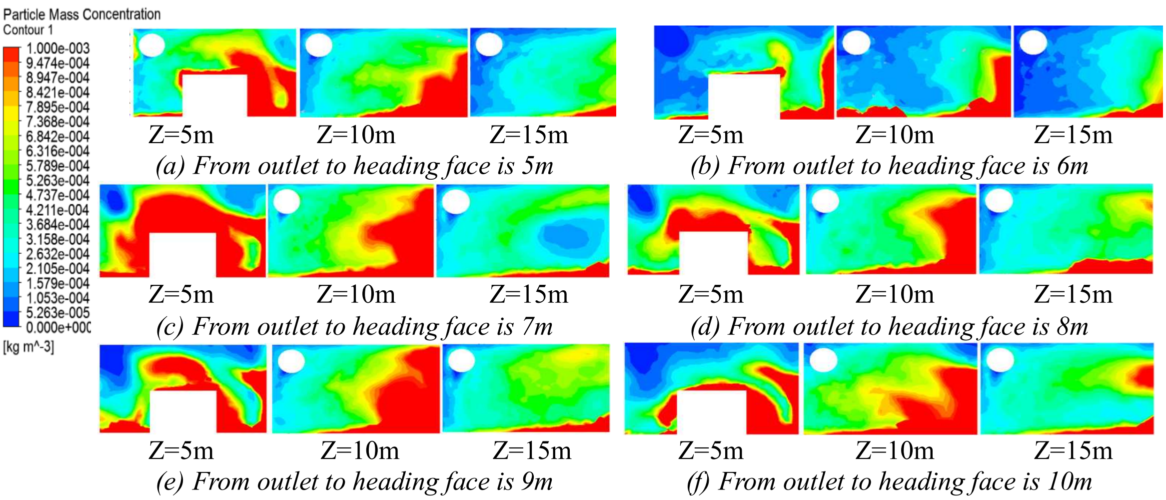
$$\overline{C}_b = C_0 + \frac{G}{Q_0} \qquad \text{(Eq.12)}$$

$$\overline{C}_c = C_0 + \frac{G}{Q_0} \qquad \text{(Eq.13)}$$

Where ,$\overline{C}_a$ (mg/m^3) is dust concentration in jet-flow zone, ,$\overline{C}_b$ (mg/m^3) is dust concentration in vortex zone, ,$\overline{C}_c$ (mg/m^3) is dust concentration in backflow zone, C_0 (mg/m^3) is dust concentration in air-flow, Q_0 (mg/m^3) is forced air volume, Q_1 (mg/m^3) is air volume discharged from heading face, Q' (mg/m^3) is suction volume in jet-flow, G (mg/m^3) is dust intensity.

According to the formula analysis, it is concluded that the dust concentration in the jet area, vortex area and backflow area are positively correlated with the dust intensity, and negatively correlated with forced air volume. And under the premise of the reasonable distribution of the airflow field, the larger the air volume is, the better effect of the dust dilution will be.

Considering that dust concentration is the main cause of pneumoconiosis and environmental pollution, the numerical simulations of dust field at different distances from the outlet to heading face are carried out, and the dust concentration at the different cross-sections of roadway are shown in *Figure 10.*



(a) From outlet to heading face is 5m
(b) From outlet to heading face is 6m
(c) From outlet to heading face is 7m
(d) From outlet to heading face is 8m
(e) From outlet to heading face is 9m
(f) From outlet to heading face is 10m

Figure 10. *Dust concentration distribution at different distances from air outlet to heading face*

As can be seen from *Figure 10*, the dust concentration on the return side wall and the air duct side gradually decreases as the distance from the outlet to the heading face increasing. According to the actual working environment, the range of 5-10 m is working positions for drivers, and within this range, affected by the roadheader, vortex is formed and dust accumulated, which obstruct the driver's vision. When the distance from the outlet to heading face is 5-6 m, the dust concentration is low in driver's position and heading face. When the distance is 7-10 m, the dust concentration increases in backflow, which is not conducive to safe production. The specific location of dust accumulation is shown in *Table 5*.

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***Table 5.** Distribution of dust-flowing field*

Distance from heading face	Dust concentration at the range of driver activity area (kg/m^3)	Dust concentration at backflow side (kg/m^3)	The position of dust accumulation
5m	$1.0\times10^{-3} \sim 5.0\times10^{-3}$	$3.2\times10^{-5} \sim 1.4\times10^{-3}$	Front, right and above of roadheader
6m	$2.4\times10^{-4} \sim 1.0\times10^{-3}$	$2.4\times10^{-5} \sim 4.0\times10^{-4}$	Wall at backflow along roadway
7m	$1.0\times10^{-3} \sim 5.0\times10^{-3}$	$2.5\times10^{-5} \sim 1.6\times10^{-3}$	Front of roadheader
8m	$5.1\times10^{-4} \sim 8.0\times10^{-3}$	$2.6\times10^{-5} \sim 1.1\times10^{-3}$	Above and right of roadheader
9m	$9.0\times10^{-4} \sim 7.8\times10^{-3}$	$6.6\times10^{-5} \sim 1.5\times10^{-3}$	Back of roadheader
10m	$7.6\times10^{-4} \sim 8.0\times10^{-3}$	$1.2\times10^{-5} \sim 2.3\times10^{-3}$	Driver position

Results

Influence of air outlet caliber change on airflow dust and gas distribution

To improve the utilization rate of underground energy, under the premise of constant air volume, we propose that the outlet caliber should be adjustable for changing the airflow speed. According to the migration mechanism of the airflow dust and gas, reasonable airflow distributions will have a direct influence on the gas and dust distribution in heading face. The distribution of airflow field at calibers of 0.7, 0.8, 0.9, 1.0, 1.1 and 1.2 m are numerically simulated, respectively. The velocity distribution of airflow field at the horizontal plane is shown in *Figure 11*.

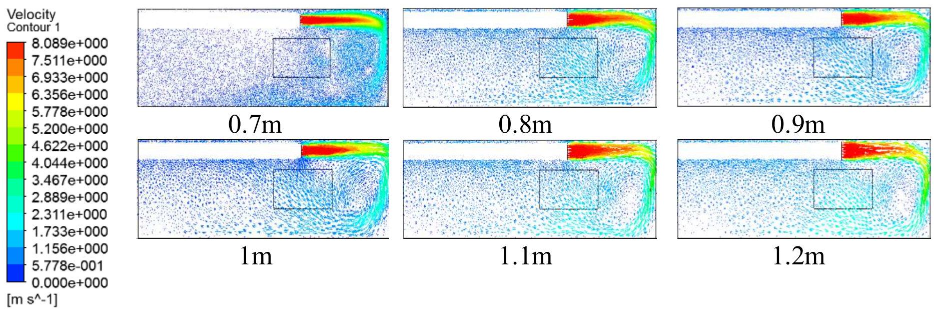

***Figure 11.** Airflow velocity distribution under different caliber*

As the caliber increases, the effective range of jet becomes short, and the airspeed at the heading face decreases. The vortex is formed at front of roadheader and is beneficial to diluting the dust and gas at the heading face; and the larger the caliber of the air outlet, the larger radius of curvature of backflow is. Under the premise of the certain total air volume, when the outlet is far away from the heading face, the axial velocity of jet can be increased by caliber necking; and when the outlet is close to the heading face, the air velocity is decreased by reducing the impact force on the heading face. In summary, the adjustment methods of outlet caliber are put forward to meet the Coal Mine Safety Regulations on the air velocity ranging of 0.25-4 m/s. When the distance from the outlet to the heading face is 5 m, the caliber should be extended to 1.1 and 1.2 m; when the distance is 6-8 m, the caliber’s unchanged (at 1 m), and it is necked to 0.8 and 0.9 m when the distance is 9-10 m. At distances of 5 m and 10 m, the distribution of the airflow dust and gas field under different caliber are shown in *Figures 12* and *13*.

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The different value of Y in *Figures 12a* and *13a* mean different X-Z plans, for example, Y = 1.0 m indicates the X-Z plan, which is 1.0 m away from ground.

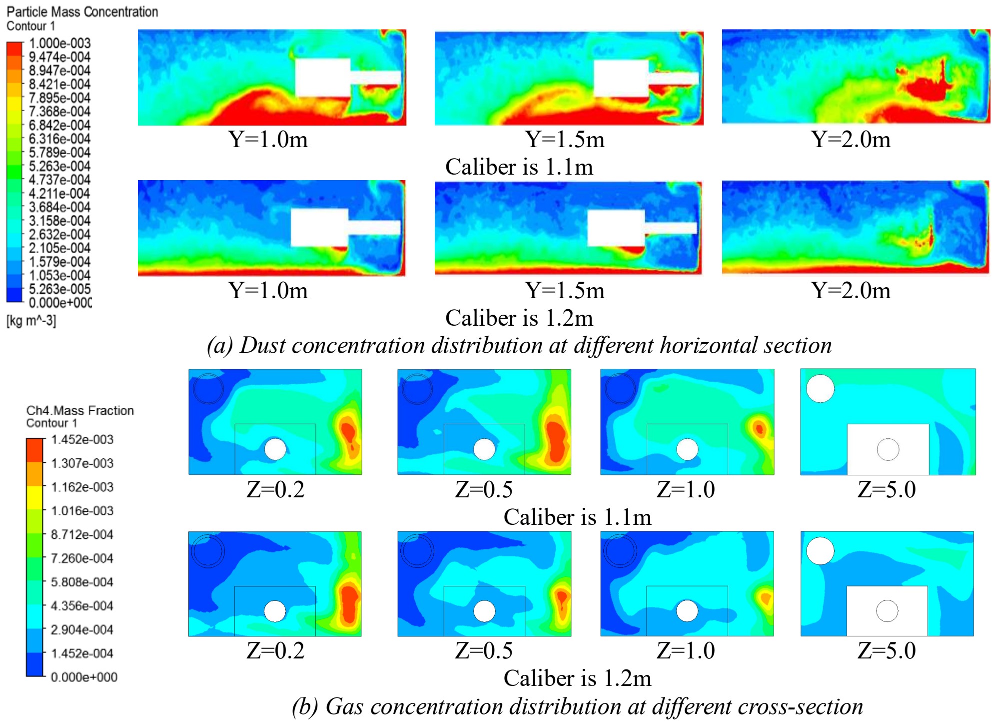

Figure 12. *Dust and gas concentration distribution under different caliber of outlet at the distance of 5 m from heading face*

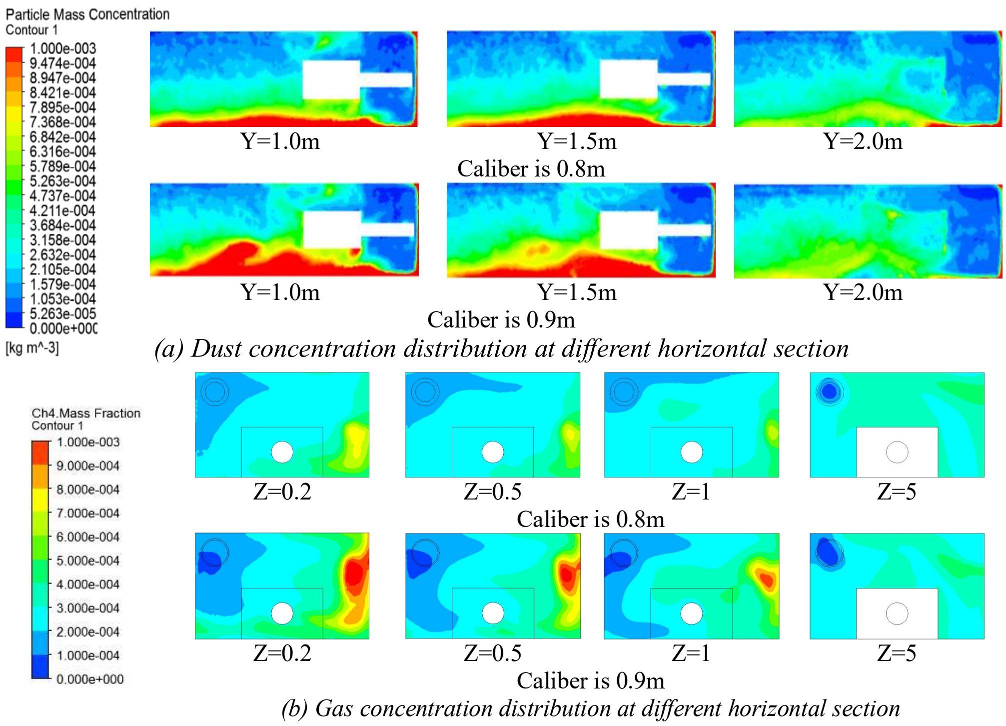

Figure 13. *Dust and gas concentration distribution under different caliber of outlet at the distance of 10 m from heading face*

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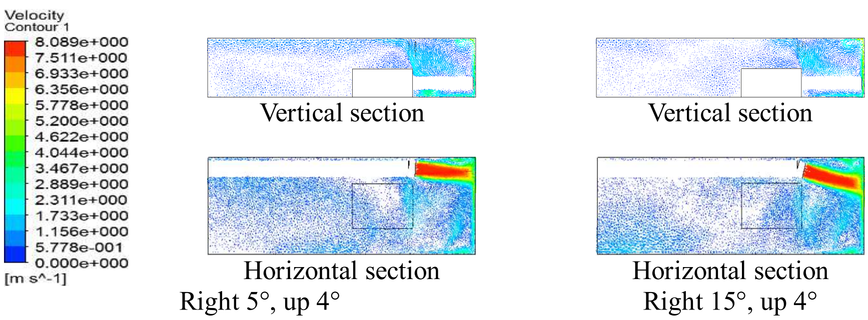
At the distance of 5 m, extending caliber can effectively decrease the dust and gas concentration (see *Figure 12*). When the caliber is 1.1 m, dust is accumulated above the heading face, which will seriously pollute the working environment of driver, and the gas concentration at the air return side is a tendency of decreasing-increasing-decreasing. When the caliber is 1.2 m, the dust concentration is low with uniform distributions, the orange area gradually gets shrunk, indicating that gas concentration decreases. At the distance of 10 m, the necked caliber can effectively improve the distribution of the dust and gas field (see *Figure 13*). When the caliber is 0.8 m, the orange area representing the dust and gas accumulation at air return side is smaller than that of 0.9 m of caliber. According to the above analysis, the reasonable range of caliber is: at the distance of 5 m, select 1.2 m of caliber; at distances of 6-8 m, select 1 m of caliber, when it is 9-10 m, select 0.8 m of caliber.

Influence of air outlet angle direction change on air-flow dust and gas distribution

Due to the limitation of the geometric size of the roadway section and the premise of normal operation of roadheader, according to the existing problems in the airflow field, dust field and gas field, the velocity in backflow should be improved to break the vortex around the roadheader and dilute the dust and gas at the heading face and dead corner area. The angle of outlet should be adjusted at the horizontal right deviation of 5°-25° and vertical upward deflection of 2°-8°. Selecting the angle of horizontal right deviation at 5°, 10°, 15°, 20° and 25° and vertical upward deflection of 2°, 4°, 6° and 8°; respectively, the numerical simulation experiments were carried out on the distribution of the air, dust and gas in fully mechanized heading face. *Figure 14* only shows the numerical simulation results of airflow velocity distribution, gas concentration distribution and dust concentration distribution under different outlet angles when the outlet is 5 m away from the heading face owing to the space constraints.

The numerical simulation results show that the problems of low velocity, dead corner of ventilation and vortex area in the local area of original field can be improved by changing the angle of outlet. Under the premise of constant caliber, the angle adjustment can improve the airflow distribution near the roadheader, but it still impacts the heading face, indicating that the velocity of outlet is too high. Therefore, the velocity of outlet should be decreased by changing the caliber. Considering the reasonable distribution of the airflow gas and dust, the reasonable range of outlet angle should be in the horizontal right-skew of 5°-15° and vertical up-skew of 2°-6°. To realize comprehensive optimization for the airflow field, dust field and gas field at the fully mechanize heading face, it is necessary to determine the specific angle parameter value of outlet.



(a) Airflow velocity distribution

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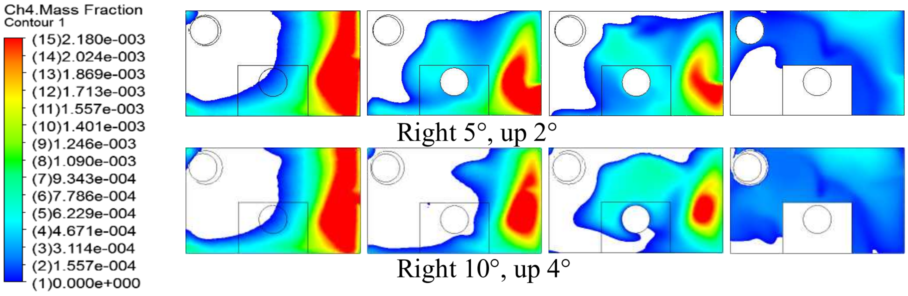
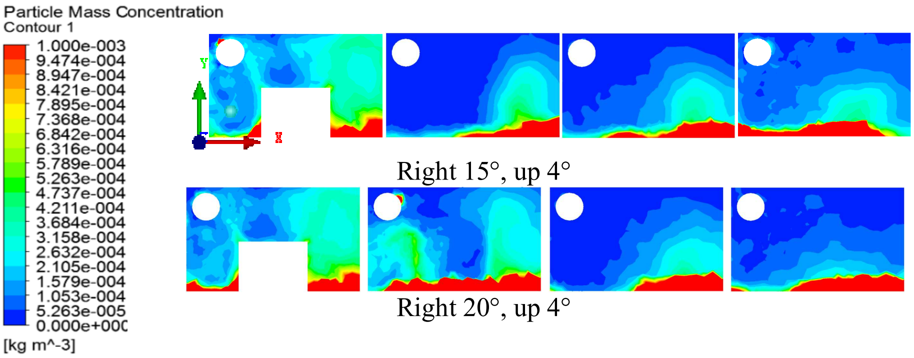


(b) Gas concentration distribution at different cross-section of heading face



(c) Dust concentration distribution at the position of the driver

Figure 14. *Airflow dust and gas distribution under different angle direction of the outlet at the distance of 5 m from heading face*

Airflow adaptability adjustment for dust and gas

Design of comprehensive optimization scheme

In light of the existing problems of the airflow field, dust field and gas field, combined with the reasonable value range of outlet parameters, we put forward a comprehensive optimization adjustment scheme for the airflow field, dust field and gas field under different distances (5, 6, 7, 8, 9, and 10 m) from outlet to heading face (see *Table 6*).

Table 6. *Design of optimization adjustment scheme*

Scheme	5m	6m	7m	8m	9m	10m
Scheme 1	Caliber 1.2m, right 5, up 4	Caliber 1m, right 5°, up 2°	Caliber 1m, right 5°, up 2°	Caliber 1m, right 5°, up 2°	Caliber 0.8m, right 5°, up 4°	Caliber 0.8m, right 5°, up 4°
Scheme 2	Caliber 1.2m, right 15, up 4°	Caliber 1m, right 10°, up 2°	Caliber 1m, right 10°, up 2°	Caliber 1m, right 10°, up 2°	Caliber 0.8m, right 10°, up 6°	Caliber 0.8m, right 10°, up 4°
Scheme 3	Caliber 1.2m, right 15°, up 6°	Caliber 1m, right 15°, up 2°	Caliber 1m, right 15°, up 2°	Caliber 1m, right 15°, up 2°	Caliber 0.8m, right 15°, up 2°	Caliber 0.8m, right 15°, up 2°

Discussion

The comprehensive optimization results of the airflow, dust and gas under different adjustment schemes are compared and analyzed. The air velocity and the concentration distribution of dust and gas in backflow side at the distance of 5 m are shown in

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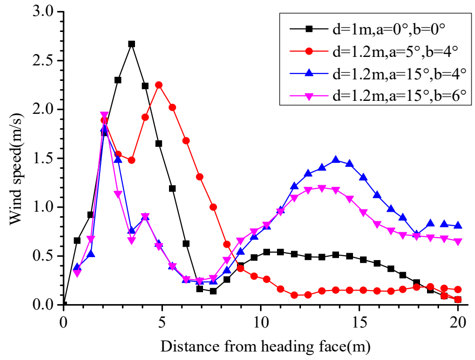
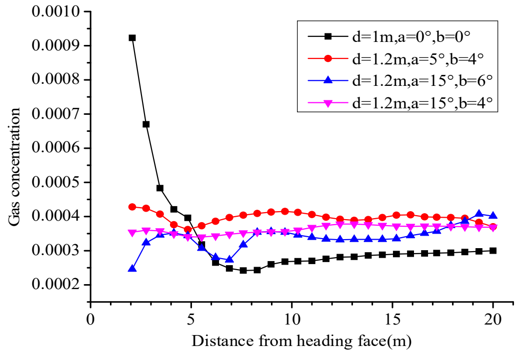
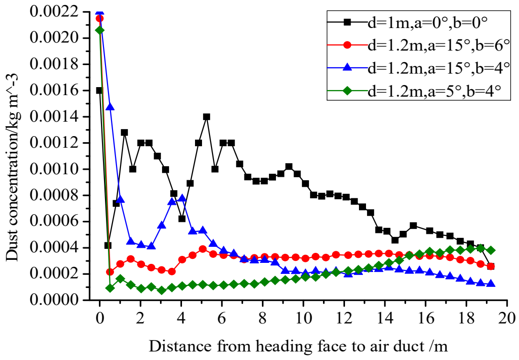
Figure 15. The optimization result of dust particle size distribution was shown in *Figure 16*.



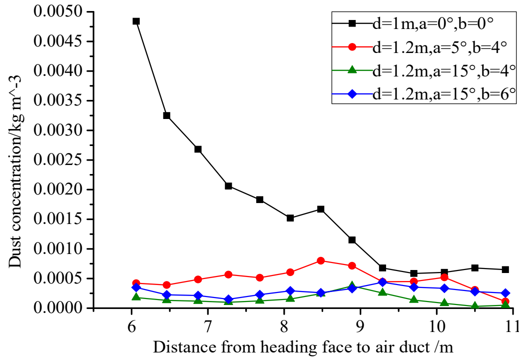
(a) Air velocity distribution at backflow side



(b) Gas concentration at backflow side



(c) Dust concentration at backflow side



(d) Dust concentration at driver position

Figure 15. *Air-flow, dust and gas distribution under different adjustment scheme (d-caliber, a-right-skewed angle value, b-up-skewed angle value)*

As shown in *Figure 15*, when the caliber of outlet is 1.2 m and the angle is at right-skewed 15° and up-skewed 6°, the impact force of air-flow on heading face is reduced, and the problem of ventilation at dead corner is solved thanks to the larger caliber of outlet. Compared with other scheme, the gas concentration can be decreased from 0.1% to 0.023% and the air velocity is at 0.3-2 m/s in the return side. The dust concentration at the air return side is substantially reduced (the lowest concentration is 0.01%), indicating that environment for drivers has signifcantly been improved.

The scheme of caliber 1.2 m at the right-skew of 15° and up-skew of 6° can effectively improve the airflow field. Compared with the original air duct, when the caliber is extended, the impact force of airflow on heading face is reduced, especially adjusting the angle of outlet at the right-skew of 15° and up-skew of 6° is conducive to breaking vortex and solving the ventilation problem in the dead area. And at velocities ranging of 0.3-2 m/s, the gas concentration is declined from 0.1% to 0.023% and the dust concentration is greatly reduced.

As shown in *Figure 16*, the dust particle size distribution is uniform at distances of 0-10 m from the heading face. At distances of 15-20 m, the large-sized particles begin to settle down, and the quantity of small-sized particles accounts for majority, especially presenting the larger percentage of respirable dust (particle size < 7μm). From

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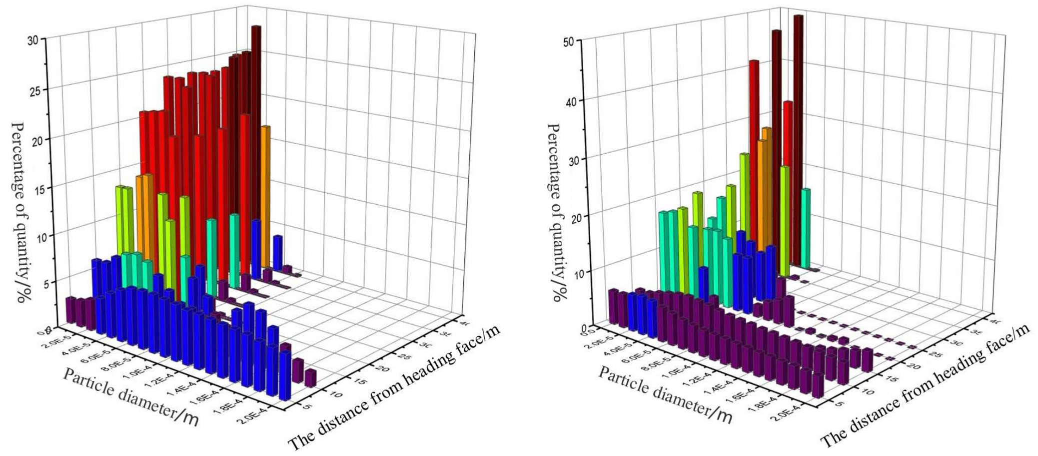
Figure 16, we can see that dust concentration is much lower, which reduces the harm for workers.



Before After

Figure 16. *Comparison of particle size distribution in roadway*

Using the same method above, the distribution of dust and gas at different distances (6, 7, 8, 9 and 10 m) between the outlet and heading face is comprehensively optimized, and the corresponding adaptability optimization adjustment method for air-flow is obtained, as shown in *Table 7*.

Table 7. *Optimization adjustment scheme*

Distance from heading face	Adjustment scheme	Velocity of air duct side	Dust concentration at driver position	Gas concentration at upper corner
5m	d=1.2m, a=15°, b=6°	Reduced by 60% at end of the jet-flow	Reduced by 35%	Reduced by 26%
6m	d=1m, a=15°, b=2°	Increased by 30% at front of roadheader	Reduced by 30%	Reduced by 28%
7m	d=1m, a=10°, b=2°	Increased by 27% at right of air duct	Reduced by 29%	Reduced by 30%
8m	d=1m, a=5°, b=2°	Increased by 31% in front of air duct	Reduced by 31%	Reduced by 32%
9m	d=0.8m, a=10°, b=6°	Increased by 56% at front of roadheader	Reduced by 29%	Reduced by 54%
10m	d=0.8m, a=5°, b=4°	Increased by 65% at front of roadheader	Reduced by 28%	Reduced by 48%

Conclusion

The caliber, angle and the distance from the outlet to heading face of the duct outlet have a certain influence on the air, dust and gas field distribution in the fully mechanized heading face. The caliber adjusting of outlet can change the effective range of airflow as well as wind the velocity of duct outlet; the angle adjusting can change the direction of wind flow, which effectively solves the problems of dust and gas accumulation and ventilation in dead corner area; different distances from outlet to

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heading face have different requirements for parameters adjustment of outlet. According to the dust and gas migration law under different distances from duct outlet to heading face, the outlet parameters are adjusted using the method of airflow adaptability. The results show that the airflow adaptability adjustment not only improves the ventilation efficiency but also reduces the dust and gas accumulation to improve the environmental conditions for miners.

Through the numerical simulation analysis of the existing problems in the air, dust and gas field, the airspeed, gas concentration and dust concentration at the side of air duct are higher than that at the backflow side. The gas is easily accumulated at the upper and lower corners of the backflow side of heading face and near the heading machine, which causes dust accumulation and more attention should be paid.

Taking Ningtiaota coal mine in Northern Shaanxi of China as an object for practical application, the airflow, dust and gas field in the fully mechanized heading face was optimized comprehensively. The optimized simulation results were compared with those of the original field. Then the various optimization adjustment schemes were obtained at the different distances from the duct outlet to heading face. The dust concentration at the at driver position was decreased by 35% and the gas concentration reduced by 54% after the optimization. When the duct outlet is at the distance of 5 m from the heading face, the caliber of outlet should be expanded to 1.2 m, which can rationally decrease airspeed at duct outlet. When the air outlet is at the distance of 9-10 m from the heading face, the outlet should be necked to 0.8 m to increase the effective range of airflow.

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ENHANCEMENT IN PLANT WATER RELATIONS AND FATTY ACID PROFILE IN SUNFLOWER (*HELIANTHUS ANNUUS* L.) THROUGH APPLICATION OF ABSCISIC ACID UNDER VARIED WATER LEVELS

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Abstract. Enhancement in yield and quality of sunflower by foliar spray of abscisic acid (ABA) under water deficit condition was studied during 2016 and 2017. Three irrigation treatments were applied. Four irrigations (at 4-6 leaf, at vegetative, at flowering and at grain formation stage), three irrigations (at 4-6 leaf, at pre flowering and at grain formation stage), three irrigations (at 4-6 leaf, at vegetative and at grain formation stage). Sunflower hybrid (S-278) was sprayed with different ABA concentrations (0, 10 µm and 20 µm) at 4-6 leaf, vegetative, flowering and grain formation stage. Foliar application of 10 µM ABA under water stress at vegetative stage significantly enhanced plant height, head diameter, achene yield and biological yield. It also enhanced sunflower water relations by increasing water potential. ABA application and water deficit showed opposite results for yield and oil quality. Water deficit at vegetative or at flowering stage maximized stearic and oleic acid contents and minimized palmitic and linoleic acid contents, whereas foliar spray of ABA under water stress at both stages reduced stearic and oleic acid but maximized palmitic and linoleic acid.
Keywords: *abscisic acid, relative water contents, turgor pressure, oleic acid, palmitic acid, linoleic acid, sunflower*

Introduction

Drought is the foremost yield-limiting feature for sunflower (*Helianthus annuus* L.) productivity in semi-arid areas (Rauf et al., 2015; Daryanto et al., 2016). In different parts of the world oil and achene yield losses due to drought stress were described (Woli et al., 2014; Yin et al., 2014). Water scarcity encourages significant alterations in physiological and biochemical processes implicated in biomass production, and alters dry matter partitioning (Fernández et al., 2012; Wu et al., 2017). Breeding for drought tolerance is thus indispensable to diminish yield losses in sunflower in drought-prone

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regions (Rauf et al., 2015). In order to recognize probable sources of drought tolerance, cultivated sunflower breeding lines from different sources were assessed on the basis of comparative performance under drought stress (Hussain et al., 2017). Nevertheless, the narrow genetic base of breeding lines was one of the major restrictions for the possibility of such studies.

Several studies showed, abiotic stresses activate several biochemical, physiological and molecular responses that effect several plant processes at cellular level (Wang et al., 2003; Hasanuzzaman et al., 2013; Tsironi and Taoukis, 2017). To fight several environmental pressures, dynamic methods and techniques should be developed (Yin et al., 2017). Among various control measures and monitoring tools (remote sensing) to assess the impacts of drought stress on crop plants (Villegas et al., 2017; Natsagdorj et al., 2017; Song et al., 2017), agronomic measures for water conservation and chemical plant growth regulators are of great interest (Ahmad et al., 2016). Phytohormone engineering could be considered as a preferable technique to increase the production. Phytohormones are the substantial regulators in plant growth and development and also intermediaries of environmental stress responses (Sreenivasulu et al., 2012). Among several phytohormones, abscisic acid is the vital regulator of abiotic stress resistance in plants and synchronizes an arrangement of roles (Finkelstein, 2013; Wani and Kumar, 2015), allowing plants to survive with diverse stresses. In the plant, the level of abscisic acid increases through abscisic acid biosynthesis when environmental conditions are harsh. The augmented abscisic acid binds to its receptor to start signal transduction causing cellular responses to different stresses (Ng et al., 2014); thus, ABA is also termed a stress hormone (Mehrotra et al., 2014). ABA was firstly suggested to be implicated in abscission and has later been revealed to play a role in plant growth and development, comprising cell division followed by elongation, embryo maturation, seed dormancy, germination, root growth, floral initiation, and responses to both biotic and abiotic stresses, such as osmotic stress, chilling, high salinity, drought, pathogen attack and UV radiation (Finkelstein, 2013; Yoshida et al., 2014; Sah et al., 2016). ABA is significantly increased under drought or salinity stress conditions, stimulating stomatal closure, change in gene expression, and adaptive physiological responses (Kim et al., 2010; Sah et al., 2016). Abscisic acid also plays a vital role in many processes at cell level including dormancy, seed development, vegetative growth, germination etc. (Finkelstein et al., 2013; Saradadevi et al., 2017) and variation in root morphology (Harris, 2015). Meanwhile the discovery of abscisic acid, numerous struggles have been dedicated to understanding how abscisic acid is produced under stress conditions. In stressful conditions like extreme temperature, drought, and high salinity, content in plants enhances significantly, stimulating stress-tolerance effects that support plants, acclimatize, and endure under these stressful conditions (Ng et al., 2014). Under non-stress situations, abscisic acid is also mandatory for plant overall growth and development.

Around the globe enormous research has been done on various crop plants to enhance water use efficiency (WUE) by sowing better varieties and improvement of drought tolerance by foliar application of abscisic acid (ABA). But little or no data is existing on the interaction of abscisic acid and use of diverse cultivars of *Helianthus annuus* to alleviate the impacts of water stress in different agro-ecological conditions of Pakistan. Therefore the current study has been planned to adjust ABA application stage for attaining greater yields of sunflower under water stress and to associate the performance of different *Helianthus annuus* hybrids under water stress environments.

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Materials and methods

Study area, soil and weather condition

Field experiments were conducted to study the response of spring planted sunflower hybrids to different irrigation schedules and foliar spray of abscisic acid. Trials were conducted in 2016 and 2017 at the Agronomic Research Farm, University of Agriculture, Peshawar, Pakistan. The climate of Peshawar region is semi-arid (34.01°N, 71.35°E) at an altitude of 350 meters above sea level. Peshawar is situated about 1600 km north of the Indian Ocean. All the analytical work associated to research was conducted in the Stress Physiology Laboratory, Nuclear Institute for Food and Agriculture, NIFA, Peshawar. Soil analysis of the experimental soil was given in *Table 1*. The climate of the area is semiarid where the mean annual rainfall is very low (300 to 500 mm), 60–70% rainfall occurs in summer, whereas the remaining 30–40% rainfall occurs in winter (*Fig. 1*).

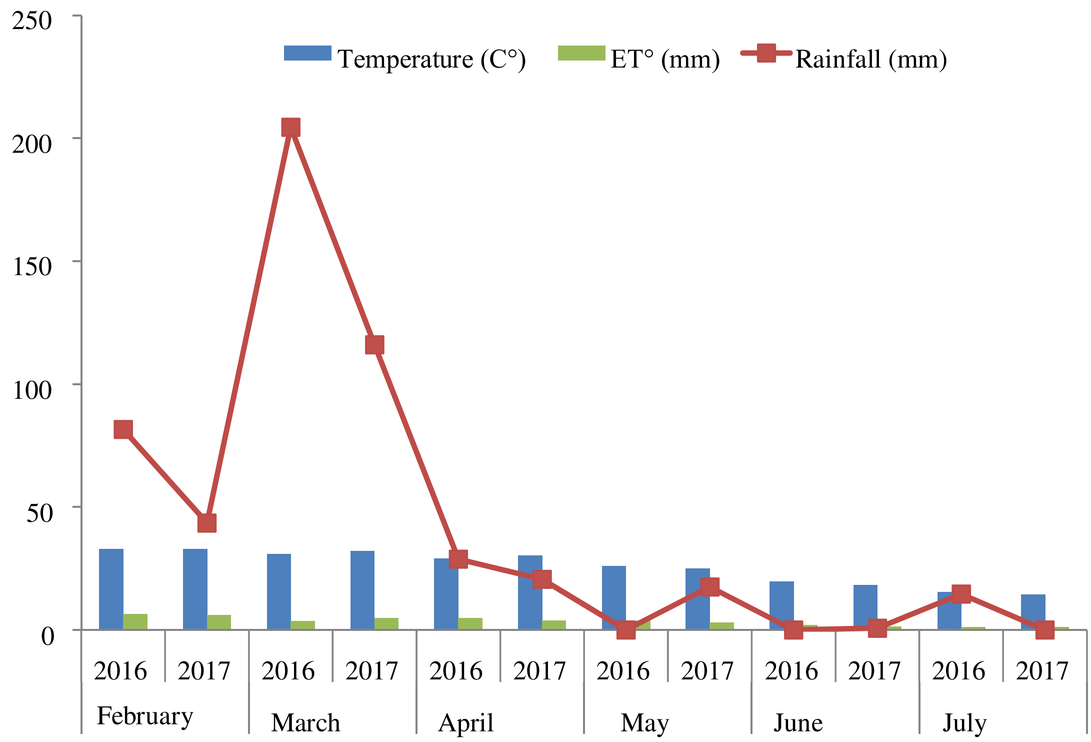

***Figure 1.** Monthly meteorological data for experimental years 2016 and 2017 of Agronomic Research Farm, University of Agriculture, Peshawar, Pakistan*

Experimental design and treatments

The layout of experiment was randomized complete block design (RCBD) with factorial arrangement with three replications. Net plot size was 3.0 m × 6.0 m. Three irrigation schedules (I) were imposed which were (I_1) four irrigations (at 4-6 leaf, at vegetative, at flowering and at grain formation stage), (I_2) three irrigations (at 4-6 leaf, at pre flowering and at grain formation stage), (I_3) three irrigations (at 4-6 leaf, at vegetative and at grain formation stage) and five ABA concentration (C_1: control, C_2: 10 µM ABA at vegetative stage; C_3: 10 µM ABA at flowering stage, C_4: 20 µM ABA at

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vegetative stage and C_5: 20 µM ABA at flowering stage. The first irrigation was applied when the plant had 4-6 leaves (25 DAS), the 2nd irrigation was applied at vegetative stage (45 DAS) excluding the plots, which were exposed to water deficit at this stage, the 3rd irrigation was applied at flowering stage (67 DAS) excluding the plots, which were exposed to water deficit at this stage. The 4th irrigation was given to all plots at grain formation stage (90 DAS). ABA was weighed according to per treatment and was added in a graduated cylinder and volume was made 1 L in volumetric flask with distilled water. Afterward Knapsack sprayer was calibrated (250 L ha^{-1}) and used to spray solution. While distilled water was sprayed on the control plots.

***Table 1.** Physicochemical analysis of soil during 2016-17*

Characteristic	Unit	Value	
		2016	**2017**
Sand	%	54	56
Silt	%	24.2	20
Clay	%	21.8	22
Textural class	-	Sandy clay loam	Sandy clay loam
Saturation percentage	%	32	30
Ec	dS/m	1.44	1.56
pH	-	7.6	7.9
Organic matter	%	0.71	0.68
Organic carbon	%	0.44	0.39
Total nitrogen	%	0.041	0.038
Available P	ppm	6.1	6.6
Available K	ppm	135	131

Crop husbandry

The seedbed was prepared by pre-soaking up to 10 cm irrigation then cultivating the field for 2-3 times with tractor-mounted cultivator. *Helianthus annuus* hybrids were sown on 19th and 13th of February 2016 and 2017, on ridges using dibbler with seed rate of 8 kg ha^{-1}. Ridges were made 75 cm distant and plant to plant distance of 25 cm was kept. Seed of sunflower hybrid S-278 were obtained from Syngenta Company. were obtained from Fertilizers were applied at the rate of 100 kg P_2O_5 ha^{-1} in the form of triple super phosphate (TSP) and 150 kg N in the form of urea. Half nitrogen and full phosphorus were applied at sowing, whereas remaining nitrogen was applied with 1st irrigation. Irrigation was done as per treatment by flooding. To keep field free from weeds field hoeing was done. Plant protection measures were conducted to keep crop free of insect pests, diseases and parrots. For control of whitefly and head rot Polo and Radomil Gold were applied, respectively. The crop was harvested on May 30, 2016 and June 6, 2017.

Measurements

Agronomic traits

From each plot, ten plants were selected randomly. The plant height was determined with measuring tape and then averaged. In order to find head diameter, 10 heads were

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randomly selected from each plot and their diameter was measured with the help of a measuring tape and then averaged. For calculation of achene yield plants were harvested at maturity, sunflower' heads were detached after drying then threshed manually to find the achene yield plot^{-1}. The moisture contents were calculated from random achene samples. The achene yield was attuned to 10% moisture content and expressed in kg ha^{-1}. Weight of air-dried plants (excluding achenes) was noted on plot basis and then converted into kg ha^{-1}. To compute total biological yield, noted weight was added to the already calculated achene yield (kg ha^{-1}).

Plant water relations

Water potential, relative leaf water content, turgor pressure and osmotic potential were recorded 10 days after the ABA foliar application. To find the relative leaf water content (RLWC), third leaf from the top (fully expanded) of two different plants from different treatment was taken. Leaves were cut at base of lamina and wrapped in plastic bags then transported to laboratory immediately. Fresh weight (FW) was calculated after two hours. After that turgid weight (TW) was found by saturating leaves in distilled water for time period of 16-18 h at room temperature. After saturation, leaves were prudently and rapidly blotted dry to find turgid weight. Dry weight (DW) was calculated after drying of leaf samples in oven for time period 72 h at 70 °C. Relative leaf water content (RLWC) was calculated through the procedure of Schonfeld et al., 1988 and then averaged (*Eq. 1*).

$$RLWC\ (\%) = (FW - DW)/(TW - DW) \times 100 \qquad \text{(Eq.1)}$$

where, FW= fresh weight of leaf; DW= dry weight of leaf; and TW = turgid weight of leaf.

Leaf water potential was ranged from 8.00 to 10.00 A.M. It was computed through Scholander pressure chamber by following the method of Scholander et al. (1965). In order to compute, water potential, leaf was kept in a freezer at -20 °C for 7 days. After that frozen leaf was defrosted in order to extract cell sap through a disposable syringe. Then osmotic potential was computed from extracted sap via an osmometer (Wescor 5500). Turgor pressure was calculated from the difference of water potential (Ψw) and osmotic potential (Ψs) (*Eq. 2*).

$$(\Psi p) = (\Psi w) - (\Psi s) \qquad \text{(Eq.2)}$$

Quality parameters

Oil content

Oil content in seeds was calculated using Soxhlet Fat Extraction method (AOAC, 1990). For about 10 h, seeds were dried in an oven at 105 °C. Seeds were weighed before and after drying for determination of moisture content. For oil content determination, two grams of achenes per thimble were crushed in a coffee mill. Thimbles were weighed individually, crushed seeds were added and the final weight was estimated. Later, the thimbles were placed in extractors. Six dry and clean round bottom 250 ml flasks were weighed and their weight noted. Solvent (petroleum ether) was added to flasks, attached to the extractors and placed on heating mantles attached to with condensers. Flasks were heated and extraction was continued for at least 6 h,

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stopped extraction, removed thimbles and then reheated the flasks, so that all of the solvent might be collected in the Soxhlet extractors. The apparatus permitted to cool and flasks dried at 105 °C for 1 h. After cooling, the flasks and oil were weighed together. Percent oil content was calculated via the following equation (*Eq. 3*).

$$Oil\ contents\ (\%) = \frac{(flask\ weight + oil\ weight) - flask\ weight}{(flask\ weight + seed\ weight) - flask\ weight} \times 100 \quad \text{(Eq.3)}$$

Fatty acid profile

Gas liquid chromatography was used to determine fatty acid composition in sunflower oil by following Martin (1979). Oil was extracted from seed of *Helianthus annuus* hybrids by using Rancy oil seed crusher. In a loop of oil, 0.5 ml petroleum and 1 ml methylating solution ether was added in test tube. Solution was swirled to disperse loop and petroleum ether was also added to rinse loop during sampling. After addition of 1 ml distilled water; it was kept for 10 min. One µl solution was taken from upper layer and injected into the gas chromatograph. The total peak area and area of each fatty acid peak was computed by an electronic integrator and expressed as percentage of the total area of the peak.

Statistical analysis

Collected data was analyzed by using Fisher's analysis of variance technique in MSTAT-C. The differences among treatments, means etc. was compared through least significant difference (LSD) test at 5% probability (Steel et al., 1997). Factorial experiment under Randomized Complete Block Design was used for analysis over years. Microsoft Excel Program was used for contrast study (Microsoft, version 2013).

Results

Response of agronomic traits of sunflower to irrigation schedules and ABA application

Effect of year on plant height was statistically found non-significant during 2016 and 2017 but taller plants were observed in 2017 over 2016 (*Table 2*). Irrigation levels and ABA application exhibited significant impact on plant height during 2016-17 while their interactions were found non-significant. Under no water stress, plant height was significantly enhanced. Water deficit significantly reduced plant height with a larger reduction in plant height when stress was enforced at vegetative over at flowering stage. Negative impact of water deficit at both stages might be mitigated by spraying of ABA. In different ABA concentrations and its application stages, 10 µM ABA spray under water deficit at vegetative or at flowering stage considerably maximized plant height (*Table 2*). Orthogonal contrasts showed that 4 irrigations produced taller plants over three irrigations; ABA applied at vegetative stage produced taller plants over when applied at flowering stage. The contrast between 10 µM and 20 µM ABA was non-significant (*Table 2*).

Effect of year on head diameter was statistically not-significant during 2016 and 2017 but larger head diameter was observed in 2017 over 2016. Head diameter was significantly influenced by irrigation schedules and foliar spray of ABA at diverse growth stages while interactive effect had non-significant impact during both years of

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study (*Table 2*). Water deficit reduced head diameter over no stress. Water stress imposed at flowering stage caused significant reduction in head diameter and this reduction was more prominent in those treatments where stress was imposed at vegetative stage. Negative impact of water stress at both stages might be declined by foliar spray of ABA (*Table 2*). Orthogonal contrasts showed that 4 irrigations vs. 3 irrigations, ABA application at vegetative stage vs. ABA application at flowering and 3 irrigations (miss at vegetative) vs. 3 irrigations (miss at flowering) were significant but orthogonal contrast 10 µM ABA vs. 20 µM ABA was statistically non-significant during 2016-17.

Table 2. *Agronomic traits of sunflower as affected by irrigation levels and ABA application*

Irrigation levels (I)	Plant height (cm)		Head diameter (cm)		Achene yield (kg ha^{-1})		Biological yield (kg ha^{-1})	
	2016	2017	2016	2017	2016	2017	2016	2017
I_1	190.99a	193.31a	16.87a	17.57a	2696.98a	2859.33a	12133.37a	12322.65a
I_2	170.71c	171.02c	12.60b	12.73b	2043.77b	2192.47b	11744.09b	11890.12b
I_3	180.90b	182.81b	10.41c	10.78c	1951.31c	2002.18c	11240.79c	11128.35c
LSD (0.05)	1.09	1.2	0.28	0.37	39.77	41.49	25.48	24.98
ABA levels and its application stages (C)								
C_1	182.78a	183.47a	13.93a	14.15a	2252.87b	2387.68a	12028.15a	11990.02c
C_2	180.60b	183.20a	13.90a	14.57a	2359.91a	2483.96a	11900.68b	12010.88b
C_3	183.49a	183.43a	13.89a	14.24a	2359.89a	2457.96a	12023.25a	12193.92a
C_4	178.04bc	179.61b	12.62b	12.94b	2111.47c	2259.34b	11289.99c	11467.38d
C_5	179.40b	181.11b	12.10c	12.56b	2069.23d	2169.17c	11288.34c	11239.66e
LSD (0.05)	1.41	1.55	0.37	0.48	51.34	53.63	32.89	32.25
Interaction	ns	ns	ns	ns	ns	ns	ns	ns

Mean values in column carrying different letters are statistically significant at 5% probability level. I_1: No stress (4 irrigations), I_2: Stress at vegetative stage (3 irrigations), I_3: Stress at flowering stage (3 irrigations), C_1: Control, C_2: 10 µM ABA at vegetative stage, C_3: 10 µM ABA at flowering stage, C_4: 20 µM ABA at vegetative stage, C_5: 20 µM ABA at flowering stage, ns: non-significant

Table 2a. *Orthogonal contrasts of agronomic traits*

Orthogonal contrasts	Plant height (cm)		Head diameter (cm)		Achene yield (kg ha^{-1})		Biological yield (kg ha^{-1})	
	2016	2017	2016	2017	2016	2017	2016	2017
4 irrigations vs. 3 irrigations	*	*	*	*	*	*	*	*
10 m ABA vs. 20 m ABA	ns	ns	ns	ns	ns	ns	ns	ns
ABA spray at vegetative vs. ABA spray at flowering stage	*	*	*	*	*	*	*	*
3 irrigations (missed at vegetative stage) vs. 3 irrigations (missed at flowering stage)	*	*	*	*	*	*	*	*

*: Significant, ns: non-significant

Effect of year was statistically significant for achene yield during both years and significantly maximum achene yield was observed in 2017 over 2016 (*Table 2*).

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Analysis of the data exhibited that achene yield was significantly influenced by irrigation levels and spray of ABA at diverse growth stages while interactive impact had not-significant impact during both years (*Table 2*). Water stress reduced achene yield over no stress. When drought was enforced at flowering stage achene yield was significantly reduced and this reduction was more over stress imposed at vegetative stage. Damaging impacts of water stress at both stages might be decreased by foliar ABA spray (*Table 2*). Orthogonal contrasts for 4 irrigations vs. 3 irrigations, ABA application at vegetative stage vs. ABA application at flowering stage and 3 irrigations (miss at vegetative) vs. 3 irrigations (miss at flowering) were significant but orthogonal contrast 10 µM ABA vs. 20 µM ABA was statistically non-significant during 2016-17.

Year impact on biological yield was statistically significant during 2016-17 and significantly maximum biological yield was observed in 2017 over 2016. Irrigation levels and ABA spray significantly affected biological yield while their interaction was found insignificant. Biological yield was significantly increased in control treatments (no water stress) over stress imposed at vegetative or flowering stage (*Table 2*). Under various irrigation schedules influence of ABA application was deviating (*Table 2*). When the crop encountered no water stress, foliar spray of 10 or 20 µM ABA at vegetative or flowering stage considerably reduced biological yield with respect to that of the control during 2016-17. Orthogonal contrasts between 4 irrigations vs. 3 irrigations, ABA application at vegetative vs. ABA application at flowering 3 irrigations (miss at vegetative) vs. 3 irrigations (miss at flowering) were significant for biological yield whereas orthogonal contrast 10 µM ABA vs. 20 µM ABA was statistically non-significant during both years.

Response of plant water relations to irrigation schedules and ABA application

Effect of year on leaf water potential was non-significant during 2016 and 2017 and leaf water potential was maximum in 2017 over 2016. Levels of irrigation and foliar ABA spray effect was prominent on leaf water potential during both years. Water deficit significantly reduced leaf water potential over control. Leaf water potential was significantly reduced when water stress imposed at flowering over vegetative stage. Mean values clearly showed that foliar ABA application significantly influenced the leaf water potential (*Table 3*). When crop encountered water deficit at vegetative stage, the application of 10 µM ABA significantly improved leaf water potential with respect to control but it was statistically comparable with 20 µM ABA spray at vegetative stage (*Table 3*). Orthogonal contrasts for 4 irrigations vs. 3 irrigations, ABA application at vegetative vs. ABA application at flowering and 3 irrigations (miss at vegetative stage) vs. 3 irrigations (miss at flowering) were significant but the contrast 10 µM ABA vs. 20 µM ABA was statistically non-significant during 2016-17.

Year impact on leaf osmotic potential was statistically significant during 2016-17 and significantly maximum leaf osmotic potential was observed in 2016 over 2017. Irrigation schedules and ABA application significantly influenced leaf osmotic potential while their interactions during both years were found insignificant. Water stress at both vegetative and flowering stage significantly reduced leaf osmotic potential over control. Leaf osmotic potential was reduced when water stress executed at flowering over vegetative stage. Mean values showed that foliar application of ABA significantly influenced the leaf osmotic potential. Under diverse irrigation schedules, performance of foliar application of ABA was divergent. When crop encountered no water stress at vegetative or flowering stage, foliar ABA spray reduced leaf osmotic potential during

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2016-17. Orthogonal contrast between 4 irrigations vs. 3 irrigations and 3 irrigations (miss at vegetative stage) vs. 3 irrigations (miss at flowering stage) for leaf osmotic potential were significant during 2016 and 2017 but orthogonal contrast for ABA application at vegetative stage vs. ABA application at flowering stage was non-significant in 2016 and statistically significant in 2017. Likewise contrast 10 µM ABA vs. 20 µM ABA was statistically non-significant during 2016-17.

Effect of year on leaf turgor pressure was statistically significant during 2016 and 2017 and statistically higher leaf turgor pressure was observed in 2017 over 2016. Leaf turgor pressure was significantly influenced by irrigation schedules and foliar application of ABA at various growth stages while their interaction had non-significant impact during 2016-17. Water deficit reduced leaf turgor pressure over no stress (*Table 3*). Turgor pressure was significantly reduced when water stress was enforced at flowering stage and this reduction was higher over when stress was enforced at vegetative stage (*Table 2*).

Application of 10 µM ABA under water deficit at vegetative stage improved leaf turgor pressure with respect to control. Water deficit at vegetative stage and application of 20 µM ABA enhanced turgor pressure than control and this enhancement was statistically comparable with 10 µM ABA application at vegetative stage. When crop confronted water deficit at vegetative stage but ABA (10 or 20 µM) was sprayed at flowering stage, it considerably reduced turgor pressure over control. Comparable observation was noted during 2016-17. Orthogonal contrasts between 4 irrigations vs. 3 irrigations, ABA application at vegetative stage vs. ABA application at flowering stage and 3 irrigations (miss at vegetative) vs. 3 irrigations (miss at flowering stage) were significant for turgor pressure but contrast 10 µM ABA vs. 20 µM ABA was statistically non-significant during both 2016-17 (*Table 3*).

Table 3. *Plant water relations of sunflower as affected by irrigation levels and ABA application*

Irrigation levels	**Water potential (-MPa)**		**Osmotic potential (-MPa)**		**Turgor potential (MPa)**		**Relative water content (%)**	
	2016	**2017**	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**
I_1	0.674a	0.681a	1.545a	1.561a	0.972a	0.881a	85.57a	86.36a
I_2	0.939b	0.949b	1.678b	1.696b	0.741b	0.748b	79.23b	79.76b
I_3	1.042c	1.053c	1.704c	1.723c	0.664c	0.671c	75.82c	76.81c
LSD (0.05)	0.015	0.015	0.019	0.021	0.11	0.014	0.28	0.43
ABA levels and its application stages								
C_1	0.829a	0.838a	1.608b	1.625b	0.779a	0.798a	80.33b	81.25a
C_2	0.831a	0.840a	1.596a	1.613a	0.766b	0.774b	80.92a	81.68a
C_3	0.847a	0.856a	1.626c	1.643c	0.780a	0.788a	80.45b	81.13a
C_4	0.965b	0.975b	1.694d	1.712d	0.726c	0.737c	79.99c	80.91ab
C_5	0.951b	0.961b	1.687d	1.705d	0.737c	0.744c	79.30d	80.19b
LSD (0.05)	0.019	0.019	0.012	0.012	0.014	0.014	0.36	0.563
Interaction	ns	ns	ns	ns	ns	ns	ns	ns

Mean values in column carrying different letters are statistically significant at 5% probability level. I_1: No stress (4 irrigations), I_2: Stress at vegetative stage (3 irrigations), I_3: Stress at flowering stage (3 irrigations), C_1: Control, C_2: 10 µM ABA at vegetative stage, C_3: 10 µM ABA at flowering stage, C_4: 20 µM ABA at vegetative stage, C_5: 20 µM ABA at flowering stage, ns: non-significant

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Table 3a. *Orthogonal contrasts of plant water relations*

Orthogonal contrasts	Water potential (-MPa)		Osmotic potential (-MPa)		Turgor potential (MPa)		Relative water content (%)	
	2016	2017	2016	2017	2016	2017	2016	2017
4 irrigations vs. 3 irrigations	*	*	*	*	*	*	*	*
10 m ABA vs. 20 m ABA	ns	ns	ns	ns	ns	ns	ns	ns
ABA spray at vegetative vs. ABA spray at flowering stage	*	*	ns	*	*	*	*	*
3 irrigations (missed at vegetative stage) vs. 3 irrigations (missed at flowering stage)	*	*	*	*	*	*	*	*

*: Significant, ns: non-significant

Effect of year on relative leaf water content was statistically not-significant during 2016-17 but relative leaf water content was slightly higher during 2017 over 2016. Analysis of the data revealed that relative leaf water content was significantly influenced by irrigation schedules and foliar application of ABA at diverse growth stages while their interaction had non-significant impact during both years (*Table 3*). Water deficit reduced relative leaf water content over no stress. When water deficit was imposed at flowering stage relative leaf water content was significantly reduced and this reduction was higher over where stress was enforced at vegetative stage. Negative impact of water deficit at both stages might be decreased by foliar spray of ABA (*Table 3*). Influence of foliarly applied ABA was different under diverse irrigation levels (*Table 3*). No water deficit at both growth stages, foliar application of ABA reduced relative leaf water content during 2016-17. Orthogonal contrasts between 4 irrigations vs. 3 irrigations, ABA application at vegetative stage vs. ABA application at flowering stage and 3 irrigations (miss at vegetative stage) vs. 3 irrigations (miss at flowering stage) were significant for relative leaf water content but orthogonal contrast for 10 µM ABA vs. 20 µM ABA was statistically no-significant during 2016-17.

Response of quality parameters of sunflower to irrigation schedules and ABA application

Effect of year on achene oil content was statistically non-significant but oil content was higher in 2017 with respect to 2016 (*Table 4*). Mean values exhibited that oil content was significant during irrigation treatments and foliar application of ABA at various growth stages while their interactions was found non-significant during both years (*Table 4*). Water stress reduced oil content compared to no stress. Oil content significantly reduced when water deficit was executed at flowering stage and this reduction was higher over when deficit was enforced at vegetative stage (*Table 4*). Under water deficit condition foliar spray of 10 µM ABA at vegetative stage improved oil content over control. Water deficit at vegetative stage and 20 µM ABA application also enhanced oil content than control but this enhancement was statistically comparable with 10 µM ABA application at vegetative stage. When crop confronted water deficit at vegetative stage but ABA (10 or 20 µM) was sprayed at flowering stage, it significantly reduced oil content than control. Comparable observation was observed during 2016-17.

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Impact of year on stearic acid content was statistically significant during 2016-17 and maximum stearic acid content was observed during 2016 over 2017. Analysis of the data revealed that irrigation levels and ABA application exhibited significant impact on stearic acid content during 2016-17. Water deficit significantly improved stearic acid content and maximum increment in stearic acid content was noted when water deficit was executed at flowering stage over at vegetative stage (*Table 4*). Foliar spray of 20 µM ABA at flowering stage by enforcing stress at vegetative stage significantly enhanced stearic acid content over all treatments. Whereas spraying 10 or 20 µM ABA at vegetative stage under water stress at vegetative stage reduced stearic acid content than control. Similar trend was noticed during 2016-17. Orthogonal contrasts between 4 irrigations vs. 3 irrigations and 3 irrigations (miss at vegetative stage) vs. 3 irrigations (miss at flowering stage) were significant for stearic acid content but orthogonal contrasts 10 µM ABA vs. 20 µM ABA, and ABA application at vegetative stage vs. ABA application at flowering stage were statistically non-significant during 2016-17.

Orthogonal contrasts for 4 irrigations vs. 3 irrigations, ABA application at vegetative stage vs. ABA application at flowering stage and 3 irrigations (miss at vegetative stage) vs. 3 irrigations (miss at flowering stage) were significant for oil content but orthogonal contrast 10 µM ABA vs. 20 µM ABA was statistically non-significant during 2016-17.

Effect of year on oleic acid content was statistically significant during 2016 and 2017 and statistically higher oleic acid was observed in 2016 over 2017. Oleic acid content was statistically influenced by irrigation levels and ABA spray while their interaction was found insignificant. Water stress significantly improved oleic acid content and maximum increment in oleic acid content was noted when water deficit was executed at vegetative stage over at flowering stage (*Table 4*). Water stress at vegetative stage and spraying of 20 µM ABA at flowering stage had significantly improved oleic acid content over all treatments. Enforcing water deficit at vegetative stage and spraying 10 µM ABA at flowering stage also significantly improved oleic acid content with respect to control but this increment was lower with respect to spraying of 20 µM ABA at the similar stage. Whereas spraying 10 or 20 µM ABA at vegetative stage under water deficit at vegetative stage reduced oleic acid content than control. Same trend was observed during 2016-17. Orthogonal contrasts for 4 irrigations vs. 3 irrigations was significant but orthogonal contrasts 10 µM ABA vs. 20 µM ABA and ABA application at vegetative stage vs. ABA application at flowering stage and 3 irrigations (miss at vegetative stage) vs. 3 irrigations (miss at flowering stage) were statistically non-significant during both years.

Palmitic acid content was significantly affected during 2016 and 2017 and statistically higher palmitic acid content was observed in 2017 over 2016. Different irrigation levels and foliar spray of ABA at diverse growth stages significantly affected palmitic acid content while their interaction was found insignificant. Water stress reduced palmitic acid content over no stress. Palmitic acid content was considerably minimized when water stress was enforced at flowering stage and this reduction was maximum than where water deficit was enforced at vegetative stage (*Table 4*). Spray of 10 µM ABA under water stress at vegetative stage statistically increased palmitic acid content over all treatments. Water stress at vegetative stage and application of 20 µM ABA also enhanced palmitic acid content than control but this enhancement was lower than spraying of 10 µM ABA at vegetative stage. When crop confronted water stress at vegetative stage but ABA (10 or 20 µM) was sprayed at flowering stage, it significantly reduced palmitic acid content over control. Same results was noted during both years.

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Orthogonal contrasts between 4 irrigations vs. 3 irrigations was significant for palmitic acid content but orthogonal contrasts 10 µM ABA vs. 20 µM ABA, 3 irrigations (miss at vegetative stage) vs. 3 irrigations (miss at flowering stage) and ABA application at vegetative vs. ABA application at flowering stage were statistically non-significant during 2016-17.

Table 4. *Oil content and fatty acid profile of sunflower as affected by irrigation levels and ABA application*

Irrigation levels (I)	Oil content (%)		Stearic acid (%)		Oleic acid (%)		Palmitic acid (%)		Linoleic acid (%)	
	2016	**2017**	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**	**2016**	**2017**
I_1	41.42a	41.61a	3.49c	3.40c	11.63c	11.52c	6.78a	6.83a	78.31a	78.37a
I_2	39.96b	40.34b	3.65b	3.55b	11.79a	11.68a	5.61b	5.769b	76.91b	76.85b
I_3	39.28c	39.46c	3.79a	3.66a	11.72b	11.67b	5.36c	5.50c	76.55c	76.65c
LSD (0.05)	0.23	0.26	0.081	0.07	0.01	0.01	0.012	0.014	0.013	0.015
ABA levels and its application stages (C)										
C_1	40.49a	40.88a	3.61c	3.51c	11.88c	11.61c	5.97a	6.06a	77.27b	77.26c
C_2	40.46a	40.70a	3.61c	3.47d	11.72b	11.65b	5.94b	6.04b	77.34a	77.45a
C_3	40.18b	40.37b	3.62c	3.52bc	11.63d	11.53d	5.97a	6.05ab	77.22d	77.30b
C_4	40.07b	40.25b	3.64b	3.53b	11.82a	11.68a	5.87c	5.96c	77.24c	77.20b
C_5	39.88b	40.13b	3.74a	3.62a	11.73b	11.65b	5.81d	5.92d	77.18e	77.23b
LSD (0.05)	0.3	0.34	0.02	0.01	0.01	0.012	0.013	0.012	0.011	0.01
Interaction	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Mean values in column carrying different letters are statistically significant at 5% probability level. I_1: No stress (4 irrigations), I_2: Stress at vegetative stage (3 irrigations), I_3: Stress at flowering stage (3 irrigations), C_1: Control, C_2: 10 µM ABA at vegetative stage, C_3: 10 µM ABA at flowering stage, C_4: 20 µM ABA at vegetative stage, C_5: 20 µM ABA at flowering stage, ns: non-significant

Table 4a. *Orthogonal contrasts of oil content and fatty acid profile of sunflower*

Orthogonal contrasts	Oil content (%)		Stearic acid (%)		Oleic acid (%)		Palmitic acid (%)		Linoleic acid (%)	
	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
4 irrigations vs. 3 irrigations	*	*	*	*	*	*	*	*	*	*
10 m ABA vs. 20 m ABA	ns	ns	ns	ns	ns	ns	ns	ns	ns	Ns
ABA spray at vegetative vs. ABA spray at flowering stage	*	*	ns	ns	ns	ns	ns	ns	ns	Ns
3 irrigations (missed at vegetative stage) vs. 3 irrigations (missed at flowering stage)	*	*	*	*	ns	ns	ns	ns	ns	Ns

*: Significant, ns: non-significant

Effect of year on linoleic acid content was significant and statistically higher linoleic acid was observed in 2017 over 2016. Linoleic acid content was significantly influenced by irrigation levels and foliar spray of ABA at various growth stages while their interaction was found non-significant effect on linoleic acid content. Water stress

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reduced linoleic acid content over no stress. Considerably lesser linoleic acid was noted when water stress was enforced at flowering stage than where water deficit was enforced at vegetative stage (*Table 4*). Spray of 10 µM ABA under water stress at vegetative stage statistically augmented linoleic acid content over control. Water stress at vegetative stage and application of 20 µM ABA also enhanced linoleic acid content than control but this enhancement was lower than spraying of 10 µM ABA at vegetative stage. When crop confronted water deficit at vegetative stage but ABA (10 or 20 µM) was sprayed at flowering stage, it considerably reduced linoleic acid content over control. Same observation was recorded during 2016-17. Orthogonal contrasts for 4 irrigations vs. 3 irrigations was significant for linoleic acid content but orthogonal contrasts 10 µM ABA vs. 20 µM ABA and ABA application at vegetative stage vs. ABA application at flowering stage and 3 irrigations (miss at vegetative) vs. 3 irrigations (miss at flowering stage) were statistically non-significant during both years.

Discussion

Agronomic and yield related traits

Water stress is one of the severe threats to crop production in semi-arid and arid areas of Pakistan. In present trial, foliar spraying of abscisic acid d to sunflower under water deficit either at vegetative or flowering stage has been noticed to considerably enhanced crop growth, yield, osmotic adjustment, development, water relations, achene quality and fatty acid profile over no spray of ABA under water stress.

Water deficit both at vegetative or flowering stage declined sunflower plant height and maximum decrease in plant height was recorded when water deficit happened at bud initiation (Unger, 1983). Exogenous ABA application under no water stress condition reduced plant height. Under no water stress condition, shoot growth reduced by ABA application in maize seedling (Zhang et al., 2012) which finally reduced plant height. ABA foliar application under water stress enhanced plant height since it perform as shoot growth regulator under water scarce environments (Zhang et al., 2012). In the current trial less concentration (10 µM ABA) supported vegetative growth under water stress whereas the greater concentration (20 µM ABA) could have facilitated in survival of plant by regulating processes like plant size growth and stomatal opening (Sah et al., 2016). Controversially foliar spray of ABA under water stress and no stress condition decreased height of shoot in *Populus davidiana* which was owed to lesser accumulation of dry matter and its maximum distribution to *Populus davidiana* roots (Chunyang et al., 2004).

Water stress significantly reduced head diameter of sunflower and maximum reduction in head diameter happened when crop encountered water deficit at flowering over vegetative stage (Hussain et al., 2015, 2017). This decrease was due to reduction in photosynthates production (Dong et al., 2017; Mila et al., 2017) and their minimum distribution to the floral parts (Dong et al., 2017). Spraying of 10 µM ABA under water deficit at flowering stage considerably enhanced head diameter because abscisic acid useful in water maintenance response of plants and may have enhanced plant growth through improvement in water use efficiency (WUE) (Wei et al., 2015; Dong et al., 2017).

For better production of crops, normal irrigations are essential but under water scarcity situation, it is significant to recognize important growth stage where irrigation can be missed without significantly reducing crop production (Wang et al. 2017). Water

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deficit both at vegetative or flower initiation stage reduced achene yield along with its attributes in Helianthus (Hussain et al., 2014, 2015). Harsh water scarcity effects were more apparent at flower initiation stage over at vegetative stage (Hussain et al., 2015). ABA foliar application during normal irrigation also considerably declined the yield, as it only enhanced yield during water deficit both at vegetative or flower initiation stage. From tolerance to dehydration, the plant hormone ABA played imperative (Wani et al., 2016) by helping dispersion of roots of maize in soil (Zhang et al., 2012) which finally augmented production of assimilates and yield through enhancing osmotic adjustment and water relation in plants like sunflower (Hussain et al., 2014, 2015; Wani et al., 2016; Dong et al., 2017). Abscisic acid sprayed under water deficit altered the sorghum physiology and growth and its application on seed enhanced grain yield (Traore and Sullivan, 1990). Contrary Ayub et al. (2000) described that foliar spray of abscisic acid during water stress to mung bean exhibited non-significant response to grain yield.

In current study biological yield of Helianthus was maximally reduced when water stress was enforced at vegetative over at flowering stage. Foliar application of 10 or 20 µM ABA under water stress at vegetative stage enhanced biomass but maximum increment in biomass was observed when 10 µM abscisic acid sprayed during this stage. This enhancement in biomass was due to decrease in shoot' water loss, roots deeper dispersion and increment in WUE which eventually augmented production of crop. Same findings were reported in Helianthus (Zhang et al., 2012; Hussain et al., 2015; Dong et al., 2017) and maize (Hartung et al., 1994). Contradictory ABA foliar application under water deficit and well-watered condition decreased biomass production in *Populus* which is owed to lesser dry matter accumulation and maximum distribution to roots of plant (Chunyang et al., 2004) ultimately may reduce above ground vegetation.

Plant water relations

Results of current trial showed that water potential became more adverse under water scarcity both at vegetative or at flowering stage and the exogenous application of abscisic acid considerably averted the negative impact of water stress by making the water potential in sunflower plants less negative. When ABA sprayed at vegetative stage over at flowering stage water potential became less negative. This enhancement in water potential indicate improvement of water shortage tolerance in helianthus by foliar application of abscisic acid, which eventually retained plant moisture through restricted and steady stomatal closure and by increasing root penetration and lastly help in plant survival through dehydrin protein production under cellular desiccation. Alfredo and Setter (2000) described that Cassava has capacity of partial stomatal closure under water stress due to abscisic acid accumulation in vegetative parts specifically in leaves which decreased leaf development and lastly continuation of growth happened after watering the crop again. Foliar application of abscisic acid responds to water stress condition by storing dehydrin protein in sunflower vegetative tissues (Aguado et al., 2014), poplar and wheat (Pelah et al., 1997). Dehydrin protein saves plants from cellular desiccation (Baker et al., 1988; Dure et al., 1989; Close, 1996). Jones and Corlett (1992) noticed contrary plant response as plant metabolic processes were more prone to cell size and turgor pressure compared to absolute water potential.

Water deficit both at vegetative or flowering stage depressed the water potential which also triggered a comparable reduction in osmotic potential. Ultimate decrease in osmotic potential was result of solutes accumulation in various plant parts through

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osmotic adjustments (Serraj and Sinclair, 2002; Hussain et al., 2015). ABA foliar application to helianthus caused osmotic potential less adverse. When ABA was sprayed at vegetative stage over at flowering stage, osmotic potential became less negative. This enhancement in osmotic potential was the result of compatible solutes accumulation like glycinebetaine (Serraj and Sinclair, 2002), proline (Hussain et al., 2015) and total soluble sugars. Such compounds are supportive in delay of desiccation through ROS detoxification, maintenance of membranes in addition to enzymes arrangement (Ludlow and Muchow, 1990; Subbarao et al., 2000). Hussain et al. (2015) exhibited that under water stress condition, key physiological mechanism was decrease in osmotic potential to keep leaf turgor pressure. Contrarily ABA foliar application may increment the action of definite enzymes such as ribonuclease and α-amylase. These enzymes may be involved in breakdown of starches plus other related materials thus making osmotic potential more adverse (Dong et al., 2017; Mila et al., 2017).

Water stress at vegetative or flower initiation stage considerably decreased turgor pressure of leaf. Decrease in turgor pressure of leaf was owed to reduction in water potential and already reported in wheat (Blum, 1997) and Helianthus (Hussain et al., 2015). ABA foliar application during water stress considerably enhanced leaf turgor pressure which was maximum at vegetative over at flower initiation stage. This enhancement in leaf turgor potential was result of plant efforts to maintain moisture. These types of findings were also reported in maize (Zhang et al., 2012) and in poplar (Pelah et al., 1997).

Results of the current study exposed that water stress at vegetative or flowering stage severely decreased sunflower relative water content of leaf. Decline in relative leaf water content in Helianthus was also noticed by Hussain et al. (2015). Further reduction in relative water content of leaf was noticed when water deficit imposed at vegetative over flower initiation stage. ABA foliar application considerably improved relative leaf water content under water deficit situations. These enhancements in relative water content of leaf was owed to plant moisture preservation through limited and steady stomata closure, root proliferation for water abstraction and dehydrin protein buildup which ultimately rescued plant from cellular desiccation. The same result was noticed in poplar, wheat and cassava (Pelah et al., 1997; Alfredo and Setter, 2000; Aguado et al., 2014). Contrary to this, ABA foliar application to maize during water stress did not increment relative water content of leaf (Unyayar et al., 2004).

Oil contents and fatty acid profile

Water stress both at vegetative and flower initiation stage decreased achene oil content. Oil content was observed to increase when water deficit was applied at flower initiation stage over vegetative stage. Water deficit applied prior to reproductive stage (Saleem et al., 2013), at flower initiation stage (Hammadeh et al., 2005) and for the duration of seed filling stage (Mekki et al., 1999; Hussain et al., 2015) of Helianthus declined oil content. In contrast the seed oil content exhibited constancy during incrementing water scarcity (Khan et al., 2000). Hussain et al. (2015) also reported no significant reduction in oil content under water deficit. ABA spraying under water deficit at vegetative or at flower initiation stage statistically enhanced oil content. Seed oil content may be improved by abscisic acid foliar spray because its spray result in limited stomata closure, reduced transpiration rate, repressed shoot development and improved root proliferations (Alfredo and Setter, 2000; Saleem et al., 2013) which eventually improved water accessibility for oil production in achene yield.

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Water deficit and ABA foliar application under water deficit or well-watered conditions influenced fatty acid profile. Results of present trial emphasized that oleic acid and stearic acid reduced whereas linoleic acid and palmitic acid improved during well-watered and ABA foliar application improved oleic acid and stearic acid whereas reduced linoleic acid and palmitic acid. Water deficit at vegetative and at flower initiation stage slightly improved oleic acid and stearic acid whereas linoleic acid and palmitic acid reduced. Further increment was noticed in stearic acid when water deficit imposed at flower initiation stage whereas same response was noticed in oleic acid when sunflower encountered water deficit at vegetative stage. Linoleic acid and Palmitic acid were reduced under water shortage situation imposed at vegetative or at flower initiation stage but further decrease in both fatty acids was noted in crop that confronted water deficit at flower initiation stage. Flagella et al. (2002) also described reduction in oleic acid and stearic acid and increment in linoleic acid and palmitic acid in Helianthus under irrigation. Contrary, in Helianthus, oleic acid decreased and palmitic acid improved under water shortages (Saleem et al., 2013; Hussain et al., 2015). ABA foliar application both at vegetative and at flower initiation stage under water shortage somewhat reduced oleic acid and stearic acid, whereas its application to some extent enhanced palmitic acid and linoleic acid. This decrease in stearic and oleic acid and enhancement in linoleic and palmitic acid revealed that abscisic acid was useful in extenuating the negative impacts of water stress through enhancing water availability to plants. Water availability may be enhanced through maintaining moisture content in plant by restricted stomatal closure, decrease in transpiration rate, increment in root proliferation and inhibiting shoot growth (Alfredo and Setter, 2000; Hoad et al., 2001; Saleem et al., 2013; Hussain et al., 2015, 2017; Dong et al., 2017;).

Conclusion

It is concluded from this experiment that foliar spray of 10 µM ABA under water sacristy at vegetative stage significantly enhanced plant height, head diameter, achene yield and biological yield with respect to no abscisic acid. It also enhanced water relations by enhancing turgor pressure and water potential which specifies that ABA foliar application was useful in improving sunflower drought tolerance.

Foliar application of 10 µM abscisic acid at flowering stage by enforcing water stress at this stage also enhanced plant height, head diameter, achene yield and biological yield as compared to no ABA but this increment was lower than with the 10 µM ABA foliar application at vegetative stage after enforcement of water deficit at this stage.

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HISTORICAL CHANGES AND VEGETATION DEVELOPMENT AFTER INTENSIVE PEAT EXTRACTION IN THE LOWLAND MIRES OF SLOVAKIA

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Abstract. Mires in the Danubian Lowland (Northern part of the Pannonian Basin) are typically overgrown with common alder (*Alnus glutinosa*). Similar stands at this location are unfortunately very rare, what is caused by continuous human intervention in landscape and its natural pattern from Early Holocene. Peatland degradation near Pusté Úľany village started in the mid - eighteenth century, what is supported by historical maps. In the previous century a huge peat extraction took place at this specific mire. Despite these anthropogenic changes, there are still some remnants of natural habitats present, mostly dependant on ground water level. Methodology includes historical map's analysis, digital terrain model and vegetation cover mapping. Actual map of biotopes was completed for the current state of the vegetation cover acquaintance. Historical map's analysis revealed changes and development in the vegetation cover as well as the further relevant landscape changes in the mire during drainage system building. The first artificial drainage channel was built in the second half of the eighteenth century. Subsequently, dense system of the drainage channels was built near Továrniky settlement, continuing the drainage of this area for meadows, pastures and arable land acquisition. This wetland habitat was probably one of the largest mires in Slovakia during Holocene. Its area used to be markedly larger and its sedimentary basin presented much more important influence on landscape development. However, the historical sources revealed higher occurrence of common alder in the past, as well as typical xerothermic oak forest in this area. 27 biotopes were identified, from which 13 biotopes was purely human induced. Most of the area is currently abandoned for its own vegetation succession.
Keywords: *biotope mapping, landuse, DTM, Pusté Úľany, Danubian lowland*

Introduction

Historical settlements on the Danubian Lowland, dated to Neolithic, means rather early human interference into natural vegetation cover in this area (Jamrichová et al., 2017; Opršal et al., 2016). Peatland and lowland mire prospection is a very important topic in the context of vegetation development research and climatic changes during Holocene (Petr et al., 2013; Szabó et al., 2016). Lowland mires present an important image about vegetation development in regional context as well (Petr et al., 2013). The important element in interpretation of vegetation development is sedimentary basin area of the mire (Sugita, 2007). Relevant factor in interpretation of vegetation development

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and landscape change is also human interventions and its influence on natural habitats. Archaeological records about Danubian lowland extends back to 7 500–7 600 cal years BP. (Jamrichová et al., 2017). Historical maps offer the cover of recent history, mostly to seventeenth or eighteenth century, however more precise and quantifiable (Pišút et al., 2007; Hrnčiarová et al., 2016). Changes of water-bounded communities reach and natural succesion process is visible precisely on the historical maps. Peatlands and mires were subject to severe mining for various purposes. Some of these areas were subject to permanent protection as protection areas. Unfortunately, as mire near village Pusté Úľany was too large in its area, there were many efforts to drain this area and use it for agriculture. Similar large areas of peat accumulation in Danubian lowland are for example Šúr in the north of the Danubian lowland near Little Carpathians Mts. (Petr et al., 2013) or Parížske močiare wetland mires in the southeast of Danubian lowland (Jamrichová et al., 2014, 2017), or Cerová-Lieskové on the Záhorská nížina lowland (East Wienna basin) (Kvaček et al., 2014).

In 1950s an intensive peat extraction took place in mire near Pusté Úľany. Drainage canal systems are visible in landscape in the surrounding area as a part of protected bird area (Protected bird Area of Úľany wetland, which is one of the 38 bird areas in Slovakia in the Natura 2000 network. The problem about this area is “no monitoring” (Kopecká, 2011, 2013). After peat extraction, degraded landscape with large ponds remained left for new vegetation succession (Kopecká, 2011, 2013; Hreško and Guldanová, 2012; Bubíková et al., 2016). So the aim of this article is to answer several inquiries about this area:

- What was the original area of the water-bounded communities of the mire near Pusté Úľany?
- How was this area changing on the historical maps?
- In what manner human intervention was part of this changes and what is the actual condition of these biotopes now?

Materials and methods

Research area

Pusté Úľany village is located in the west part of Slovakia between towns of Senec and Galanta. Mire is located between the villages Pusté Úľany and Veľký Grob (*Fig. 1*). Currently the remains of the mire are visible mostly from orthophotomaps as peat extraction depressions. This mire is however tectonic interface between slightly curved Upland of Trnava loessic plain and Danubian plain (Mazúr and Lukniš, 1978). Geomorphologically it is a subassembly of Trnavsko-dubnická panva plain and Blatnianska priehlbina depression (INGEP, 2007; Maglay, 2005). In the residues of this Trnava looesic plain there are several depressions and depression valleys present including mire depression near Pusté Úľany (INGEP, 2007; Maglay, 2005).

Geologically is the origin of the depressions scheme fixed to the complex of Trnava Upland, which is leaning towards south-east, where underground water is reaching the topsoil and produced organic sediments (peat). They are bounded to the local depressions with mires such as mire near Pusté Úľany or Šúr near Little Carpathians Mts. (Maglay, 2005). Research area is restricted to the boundary connecting loose sediments and dominating alluvium. Mentioned alluvium is formed by grey and bright clays, silt, sand and gravels. Gravel sediments of the lower fluvial terraces of Váh River

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are located as well near Pusté Úľany (Maglay, 2005). Research area is a depression part with gently sloping tectonic subsidence. Tectonic faults are usually directed NE–SW. Hydrogeologically it is district QN 050, named Quaternary of the Trnava Upland as a part of looesic sediments on fluvial terraces (Hrašna, 2002).

Nowadays, the hydrological surrounding is formed by Stoličný stream basin flowing into the Čierna voda river. Stoličný stream is 38.9 km long and it is flowing from the Little Carpathians Mts. Near wetland area in the Uľanská mokraď it is crossed by Čatajský stream near Veľký Grob village.

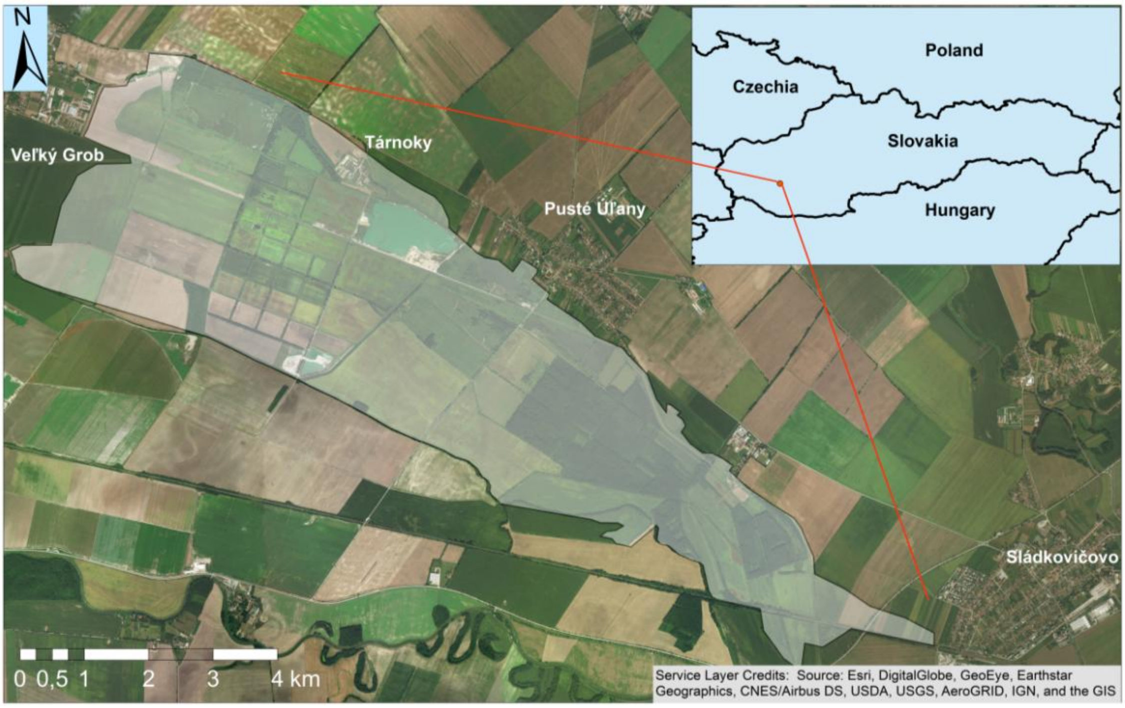

Figure 1. *Current area of water-bounded communities reach in the mire near Pusté Úľany*

Secondary landcover structure reflects the changes of the last century, mostly intensification in landuse with typical deforestation for crops, which after 1. and 2. world wars started to be cultivated with automated machines and it formed the highest area of use in 1950s (Kopecká, 2011). Soil substrate is characteristic by the very fertile soil types like chernozem or fluvic mollisols, so the cultivated land is in the direct proximity of the mire. Area of mire that is too waterlogging is not suitable for cultivation and that is why even more intensive draining of this area took place in the past (Hreško and Guldanová, 2012). In 1950s the intensification was finalised by peat extraction (Krippel, 1956), which degraded the landscape of this mire and large water areas – peat extraction lakes were formed with accumulated water.

Methodology

DEM

Digital elevation model was obtained from National Institute of Forestry (Zvolen). Measuring took place with terrestrial laser scanner (TLS) with 50 cm cell size of raster resolution. Outputs were processed in AcrGIS environment, version 10.3.1.

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Historical maps

Series of historical maps were analysed and vectorised in ArcGIS environment (version 10.3.1.) and on its basis the spatial differentiation of landuse was reconstructed in several time horizons. Also original area of the mire was determined. Maps of greater and middle scales from the eighteenth and nineteenth century were used, including military surveys (1.m.m. in 1782-1785 and 2.m.m. in 1839) and map based on cadastral maps in the end of nineteenth century. Separated cartographic sources are cited in the list in the end of this article. In text interpretation we used map portal (Topographical Institute in Zvolen) – aerial scanning orthophotomaps in 1949 (http://mapy.tuzvo.sk/HOFM/). Actual state of the landcover was evaluated with orthophotomap in basemap 2018 (©ArcGIS).

After transformation of the historical maps to currently used S-JTSK coordination system, selection of the maps was used. Work with map of „*Vestigium operationis*...(number 1 in the list of cartographic sources cited in the end of this article in bibliography) was possible to rectify only with points of village establishments (churches) as main orientation points or for example Esterházy palace near town Senec. Anyway the mean error is too excessive. Map *Comitatus Posoniensis*…., published in 1757, is of better precision. However, the error of rectification was still notable (*Table 1*). Hence these too maps were visualized in text only as cut out section figures from the original maps.

The first and the second military surveys was available in better form already rectified on mapportal mapire (http://mapire.eu/en/), so it was easier to rectify map sheets already obtained from Hungarian Library (Hungaricana – Hungarian Cultural Heritage Portal) in digital form.

Table 1. *Residual mistake of rectification (first polynomial transformation) in georeferenced historical maps (years 1757 and 1760)*

Year	Name	Number of maps	Mean error of transformation	Number of control points for transformation	Type of transformation
1757	Map of Bratislava coun ty *(Comitatus Posoniensis...) [S 68 / X. / No. 94.]*	1 cut out section	1345.54	11	First order polynomial
1760	*Csádé* wetland mapping in Bratislava district	Whole map	133.242	5	First order polynomial
1782-1785	1. Military Survey	2 sheets	182.642	11	First order polynomial
1839	2. Military Survey	3 map sheets	50.750	9	First order polynomial
1890-1897	Cadastral maps of Bratislava district *(Pozsony megyei)* [001-091]	4 map sheets [56, 57, 64, 65]	67.088	17	First order polynomial
1949	Orthophotomap of Slovakia in 1949	1 cut out section	23.569	6	First order polynomial

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Biotope mapping

Actual state of biotopes was reviewed directly in ArcGIS environment (version 3.5.1.) using basemap. Standard methodology for biotopes mapping in Slovakia was used by Ružičková et al. (1996) by or photomaps interpretation and terrain confirmation. At first a preparation phase took place, obtaining necessary sources about research area forming the initial list of legend items. Boundaries of individual polygons were mapped from orthophotomaps and corrected during field work.

Boundary of the mire was depicted with consideration to the current area of depending biotopes with a ground water as the main factor of its origin. Since the biotopes mapping methodology in Slovakia came through some development in recent years and it has several specifics adapted to our landscape (different type of biotopes with range from lowlands to highlands), we decided to use the older methodology of biotopes mapping (Ružičková et al., 1996), as it differentiates the anthropogenic biotopes on the very detailed level that were necessarily needed. Appertaining English name was referred to the original Slovak names and codes of the biotopes. Finally, corresponding codes of biotopes for new methodology of mapping was attached to the table as well as NATURA 2000 code equivalent if available.

Results

Notes to the georelief topography

Mire near Pusté Úľany is a part of the Úľany depression. Digital elevation model (DEM) displays the Úľany depression and shows the higher position of adjacent Trnava Upland (about 15 m higher) in the NE position of the allocated research area (*Fig. 2*).

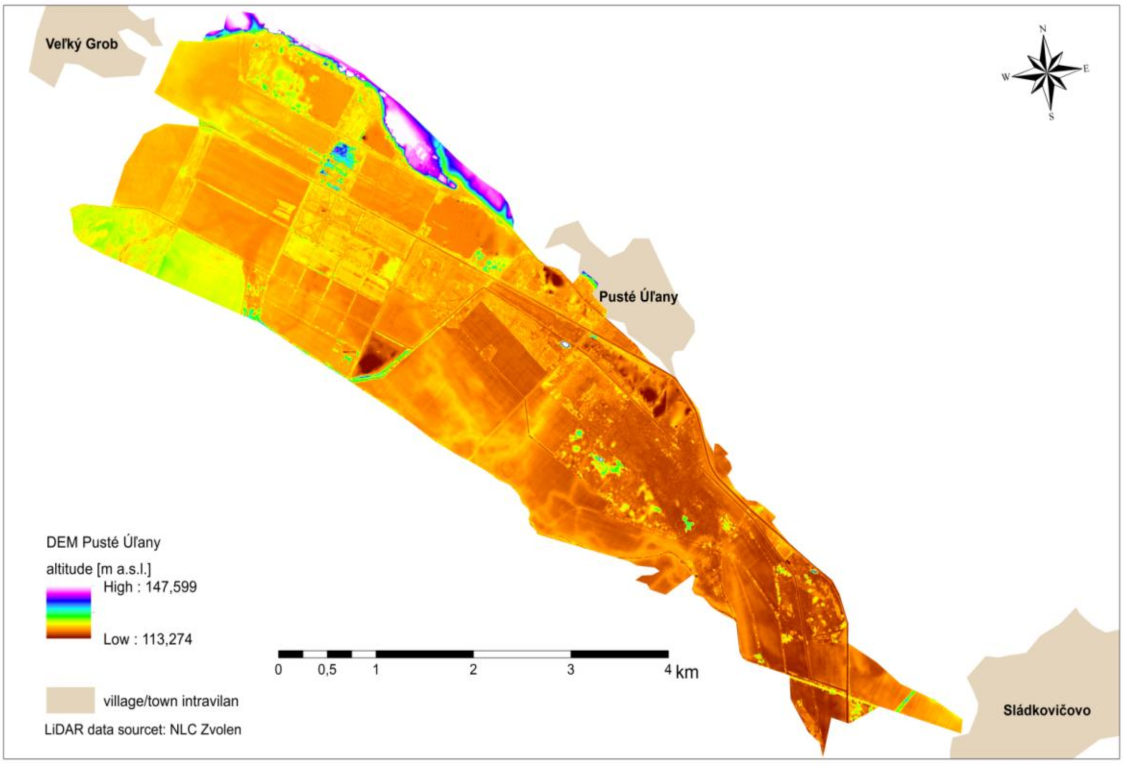

***Figure 2.** Digital elevation model of the wetland area near Pusté Úľany*

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Depression is descending in SE direction along the Pusté Úľany village continuing down to the south. The vertical segmentation of the terrain is 34.3 m. Sedimentary basin was probably much larger than today, however the remains of the depression influenced by ground water is concentrated in the part close to the boundary of the tectonic slit between the Upland and the lowland. Generally, the highest parts of the area over 135 m a.s.l. are located in the northern part and the lowest parts below 120 m a.s.l. are concentrated in the south of the research area. NE part of the area on *Figure 2* is showing the edge of Trnava Upland part. The actual mire is located in the elevation between 128 m a.s.l. and 114 m a.s.l. The bottom parts of the extraction lakes (116–113 m a.s.l.) are ascending on the colour spectrum as a result of peat extraction and consequential terrain subsidence. Gravel extraction is still in progress in the area. Some of the appeared upper parts of the DEM are caused by anthropogenic structures and others by vegetation cover, therefore these were further investigated directly during field research. This area represents the humid alluvial depression between merging two river flows: Stoličný stream around, which this wetland area is formed now, discharges into the Čierna voda River. This area between two water flows is contributing to the humidity storage.

Historical maps and literature sources

First detailed information about the mire near Pusté Úľany came from Matthias Bel and his work *Notitia…* (Bel, 1736, Volume 2, Section VI.) which contains a detailed descriptions of villages located in administrative region of the Outer Bratislava District (*Processus Superior Externus*). Significant is a passage about Pusté Úľany village where it stands that to the south of the village there is an area suffering from frequent floods. "*There is a low-lying ground so boggy, that it is not able to hold even people, nor cattle on the ground, that are diving into it like into the swirls. This wetland is locally named Csádék. But after all, it brings some benefits to the local people as well, as it grows alders, suitable for extraction*" (page 189). Important information about the mire are as well in description of Blatné village: „*From this point* (= i. e. Červený vrch Hill) *a large wetland plain starts to extend with numerous lakes between the villages Čataj, Nemecki Grob, Senec, Kráľová, Réca, Boldogfalva and Pusté Úľany, or even further. In the favourable years it is typical with rich grasslands, but if the weather is too rainy it is easily covering in mud. From* (this swamp) *some people also derive the name of the village Blatné* (*Sárfö* in Hungarian; *sár* means mud, therefore *Sárfö means "head of a swamp*"*).* Matthias Bel also mentions the possible benefits of regulation of the stream Sisak (*Sziszak*) and its conversion from wetland to meadows (Bel, l.c., page 195 and 196).

The first volume of the work of Mathias Bel (1735) also contains the first and in this time very geometrically precise map, covering the area of the mire. It is the **map of Bratislava County** authored by Samuel Mikovíni in 1735 (number 2 in the maps citation list). For the aim of our work, its easily available Homann´s edition from 1757 (number 3 in the maps citation list) was used. Map of Mikovíni as a cartographic source significantly integrates the text information from M. Bel. It also directly relates to another, somewhat older Mikovíni map ***Vestigium Operationis Astronomico-Geometricae*** (number 1 in the maps citation list), which is probably the oldest and relatively accurate projection of the mire (*Fig. 3*). This map was originally part of a letter, where Mikovíni drafted the principles of his method of the maps compliance (Purgina, 1958). According to these maps the waterlogged area and/or wet meadows

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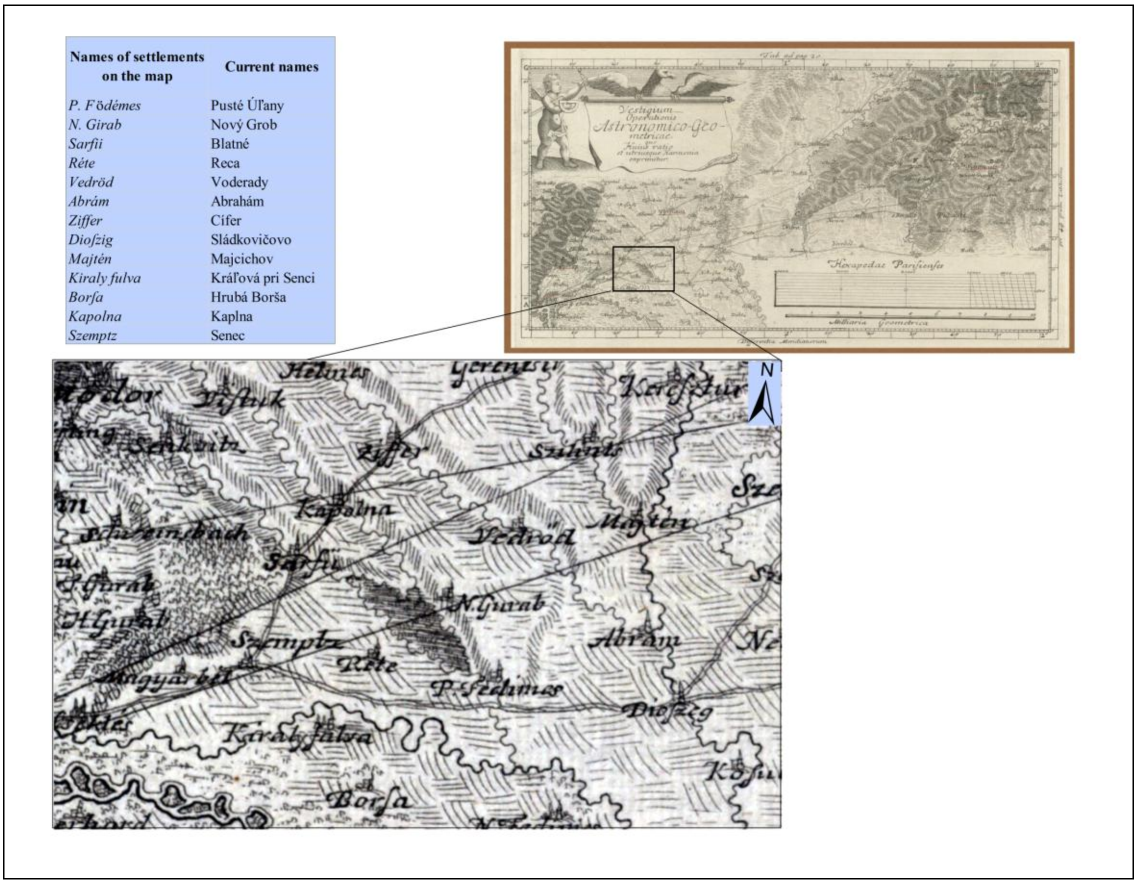
stretched from the Veľké Úľany village neighbouring the Pusté Úľany village continuing to the Čataj and Blatné, while from the western part it was protruding to the Boldogfalva. In general, it covered much larger area as it is nowadays. It is interesting that this wetland area was the very factor of connection for currently two separate settlements – villages Veľké Úľany and Pusté Úľany. Originally both were a part of one larger settlement during the Middle Ages, its inhabitants first mentioned as "*apiary*" (meaning beekeepers), later in 1221 as "*Fudemus*" in Hungarian language. The whole area reaching also the disappeared village of Dudvágszeg was later divided between several owners (Sedlák, 1994). Even map of Mikovíni (1735) has an interesting detail marked near Pusté Úľany village – map sign for the lonely standing church and farmstead (*Tárnok*). It was a remnant of the additional disappeared medieval village of *Tovarníky, that existed in thirteenth century (mentioned in 1301 as a nobleman´s settlement). The assumption that the villagers were dealing with trade is also supported by the patronicium of the local church: St. Jacob was the patron of traders (Sedlák, 1994).



Names of settlements on the map	Current names
P. Födémes	Pusté Úľany
N. Girab	Nový Grob
Sarfii	Blatné
Réte	Reca
Vedröd	Voderady
Abrám	Abrahám
Ziffer	Cífer
Diofzig	Sládkovičovo
Majtén	Majcichov
Kiraly fulva	Kráľová pri Senci
Borfa	Hrubá Borša
Kapolna	Kaplna
Szemptz	Senec

Figure 3. *Mire near Pusté Úľany plotted on the cut out section from the map "Vestigium Operationis...". (Cited as number 1 in cited maps section) authored by Mikovíni, 1732)*

For the history of draining the studied territory a **map from the second half of the eighteenth century** (approximately dated to 1760, *map number 4 in the maps citation list*) is of key importance. This map represents the oldest detailed cartographic depiction of mire, or in other words the area from the mouth of the **Čády stream into the Little**

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Danube (i. e. in to the Čierna voda river: "*Influxus aquae Csády ad minorem Danubium"*) up to the Blatné village (*Sarfö*) in the North. The most humid terrain was in the middle and lower parts of the bounds of Veľký Grob, Továrniky and Pusté Úľany. From the North the wetland was supplied by the waters from the Little Carpathians Mts. including the Vištuk brook, that was dividing into two separate branches near Čataj village and these two streams were surrounding the wetland area by each side. The key element of the map is a depiction of new drainage canal – *Novus Canalis exsiccatorius*, that begins on the boundary of the bounds of Borša, near Pusté Úľany copying the edge of Trnava loessic pseudoterrace and flowing into the side river channel near the town Sládkovičovo - *Dioszég* (*Naturalis Alveus Dioszegiensis*). The main straight channel also had a three shorter side branches. Four bridges were built through the channel to facilitate passing communications. Even the map has no legend at all, it is possible to conclude it actually represents the first project of draining this mire or it was made shortly after excavation of the canal as the previous maps did not contain this structures.

The identical condition of man-made canal is shown on the map of the **First Military Survey** two decades later (*maps number 5 and 6 in the maps citation list*). It is obvious from these maps that also a stream called Sisak from Šenkvice village was flowing into the wetland from the North as well and this very stream gave a name to the artificial drainage canal - *Sissak Graben* (ditch "Shishak"– Šišak in Slovak language). The hydronyme comes from the Croatian colonists from Šenkvice village and its variable name is still being used as Šifák, Sifák, Sisak or Sisek (Beláková, 2014). New element on this map are two additional narrow drainage canals marking the boundaries of *Továrniky settlement, flowing into the main drainage canal. The area of the mire in this stretch was thus changed to meadows and pastures. In the southern parts there are visible at least two open water areas, marked on this map. One of this water areas is named *See Tsadek* – lake Čádek (*Figs. 4* to *8; Table 2*).

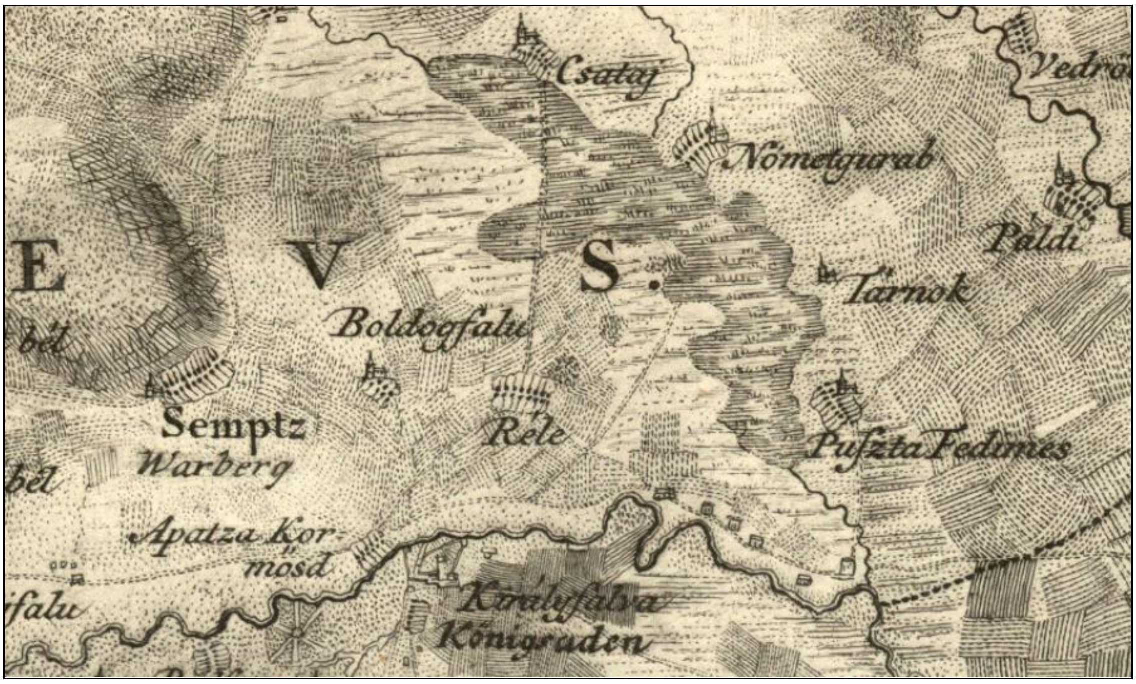

Figure 4. *Cut out section of "Comitatus Posoniensis....." map [S 68 -X. - No. 94] in 1735 showing mire near Pusté Úľany. (Cited as number 2 in cited maps section)*

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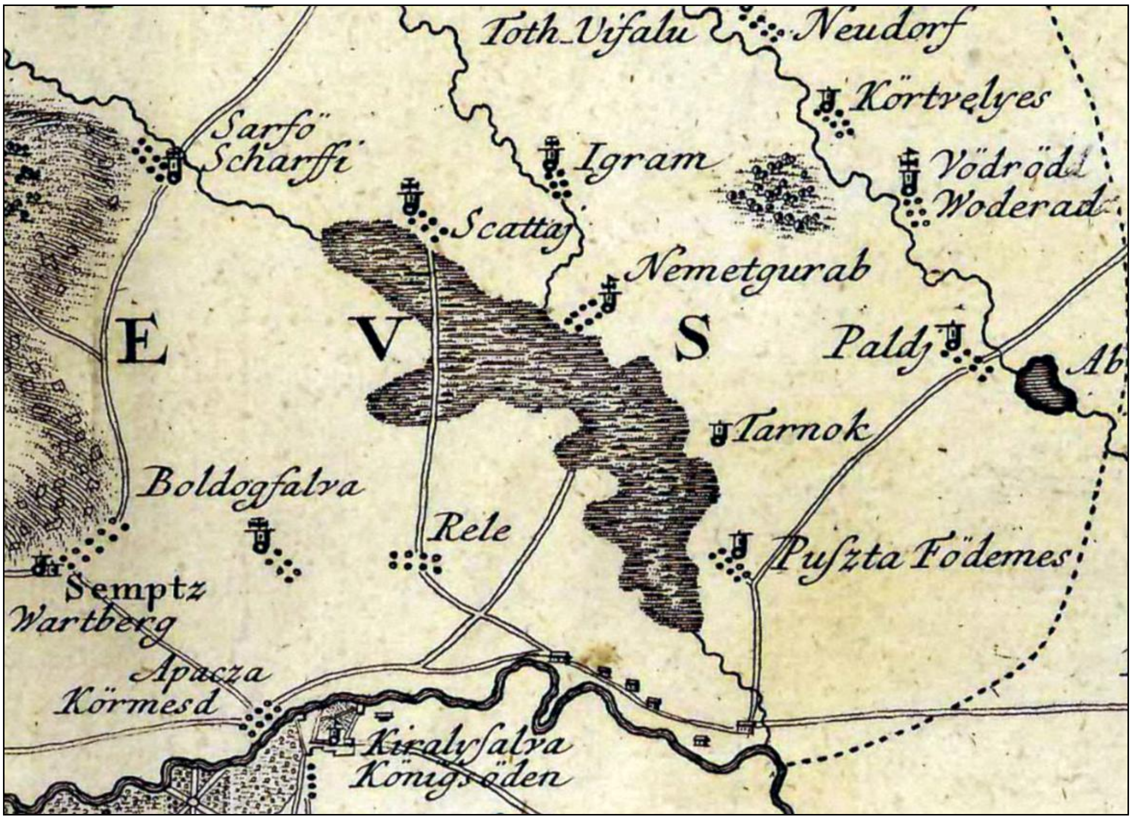

Figure 5. *Cut out section of "Comitatus Posoniensis..." map [S 68 -X. - No. 94] in 1757 showing mire near Pusté Úľany. (Cited as number 3 in cited maps section)*

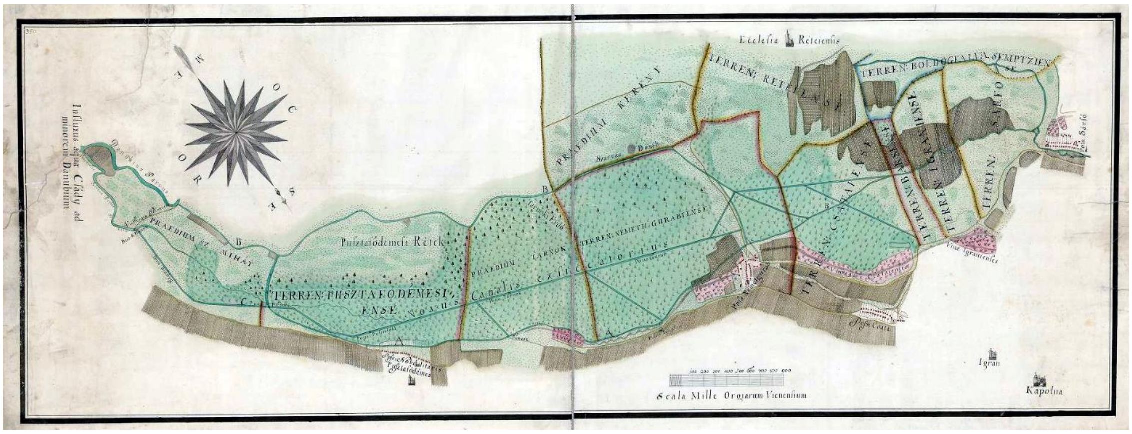

Figure 6. *Map of the mire near Pusté Úľany from second half of the eighteenth century with a first sketch of the drainage canal system*

Table 2. *Local names on the 1760 map and their meanings (Fig. 4)*

Local Latin name on the map in 1760	Present name of location	Accurate English translation
Danubius Parvus	Malý Dunaj	Little Danube
Ecclesia Réteiensis	Reciansky kostol	Church of the Réca village
Naturalis Alveus Dioszegiensis	Prirodzené rameno pri Sládkovičove	Natural channel of Sládkovičovo
Novus Canalis exficcatorius	Nový odvodňovací kanál	New draining channel
Pons Dioszeg(iensis)	Sládkovičovský most	Bridge of Sládkovičovo

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Pons Gurab(iensis)	Most k Veľkému Grobu	Bridge od Veľký Grob
Pons Pusztaföld(iensis)	Pusto-Úľanský most	bridge of Pusté Úľany
Pons Tárnok(iensis)	Tovarnícky most	Bridge of Tárnok
Poss: (essio)	Obec, dedina	Village
Poss: (essio) *Csata*	Obec Čataj	Village Čataj
Poss: (essio) *Nemetgurab*	Obec Veľký Grob	Village Veľký Grob
Poss: (essio) *Nobilitaris Pusztafödémes*	Šľachtická obec Pusté Úľany	Aristrocratic village Pusté Úľany
Praedium	Osada, majer	Farmstead, manor
Praedium Kerény	Osada Kerény	Farmstead Kerény
Praedium SZ Mihály	Osada Sv. Michal	Farmstead SZ Mihály
Praedium Tárnok	Osada Tovarníky	Settlement Tárnok
Pusztafődémeti Rétek	Pusto-Úľanské lúky	Meadows of Pusté Úľany
Puszta Temptom	Pustý kostol	Lonely Church
Stat: (ua) *S. Ioannis*	Socha sv. Jána	Statue of the Saint John
Szarvas Domb	Dobytčí pahorok	Cattle hill
Tárnok	Tovarníky	Tovarníky
Tárnoki Erdó	Tovarnícky les	Tárnok's forest
Terren (um)	chotár	Bounds, land area
Terren (um) *Boldogafalva*	Chotár Boldogu	Bounds of Boldog
Terren Németh Gurabiense	Chotár Veľký Grob	Bounds of Veľký Grob
Terren (um) *Pusztafődémesiense*	Chotár Pusté Úľany	Bounds of Pusté Úľany
Terren (um) *Sarfő*(iense)	Chotár Blatného	Bounds of Sárfő
(Terrenum) *Semptsiense*	Chotár Senca	Bounds of Senec
Via Regia Posonium	Hradská do Bratislavy	Highway to Bratislava
Vinae	Vinohrady	Vineyards
Vineae Csataienses	Čatajské vinohrady	Vineyards of Čataj
Vinae Igranienses	Igramské vinohrady	Vineyards of Igram

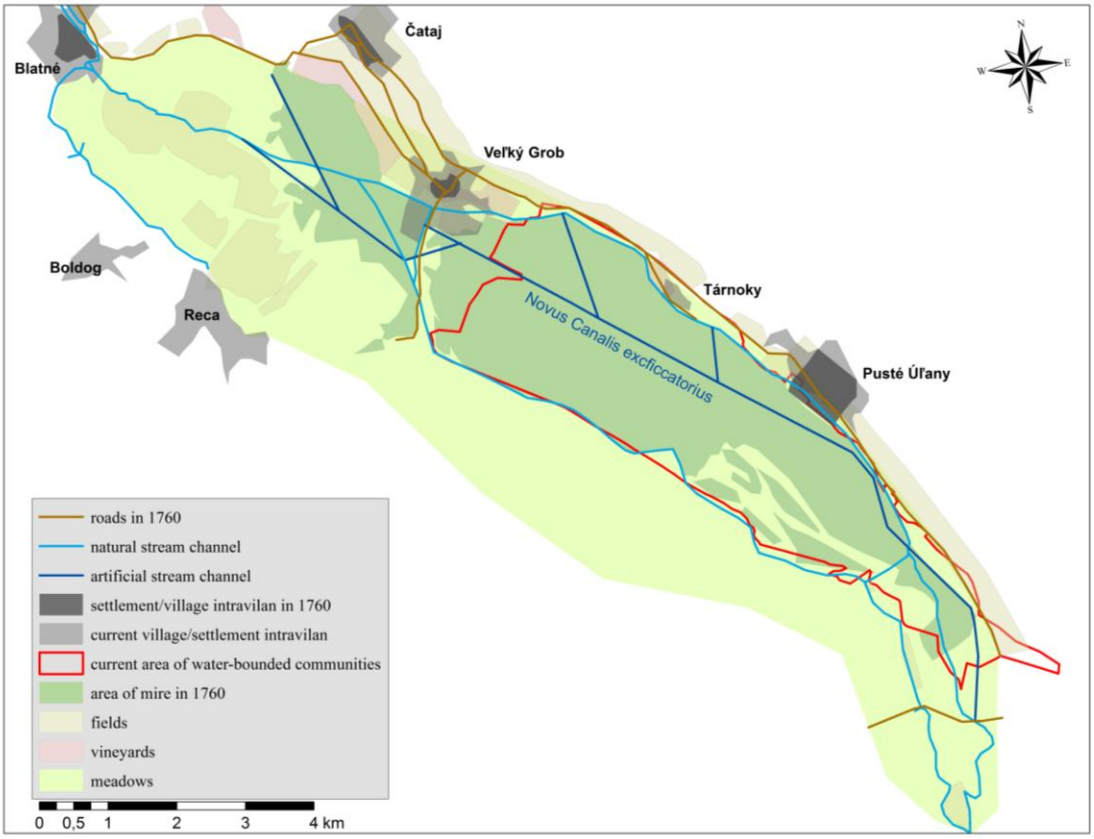

Figure 7. *Area and Landuse of the mire near Pusté Úľany around 1760*

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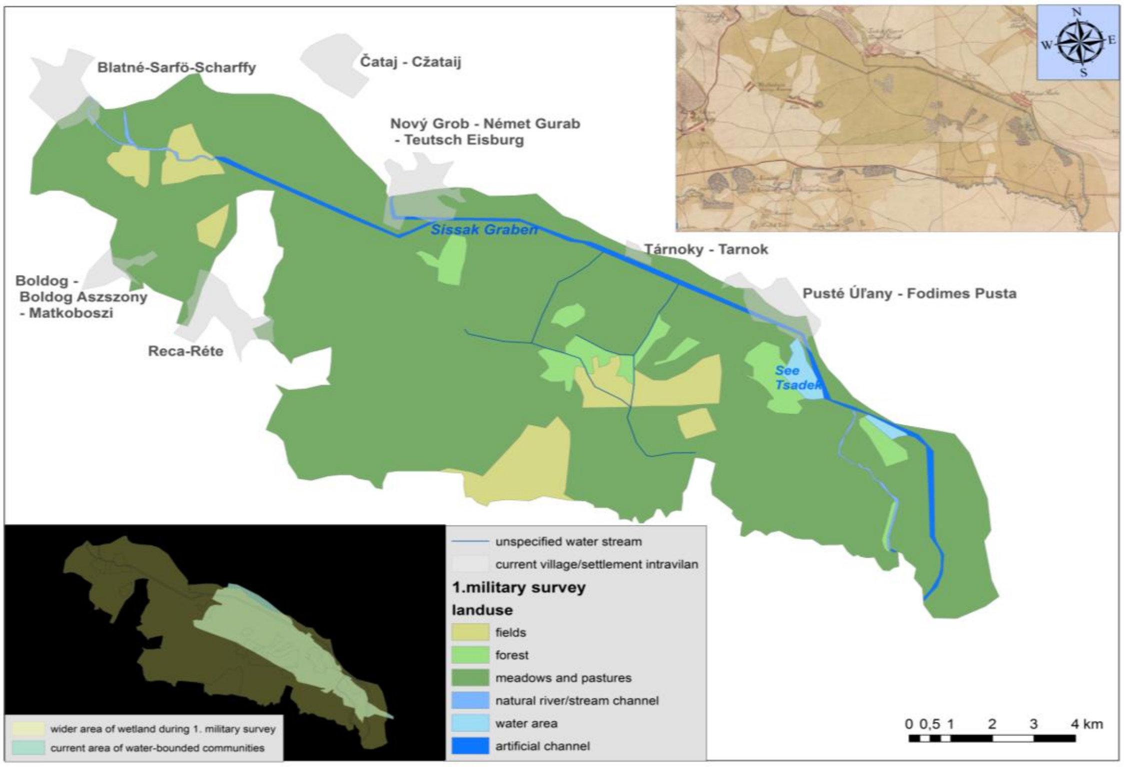

Figure 8. *Area and landuse of the mire near Pusté Úľany during 1. Military Survey (1782-1785)*

The descriptive part of the 1st military survey also contains some notes available to the specific map sheet. At the description of Pusté Úľany village (*Pusta Födimes*) and the *Tovarníky farmstead (*Tarnok*) is mentioned, that "the local meadows are mostly dry, gaining moisture only after rainy periods" (In German: "*Die Wiesen sind nur bey Regen Wetter sumpfigt, ausser den aber trocken.*"). In contrast, the meadows of Veľký Grob village are usually dry (*meist Trocken*). In the "roads" column which mainly characterised the passability of the country for wagon convoys there is a statement that roads near Továrniky are "dry on the upland parts (Trnava Upland) but good and/or bad on the meadows in the lower grounds (= Úľany depression), depending on the weather" ("*...so wie das Wetter, gut und auch schlecht.*"). Also a mentions abouth local forests merits attention. For example, woods of the Veľký Grob village, located outside the artificial canal consists of young oak stands ("*Das jenseits das Wasser befindliche kleine Wäld bestehet aus jungen Eichen.*"). Forests of Úľany and Tovarníky situated between the meadows partly consist of thin scrubland (= cippiced alderwood?), and partly of the oak thickets (*Die Waldung auf der Wiesen ... und ist theils schütteres Buschwerk, theils junge Eichenwaldung;* Arcanum, 2004).

Interestingly enough, also a sketch of the mire on the respective map sheet from the Atlas of Korabinsky from 1804 (*map number 7 in the maps citation list*) is provided. In the area of Úľany map suggests a wetland area along the stream flowing into the Čierna voda (*Schwartz Wasser*). Mire, respectively the stream is named *Tsadek* (Čadek). Map has a sketch of drainage canal as well – *Sissak Graben*, which is marked incorrectly as it is heading from the Čadek influx into Čierna voda river to the Sládkovičovo town (*Dioszeg*), respectively Košúty village (*Kossut*) in *Figure 9*.

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Figure 9. *Stream Čádek (Tsadek) and the draining channel on the section selected from the Atlas of Karabinsky*

On the **map sheet of the 2. Military Survey** (*Fig. 10*) from 1839 (*map number 8-10 in maps citation list*) there is the main drainage canal named *Comitats Kanal*, i. e. County Canal. This proves that the draining of the mire in the eighteenth century was organised and financed by the official bodies of Bratislava County. The main identifiable change on the map is a systematic draining of mire in the bounds of Továrniky by combined system of rectangular canals. These structures divided the area into square and rectangle shapes. It helped to convert the mire in this part from mire to meadows and pastures without no alder forest stands (*Fig. 11*).

On the **map from the last 1890s** (Map number 11 in the maps citation list), that originated by compilation of cadastral map sheets, the main drainage canal has a Hungarian name Megyei csatorna. The new element of the drainage system is except for more drainage canals, added to the existing system, is a new channel (Uj patak) localised from the Pusté Úľany village in the southern part of the mire area and other parallel channel in the bounds of Sládkovičovo (Magyar ée Német Diószeg in Hungarian language, *Figs. 11* and *12*).

Therefore, the mire used to reach more to the North in the past, but after draining of the territory most of the water was concentrated in the lowest parts of the mire and the upper parts were drained as the water flow distribution and outflow was accelerated.

During aerial scanning in 1949, the area consisted mostly of the arable land. After collectivisation the drainage of the area was more intensified and every possible part od the landscape was used as arable land. Mire was not really visible on these orthophotomap (*number 12 in the maps citation list, Fig. 13*). Krippel in his work from 1957 near Tovarníky settlement describes, that the top soil layer is drained and changed into typical fluvic mollisols, however there are rich peat sedimentary accumulation underneath this layer just about 90 cm to 270 cm deep (Krippel, 1957).

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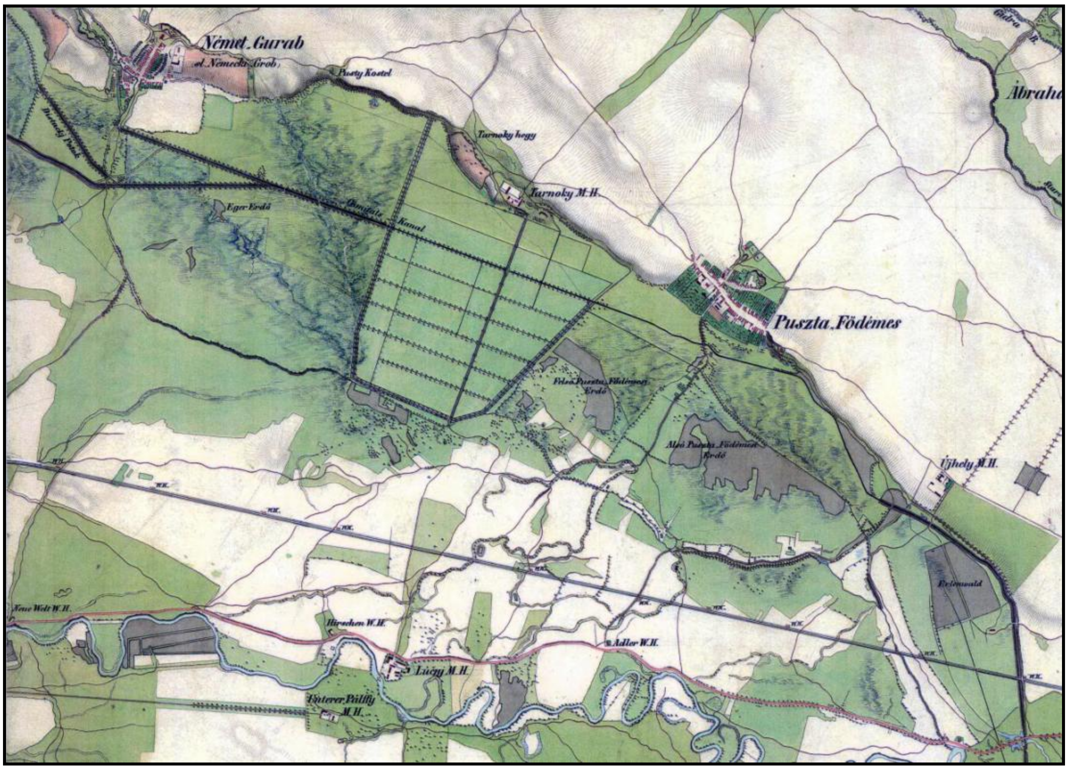

Figure 10. *Selection from the 2. military survey showing the system of draining channel in bounds of Továrniky settlement built before year 1839*

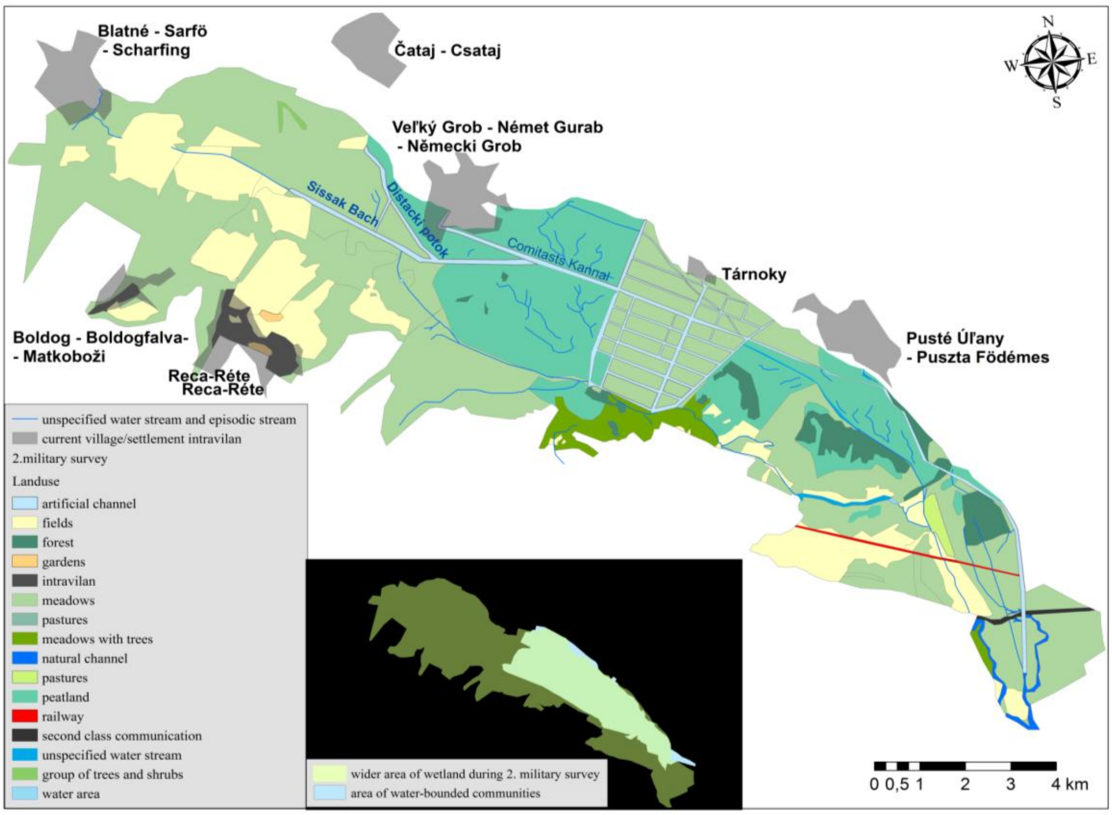

Figure 11. *Area and landuse of the mire near Pusté Úľany during 2. Military Survey (1839)*

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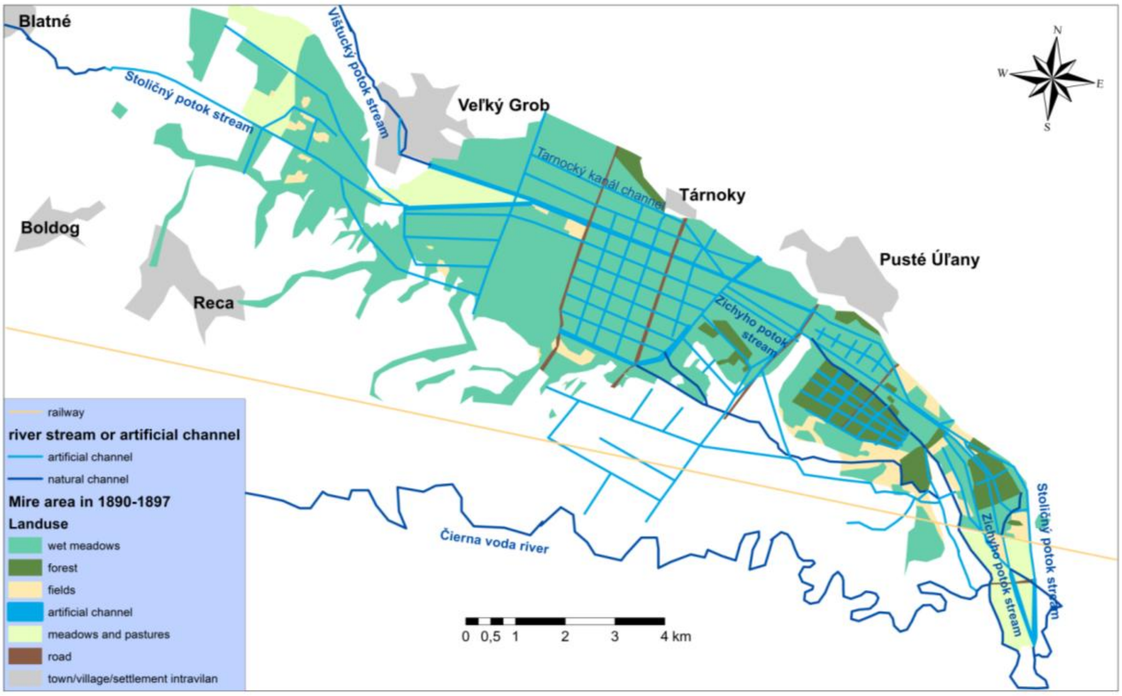

Figure 12. *Area and landuse of the mire near Pusté Úľany on the Bratislava County historical map (1890-1897)*

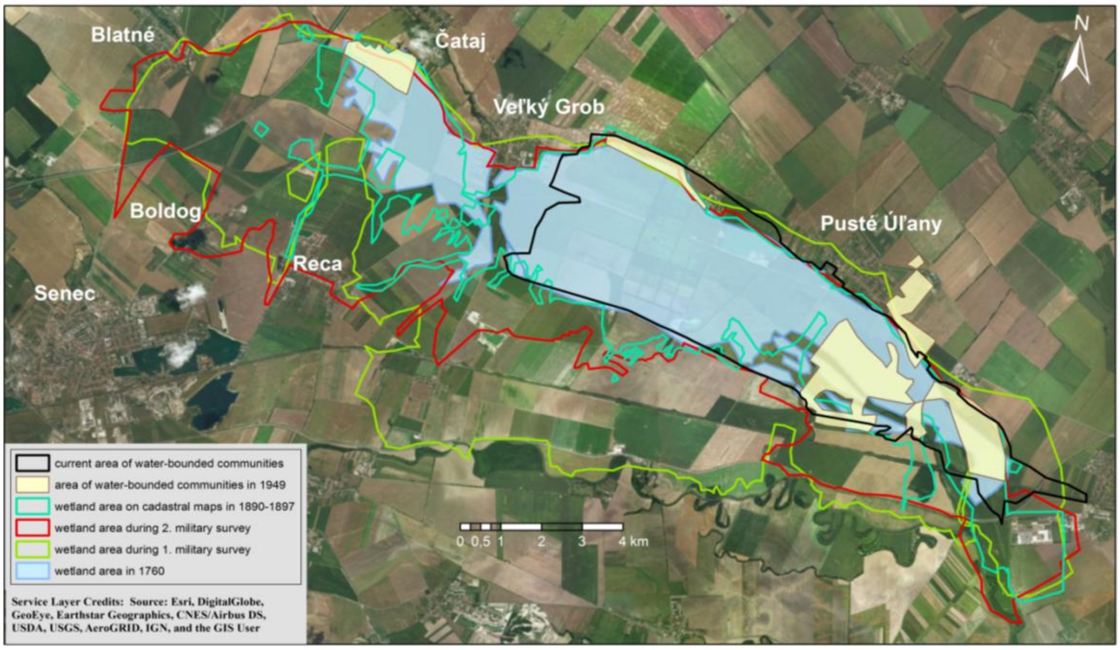

Figure 13. *Change in the area of the mire near Pusté Úľany in different time scales*

Biotope mapping

By our research of current vegetation cover, the research area was set as the reach of ground-water influenced stands. The southern boundary was more difficult to isolate

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from the alluvium of Čierna voda stream, where originally the wetland area was probably stretched. The most of the area was covered with crops in 1949, however the ground water was still high in some years so it was difficult for tillage. The research area was mapped in terrain in the distance of the water areas until the signs of any humidification by ground water was present in the vegetation cover. This area is very reduced in present day as a result of long term human interference.

During the terrain research, several polygons of different water biotopes were identified. In our notes the bottom-rooted macrophytes and the bottom-rooted surface-flowing macrophytes were determined. In the Tovarníky pond the species of *Salvinia natans* was identified. Large area of these extraction ponds is covered with hedges and reeds stands, especially on the edge of the open water areas of these ponds. Though, these areas are disturbed and degraded by local fishermen club as they are cutting reed stands in order to get the access to the open water area for fishing. Generally, these ponds are following the water succession and they are continuously overgrowing the open water area depending on the date of the extraction works shutdown. Originally, the mire was typical by alder and oak forest stands. Characteristic biotope with common alder (*Alnus glutinosa*, code 2111500) is present only marginally in the Tovarníky part of the mire. Close to this polygon the representative alluvial forest with *Fraxinus excelsior* and *Ulmus carpinifolia* at least fifty years old (present in the area on the orthophotomap in 1949 as well). Nevertheless, the typical alluvial stand of this type is missing the oak species (*Quercus sp.*).

The ground water level during field research in November (2016) was stable around 20 cm to 2 meters or more. The mean level was around 90 cm (found out during soil probes measuring near Tovarníky pond). Several drainage canals are overgrown by vegetation and they are ideal environment for bird nesting such as *Asio flammeus* (Vďačný, 2001) and other types of protected fauna. In the vicinity area of the extraction ponds the underground water level is higher and surrounding crops are currently not cultivated as they are subject to succession of different types of shrubs. In the southern parts of the wetland area, near Pusté Úľany village the forested area is managed by forest management as poplar monocultures. This management supports the prevalence of invasive species in the herb vegetation layer, what is degrading the natural communities. Number of identified biotopes is 27 (*Fig. 14; Table 3*), from which the 13 types is purely of anthropogenic origin. It is remarkable there are several marginal areas of natural vegetation after major degradation in this area (2111500). Most of the area is used as arable land and major abandonment of the agricultural landscape is situated near the open water areas.

Table 3. *List of mapped biotopes in the research area*

Biotope code (Ružičková et al., 1996)	Biotope name (Ružičková et al., 1996)	Biotope code (Stanová and Valachovič, 2002)	NATURA2000 code	Area [ha]
A110000	Intensive crops	X7	-	1 021.794
8234000	Material pit	V	-	108.8369
8150000	River/stream channel	Vo4	3260	24.5387
8160000	Regulated water flow	Vo4	3260	31.067
A520000	Roads	X3	-	1.6561
2111200	Riparian forest with *Quercus robur, Ulmus laevis* and *Fraxinus excelsior*	L.s1.2	91F0	47.9479

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2122100	Artificial broadleaved forest (*Chelidonio-Robinion*)	X9	-	4.6462
2122200	Artificial broadleaved forest with *Populus*	X9	-	194.545
8A10000	Floating aquatic plants (*Lemnion minoris, Hydrocarition* and *Utricularion vulgaris*)	Vo6	-	0.2883
8A20000	Submerged bottom-rooted aquatic plants (*Parvopotamion, Magnopotamion p.p.*)	Vo6	-	1.5137
8A30000	Surface floating bottom-rooted aquatic plants (*Nymphaeion, Callitricho-Batrachietalia*)	Vo6	-	0.0089
8B10000	Reeds in water bodies and mires (*Phragmition*)	Lk11	-	11.2704
8B22000	Large *Carex* beds	Lk10	-	0.744
3522100	Alluvial meadows and meadows in wet depressions in lowlands and Uplands	Ra7	-	17.8485
A521000	Cart-road on the field	X4	-	10.3308
A522000	Forest cart-road	X4	-	0.1425
A113000	Ruderal weed communities near fields and roads	X4	-	26.2931
A300000	Building structures outside settlements	Z	-	11.2375
A430000	Abandoned mining sites	X3	-	159.1613
2163000	Self-seeding tree formations	X2	-	0.6939
2162500	Other type of shrubs	Kr6	40A0	53.1088
2161100	Temperate thickets with *Prunus spinosa*	Kr7	-	5.9694
2162300	Glades with *Sambucus ebulus* thickets	X1	-	8.4551
A410000	Weed communities od recently abandoned urban and suburban constructions	X4	-	9.7343
2118000	Pioneering tree formations	X2/X9	-	24.7877
A200000	Anthropogenic forests	X9		0.2886
2111500	Alder swamp forests (*Alnion-glutinosae*)	Ls.7.4	-	4.6239

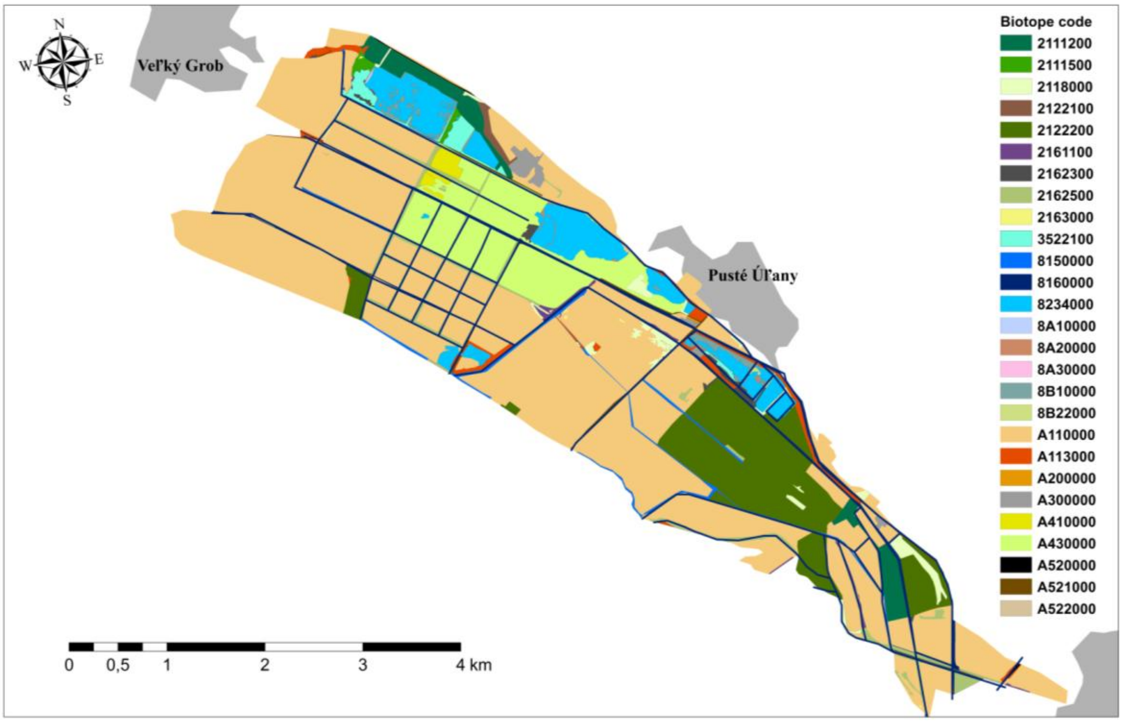

Figure 14. *Map of biotopes covering the area of mire near Pusté Úľany*

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Discussion

Human intervention can change the wetland landscape very dramatically (Guette et al., 2018). The similar changes in landuse of wetlands were mentioned as a result of collectivisation also in other countries of the Eastern bloc (Deak, 2007; Skalos et al., 2017; Jepsen et al., 2015).

Based on historical maps analysis it was found out that the area of mire had its active part in the north as well covered the area up to Blatné village, it would be better to have larger DEM in our possession. The mire was presented more as the wetland than the open water body mire area, and that is also the reason for difficulty of evaluating the mire activity in different time scales. As this area started anthropically induced drainage in eighteenth century it is really possible that a further research such as palynology or macrofossil analysis is needed to understand the genesis and the origin of the mire. However, it is depending on the further suitability of the sediment.

According to historical sources it was concluded that the mire was supplied by not only the Little Carpathians Mts's streams, but also by the high flows of the Little Danube. Historical name of the mire "Csádé" or Csádek respectively is probably an older Hungarian hydronyme (*csádé, csáté*) with meaning of "wetland" or "shrubs dominated land" (Czuczor and Fogarasi, 1862-1874). In southern part, the alder stands were historically prevailing for the longest time (*Felsö Puszta Födémesi Erdö, Erlenwald*, 1839; *Egresi Erdö*, 1897). Area of the mire and especially its extent to the south-west direction was dynamically changing. In dry years the meadows were productive, but in the wet years, meadows were more characterised as wetlands and deposition of the mineral sediments took place and the major part was impassable. In the deepest parts the open water areas were formed (lake Tsadek). At first the western part of the mire has undergone degradation (Veľký Grob – Blatné) after drainage canal construction in the second half of the eighteenth century. Hence the research was focused on the southern part as our major part for research of current biotopes (Veľký Grob – Pusté Úľany). Yet historical maps need to be considered, as they were the part of the larger image, for example the historical map of whole Bratislava County (1957), where the mire drawing could be only estimation.

The drainage in the eighteenth century was very standard solution in the Austro-Hungarian empire. For the comparison the similar degradation was proceeded in Parížske močiare wetlands were the first drainage took place in 1819 (Procházka et al., 2015), but in the lake Šúr near Svätý Jur the drainage took place only in 40. of the twentieth century (Pišút et al., 2010).

Currently the major problem of the prospected area is as well the abandonment of agricultural landscape, what is quite characteristic for the Eastern bloc countries (Lieskovský et al., 2015; Jepsen et al., 2015; Burgi et al., 2017). Landcover analysis in this area was described by Kopecká (2011) with emphasis to the protected bird's occurrence. The secondary landscape structure during the twentieth through to the twenty-first century was prospected in the Úľanská wetland bird's area (Hreško and Guldanová, 2012) and it is providing the similar results with stable major areas of arable land with considerable changes during intensification and collectivisation. Different ongoing studies in Slovakia (Špulerová et al., 2011; Skokanová et al., 2017) and in Czechia (Kilianová et al., 2017) are copying similar landcover changes and development, especially covering the arable land areas. Currently, this trend in the research area and Slovakia as a country, is turning to land fragmentation and numerous ownerships of the arable and as well non-arable land (Muchová and Lusková, 2017;

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Muchová, 2017). Future management is therefore necessary only with consideration to specifics of landcover changes in this region (Bezák et al., 2017; Klusáček et al., 2018; Jepsen et al., 2015).

Conclusion

Mire near Pusté Úľany was originally much larger and probably the biggest mire area in the Danubian lowland during Holocene. The theories of its origin are uncertain. Process of degradation of the mire started by drainage canal building around mid-eighteenth century, when from the Bratislava County financed a sophisticated drainage system construction to obtain land for pasture and cultivation. Since then, the human interference was huge into this ecosystem. Following peat extraction degraded the local landscape and destroyed the natural biotopes. Besides further research, appropriate protection and monitoring is needed to establish in this area as well as its further management. Some of the anthropogenic biotopes could reverse to a more natural state by this support and the favourable service of this area could be even recreation in the future and not only intensive forest management promoting the invasive species dominance. Currently the grasslands areas are forming in the direct proximity of this water areas as a result of fields desolation, which were cultivated in 1949. Water areas after peat and gravel extraction enables the repeated hydroserie succession forming rich sedge and reed stands that are seasonable biotope for water fowl and other endangered animal species.

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MN^{2+} AND CU^{2+} ADSORPTION WITH A NATURAL ADSORBENT: EXPANDED PERLITE

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Abstract. Water containing heavy metals causes many diseases including, in particular, cancer. For this reason, it must be completely purified before being discharged. In this study, the surface area of expanded perlite (EP) was determined to be 406.259 m^2/g by the method proposed by Brunauer, Emmet and Teller (BET). The removal efficiencies in terms of the heavy metals Mn^{2+} and Cu^{2+} were investigated according to this value. The optimum point of adsorption of manganese and copper with expanded perlite is 30 min and 5 min, respectively. The optimum absorbent dosage is 0.5 g for manganese and 0.4 g for copper. In the case of Mn^{2+} and Cu^{2+} adsorption, Log_q/Log_C values were plotted to determine the Freundlich isotherm on the expanded perlite. In addition, Langmuir isotherm constants were determined by plotting 1/q and 1/C values. It has been found that with 5 mg/L Mn^{2+} and Cu^{2+}, the Mn^{2+} heavy metals are compatible with the Langmuir isotherm model with $R^2 = 0.93$, and the Cu^{2+} heavy metals are compatible with the Langmuir isotherm model with $R^2 = 0.99$.
Keywords: *adsorption isotherms, heavy metal treatment, expanded perlite, Mn and Cu treatment*

Introduction

Industrial wastewaters contain various heavy metals, pesticides, salts and detergents, which cause chemical and physical changes in the water. Industrial waste pollutes not only the receiving environment, but also agricultural land and forests, and the surface and groundwater resources around it. The heavy metals in the wastewater resulting from industrial activities are often found in waste water from minefields, leakage waters of solid waste sources. These wastewaters accumulate in sediments mixed with rivers, lakes and underground waters. Even in a region remote from the discharge point, the level of heavy metal pollution is preserved. At the same time, metal compounds can be transformed into other compounds during transport. Many economical and effective methods for heavy metal removal have been used and new treatment methods have been developed. From these methods; ion exchange, chemical precipitation, reverse osmosis, evaporation, membrane filtration and biosorption are widely used, but the most effective method is seen as adsorption. The advantages and disadvantages of these methods used in heavy metal removal in *Table 1* are explained in detail (Hamutoglu et al., 2012).

It can cause pollution in agricultural land within the area affected by industrial establishments, the accumulation of heavy metal in soil, a decrease in productivity with regard to growing crops, and accordingly, the devaluation of agricultural land (Gonullu, 2004).

Copper and manganese, which are found in trace amounts in nature and in living things, are stored as heavy metals, especially in human and animal livers. Exposure to high doses of copper and manganese for long periods are harmful to health (Mudhoo et al., 2012). Regulations in Turkey with regard to water intended for human consumption in order to ensure the quality of the water, is regulated by technical and hygiene conditions (TS-266). The maximum values for copper Cu (II) and manganese Mn (II) were determined to be 2 mg/L and 0.05 mg/L, respectively (Published Official Gazette,

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2012). In studies carried out for the removal of heavy metals which cause water pollution as a result of industrial activities, Xie et al. (2017) used sulfur micro-particles to remove copper from the aqueous solution. The adsorption process reached an equilibrium in 30 min and the optimum removal occurred at pH 4.5, ensuring compliance with the Freundlich isotherm model (Xie et al., 2017). Dai et al. compared two different carbon materials in their work. In addition, they investigated adsorption isotherms, contact time, pH and ionic strength. The Langmuir isotherm model is suitable for copper and zinc (Yingjie, et al., 2017). Mishra et al. (2017) in experiments involving Cu (II) and Ni (II) in red and black soil, kinetic and isothermal studies of metal ions were carried out to determine contact time, adsorbent dosage and metal concentration. Cu (II) and Ni (II) reached equilibrium after 480 min and 300 min of shaking, respectively. The maximum removal efficiency for Cu (II) by red soil was 97.3%, for Ni (II) by black soil was 99.9%. An examination of kinetic and adsorption isotherms has shown that adsorption experiments involving the pseudo-second-order equation and the Freundlich isotherm model, respectively (Mishra et al., 2017). Zahar et al. (2015) studied various variables in experiments to evaluate the ability of steel slag to remove manganese from an aqueous solution. The variables included contact time, adsorbent dosage, pH and initial manganese concentration. The equilibrium contact time was 10 h. An absorbent dosage of 1 g was found to be sufficient for removing heavy metals from the aqueous solution. A pH of 6 for manganese adsorption on steel slag was found to be the optimum. The Langmuir isotherm model adapted to Mn (II) adsorption by steel slag. In the Mn (II) adsorption of steel slag, the Langmuir isotherm was conformed (Zahar et al., 2015). Ateş and Akgül (2016) used natural zeolite obtained from the Manisa-Demirci region, modified with NaOH aqueous solution (0.5-2.0 mol/dm^3), and worked on manganese adsorption. The maximum manganese adsorption was obtained using NaOH-modified zeolite. Manganese adsorption with natural zeolite was consistent with the Langmuir isotherm model, whereas the use of manganese adsorption on modified zeolite was consistent with the Freundlich isotherm model (Ateş and Akgül, 2016). In 2010, Mesci and Turan investigated the removal of Cu (II) and Zn (II) by adsorption in the leachate of industrial using illite. They investigated the effect of illite, a natural clay species, as an adsorbent on the adsorption process in terms of pH, dosage and contact time. Experimental studies have shown that illite is effective in adsorbing Cu (II) and removing Zn (II), and can be used as an alternative to other adsorbents in the treatment of leachate water due to its low cost. Although active carbon is widely applied in adsorption processes, it is very costly. They found that illite, an inexpensive material that could be an alternative to activated carbon, had sufficient binding capacity for the removal of Cu (II) and Zn (II) from the leachate (Mesci and Turan, 2010). Xu et al. investigated the effect of operating parameters and electrolytes on the removal of toxic metals (cadmium, zinc and manganese) from synthetic mine molten effluent by bulk electrocoagulation. The results have shown that the removal of heavy metals increases the efficiency of the solution by increasing the pH and the current density of the solution. Fe-Fe electrode combination is more effective than other combinations (Al-AI, Al-Fe and Fe-Al). The interaction of heavy metal ions negatively affected the removal of Zn^{2+} by increasing the concentration of Zn^{2+} at the beginning. The single chlorine system confirms the optimized removal over Mn^{2+}. Xu et al. (2017) suggested that adding a small amount of sodium chloride to a high sulphate and hardness solution could accelerate the removal of heavy metals. In 2014, Ardali et al. used expanded perlite as an adsorbent in the removal of Cu (II) in

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industrial waste leachate. In the study, the Cu (II) removal from industrial waste leachate was investigated by the discontinuous adsorption technique. The expanded perlite industrial leachate can be successfully used as an adsorbent for Cu (II) removal. Particle-to-particle diffusion and Elovich kinetic models are used. The removal rate of Cu (II) increases with an increase in the concentration of expanded perlite. The results have shown that expanded perlite leachate is a potential adsorbent for Cu (II) removal (Ardalı et al., 2014).

Table 1. *Advantages and disadvantages of methods used in heavy metal removal (Hamutoglu et al., 2012)*

Methods	Advantages	Disadvantages
Chemical precipitation and filtration	Simple and cheap	Difficult separation at high concentrations, ineffective, waste sludge formation
Electrochemical methods	Recovery of metal	Expensive, only active at high concentrations
Chemical oxidation and reduction	Inactivation	Ambient sensitivity
Ion exchange	Effective treatment and recovery of pure waste metal	Sensitive to particles and expensive resins
Evaporation	Pure waste disposal	Additional energy requirement, expensive, waste sludge formation
Reverse osmosis	Pure waste for recycling	High pressure, membrane size, expensive
Adsorption	Activated carbon use of sorbents	Application for all metals

In this study, the expanded perlite adsorption potential of manganese and copper heavy metals were evaluated. The adsorption experiments were carried out according to various parameters such as contact time, initial heavy metal concentration and adsorbent dosage. Isotherm models were applied to the data obtained from the experiments to compare the adsorption capacity of expanded heavy metal with expanded perlite.

Material and method

Adsorbent

The perlite used as an adsorbent material is an amorphous glassy volcanic rock with a pearl luster. When the perlite is heated to a softening temperature range of 760-1090 °C, it expands to about 20 times its constant volume. This expansion is due to the presence of 2-5% trapped water in the structure of the raw pearlite rock. As a result of this expansion, small amounts of granules form as hot and soft glassy particles. Turkey contains approximately 40% of the world reserves of perlite (Şahinoğlu, 2013). In Turkey, expanded perlite has a great variety of applications, while crude perlite is more limited in terms of fields of application. This property is mainly related to the chemical and physical properties of perlite (*Table 2*).

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Table 2. *Chemical and physical properties of expanded perlite*

Substance	Rate (%)
SiO_2	71-75
Al_2O_3	12.5-18
Na_2O	2.9-4
K_2O	4-5
CaO	0.5-0.2
Fe_2O_3	0.1-1.5
MgO	0.03-0.5
Humidity	Max 0.5%
pH	6
Softening point	890-1100 °C
Melting point	1280-1380 °C
Color	White, gray

Adsorption study

The adsorption isotherms of the expanded perlite manganese and copper were made according to the Batch method (Atasoy and Yesılnacar, 2017). 1000 mg/L Mn^{2+} and Cu^{2+} stock solutions were prepared from $MnSO_4.7H_2O$ (Merck) and $CuSO_4.5H_2O$ (Merck) heavy metal salts, and aqueous solutions were prepared at 8 different concentrations between 1 and 250 mg/L. Removal of heavy metals was carried out in a laboratory environment. All aqueous solutions were prepared using deionized water (ELGA Purelab Option-Q). The prepared samples were supplemented with 0.1, 0.2, 0.3, 0.4, 0.5 g of expanded perlite in 500 mL beakers, respectively. The samples were mixed at 500 rpm using a magnetic stirrer (2 mag Mix 15 Eco) for different amounts of time (5, 10, 20, 30 min). Examples were measured on the Perkin Elmer Optima 5300 DV Optical Emission brand ICP-OES instrument. The recovery efficiencies were also calculated using the following formula (*Eq. 1*).

$$\text{Efficiency } (\%) = \frac{E_i - E_e}{E_i} \times 10 \qquad \text{(Eq.1)}$$

where:
E_i = Initial concentration (mg/L)
E_e = Exit concentration (mg/L)

Data analysis

Adsorption data were calculated using the Langmuir and Freundlich isotherm models. The analysis of the adsorption data is made according to the Langmuir equation (*Eq. 2*) and the Freundlich isotherm equation (*Eq. 3*).

$$\frac{1}{q_e} = \frac{1}{q_m \times K} + \frac{1}{C_e} \qquad \text{(Eq.2)}$$

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$$\log C_s = \log K_f + \frac{1}{nf} \times \log C_e \qquad \text{(Eq.3)}$$

The equation indicates a line between the values of C_e/C_s, and the angle of the slope gives $1/q_e$, whereas the angle of the slope gives 1/Ce in the slope on the *y* axis. K_f and $1/n_f$, Freundlich constants, C_s (mg/g) give the amount of expanded perlite adsorber, while C_e (mg/mL) gives the amount of Mn^{2+} and Cu^{2+} in solution. Since the equation specifies a straight line between logC_e and logC_s, the slope of the rectangle will give the value $1/n_f$, and the point on the y-axis the slope point, logK_f (Cooney, 1998).

Results and discussion

Effect of pH

To investigate the effect of pH on the adsorption of Mn (II) and Cu (II) with expanded perlite, the initial heavy metal concentration was 5 mg/L and the temperature was 25 ° C. The changes in pH value are shown in *Figure 1*. In *Figure 1,* the adsorption efficiency is increased at pH = 5, where adsorption is less at pH = 2. When the values in the graph are taken into account, the best pH-dependent removal efficiencies for Mn (II) and Cu (II) ions are; For Mn (II), pH = 5 – 81%, pH = 7 – 86%, pH = 9 – 87%, pH = 11 – 85%, and also for Cu (II), pH = 5 – 85%, pH = 7 – 84%, pH = 9 – 80% and 81% at pH = 11. Since the removal efficiency is lower than the higher pH values at low pH values, the excess H^+ ion positively expands the expanded perlite surface of the released H^+ ion, which affects the pushing force between the molecules, thereby causing a decrease in the amount of heavy metal adsorbed. Xie et al. Sulfur microparticles were used as adsorbents in copper removal experiments from aqueous solutions. In the adsorption experiments, the pH effect was investigated, and the optimum removal was carried out at pH 4.5 (Xie et al., 2017).

As shown in *Figure 1,* the pH adsorption study of Mn^{2+} and Cu^{2+} heavy metals was carried out at pH 2-11. The best adsorption for Mn^{2+} heavy metal was achieved at pH 5.5-6. As well as the best adsorption at pH 5 in Cu^{2+} heavy metal.

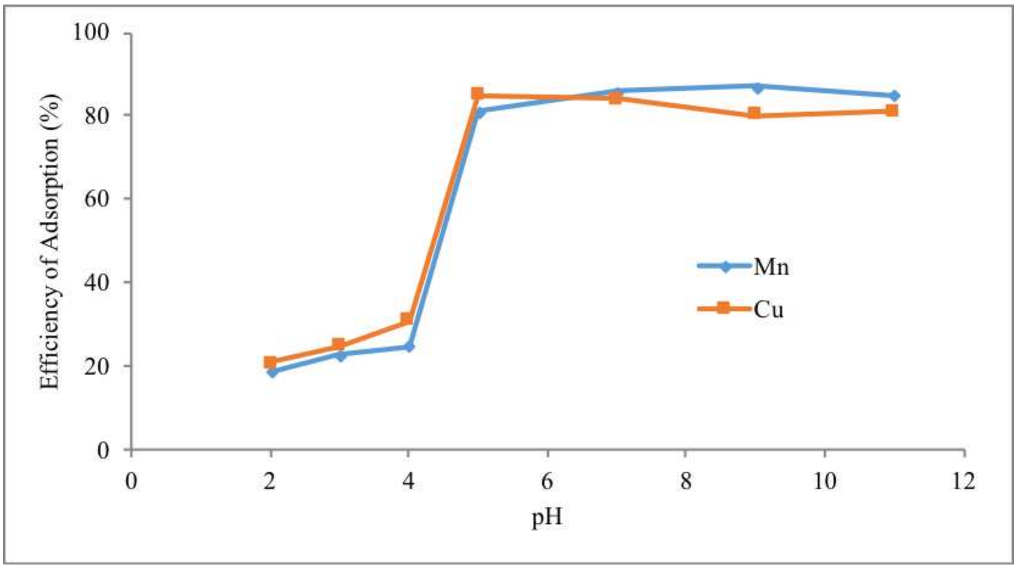

Figure 1. *Adsorption ratio depending on pH of Mn^{2+} and Cu^{2+} heavy metals*

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Effect of contact time

According to the data obtained during the contact period, Mn^{2+} and Cu^{2+} ions showed a tendency to adsorb rapidly in the first 5 min on the expanded perlite. The initial concentration was chosen to be 5 mg/L, which is the best yield in terms of the two heavy metals. The optimum time point in terms of the best adsorption values for Mn^{2+} and Cu^{2+} was assumed to be 30 min and 5 min respectively. The contact times needed to achieve the optimum point were also identified in the isotherm study. As the adsorbent and adsorbed molecules increase the collision time, the efficiency of adsorption increases with the increase of contact time, and after a while, it reaches equilibrium. Compared to the amount of ions adsorbed at the time of initial adsorption by rapid adsorption, it is easy to see that the adsorption level drops. The contact time at which the turning point was reached (30 min for Mn (II), 5 min for Cu(II)) was then used to determine the adsorbent dosing and isotherm studies. Mishra et al. (2017) reported that copper reached equilibrium after 300 min of contact with copper. *Figure 2* shows the percentages of Mn^{2+} and Cu^{2+} adsorbed by the expanded perlite.

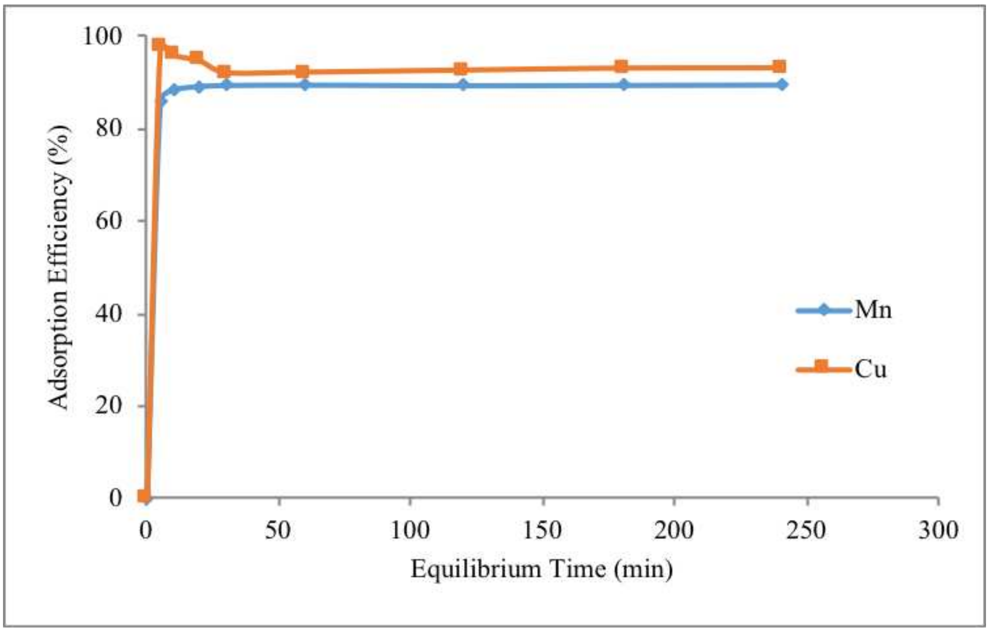

Figure 2. *Adsorption ratio depending on contact time of Mn^{2+} and Cu^{2+} heavy metals: adsorbent dosage - 0.5 g for manganese, 0.4 g for copper, 5 mg/L for initial concentration, pH - 5, T - 25 °C and shaking speed - 500 rpm*

Optimum adsorbent dosage

The adsorbate dose was studied by taking 0.1, 0.2, 0.3, 0.4, 0.5 g respectively. The adsorbent dosage experiment was carried out depending on the results obtained as a result of the experiments performed to determine the contact period. The amount of heavy metals adsorbed per gram of adsorbent was higher in the lower adsorption dose, higher in the higher adsorption dose. Although the removal efficiencies for Cu (II) were nearly similar, the best efficiency was 0.4 g of expanded perlite and the best efficiency of Mn was 0.5 g of expanded perlite. Zahar et al. found that 1 g of adsorbent dosing was sufficient for heavy metal removal from aqueous solutions (Zahar et al., 2015). The

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effect of the adsorbent dose in terms of heavy metal removal at an initial concentration of 5 mg/L manganese and copper is shown in *Figure 3*.

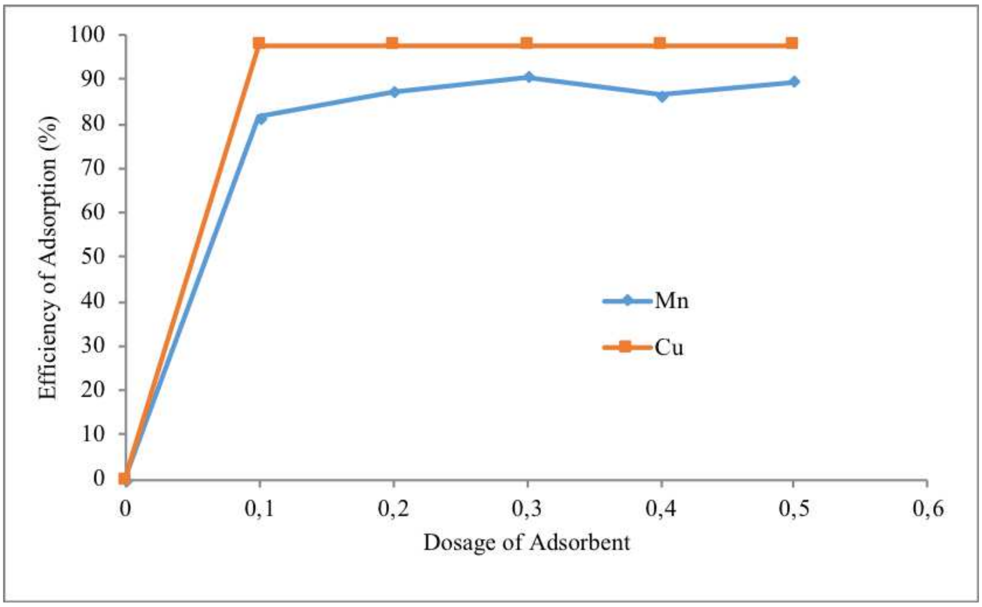

Figure 3. *The effect of the adsorbent dosage on the adsorption of Mn^{2+} and Cu^{2+} heavy metals: initial concentration - 5 mg/L, pH - 5, T – 25 °C and agitation speed - 500 rpm*

Effect of initial concentration

At different initial concentrations for each metal, 0.5 g adsorbent for manganese and 0.4 g adsorbent for copper, 25 °C of temperature, pH 5 for each heavy metal, shaking speed of 500 rpm, contact time of 30 min for manganese, 5 min for copper. The effect of the initial concentration on the adsorption yield according to the determined properties is shown in *Figure 4*.

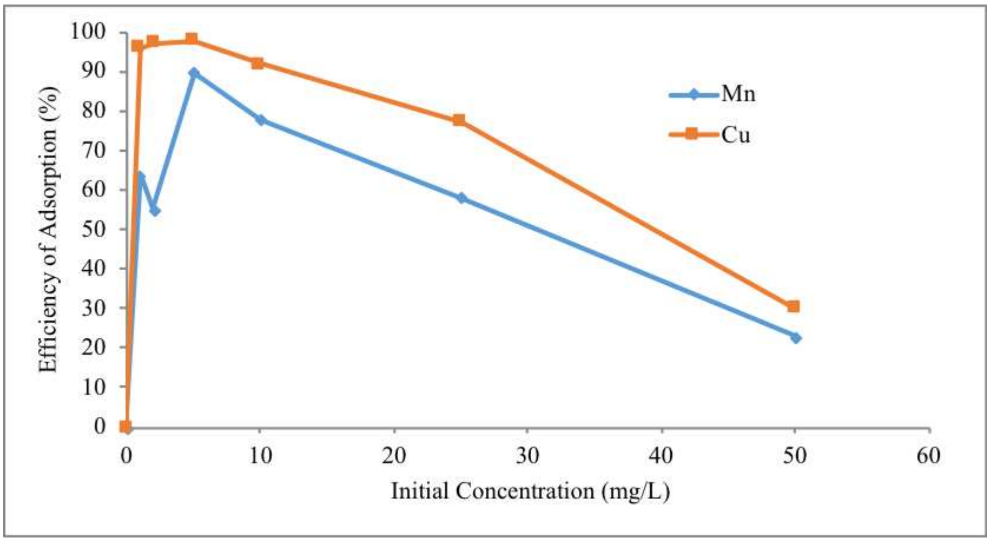

Figure 4. *Effect of initial concentration on the adsorption of Mn^{2+} and Cu^{2+} heavy metals: Adsorbent dosage - 0.5 g for Mn^{2+}, 0.4 g for Cu^{2+}, shaking time - 30 min for Mn^{2+}, 5 min for Cu^{2+}, pH-5, T- 25 °C and shaking speed - 500 rpm*

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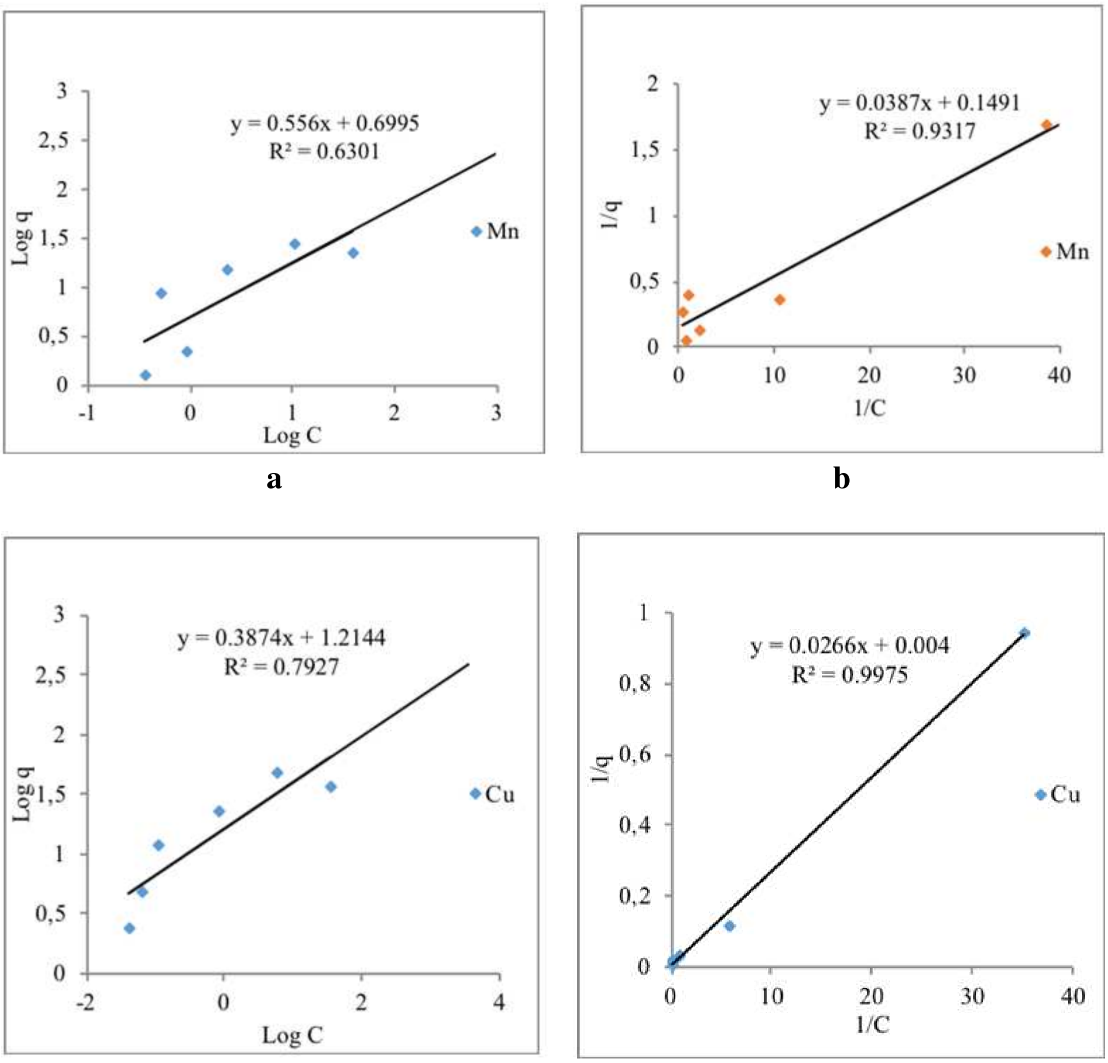
Adsorption isotherms

Manganese adsorption isotherm

Freundlich and Langmuir isotherm curves and equations for manganese adsorption applied with regard to the expanded perlite are shown in *Figure 5a* and *b*. In *Table 3,* isotherm constants and correlation coefficients are given. At the initial concentration of 5 mg/L of Mn^{2+} heavy metal, the turning point was assumed to be 30 min since 0.5 g of expanded perlite achieved the highest adsorption value. In the study, Mn^{2+} heavy metals were found to conform to the Langmuir isotherm model with R^2 = 0.93.



a **b** **c** **d**

Figure 5. ***a*** *Mn^{2+} Freundlich isotherm.* ***b*** *Mn^{2+} Langmuir isotherm.* ***c*** *Cu^{2+} Freundlich isotherm.* ***d*** *Cu^{2+} Langmuir isotherm*

A correlation value of 0.93 for Langmuir isotherm was obtained. It can be said that Langmuir isotherm is compatible with this high correlation value. Langmuir isotherms indicate that the adsorbent surface is homogeneous, and the surface is covered with a single layer.

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Table 3. *Freundlich and Langmuir isotherm constants for manganese*

Freundlich isotherm	**K_f**	**$\log_{Kf}$**	**n_f**	**$1/n_f$**	**R^2**
	5.006	0.6995	1.7985	0.556	0.63
Langmuir isotherm	**$1/b.Q_0$**	**$1/Q_0$**	**b**	**Q_0**	**R^2**
	0.1491	0.0387	0.259	25.83	0.93

Copper adsorption isotherm

The Freundlich and Langmuir isotherm curves and equations for copper adsorption on the expanded perlite are shown in *Figure 5c* and *d,* respectively. In *Table 4,* the isotherm constants and correlation coefficients are given. At an initial concentration of 5 mg/L Cu^{2+}, the turning point was assumed to be 5 min, since 0.4 g of expanded perlite achieved the highest adsorption value at this time. In the study, Cu^{2+} heavy metals were found to conform to the Langmuir isotherm model with $R^2 = 0.99$.

Table 4. *Freundlich and Langmuir isotherm constants for copper*

Freundlich isotherm	**K_f**	**$\log K_f$**	**n_f**	**$1/n_f$**	**R^2**
	16.38	1.2144	2.58	0.38	0.79
Langmuir isotherm	**$1/b.Q_0$**	**$1/Q_0$**	**b**	**Q_0**	**R^2**
	0.004	0.0266	0.15	37.59	0.99

BET surface area of expanded perlite

BET surface area analysis was performed with the Quantachrome Nova Touch LX4 instrument. The BET device can detect surface area measurements, micro, meso and macro pore size and pore size distribution at low pressures and high resolution by physical adsorption method in solid or powder samples. Prior to the test, the samples are placed in the degas unit (up to 300 °C) which is vacuum-heated for purification and sludge treatment, after which the samples are analyzed with nitrogen gas, which is used as adsorbate in the liquid nitrogen temperature. As a result of these experiments, an "adsorption isotherm" is obtained which indicates how much nitrogen the substance holds at which pressure. Once the adsorption isotherm has appeared, parameters such as BET Surface Area (Single or Multipoint), Micropore Size Distribution (0.5 nm - 2 nm), Mesopore Size Distribution (2 nm - 50 nm), Total Pore Volume, Average Pore Size can be calculated. The surface area of the expanded perlite, adsorption, desorption and average pore width were measured. According to the results in *Table 5,* single point surface area of expanded perlite is 406.259 m^2/g, multipoint surface area of expanded perlite is 1171.73 m^2/g and the average pore size is 0.823 nm. The high surface area of the expanded perlite allows for the absorption of the absorbent feature. At the same time, the pore size is directly related to the diameter of the desired heavy metal molecule.

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Table 5. *Expanded of perlite values according to BET surface area analysis results*

BET analysis	Expanded perlite
Surface area	406.259 m^2/g
Pore volume	0.4605 cc/g
Total pore volume	0.4826 cc/g
Average pore size	0.823 nm
Molecular weight	28.0134 g
BJH* adsorption	143.344 m^2/g
BJH desorption	212.578 m^2/g
DH** adsorption	146.805 m^2/g
DH** desorption	221.815 m^2/g

*Barrett-Joyner-Halenda adsorption and desorption (BJH calculation is used for plotting a pore size distribution graph)
**Dollimore heal method (adsorption and desorption)

Conclusion

In this study, the expanded perlite was used as an adsorbent for the adsorption of heavy metals such as Mn (II) and Cu (II). The effects of contact time, pH, adsorbent dosage and inlet concentration parameters were investigated in adsorption experiments. According to the batch method, Mn^{2+} and Cu^{2+} ions showed fast adsorption tendency in 30 min for Mn^{2+} and 5 min for Cu^{2+} on the expanded perlite used as the adsorbent material as a result of the experiments to determine the turning point depending on the contact time. For both heavy metals these values were accepted as turning point. In the study where the amount of adsorbent is determined, the adsorbent amount in which a large part of the Mn^{2+} and Cu^{2+} concentrations are adsorbed is determined as 0.5 g/L for Mn and 0.4 g/L for Cu. It has been observed that the increase in the removal of the expanded perlite Mn^{2+} and Cu^{2+} is at pH 5. In the adsorption of Mn^{2+} and Cu^{2+}, $\log_q/\log_C$ values were plotted to determine the Freundlich isotherm, while Langmuir isotherm constants were determined by plotting $1/q$ and $1/C$ values. At an initial concentration of 0.5 mg/L Mn^{2+} heavy metal, the optimum point was assumed to be 30 min since 0.5 g of expanded perlite achieved the highest adsorption value at this time. In the study, Mn^{2+} heavy metals R^2 = 0.93 were obtained. It can be said that Langmuir isotherm is compatible with this high correlation value. Langmuir isotherm compliance indicates that the adsorbent surface is homogeneous and that the surface is covered by a single layer. At a concentration of 5 mg/L Cu^{2+}, the optimum point was assumed to be 5 min since 0.4 g of expanded perlite achieved the highest adsorption value at this time. In the study, Cu^{2+} heavy metals were found to conform to the Langmuir isotherm model with R^2 = 0.99. In this study, it has been found that economically expanded perlite can be used as an effective adsorbent material in the removal of heavy metals. Different processes can be developed by undertaking additional laboratory studies.

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A COMPARISON OF THE CATALYTIC OXIDATION OF FORMALDEHYDE OVER THREE TYPES OF CATALYSTS AT AMBIENT TEMPERATURE: THE EFFECT OF SUPERFICIAL VELOCITY

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Abstract. Catalytic oxidation of formaldehyde (HCHO) at ambient temperature is regarded as a promising technology for removal and destruction of HCHO contaminated air. In this study, three types of catalyst which are commercial HCHO catalyst, the catalyst of granular activated carbon supported Pt (Pt/GAC catalyst) and the catalyst of granular activated carbon supported MnOx (MnO_x/GAC catalyst) were chosen for oxidizing HCHO. The impact of superficial velocity, reaction bed height and the type of catalyst to the removal efficiency of HCHO was investigated. The fact that removal efficiency of HCHO increased firstly and then decreased with the decrease of superficial velocity illustrates its effect on the catalytic oxidation existed at an optimum value, depending on the nature of different catalysts, design of the reaction bed and the operation conditions. The steady state removal efficiencies of HCHO were all above 99% when the superficial velocity was 0.5, 1.0 and 1.5 m/s respectively, yet the needed height shortened with decreasing the superficial velocity. Among the three types of catalysts, Pt/GAC catalyst shows the effective and stable catalytic activity, whose removal efficiencies reached over 90% and the conversion did not change too much with the time increasing.
Keywords: *Pt/GAC catalyst, MnO_x/GAC catalyst, commercial HCHO catalyst, HCHO removal, reaction bed height, room temperature*

Introduction

Formaldehyde (HCHO) is regarded as one of the most common indoor air pollutants, which is emitted from building and furnishing materials (Perry, 1995). Owing to the teratogenicity and carcinogenicity, its pollution problem gets more and more attention (Salthammer et al., 2010; Lu et al., 2017). A series of efforts have been made for abating its emission at room temperature to meet the environmental regulations. Although the physical adsorption or chemical reaction has been proven to remove HCHO, the effectiveness is limited by the material's capacities (Nakayama et al., 2002). On the contrary, due to its low energy consumption and environment-friendly reaction conditions, room-temperature catalytic oxidation, which can completely oxidize HCHO into harmless CO_2 and H_2O, has become a promising technology for removal and destruction of HCHO contaminated air and has attracted extensive attention (Spivey, 1989; Qi et al., 2015; Yan et al., 2016; Ye et al., 2016; Yang et al., 2017).

Recently, dozens of studies have shown that the supported noble metal catalysts (such as Pt, Ru, Pd, Au and so on) exhibit high catalytic activity at room temperature (Yang et al., 2017; Zhang et al., 2006; Huang et al., 2011; An et al., 2013; Quiroz et al., 2013). Among them, the high efficiency of catalytic oxidation by supported Pt catalysts has been proven (Peng and Wang, 2007). It was reported by Zhang et al. (Zhang et al.,

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2006) that 100% HCHO decomposition into CO_2 and H_2O was achieved over 1 wt% Pt/TiO_2 catalyst at ambient temperature. However, because of high cost, this catalyst cannot be widely applied in the engineering field (Yusuf et al., 2017).

Another approach for removing HCHO is to combine activated carbon adsorption with catalytic oxidation (Granqvist et al., 2007). Many catalysts have been shown to have high removal efficiency when applied with activated carbon, such as photo-catalysts (Bashkova et al., 2011), silver catalysts (Li et al., 2016), metal oxides catalysts (Fang et al., 2017) and so on. Li et al. (Li et al., 2016) reported that the catalyst of granular activated carbon supported MnO_x had a high activity to remove HCHO at ambient temperature, but its removal efficiency dropped from 75% to 10% after running continuously for 30 h. Fang et al. (Fang et al., 2017) investigated the effectiveness of coconut shell activated carbon supported MnO_x for catalytic oxidation of HCHO at room temperature. The HCHO removal efficiency was kept almost at 100% during a 1000 min period.

For HCHO, the efficiency of the catalytic reaction is strongly affected by factors, such as temperature, humidity, gas hourly space velocity (GHSV), inlet HCHO concentration and the properties of the catalyst (preparation method, morphology and structure, specific surface active area, active sites, low-temperature reducibility and surface active oxygen species) and so on. It was reported that the removal efficiency of HCHO decreased with the increase of the inlet HCHO concentration and GHSV (Xia et al., 2010; Han et al., 2016). Chen et al. (Chen et al., 2013) examined the effect of GHSV on HCHO conversion over 1 wt% Au/CeO_2 (DPU) at room temperature in the range of 34000-143000 h^{-1}. The steady conversion of HCHO reached 100% at all GHSV (34000, 95500, 143000 h^{-1}), but the reaching time was reduced with the increase of GHSV. Besides, Han et al. (Han et al., 2016) investigated that HCHO catalytic efficiencies for all the prepared catalysts increased with the rising reaction temperature. Though various factors influencing the HCHO decomposition have been reported in many kinds of literature, there are few studies related to the effect of superficial velocity. Besides, finding high activity of catalyst to oxidize low-concentration HCHO at room temperature also needs to be investigated further (Zhang et al., 2005; Tang et al., 2008).

On the bases of the reports above, studies on the catalytic oxidation of low-concentration HCHO at room temperature still need to be further developed and completed. Therefore, in this study, we investigated the properties and efficiencies of two different typical catalysts (Pt/GAC catalyst and MnO_x/GAC catalyst) for removing HCHO, compared with the commercial HCHO catalyst. Moreover, the effects on catalytic oxidation were studied concerning the factors of superficial velocity, reaction bed height and the types of catalysts.

Materials and methods

Catalyst preparation

The catalysts used in this study were commercial HCHO catalyst ($10 \times 10 \times 10$ cm^3, five blocks), Pt/GAC catalyst ($10 \times 10 \times 10$ cm^3, one block), and the MnO_x/GAC catalyst. The activated carbon used in this study was coal-based columnar carbon with an averaged diameter of 4 mm. The carbon tetrachloride adsorption activity of GAC was 82.95%. The details of Pt/GAC catalyst preparation were as follows: Chloroplatinic acid solution with Pt was prepared and used as the impregnation liquid, then GAC ($10 \times 10 \times 10$ cm^3 per block) was immersed in the solution at room temperature till they

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are mixed completely. A series of steps including dehumidification, purification and reduction reaction were completed in the preparation (Zhang et al., 2015; Zhu et al., 2016). The MnO_x/GAC catalyst was obtained via in-situ reduction of potassium permanganate with GAC. The MnO_x/GAC catalyst was prepared by the reaction of in-situ synthesis. The details of MnO_x/GAC catalyst preparation were as follows: the GAC was immersed in the $KMnO_4$ solution (concentration of 1%) for 1 h. The obtained MnO_x/GAC catalyst was then filtered and dried at 120 °C for 1 h (Fang et al., 2017).

Experimental setup

The general configuration of the apparatus used to test the catalytic performance with the commercial HCHO catalyst or the Pt/GAC catalyst is depicted in *Figure 1.* The apparatus consisted of three parts: gas distribution system, catalytic system and detection system. Liquid HCHO (ACS reagent grade) was delivered by a syringe pump (LongerPump, LSP01-2A, United Kingdom), evaporated and mixed with contaminant-free compressed air. The air mixture flowing through a rotameter and a buffer to measure and regulate the gas flow contained 9 ppm HCHO and clean air, which was introduced as the reactants. The catalytic system included a wind turbine and catalytic reactor. Owing to the large catalyst resistance, the catalytic system had a wind turbine. The catalytic oxidation of HCHO was performed in a quartz tubular fixed-bed reactor (five levels) under atmospheric pressure at room temperature (25 ± 1 °C). The catalyst was respectively loaded in the five-story reactor, installing a perforated stainless-steel plate at the bottom of each section. Valves were installed in the front of and behind every catalytic unit to measure contaminant concentrations.

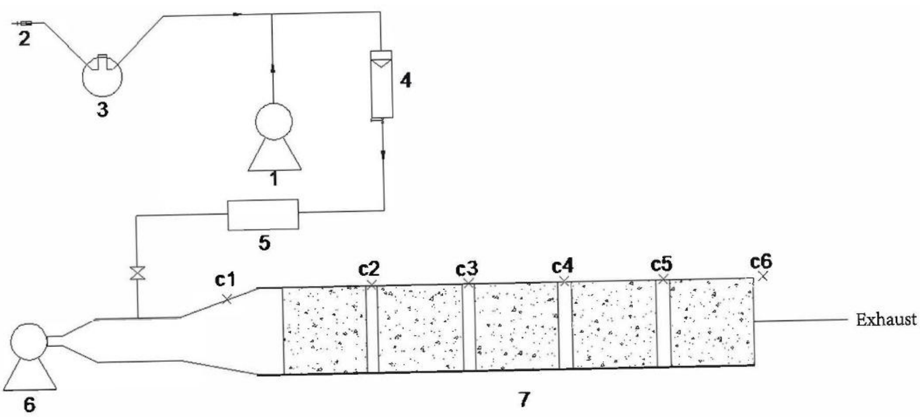

Figure 1. *Schematic diagram of apparatus used for catalytic performance with the commercial HCHO catalyst or the Pt/GAC catalyst. (1, 6: blower; 2: syringe pump; 3: temperature-controlled magnetic stirrer; 4: rotameter; 5: surge flask; 7: catalytic unit; c1-c6: sampling port)*

The Catalytic performance with the MnO_x/GAC catalyst in the experiment was set up as shown in *Figure 2.* As in the above process, the air mixed with HCHO, then adjusted to the desired concentration. The catalyst (the depth of 10 cm) was installed in the packed-columns which were made of glass (i.d. 40 mm) and operated in the downflow mode. Valves were installed before the catalytic unit inlet and after the catalytic column to measure contaminant concentrations.

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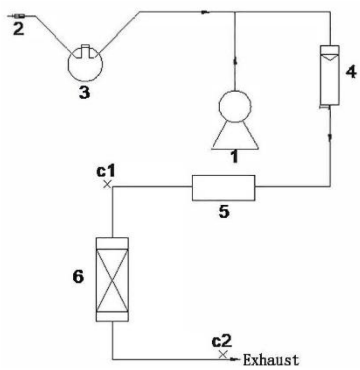

***Figure 2.** Schematic diagram of apparatus used for catalytic performance with the MnOx/GAC catalyst. (1: blower; 2: syringe pump; 3: temperature-controlled magnetic stirrer; 4: rotameter; 5: surge flask; 7: catalytic unit; c1-c2: sampling port)*

Experimental testing and analytical techniques

During the catalytic oxidation of HCHO, ~9 ppm HCHO was injected mixed with clear air as a contaminated gas. The superficial velocity was set by 0.5, 1.0, 1.5 m/s for the commercial HCHO catalyst and Pt/GAC catalyst and 0.2, 0.5, 1.0 m/s for the MnO_x/GAC catalyst. The HCHO in the airstream was analyzed by an HCHO monitor (Formaldemeter 400, PPM Technology, United Kingdom).

The results of removal efficiency of HCHO under different parameters were compared and analyzed statistically. The multivariate analysis was applied to identify the significance of selected differencing factors.

Equation 1 below shows the complete reaction process of HCHO decomposition in the catalytic oxidation:

$$HCHO + O_2 \rightarrow CO_2 + H_2O \tag{Eq.1}$$

The results of each selected influencing factors of removal efficiency were analyzed statistically. The HCHO removal efficiency was calculated by *Equation 2* as follows:

$$\text{The Removal efficiency of HCHO} = \frac{[HCHO]_{in} - [HCHO]_{out}}{[HCHO]_{in}} \times 100\% \tag{Eq.2}$$

where $[HCHO]_{in}$ and $[HCHO]_{out}$ are the inlet and outlet HCHO concentration respectively (both expressed in ppm).

Results and discussion

The influence factors of the catalytic reaction, including superficial velocity, reaction bed height, the types of catalysts, were studied to evaluate the catalytic activity and stability of the catalysts.

Effect of superficial velocity on removal efficiency of HCHO

Superficial velocity has a vital effect on the adsorption time and catalytic oxidation time, but only limited studies focused on it. Keeping the inlet HCHO concentration of

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9 ppm, the impact of superficial velocity on the catalytic performance was investigated by setting the velocity from 0.5 to 1.5 m/s for the commercial HCHO catalyst and Pt/GAC catalyst and from 0.2 to 1.0 m/s for the MnO_x/GAC catalyst. The results are shown in *Figure 3*. For the Pt/GAC catalyst (*Fig. 3b*) and the MnO_x/GAC catalyst (*Fig. 3c*), when the superficial velocity was increased, the removal efficiency for HCHO would be decreased. However, for the commercial HCHO catalyst (*Fig. 3a*), the removal efficiencies from the highest to the lowest were SV = 1.0 m/s > SV = 1.5 m/s > SV=0.5 m/s.

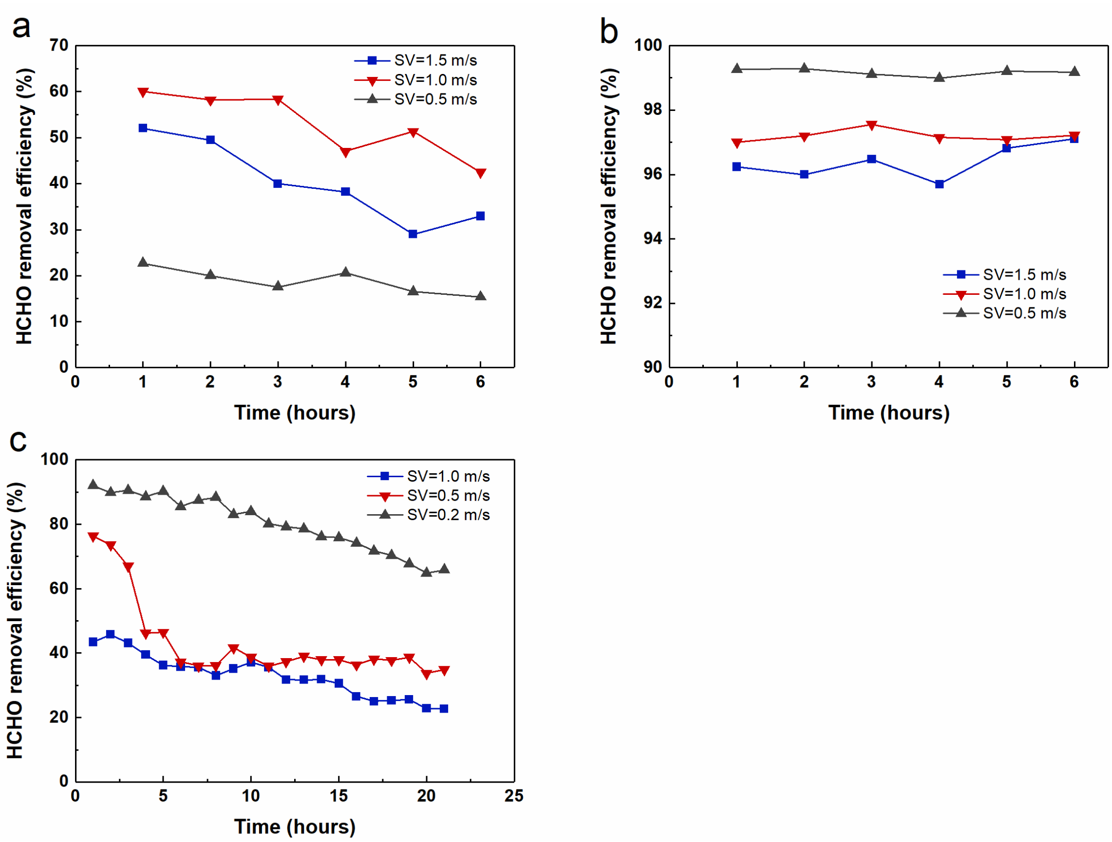

Figure 3. *Removal efficiencies of HCHO over three types of catalysts under different superficial velocities. (a: the commercial HCHO catalyst; b: the Pt/GAC catalyst; c: the MnOx/GAC catalyst; SV refers to superficial velocity)*

According to the *Figure 3,* the nature of different catalysts showing different catalytic activities was related to the influence of superficial velocity on removal efficiency of HCHO. For the commercial HCHO catalyst, in a certain scale, the removal efficiency increased with the reduction of superficial velocity. Further decrease of the superficial velocity to 0.5 m/s appeared no positive effect on HCHO conversion, even a sharp decrease. These results can be understood by considering the fact that HCHO molecules fully exposed to the catalyst and participated in the removal reaction per unit would increase with the decrease of superficial velocity, which resulted in improving the removal efficiency correspondingly. Zhang et al. (Zhang et al., 2005) investigated the impact of air-flow rate on catalytic performance, which illustrates that the removal efficiency of HCHO in the catalytic oxidation was inversely proportional to the air-flow

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rate and the correlation coefficient (R^2) reached 0.99. However, below a certain range of superficial velocity, because of the increase of residence time and HCHO molecules, the competition for the active catalytic sties on the catalyst surface would strengthen, leading to a decline of the removal efficiency. The Pt/hydrophobic catalyst was tested under different gas flow rates by Chuang et al. (1994), where HCHO conversion percentage showed a small increase when the gas flow rate increased from 6 to 18 L/h. The fact shows that the catalytic oxidation was dominated by the diffusion rate of HCHO molecules onto the surface of catalyst. For the Pt/GAC catalyst and the MnO_x/GAC catalyst, the fact that removal efficiency of HCHO rose with a decrease in the superficial velocity indicated that the catalytic oxidation was dominated by HCHO molecules onto the catalyst surface, and the competitive reactions between molecules were not obvious. Therefore, the optimal superficial velocity of the Pt/GAC catalyst and the MnO_x/GAC catalyst needed to be investigated further. Huang et al. (Huang et al., 2011) confirmed the conversion of HCHO decreased with the increase of GHSV through examining the effect of GHSV on the catalytic oxidation of HCHO in the range of 40000-240000 h^{-1}. The steady conversion of HCHO was 100% at 40000 h^{-1} which dropped to 90.5% at 240000 h^{-1}.

Overall, these results show that superficial velocity is an important factor which influences the removal efficiency of HCHO, which has the two-side influence on the catalytic oxidation. For one thing, due to the external mass transfer, higher superficial velocity can increase the removal efficiency of HCHO. For another thing, the residence time reduces with the increase of the velocity, resulting in the decrease of removal efficiency. Therefore, the effect of the superficial velocity on the catalytic oxidation existed an optimum value, depending on the nature of different catalysts, design of the reaction bed and the operation conditions (Pei and Zhang, 2011).

Effect of reaction bed height on removal efficiency of HCHO

The effect of reaction bed height on the removal efficiency over the commercial HCHO catalyst at ambient temperature was further investigated by keeping the inlet HCHO concentration at 9 ppm. A plot of removal efficiencies versus reaction bed height determined in the present study operated under different operation times and superficial velocities were conducted as shown in *Figure 4*. The reaction bed heights of 0-50 cm correspond to the height of measured sampling point 1-6, respectively.

For each superficial velocity, the similar trend of removal efficiency appears at different operation time – that the removal efficiency of HCHO at different operation time increased with the rise of reaction bed heights and kept almost unchanged over a certain height. This result suggests that higher reaction bed height provides more reaction time as a result of the fact that the removal efficiency of HCHO increases more.

From *Figure 4a,* keeping the superficial velocity of 1.5 m/s, removal efficiency of HCHO in the height of 10 cm follows the order: T = 1 h > 2 h > 3 h > 4 h > 6 h > 5 h at different operation time, and the efficiency was 52.06% at 1h, dropping to 29.01% at 5 h. With the increase of reaction bed height, the difference of removal efficiency at different operation time decreased gradually, and the efficiencies all reached to above 98% in the height of 40 cm.

As shown in *Figure 4b,* keeping the superficial velocity of 1.0 m/s and removal efficiency of HCHO in the height of 10 cm follows the order: T = 1h ≈ 2h ≈ 3h > 5h > 4 h > 6 h at different operation time, and the efficiency was 60.06% at 1 h, dropping to

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42.52 % at 6 h. Compared with the efficiency under the superficial velocity of 1.5 m/s, the difference among different operation time narrowed, and the efficiency had improved some. When the height was above 20 cm, the efficiency has little difference at different operation time, reaching to about 95%.

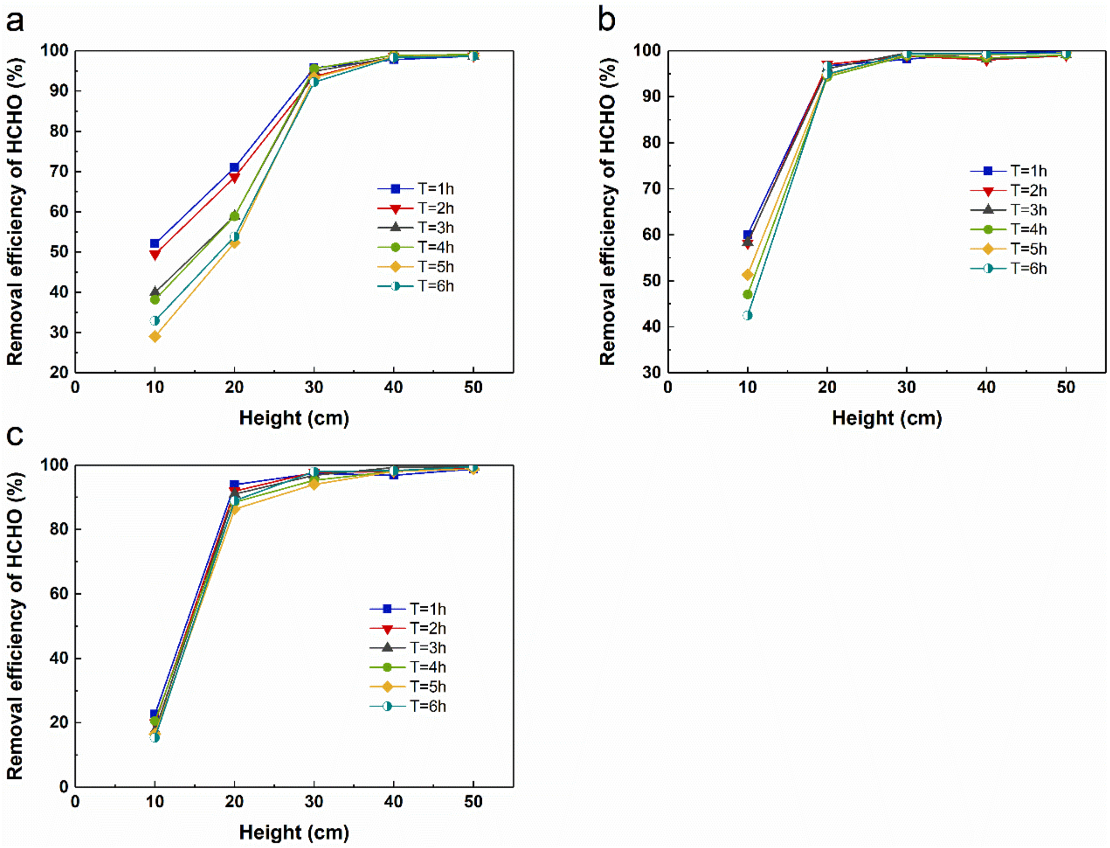

Figure 4. *Removal efficiencies of HCHO over the commercial HCHO catalysts under different reaction bed heights. (a: superficial velocity = 1.5 m/s; b: superficial velocity = 1.0 m/s; c: superficial velocity = 0.5 m/s)*

The change of removal efficiency with reaction bed height under the superficial velocity of 0.5 m/s was seen in *Figure 4c*. There was little difference at the different operation time in the same height. However, the removal efficiency of HCHO decreased when superficial velocity dropped to 0.5 m/s, only 20% or so at the height of 10 cm.

Overall, the steady state removal efficiencies of HCHO were all above 99% when the superficial velocity was 0.5, 1.0 and 1.5 m/s respectively, yet the needed height shortened with decreasing the superficial velocity, which means that superficial velocity and reaction bed height both play important roles in the catalytic oxidation for HCHO.

To further discuss the effect of reaction bed height to the HCHO destruction, experimentally measured HCHO concentration changing curves, conducted under different superficial velocities and reaction bed heights are shown in *Figure 5*. Each data point in the graph illustrates the average of outlet concentration measured when it ran for one hour.

From *Figure 5,* it is evident that HCHO concentration collected when superficial velocity was 0.5 and 1.0 m/s reduced to 0.2 ppm under the height of 20 cm. However,

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HCHO concentration measured when the superficial velocity was 1.5 m/s dropped to 0.2 ppm under the height of 30 cm. With the increase of superficial velocity, the residence time and contact time reduced, which led to the fact that it decreased the removal efficiency of HCHO and needed higher reaction bed to meet the emission standard. After the contaminated air was treated under a certain value, no influence of reaction height was observed on the HCHO concentration.

From the micro-perspective, the slope of that curve represents removal rate of HCHO. According to the results, in the certain height range, the removal rate of HCHO increased with the rise of reaction bed height. When the concentration dropped to certain levels, the removal rate began to decrease rapidly and down to zero finally. This result was consistent with the variation of removal efficiency with reaction bed height.

Multivariate analysis was applied to determine the significance of height and superficial velocity which indicates that reaction bed height has important influence in the removal efficiency of HCHO ($F = 46.362 > F$ crit $= 3.326$) and superficial velocity has no reliable effect ($F = 0.969 < F$ crit $= 4.103$).

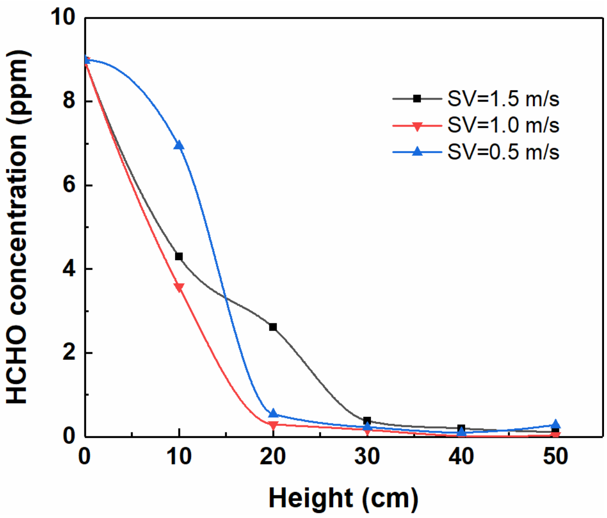

Figure 5. *HCHO concentration over the commercial HCHO catalysts under different reaction bed heights. (SV refers to superficial velocity)*

Effect of catalyst types on removal efficiency of HCHO

The effect of catalyst types on the removal of HCHO was studied to enhance the removal efficiency and improve the catalytic oxidation method. The catalytic activities of three types of catalysts were evaluated for HCHO conversion under the same superficial velocity (0.5 and 1.0 m/s respectively) as shown in *Figure 6*.

In the case of Pt/GAC catalyst, HCHO could be almost degraded into CO_2 and H_2O under both superficial velocities, whose removal efficiencies reached over 90% and the conversion did not change too much with the time increasing. However, the other catalysts showed lower effects in the same conditions. While the superficial velocity was 1.0 m/s, the commercial HCHO catalyst had a better removal effect than the MnO_x/GAC catalyst, but the conclusion was opposite when superficial velocity dropped to 0.5 m/s. As shown in *Figure 6a,* the removal efficiency of commercial HCHO catalyst dropped slowly from 60.06% to 42.52% after continuously running 6 h, while the efficiency over MnO_x/GAC catalyst fluctuated around 40%. From the *Figure 6b,* the

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removal efficiency of HCHO over MnO_x/GAC catalyst at the superficial velocity of 0.5 m/s dropped dramatically from 76.42 to 37.27% within 6 h, while the efficiency over commercial HCHO catalyst changed little, always taking up 20% or so. Therefore, it can be concluded that the influence of catalyst on HCHO removal rate was related to superficial velocity. Besides, these findings illustrated that different activities of different types of catalysts depend on the different capacities for oxidizing HCHO (Zhang and He, 2007). In addition, the support makes a difference in the catalytic oxidation of HCHO at ambient temperature (Colussi et al., 2015).

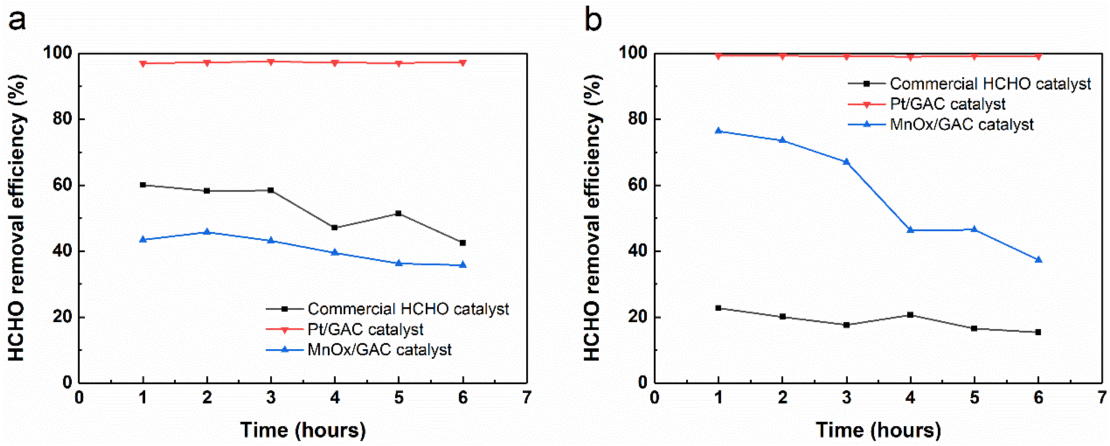

Figure 6. *Removal efficiencies of HCHO over three types of catalysts under different superficial velocities. (a: superficial velocity = 1.0 m/s; b: superficial velocity = 0.5 m/s)*

Among the three catalysts, the fact that Pt/GAC catalyst shows the effective and stable catalytic property suggests that it is a promising catalyst for HCHO oxidation, but the expensive cost blocked its application from commercialization. The commercial HCHO catalyst possessed the best economical interest and certain catalytic ability but had poor stability. There was no advantage in both the price and catalytic performance of commercial HCHO catalyst. Hence, the selection of catalyst needs to consider the effects of multiple factors such as removal performance, residence time and economy comprehensively.

Conclusions

In this study, compared with the commercial HCHO catalyst, two typical catalysts (Pt/GAC catalyst and MnOx/GAC catalyst) were studied for catalytic oxidation of HCHO at ambient temperature. Different influencing factors of catalytic oxidation on the removal efficiency such as superficial velocity, reaction bed height and the type of catalyst were investigated.

According to the results, the superficial velocity influenced the removal efficiency of HCHO, and there was an optimum value for catalytic oxidation which depends on the type of catalyst. The removal efficiency of HCHO increased firstly and then decreased with the decrease of superficial velocity. For the commercial HCHO catalyst, the removal efficiencies of HCHO from the highest to the lowest were SV = 1.0 m/s > SV = 1.5 m/s > SV = 0.5 m/s. The removal efficiency over the commercial HCHO catalyst could be enhanced by increasing the reaction bed heights.

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HCHO concentration measured when superficial velocity was 0.5 and 1.0 m/s reduced to 0.2 ppm under the height of 20 cm, but when the superficial velocity was 1.5 m/s, the height has to be 30 cm to meet the emission standard. Among the three types of catalysts, Pt/GAC catalyst shows the effective and stable catalytic property, whose removal efficiencies reached over 90% and the conversion did not change too much with the time increasing.

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THE EFFECT OF MANURING WITH UNDERSOWN WHITE MELILOT ON THE CONTENT OF MICROELEMENTS IN THE TUBERS OF POTATO CULTIVATED IN VARIOUS PRODUCTION SYSTEMS

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Abstract. The work presents results of studies conducted from 2008 to 2011 to determine the effect of undersown catch crops, which were either autumn-incorporated or left on the soil surface as mulch for spring incorporation, on the content microelements in potato tubers cultivated in various production systems. The following two factors were examined: I. − manuring with an undersown catch crop: control, farmyard manure, white melilot, white melilot + westerwolds ryegrass, westerwolds ryegrass, white melilot applied as mulch, white melilot + westerwolds ryegrass applied as mulch, westerwolds ryegrass applied as mulch; II. − production system: integrated and organic. Potato tuber samples were taken to determine microelements. The results demonstrated that manuring of potato with undersown catch crops, in particular autumn-incorporated white melilot, increased iron, zinc and boron contents, and reduced copper and manganese contents in potato tubers. Potatoes cultivated in the integrated production system had a higher concentration of copper, manganese and zinc whereas organic tubers had more iron and boron.
Keywords: *root crop, green manure, mulch, integrated cultivation, organic cultivation, minerals*

Introduction

Potato is undoubtedly one of our most valuable crop plants and farmyard manure is the basic natural manure applied in potato cultivation. As farmyard manure production has been declining due to a decreasing number of livestock and the development of integrated and organic potato growing, alternative solutions are being sought. In these circumstances, green manures are increasing in popularity (Redulla et al., 2005; Rożyło and Pałys, 2009; Płaza et al., 2015), and undersown catch crops seem to be the cheapest source of organic matter (Płaza et al., 2017). It has been observed that they beneficially affect yields and chemical composition of potato tubers (Różyło and Pałys, 2009; Wierzbicka and Trawczyński, 2011; Płaza et al., 2017). The nutritional value of potato tubers is also determined by minerals, including micronutrients, which after digestion and absorption into the blood are used by the body as a building material or a factor regulating life processes (Wszelaki et al., 2005). In order for the human body to function properly, it must receive from the outside all the necessary micronutrients, especially iron, zinc, boron, copper and manganese. However, there is a paucity of Polish studies on the influence of undersown white melilot on the potato tuber content of microelements. Consumers expect potato producers, in particular organic potato growers, to provide more nutritious tubers, but there is still little research comparing the chemical composition of potato tubers produced in various production systems. Hence,

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the need arises to conduct this type of research. The purpose of the study reported here was to determine the effect of undersown catch crops, which were either autumn-incorporated or left as mulch on the soil surface for spring incorporation, on the content of microelements in the tubers of potato grown in various production systems.

Materials and methods

A field experiment was carried out at the Zawady Experimental Farm (50°20’ N, 22°30’ E) which belongs to Siedlce University of Natural Sciences and Humanities, Poland in 2008-2011. The experimental soil was Albic Luvisol (Arenic), soil valuation class IVa. The soil contents of available minerals were as follows: P 5.29 $mgkg^{-1}$, K 11.59 $mgkg^{-1}$, Mg 5.60 $mgkg^{-1}$, Mn 114 $mgkg^{-1}$, Cu 1.8 $mgkg^{-1}$, B 0.54 $mgkg^{-1}$, Zn 7.5 $mgkg^{-1}$, Fe 654 $mgkg^{-1}$. Soil reaction was neutral and humus content amounted to 1.37%. The experiment was a split-block arrangement with three replicates. The following two factors were examined: I. − manuring with an undersown catch crop: control (no undersown catch crop manure), farmyard manure (30 tha^{-1}), *Melilotus albus* (seed sowing rate 26 $kgha^{-1}$), *Melilotus albus* + *Lolium westerwoldicum* (seed sowing rate 13 + 10 $kgha^{-1}$), *Lolium westerwoldicum* (seed sowing rate 20 $kgha^{-1}$), *Melilotus albus* applied as mulch (seed sowing rate 26 $kgha^{-1}$), *Melilotus albus* + *Lolium westerwoldicum* applied as mulch (seed sowing rate 13 + 10 $kgha^{-1}$), *Lolium westerwoldicum* applied as mulch (seed sowing rate 20 $kgha^{-1}$); II. − production system: integrated and organic.

In autumn, fresh matter yield of undersown catch crops, including their root mass to the depth of 30 cm, was determined in a 1 m^2 area in each plot. The average yields calculated across three years in the integrated and organic production systems were, respectively, 27.5 and 22.9 tha^{-1} for *Melilotus albus*, 31.7 and 25.8 tha^{-1} for *Melilotus albus* + *Lolium westerwoldicum*, and 35.4 and 27.6 tha^{-1} for *Lolium westerwoldicum*.

Table potato was preceded by spring triticale grown for grain which was undersown with catch crops. In the integrated production system, mineral fertilisers were applied to the whole experimental area in early spring. Their rates per 1 ha were as follows: 90 kg N, 36.9 kg P and 99.6 kg K. The rates were adjusted to soil availability and projected yield levels. In autumn-ploughed plots, mineral fertilisers were spread in the spring and mixed with the soil by means of a cultivator with a harrow attached to it. In mulched plots, a disc harrow was followed by a cultivator. In the organic system, instead of mineral fertilisers farmyard manure was applied at the rate of 30 tha^{-1} to the whole experimental area, and followed by spring triticale which was undersown with catch crops. Potatoes were planted in late April and harvested in mid-September. In the integrated production system, weeds, pests and diseases were controlled using mechanical practices and chemicals. Prior to emergence, potatoes were hilled and harrowed every 7 days, and just before emergence, they were sprayed with a mixture of the herbicides Afalon 50 WP + Reglone Turbo 200 SL (1 kg + 1 dm^3ha^{-1}). Colorado potato beetle and late blight were controlled by means of two applications of, respectively, the pesticide Fastac (0.1 dm^3ha^{-1}) and the fungicide Ridomil MZ WP (2 dm^3ha^{-1}). Organic potatoes were mechanically protected against weeds. Colorado potato beetle was controlled using two applications of Novodor SC (2.5 dm^3ha^{-1}), and Miedzian 50 WP (4 $kgha^{-1}$) was applied three times to keep late blight in check. During potato harvest, after 10 tuber samples were collected in each plot to analyse for microelements. Cu, Fe, Mn, Zn and B contents were determined in the dry matter of potato tubers by means of the Inductively Coupled Plasma Optical Emission

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Spectrometry (ICP-OES) at wavelength: Cu 327.393 nm, Fe 238.204 nm, Mn 257.601 nm, Zn 206.200 nm, B 249.677 nm.

Data obtained for each of the characteristics studied was analysed by means of ANOVA suitable for the split-block mathematical model, according to the model: yijl = n + ai + gj + eij (1) + bl + ejl (2) + abil + eijl (3), where a = 1.2; b = 1, 2..8; n = 1, 2, 3 (number of repetitions); yijl – value of the examined feature; ai - the effect of the i-th factor A; gj - the effect of repetitions (blocks); eij (1) - error 1 resulting from factor A interaction and repetition; bl - the effect of the 1st level factor B; ejl (2) - error 2 resulting from interaction factor B and repetitions; abil - effect of factor interaction A and B; eijl (3) - random error. When significant sources of variation were detected, Tukey test was applied to separate the means.

Results

The copper content of potato tuber

Copper content in potato tubers was significantly affected by the experimental factors and their interaction (*Table 1*). The highest copper content was recorded in the tubers of control potato. An application of farmyard manure and undersown catch crops contributed to a significant decline in the potato tuber content of copper. The highest content was recorded in the tubers of potato following autumn-incorporated white melilot. Also production system had a significant effect on the concentration of copper in potato tubers, it being higher in the tubers of potato grown using integrated farming practices than in organic tubers. An interaction between the experimental factors was confirmed. It indicated that the highest copper content was found in the tubers of control potato following mineral fertiliser only, in the integrated production system, and the lowest concentration was determined in the tubers of organic potato following farmyard manure and undersown catch crops.

Table 1. *Copper content in potato tubers depending on the manuring with undersown catch crop and production system (means over 2009-2011), mgkg^{-1} d.m.*

Manuring with undersown catch crop	Production system		Means
	Integrated	**Organic**	
Control	5.085	4.763	4.924
Farmyard manure	4.850	4.635	4.743
White melilot	4.737	4.554	4.646
White melilot + westerwolds ryegrass	4.794	4.605	4.699
Westerwolds ryegrass	4.839	4.613	4.726
White melilot – mulch	4.743	4.601	4.672
White melilot + westerwolds ryegrass – mulch	4.801	4.617	4.709
Westerwolds ryegrass – mulch	4.848	4.622	4.735
Means	4.837	4.626	-
ANOVA	P-value		$LSD_{0.05}$
Manuring with undersown catch crop	<0.001		0.077
Production system	<0.001		0.071
Interaction	0.003		0.095

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The iron content of potato tuber

Statistical analysis revealed a significant influence of the experimental factors and their interaction on iron content in potato tubers (*Table 2*). The lowest iron content was recorded in the tubers of potato harvested in the control unit. A application of farmyard manure and undersown catch crops resulted in a significant increase in the potato tuber content of iron. The highest concentration of this element was recorded in the tubers of potato following spring-incorporated white melilot. Iron content in the tubers of potato manured with an autumn-incorporated mixture of white melilot and westerwolds ryegrass, and white melilot left as mulch on the soil surface for spring incorporation differ insignificantly from the concentration of iron in the tubers of potato following farmyard manure. In the remaining plots, iron content was lower compared with farmyard manure. Production system significantly affected iron content in potato tubers, it being higher in the tubers of organic potato. An interaction between the experimental factors was confirmed, which demonstrated that the highest iron content was recorded in the tubers of potato manured with autumn-incorporated white melilot in the integrated and organic potato system, it being the lowest in the tubers of control potato, following mineral fertilisers only, in the integrated production system.

Table 2. *Iron content in potato tubers depending on the manuring with undersown catch crop and production system (means over 2009-2011), mgkg^{-1} d.m.*

Manuring with undersown catch crop	**Production system**		**Means**
	Integrated	**Organic**	
Control	43.39	45.44	44.42
Farmyard manure	51.80	53.24	52.52
White melilot	54.86	56.01	55.44
White melilot + westerwolds ryegrass	50.77	51.84	51.31
Westerwolds ryegrass	46.44	47.52	46.98
White melilot – mulch	51.33	53.40	52.37
White melilot + westerwolds ryegrass – mulch	49.48	50.56	50.02
Westerwolds ryegrass – mulch	45.39	46.81	46.10
Means	49.18	50.60	-
ANOVA	P-value		$LSD_{0.05}$
Manuring with undersown catch crop	<0.001		1.29
Production system	<0.001		0.83
Interaction	0.001		1.43

The manganese content of potato tuber

Manganese content in potato tubers was significantly affected by the experimental factors and their interaction (*Table 3*). The lowest concentration of manganese was determined in the tubers of control potato. An application of farmyard manure and undersown catch crops contributed to a significant decline in the potato tuber content of manganese. The lowest concentration of this element was recorded in the tubers of potato following undersown catch crops, whether they had been ploughed down in autumn or left as mulch on the soil surface for spring incorporation. Production system

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had a significant influence on the potato tuber content of manganese, it being higher in the tubers of potato grown in the integrated versus organic production system. An interaction between the experimental factors was confirmed. It explains why the highest manganese content was found in the tubers of control potato, which followed mineral fertiliser only, in the integrated production system, and the lowest concentration was determined in the tubers of organic potato manured with *Melilotus albus*, regardless of when the undersown catch crop had been incorporated.

Table 3. *Manganese content in potato tuber depending on the manuring with undersown catch crop and production system (means over 2009-2011), mgkg^{-1} d.m.*

Manuring with undersown catch crop	**Production system**		**Means**
	Integrated	**Organic**	
Control	8.868	8.635	8.752
Farmyard manure	8.653	8.466	8.559
White melilot	8.260	8.135	8.198
White melilot + westerwolds ryegrass	8.361	8.244	8.303
Westerwolds ryegrass	8.448	8.341	8.395
White melilot – mulch	8.319	8.204	8.365
White melilot + westerwolds ryegrass – mulch	8.411	8.318	8.365
Westerwolds ryegrass – mulch	8.508	8.410	8.459
Means	8.479	8.344	-
ANOVA	P-value		$LSD_{0.05}$
Manuring with undersown catch crop	<0.001		0.084
Production system	<0.001		0.079
Interaction	0.001		0.095

The zinc content of potato tuber

Statistical analysis demonstrated a significant effect of the factors examined in the experiment and their interaction on zinc content in potato tubers (*Table 4*).

Table 4. *Zinc content in potato tuber depending on the manuring with undersown catch crop and production system (means over 2009-2011), mgkg^{-1} d.m.*

Manuring with undersown catch crop	**Production system**		**Means**
	Integrated	**Organic**	
Control	11.71	10.87	11.29
Farmyard manure	13.33	12.46	12.90
White melilot	14.66	13.13	13.90
White melilot + westerwolds ryegrass	13.88	12.95	13.42
Westerwolds ryegrass	13.03	12.66	12.85
White melilot – mulch	13.91	13.20	13.56
White melilot + westerwolds ryegrass – mulch	13.39	12.55	12.97
Westerwolds ryegrass – mulch	12.80	12.02	12.41
Means	13.34	12.48	-
ANOVA	P-value		$LSD_{0.05}$
Manuring with undersown catch crop	<0.001		0.69
Production system	<0.001		0.50
Interaction	0.003		0.91

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The highest zinc content was recorded in the tubers of potato harvested in the control unit. An application of farmyard manure and undersown catch crops resulted in an increase in the concentration of zinc in potato tubers. The highest zinc content was determined in the tubers following autumn-incorporated white melilot. There were no significant differences between the zinc contents in the tubers of potato following farmyard manure and the remaining undersown catch crops. Production system significantly affected the potato tuber content of zinc, too. The concentration of this element was higher in the tubers of potato cultivated using integrated farming practices rather than organic management. An interaction between the experimental factors was confirmed. It explained why the highest zinc content was recorded in the tubers of potato manured with autumn-incorporated *Melilotus albus* and a mixture of *Melilotus albus* and *Lolium westerwoldicum* in the integrated production system, and the lowest concentration of zinc was in control tubers grown in both the production system.

The boron content of potato tuber

Boron content in potato tubers was significantly affected by the experimental factors and their interaction (*Table 5*).

Table 5. *Boron content in potato tuber depending on the manuring with undersown catch crop and production system (means over 2009-2011), mgkg^{-1} d.m.*

Manuring with undersown catch crop	Production system		Means
	Integrated	Organic	
Control	4.635	4.744	4.689
Farmyard manure	5.337	5.473	5.405
White melilot	5.631	5.837	5.734
White melilot + westerwolds ryegrass	5.395	5.578	5.487
Westerwolds ryegrass	5.131	5.297	5.214
White melilot – mulch	5.546	5.701	5.624
White melilot + westerwolds ryegrass – mulch	5.293	5.442	5.368
Westerwolds ryegrass – mulch	5.029	5.170	5.099
Means	5.250	5.405	-
ANOVA	P-value		$LSD_{0.05}$
Manuring with undersown catch crop	<0.001		0.063
Production system	<0.001		0.044
Interaction	0.001		0.087

The lowest boron content was found in the tubers of control potato. An application of farmyard manure and undersown catch crops contributed to an increase in the potato tuber content of boron. The highest concentration of this element was recorded in the tubers of potato grown in plots amended with *Melilotus albus*, whether it was autumn-incorporated or left as mulch on the soil surface for spring incorporation. Boron content in the tubers of potato following a mixture of *Melilotus albus* and *Lolium westerwoldicum* differ insignificantly from the value recorded determined in the tubers harvested in farmyard manure-amended plots. In the remaining units manured with undersown catch crops, boron content in potato tubers was significantly lower compared with the tubers of potato following farmyard manure. Production system had

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a significant impact on the potato tuber content of boron. The concentration of this element was higher in the tubers of organic potato compared with the integrated production system. There was a confirmed interaction between the experimental factors which provided an explanation why the highest boron content was recorded in the tubers of organic potato following autumn-incorporated *Melilotus albus*, and the lowest concentration was determined in control tubers, following mineral fertiliser only, in the integrated production system.

Discussion

Microelements are as important as macroelements. However, their content in potato tubers is much lower. They are components of enzymes which activate a range of biochemical processes (Różyło and Pałys, 2009; Wierzbicka and Trawczyński, 2011; Wierzbicka, 2012).

In this study, the highest copper content was determined in control tubers which were harvested in plots where only mineral fertilisers had been applied. Also Klikocka (2009) as well as Braun et al. (2011) obtained a higher copper content in the tubers of potato following mineral fertiliser. In the experiment reported here, an application of farmyard manure and undersown catch crops prior to potato cultivation contributed to a significant decline in the tuber content of copper. Trawczyński and Bogdanowicz (2007), Wierzbicka and Trawczyński (2011), Wierzbicka (2012) and Osvalde et al. (2016) found that vermicompost and green manures reduced copper content in potato tubers. Wierzbicka and Trawczyński (2011) in organic potato cultivation with the use of stubble catch crops with white mustard, field pea and serradella in irrigation conditions showed a decrease in copper content in tubers. This is in line with the results of the Wierzbicka study (2012), which investigated the copper content in potato tubers of cultivated also in the organic production system of manured with stubble catch crops without irrigation. However, in the discussed experiment a different form of green manure was used, undersown catch crop, both plowed in autumn and left to spring in the form of mulch, which in Polish conditions has not been studied so far. It was shown that manuring the potato with *Melilotus albus* also caused a decrease in copper content in tubers. The experiment was carried out in two production systems, not only in organic one, as in the studies of Wierzbicka and Trawczyński (2011) or Wierzbicka (2012).

Similarly to Hajšlovă et al. (2005) Wang et al. (2008), Hunter et al. (2011) and Sawicka et al. (2016), in the present study, copper content was lower in organic potato tubers.

In the experiment reported here, the lowest iron content was determined in control tubers, which supports findings reported by Braun et al. (2011), Hadi et al. (2014) and Ashafzadeh et al. (2017) who demonstrated that iron content was the lowest in the tubers of potatoes following mineral fertiliser only. In the current study, farmyard manure and undersown catch crops contributed to an increase in the potato tuber content of iron. It follows from the fact that farmyard manure and green manures introduce additional amounts of macroelements and microelements into the soil (Wierzbicka and Trawczyński, 2011; Płaza et al., 2017). In the experiment discussed here, tubers of organic potato following green manures and farmyard manure contained significantly more iron compared with the integrated system. Such a relationship was also observed

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in the studies by Wiśniowska-Kieljan and Klima (2007), Wierzbicka (2012) and Sawicka et al. (2016).

In the present study, the highest manganese content was determined in the tubers of control potato following mineral fertiliser only. Similar findings were reported by White et al. (2009), Braun et al. (2011), Hadi et al. (2014) and Baranowska et al. (2017). Undersown catch crops and farmyard manure applied in the experiment reported here contributed to a significant decline in the potato tuber content of manganese, which corresponds with results obtained by Redulla et al. (2005) as well as Różyła and Pałys (2009). Also Baranowska et al. (2017) demonstrated that soil conditioner application was associated with a decline in manganese content in potato tubers. In the present work, a higher manganese content was determined in the tubers of potato cultivated in the integrated versus organic production system, the finding being similar to results reported by Wszelaki et al. (2005) and Sawicka et al. (2016).

In the trial discussed here, the lowest zinc content was determined in the tubers of control potato. Also Braun et al. (2011), Hadi et al. (2014) and Ashrafzadeh et al. (2017) found the lowest concentration of zinc in the tubers of potato following mineral fertiliser. In the present study, farmyard manure and undersown catch crops, which provide potato plants with macroelements and microelements, contributed to an increase in the potato content of zinc. Trawczyński and Bogdanowicz (2007), Różyło and Pałys (2009), White et al. (2009) as well as Wierzbicka and Trawczyński (2011) have demonstrated that green manures increase zinc content in potato tubers. In this study, zinc content was higher in the tubers of potato grown in the integrated versus organic production system. A similar relationship was found by Hajšlovǎ et al. (2005), Wang et al. (2008) and Sawicka et al. (2016).

In the present work, control potatoes contained the lowest amounts of boron, which agrees with reports by Sayed et al. (2015) and Osvalde et al. (2016). Farmyard manure and undersown catch crops contributed to an increase in the potato tuber content of boron. Also Wierzbicka and Trawczyński (2011) as well as Osvalde et al. (2016) observed that green manures and vermicompost increased the concentration of boron in potato tubers. In the present study as well as in the works by Hajšlovǎ et al. (2005), Wierzbicka and Trawczyński (2011) and Wierzbicka (2012), a higher boron content was recorded in the tubers of organic potato.

In the conducted study, it was discovered that potato manuring with the form *Melilotus albus*, both plowed in autumn and left to the spring in the form of mulch, most preferably affects the content of micronutrients in tubers. Increases the content of iron, zinc and boron in potato tubers, and decreases the content of copper and manganese. Potato from the organic production system contained more iron and boron, and from the integrated production system more copper, manganese and zinc, which indicates a more favorable content of micronutrients in tubers from the organic production system.

Conclusions

1. Potato manuring with undersown catch crops, and especially with the little clover plowed in autumn, increases the content of zinc and boron iron in potato tubers, and reduces the content of copper and manganese (micronutrients of lesser nutritional importance).

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2. Potatoes cultivated in the integrated production system were characterized by a higher concentration of copper, manganese and zinc, and cultivated in the organic production system a higher content of iron and boron.

3. In the conducted research, it was discovered that potato manuring with one year's form of *Melilotus albus*, both plowed in autumn and left to spring in the form of mulch in an ecological production system, provides the most beneficial micronutrient content in tubers.

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VARIATION IN HEAVY METAL CONTENT IN PLANTS GROWING ON A ZINC AND LEAD TAILINGS DUMP

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Abstract. Industrial waste landfills might cause contamination of agricultural land, forests, surface water and groundwater through migration of toxic substances, including heavy metals, posing a threat to all living organisms, including humans. The aim of the study was to assess the variation in Cd, Pb, Zn and Cu content in plants growing on a zinc and lead post-flotation tailings dump. The concentrations of the elements in the shoots and leaves of silver birch, black locust, common rowan, herbaceous plants and soil were determined by the flame atomic adsorption spectrophotometry (FAAS) method. The statistical nalysis indicates a significant relationship between the Pb and Zn concentrations and the plant species, plant part, and location on the dump. Differences in cadmium concentrations depending on the plant species were also statistically significant. None of these relationships was significant in the case of copper content. The mean biological concentration factors (BCF) were higher in plants growing on the southern slope of the dump. Of the elements analysed, zinc and cadmium were accumulated in larger quantities than lead and copper.
Keywords: *post-flotation tailings, heavy metals, bioindicators different plant species, ore mining residues*

Introduction

Human activity is associated with the generation of waste. Its quantity increases every year as a result of continuous technological progress and the development of civilization. It should be noted that new technologies used for extraction and processing of raw materials increase the diversity of generated waste (Salihoglu, 2010; Janas and Zawadzka, 2017; Koolivand et al., 2017). By-products of human activity generated during extraction and industrial processing of raw materials are known as industrial waste. The composition and quantity of industrial waste are linked to the type of production and the specific industry. An important consideration is the environmental harm and nuisance caused by this waste. According to Eurostat data, the total amount of waste from mining and extraction activity in the EU in 2014 was 700.8 billion kg. At the end of that year, 2,123.9 billion kg of mineral waste remained deposited in landfills (Eurostat, 2017). In Poland, 31.2 billion kg of waste was generated in flotation enrichment of non-ferrous ores in 2016. At the end of that year, 635.4 billion kg of this type of waste remained deposited on landfills, of which 38.4 million Mg was in sites located in Małopolska (PCSO, 2017). Industrial and post-industrial sites are found all over the world, and more attention and research should be devoted to them due to the threat they pose to the environment (Grattan et al., 2003; Pyatt et al., 2000, 2005; Stefanowicz et al., 2014; Teršič et al., 2009; Alloway, 2013). Industrial waste landfills could cause contamination of agricultural land, forests, surface water and groundwater through the migration of toxic substances, including heavy metals, thereby posing a threat to all living organisms, including humans

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(Tordoff et al., 2000; Wong, 2003; Klojzy-Kaczmarczyk et al., 2005). Heavy metals can persist in high concentrations for decades or even millennia. Their transport to agricultural soils, sediments and watercourses occurs gradually and is associated with high levels of pollution of the natural environment. None of the technologies currently in use completely protects the environment from the negative impact of landfills. Both external factors (topography, microclimate conditions, vegetation or type of protective barrier) and internal ones (deposition technology, physical and chemical properties of the waste, or means of protecting the substrate) affect the type and degree of the nuisance, as well as the amount of pollutants coming from a given site (Michałkiewicz, 2009; Adeolu et al., 2011). The aim of the study was to evaluate the variation in cadmium, lead, zinc and copper concentrations in plants growing on the reclaimed Trzebionka zinc and lead post-flotation tailings dump and to analyse factors generating this variation.

Material and methods

Study location

The research area was the reclaimed zinc and lead post-flotation tailings dump of the former “ZG Trzebionka S.A” (*Fig. 1*). Mining Plants, located on the administrative border of the Chrzanów and Trzebinia communes, in the western part of the Małopolska Voivodeship, south of Poland (Klojzy-Kaczmarczyk et al., 2012).

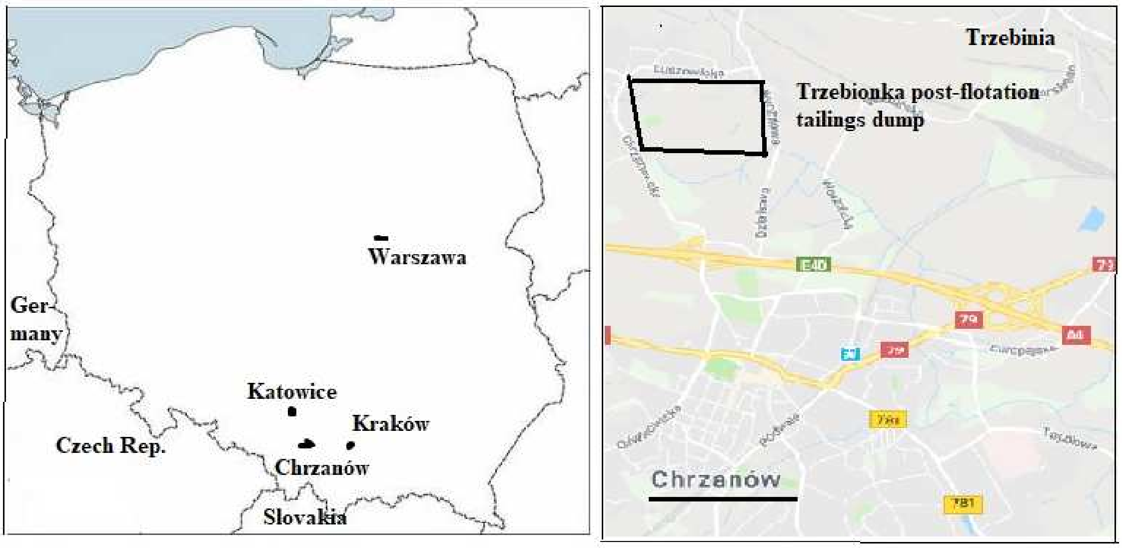

Figure 1. *Localisation of research area within the border of Poland (European Union)*

The dump, which was established in 1966, is one of the largest ponds collecting tailings from the production of zinc and lead ore concentrates in Poland. It covers an area of about 64 ha and rises about 60 m above the ground, and the surface of its crown is 20 ha. Waste was transported in the form of an aqueous suspension of finely ground dolomite with zinc and lead carbonates and sulphides with a particle size of less than 0.2 mm. The content of Zn and Pb was low – 0.92% and 0.41%, respectively. The bottom of the pond consists of concentrated and compact sediments whose

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average particle size decreases towards the centre (Klojzy-Kaczmarczyk et al., 2005; Nowak, 2008). During deposition, the coarsest fraction of the tailings was used to build the embankments by means of clay grouting, the finest fraction was used to seal the bottom of the pond, and the rest was placed inside the pond, where the solids underwent sedimentation. The finest waste covered the outermost layer on the crown, and when operation of the landfill was discontinued, the tailings dump underwent reclamation (Neya, 1997; Klojzy-Kaczmarczyk et al., 2009, 2012). The reclamation work consisted in covering the active surface of the dump with latex and creating a sprinkler system (measures to reduce airborne dust), covering the slopes with humus, planting trees and shrubs, sowing grass and legumes, application of stabilized sewage sludge, and pumping water to the irrigation system (the pond was drained prior to reclamation of the top). Vegetation for reclamation was chosen taking into account the difficult conditions (Klojzy-Kaczmarczyk et al., 2009, 2014).

Sampling and analyses

The research material was obtained by moving about the dump study area from the base of the embankment to the crown and collecting samples in three characteristic research areas (the base of the embankment, the middle of the embankment and the crown of the dump), on the south-west and north-east slopes. Three sampling plots were selected for each research areas. Each sampling plot was an area of about 100 m^2, from which we collected five primary samples of the shoots of herbaceous plants and the shoots and leaves of trees – silver birch (*Betula pendula* Roth), black locust (*Robinia pseudoacacia* L.), and common rowan (*Sorbus aucuparia* L.), as well as soil samples. Following homogenization, these samples constituted an average sample of about 1000 g fresh matter in the case of the plants, and about 500 g in the case of soil. The aerial parts of the plants were collected using stainless steel secateurs, and the soil samples were collected using a soil sampler from a depth of 0–0.1 m. After air-drying, the plants were ground in a high-speed rotor mill.

The collected soil samples were dried and then sifted through a sieve with a 1 mm mesh. In soil prepared this way, basic soil properties were determined: pH in 1 mol KCl by the potentiometric method, content of organic carbon using annealing method (Ostrowska et al., 1991). Besides that, total concentrations of cadmium, lead, zinc and copper along with forms of these elements extracted with 1 mol dm^{-3} HCl. Dry mineralization was performed using a mixture of concentrated HNO_3 and $HClO_4$ for plant material (Ostrowska et al., 1991; Haluschak, 2006). Concentrations of cadmium, lead, zinc and copper were determined by atomic flame absorption spectrometry, using a Unicam Solaar M6 spectrometer. Samples were collected at late summer 2017 year.

The migration conditions of trace elements in the soil–plant system were analysed based on the bioaccumulation factor (BCF) (Nannoni et al., 2016; Li et al., 2007; Marchiol et al., 2013), which illustrates the average ability of plants to accumulate metals from the soil and is calculated as the ratio of the metal concentration in the plant to the metal content of the soil. BCF values greater than 1 indicate high accumulation of metals in the plant, values from 0.1 to 1 indicate moderate accumulation, 0.01 to 0.1 – low accumulation, and below 0.01 – no accumulation (Laskowska and Wiechuła, 2015; Marchiol et al., 2013; Łaszewska et al., 2007). In addition, the coefficient of specific relative accumulation (CSRA *Eq. 1*) was calculated as the quotient of the concentration of a given element in a given plant and

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the content of this element in other plant species present in the area (Stawinoga et al., 2007).

$$CSRA = \frac{c_r}{c} \quad [-] \qquad \text{(Eq.1)}$$

where:
c_r – mean quantity of element in the plant
c – mean quantity of element in all plant species present in the area

The Shapiro–Wilk test showed that the variables did not have normal distribution, so a generalized linear model was used for Poisson distributions to test the relationship between the plant species (black locust, silver birch, rowan and herbaceous plants) and plant part (leaves or shoots) and the location on the landfill (side) and height (base of the embankment, middle or crown). Statistical analyses were performed in Statistica 12.0 software.

Results

In the present study, cadmium content ranged from 0.14 mg·kg^{-1} to 4.49 mg·kg^{-1}, with the lowest content in the rowan and successively higher content in the black locust, silver birch and herbaceous plants growing on the crown of dump on the north-east side. In most of the samples, this metal was accumulated mainly in the leaves of the plants *(Figure 2a)*. An exception was the black locust growing on the middle of the embankment on the south-west side, which had higher cadmium content in its shoots, amounting to 1.05 mg·kg^{-1} (*Table 1*). The cadmium concentrations were also higher in the shoots of the rowan trees growing on the base and crown of the embankment on the north-east side: 0.63 mg·kg^{-1} and 0.21 mg·kg^{-1}, respectively (*Table 2*). Among all the trees, the rowan had the lowest mean cadmium content, similar on all sampling plots, in both the leaves (0.16 mg·kg^{-1}–0.54 mg·kg^{-1}) and the shoots (0.18 mg·kg^{-1}–0.63 mg·kg^{-1}). In silver birch, irrespective of the sampling location, the cadmium content was similar and higher than in the other trees (0.91 mg·kg^{-1}–1.93 mg·kg^{-1}) (*Figure 2a*). The highest variation in this metal was recorded in the herbaceous plants – 89%, followed by the black locust shoots – 55%, and the lowest in the black locust leaves and silver birch leaves – 3%. The analysis confirms statistically significant variation in the content of this element in the plant species tested *(Figures 2a* and *3a (p < 0.001); Table 3)*.

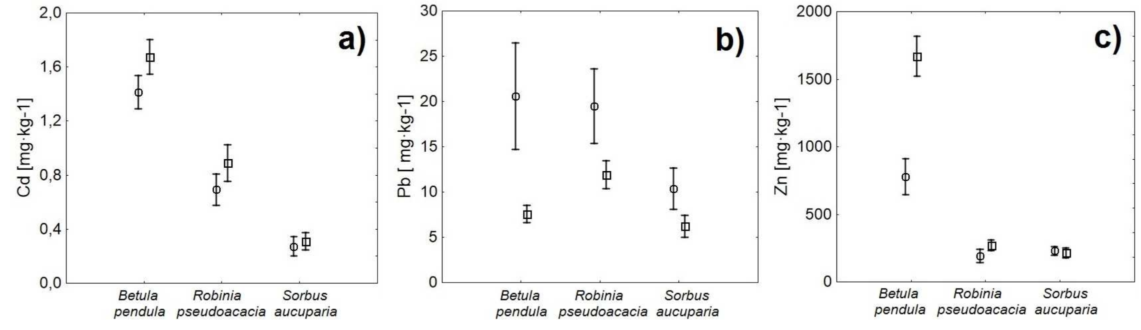

Figure 2. *Distribution of mean concentrations of heavy metals in the shoots (circles) and leaves (squares) of three species: silver birch, black locust and common rowan*

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Table 1. *Mean concentrations of heavy metals in plants and soil for south-west site*

			Content in plants mg·kg^{-1}				Content in soil mg·kg^{-1}				%	
Site/slope	**Plant**	**Organ**	**Cd**	**Pb**	**Zn**	**Cu**	**Cd**	**Pb**	**Zn**	**Cu**	**org**	**pH (KCl)**
Base	*Betula pendula*	shoot	1.26	27.50	1207.40	5.79	16.78	645.60	2253.00	78.35	3.20	7.50
		leaf	1.45	7.40	1845.40	4.90						
	Robinia pseudoacacia	shoot	0.36	11.10	113.40	4.78						
		leaf	0.62	8.30	254.60	3.46						
	Sorbus aucuparia	shoot	0.18	8.60	182.60	5.82						
		leaf	0.18	4.10	201.80	3.93						
	Herbaceous plants	shoot	0.91	13.90	426.60	3.09						
Middle	*Betula pendula*	shoot	0.91	17.80	1073.90	6.18	17.06	621.20	2166.00	72.15	3.00	7.40
		leaf	1.12	5.30	2226.50	5.01						
	Robinia pseudoacacia	shoot	1.05	23.30	442.90	2.91						
		leaf	0.65	17.50	212.20	3.79						
	Sorbus aucuparia	shoot	0.16	12.90	277.70	3.27						
		leaf	0.31	6.20	245.30	3.15						
	Herbaceous plants	shoot	0.42	39.2	264.30	3.11						
Crown	*Betula pendula*	shoot	1.35	10.60	427.60	4.74	12.56	3415.70	5494.00	93.55	4.75	7.50
		leaf	1.88	5.60	1172.30	4.86						
	Robinia pseudoacacia	shoot	0.36	8.0	96.50	5.46						
		leaf	0.6	8.10	154.80	4.05						
	Sorbus aucuparia	shoot	0.26	5.20	176.80	6.56						
		leaf	0.47	4.40	355.70	4.43						
	Herbaceous plants	shoot	4.03	16.70	292.10	4.45						

org = content of organic matter

Table 2. *Mean concentrations of heavy metals in plants and soil for north-east site*

			Content in plants mg·kg^{-1}				Content in soil mg·kg^{-1}				%	
Site/slope	**Plant**	**Organ**	**Cd**	**Pb**	**Zn**	**Cu**	**Cd**	**Pb**	**Zn**	**Cu**	**org**	**pH (KCl)**
base	*Betula pendula*	shoot	1.74	47.70	932.40	5.20	48.05	1499.50	5592.00	76.45	4.05	7.50
		leaf	1.8	12.10	1808.50	3.75						
	Robinia pseudoacacia	shoot	0.96	32.80	184.80	4.04						
		leaf	1.26	15.40	284.30	3.61						
	Sorbus aucuparia	shoot	0.63	13.50	383.30	4.34						
		leaf	0.54	11.80	258.70	4.26						
	Herbaceous plants	shoot	1.53	3.00	145.80	5.39						
middle	*Betula pendula*	shoot	1.51	10.20	529.90	3.82	55.27	4414.75	12,443.50	407.85	5.75	6.80
		leaf	1.87	7.80	1384.50	3.65						
	Robinia pseudoacacia	shoot	0.71	29.40	156.90	4.87						
		leaf	0.83	9.30	279.50	3.34						
	Sorbus aucuparia	shoot	0.20	18.70	181.80	4.31						
		leaf	0.21	7.30	127.80	5.01						
	Herbaceous plants	shoot	1.57	15.00	290.60	5.87						
crown	*Betula pendula*	shoot	1.71	9.80	495.20	4.22	26.03	5101.60	1710.50	375.45	5.80	7.10
		leaf	1.93	7.20	1585.70	3.77						
	Robinia pseudoacacia	shoot	0.72	12.30	151.90	4.39						
		leaf	1.38	12.90	441.10	3.66						
	Sorbus aucuparia	shoot	0.21	3.40	176.20	4.07						
		leaf	0.16	3.60	100.70	4.41						
	Herbaceous plants	shoot	4.46	7.10	229.60	6.24						

org. =content of organic matter

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Table 3. *Results of generalized linear model*

Cd	df	Wald's statistic	p
Residual	1	11.72	**
Location	5	5.29	ns
Species	2	35.53	***
Plant part	1	0.66	ns
Location x species	10	4.30	ns
Location x plant part	5	0.38	ns
Species x plant part	2	0.06	ns
Location x species x plant part	10	1.35	ns
Pb	**df**	**Wald's statistic**	**p**
Residual	1	6811.40	***
Location	5	187.69	***
Species	2	89.35	***
Plant part	1	100.03	***
Location x species	10	64.46	***
Location x plant part	5	21.85	**
Species x plant part	2	14.40	**
Location x species x plant part	10	38.28	***
Zn	**df**	**Wald's statistic**	**p**
Residual	1	1,291,481.39	***
Location	5	1921.66	***
Species	2	35,429.32	***
Plant part	1	1383.08	***
Location x species	10	1661.00	***
Location x plant part	5	624.25	***
Species x plant part	2	1767.54	***
Location x species x plant part	10	1237.76	***
Cu	**df**	**Wald's statistic**	**p**
Residual	1	1296.97	***
Location	5	4.59	ns
Species	2	2.16	ns
Plant part	1	2.88	ns
Location x species	10	7.30	ns
Location x plant part	5	1.50	ns
Species x plant part	2	0.12	ns
Location x species x plant part	10	3.94	ns

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant

The variation in cadmium concentrations in the plants was associated to a lesser degree with the height gradient (*Figure 3a; p < 0.01*). No statistically significant

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differences were found in the content of this element in the plant parts or its content in the substrate depending on the research (slope) area where the samples were taken.

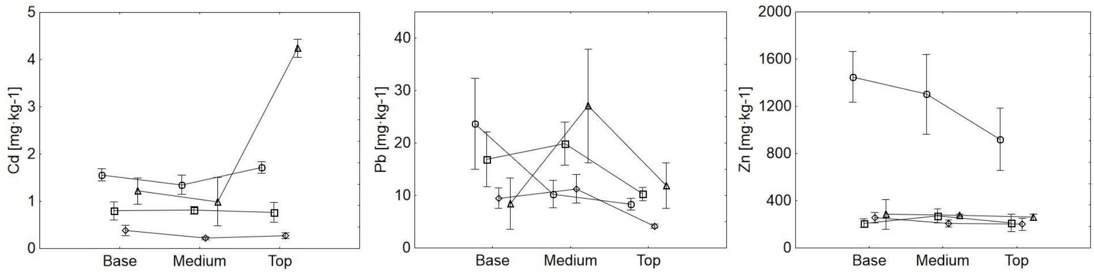

Figure 3. *Distribution of mean heavy metal concentrations in the plants: silver birch (circles), black locust (squares), common rowan (diamonds) and herbaceous plants (triangles)*

The lead content in the vegetation growing in the study area ranged from 3.0 mg·kg^{-1} (north-eastern part, base of embankment, herbaceous plants) to 39.5 mg·kg^{-1} (south-western part, middle of the embankment, herbaceous plants). The highest coefficient of variation on the south-west slope was found for the herbaceous plants (49%) and black locust shoots (47%), and on the north-east side also for the herbaceous plant samples (60%), as well as for the shoots of silver birch (79%). In most of the samples, on both the south-west and north-east embankments, the shoots accumulated higher lead content. In the case of herbaceous plants, rowan, and black locust, higher lead content was found in the middle of the embankment on the south-west slope than in the other sampling plots. Moreover, as the height increased, the metal content decreased in silver birch (base > middle > crown). On the north-eastern embankment, the concentration of lead in silver birch and black locust decreased in this order: base > middle > crown of embankment. The rowan and herbaceous plants collected from the middle of the embankment had higher content of this metal in comparison with the other sampling plots (the base and crown of the embankment). The analysis confirms a statistically significant relationship between the Pb concentration and the plant species, plant part, and location on the landfill (both the side of the slope and the height) *(Figures 2b* and *3b (p < 0.001); Table 3)*. The zinc content on the Trzebionka post-flotation tailings dump ranged from 96.5 mg·kg^{-1} (south-west side, crown, black locust shoots) to 2226.6 mg·kg^{-1} (south-west, middle, silver birch leaves). On the southern slope, the highest variability in the content of zinc was observed in the shoots of black locust (73%). On the northern embankment, the highest variability was recorded for common rowan (42%). In the silver birch and black locust trees growing on the north-east embankment, higher concentrations of this element were found in the leaves. The reverse was true in the case of rowan, in which the shoots contained higher amounts of zinc. A similar tendency was noted in most of the samples collected on the south-west slope. The analysis confirmed the statistically significant impact of the sampling location, plant species and plant part on the content of this metal *(Figures 2c* and *3c (p < 0.001); Table 3)*. Copper content in all plants was similar on individual sampling plots and ranged from 2.91 mg·kg^{-1} (south-west, middle of embankment, black locust shoots) to 6.58 mg·kg^{-1} (south-west, crown, rowan shoots). In most of the samples in the study area, this element was stored mainly in the shoots. There was also an increase in the content of this metal in the herbaceous plants along the height transect, from 3.08 mg·kg^{-1} to 4.48 mg·kg^{-1} on the south-west embankment and 5.38 mg·kg^{-1} to

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6.30 mg·kg^{-1} on the north-east side. The highest coefficient of variation for the south-west side of the landfill was 27% for rowan shoots and 25% for black locust shoots, while for the north-east side of the site it was 13% for silver birch shoots. This element showed the least variation of all metals analysed. The statistical analysis did not indicate a significant relationship between the plant species, plant part or location on the landfill and the content of this metal *(Table 3)*.

Discussion

Areas where waste is deposited are usually unfavourable substrates for plant growth, due to the lack of organic matter, unsuitable pH, high concentrations of toxic metals, high salinity, low water retention capacity, low nutrient content, lack of micro-organisms, poor physical structure and susceptibility to erosion (Krzaklewski and Pietrzykowski, 2002; Szarek-Łukaszewska and Niklińska, 2002; Wong, 2003; Ye et al., 2002). These properties exert strong selective pressure on colonization by plants, and consequently the waste is often completely devoid of vegetation or exhibits a specific and unique composition of plant species spontaneously appearing there (Woch et al., 2016).

Nadgórska-Socha et al. (2016) tested the efficiency of accumulation of trace elements in black locust leaves. The research was carried out in areas contaminated with heavy metals and the results were compared to uncontaminated areas. The results indicated that this species can grow in metal-contaminated areas and that the contamination affects the physiological parameters of the plant. The average content of elements in the black locust leaves from the Trzebionka dump was 0.89 mg·kg^{-1} for Cd, 11.92 mg·kg^{-1} for Pb, 271.0 mg·kg^{-1} for Zn and 3.65 mg·kg^{-1} for Cu. In the immediate vicinity of the Miasteczko Śląskie Zinc Smelter, Nadgórska-Socha et al. (2016) reported concentrations of 3.01 mg·kg^{-1} for Cd, 17.69 mg·kg^{-1} for Pb, 109.64 mg·kg^{-1} for Zn, and 13.11 mg·kg^{-1} for Cu. As in our research, the levels of Cd accumulated in the trees were the lowest; Cu, Pb and Zn were accumulated in higher concentrations. Hu et al. (2014) also assessed the content of heavy metals in various plant species, including black locust, in an industrial region, in a residential area, and by a motorway. The concentrations of elements differed depending on the plant species and on the sampling site. The highest concentrations were recorded near the motorway and in the industrial area. The authors presented the mean content of Cd (1.10 mg·kg^{-1}), Pb (4.55 mg·kg^{-1}), Cu (3.88 mg·kg^{-1}) and Zn (5.18 mg·kg^{-1}) for black locust. The concentrations for Pb and Zn were very different from the results obtained in our study, but are arranged in the same, the most common hierarchy of accumulation: Cd < Cu < Pb < Zn. The results obtained by Hu et al. (2014) and from the Trzebionka dump indicate that the black locust accumulates lower content of harmful substances than other analysed plant species. Tzvetkova and Petkova (2015) examined black locust leaves in industrial regions of Bulgaria in order to determine the accumulation characteristics of this tree and to evaluate it as a biomonitor of heavy metal pollution. The study was carried out on plantations near the industrial area of Devnya in eastern Bulgaria, which is heavily contaminated by cement plants, nitrogen fertilizers and polyvinyl chloride factories. The leaves of damaged and control plants were tested. The concentrations of heavy metals were higher than those obtained in the Trzebionka tailings dump for lead (30.7 mg·kg^{-1}) and copper (17.2 mg·kg^{-1}); the exception was zinc, with a concentration of 19.0 mg·kg^{-1}. The authors showed that the black locust leaves accumulated Pb, Zn and Cu as the

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concentration of these metals increased in the soil. The average content of heavy metals in the rowan tree leaves (*Sorbus aucuparia*) was 0.31 $mg \cdot kg^{-1}$ for Cd, 6.23 $mg \cdot kg^{-1}$ for Pb, 4.20 $mg \cdot kg^{-1}$ for Cu, and 215.00 $mg \cdot kg^{-1}$ for Zn, while the concentrations in the shoots were 0.27 $mg \cdot kg^{-1}$ for Cd, 10.38 $mg \cdot kg^{-1}$ for Pb, 4.73 $mg \cdot kg^{-1}$ for Cu, and 229.7 $mg \cdot kg^{-1}$ for Zn. In comparison with other trees, this species did not accumulate the highest heavy metal concentrations. Woch et al. (2017), who carried out research on waste heaps left by historical Zn-Pb mines, reported that rowan was one of the most common trees in the contaminated area. The authors also state that such areas have diverse species of woody vegetation. Compared with other tree species, silver birch (*Betula pendula*) shows much greater affinity for zinc, accumulates high concentrations of heavy metals – above the average values given for plants in the literature, and grows rapidly, even in areas with difficult conditions. This is an example of the features necessary in a species suitable for phytoremediation. Research on this species has been carried out by Wisłocka et al. (2006), Margui et al. (2007) and Dmuchowski et al. (2012, 2014). Dmuchowski et al. (2014) conducted work to assess the suitability of silver birch for phytoremediation of zinc contaminated soils. The trees grew on contaminated metallurgical waste heaps and under control conditions. The study showed the potential of this species to accumulate large amounts of zinc in the leaves in both controlled and polluted environments. The authors conclude that silver birch is a species that meets the requirements for plants used for phytoremediation and can be used to clean a zinc contaminated substrate. The analysis showed that in the environment contaminated with zinc, the soil near the trunks of *Betula pendula* contained much less of the heavy metal than the soil at a distance of 7 m. Dmuchowski et al. (2014) suggested that *Betula pendula* can be included among zinc hyperaccumulators. Analysis of the results of our research indicates that silver birch has the capacity to store large amounts of zinc (2226.5 $mg \cdot kg^{-1}$ in leaves collected from the middle of the embankment on the south-west slope). It should be noted here that among all substrate samples, the lowest Zn content was found in a sample from the middle of the slope on the south-west side of the landfill. Dmuchowski and Sołtykiewicz (2007) reported that the leaves of silver birch contained on average from 300 $mg \cdot kg^{-1}$ (Białowieża Forest) to 1344 $mg \cdot kg^{-1}$ of zinc (waste heap of a non-ferrous metal smelter near Olkusz), and in their final conclusion classified the tree as a zinc hyperaccumulator. On the Trzebionka dump, the silver birch leaves accumulated much higher concentrations of this metal (from 1172.3 $mg \cdot kg^{-1}$ to 2226.5 $mg \cdot kg^{-1}$), thus allowing the species to be counted among hyperaccumulators that can be used in soil phytoremedation processes in areas enriched with trace elements (Krzciuk, 2015). The mean content of zinc in silver birch on the Trzebionka dump was 1670.48 $mg \cdot kg^{-1}$ in the leaves and 666.63 $mg \cdot kg^{-1}$ in the shoots, which were the highest values among the plants analysed. Marguí et al. (2007) determined the content of zinc in silver birch in an abandoned lead-zinc mining area in northern Spain. To ensure maximum metal accumulation, the samples were collected in autumn, as in the present study. The concentration of zinc was also high, ranging from 1660 to 3100 $mg \cdot kg^{-1}$, while the sample from the control area contained 225 $mg \cdot kg^{-1}$ Zn. Chaabani et al. (2017) conducted research on the content of Pb, Zn and Cd in various species of herbaceous plants found on the site of former zinc and lead ore mines in northern Tunisia. The concentrations of these elements reported in the aerial parts of the plants were in the following ranges: Cd 0.3–886 $mg \cdot kg^{-1}$, Pb 2.2–341 $mg \cdot kg^{-1}$, and Zn 30.1–1048.5 $mg \cdot kg^{-1}$. The maximum concentrations of cadmium and lead are many times

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higher than those found in our study. However, it should be noted that the soils in those locations also have extremely high concentrations of these elements. Wójcik et al. (2014) analysed the content of heavy metals in the shoots of 38 species of herbaceous plants found on mining and metallurgical waste landfills. Zinc in the shoots of the plants ranged from 59.24 to 901.5 mg·kg^{-1}, lead from 3.23 mg·kg^{-1} to 116.92 mg·kg^{-1}, and cadmium from 0.54 mg·kg^{-1} to 26.86 mg·kg^{-1}. In this case as well, the maximum concentrations of these elements were significantly higher than in our research. Bech et al. (2012) report the following average concentrations for the stems of herbaceous plants growing on the site of an antimony mine in the eastern Pyrenees: for Pb 3.3–289 mg·kg^{-1}, Zn 17–81 mg·kg^{-1}, Cu 12–36 mg·kg^{-1}, and Cd < 0.1–0.2 mg·kg^{-1}. One of the factors influencing the accumulation of heavy metals in plants is their species. Calculations of coefficients such as the enrichment factor and translocation factor are used to obtain information on the suitability of plants for removing metals from contaminated soil. Plant testing can be used in remediation of contaminated soils, e.g. by lead and zinc. In addition, among a set of analysed species we can choose the ones that are suitable for reclamation and those that are candidates for stabilization of metals in the soil and for soil protection (Kirat and Aydin, 2015; Zhao and Duo, 2015). Zhao and Duo (2015) draw attention to the risk of heavy metals returning to the environment in the phytoextraction process. According to the authors, shoots of plants with accumulated harmful substances should undergo appropriate recovery and treatment. Neither the herbaceous plants nor the trees included in the study accumulated Cd, Pb, Zn and Cu intensively (BCF < 1). The silver birch and herbaceous plants had a moderate biological concentration factor (0.1 ≤ BCF ≤ 1.0) for Cd and Zn. Black locust and rowan growing on the middle of embankment and on crown of the on the south-west side had moderate zinc biological concentration factor. Regarding lead content, it should be noted that the herbaceous plants on the south-west embankment of the landfill had a low level of bioaccumulation of this metal (0.01 ≤ BCF ≤ 0.06). Bioaccumulation of this element was also low in the silver birch (0.01 ≤ BCF ≤ 0.03) and black locust (0.01 < BCF < 0.02) growing at the base and middle of the embankment on the same side. On the north-east side, weak accumulation of lead (BCF = 0.02) was found in a couple of samples of silver birch and black locust growing at the base of the embankment, but no accumulation of this metal (BCF ≤ 0.01) was noted in the remaining plants. Regarding copper content, accumulation of this element was weak in all tested plants growing on the southern side of the embankment: herbaceous plants (0.03 ≤ BCF ≤ 0.06), silver birch (0.05 ≤ BCF ≤ 0.08), black locust (0.04 ≤ BCF ≤ 0.07) and common rowan (0.03 ≤ BCF ≤ 0.08). On the north side, copper accumulation was weak in herbaceous plants growing in the middle of the embankment and on the crown (BCF = 0.02).

Higher mean values for the biological concentration factor were found in plants growing on the south-west slope of the dump. Of the elements analysed, zinc and cadmium were accumulated in greater quantities than lead and copper. Comparison of BCF valuesbetween plants reveals that the silver birch trees and herbaceous plants had higher average values for this factor than the black locust and rowan *(Table 4)*. To determine the selective metal accumulation capacity, the coefficient of specific relative accumulation (CSRA) was calculated. This coefficient can be used to categorize plant species according to their ability to accumulate individual elements (Łaszewska et al., 2007; Parzych et al., 2016). Furthermore, calculation of this coefficient enables a more complete illustration of the problem of metal accumulation, as it provides information

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relating to other plants growing in a given area (Stawinoga et al., 2007). Comparison of the CSRA coefficients for the plant species included in the study shows that silver birch had the highest zinc accumulation capacity (CSRA = 2.39 and 2.33), while the herbaceous plants had the highest capacity to accumulate cadmium (CSRA = 2.02 and 2.04). In the case of lead, the greatest storage capacity was found for the herbaceous plants on the southern slope (CSRA = 1.87) and for black locust (CSRA = 1.80) and silver birch (CSRA = 1.63) on the north-east slope. In the case of copper, the CSRA values were similar (0.80-1.33). It can also be seen that the rowan trees had the lowest capacity to accumulate Cd, Pb, Zn and Cu.

Table 4. *Comparison of mean BCF values on the northern and southern embankments of the dump*

	Cd		Zn		Pb		Cu	
	SW	**NE**	**SW**	**NE**	**SW**	**NE**	**SW**	**NE**
Betula pendula	0.09	0.05	0.48	0.13	0.01	0.01	0.07	0.02
Robinia pseudoacacia	0.04	0.03	0.08	0.03	0.01	0.01	0.05	0.02
Sorbus aucuparia	0.02	0.01	0.09	0.02	0.01	0.00	0.06	0.03
Herbaceous plants	0.12	0.06	0.12	0.03	0.03	0.00	0.04	0.04

Conclusions

The variation in accumulation of the investigated metals between the analysed plant species, plant parts, and locations on the landfill is particularly evident in the case of lead and zinc. These features are less significant in the case of the distribution of cadmium and copper concentrations. Neither the herbaceous plants nor the trees included in the study showed intensive accumulation of the metals tested (BCF < 1). Higher mean values for the bioaccumulation factor were found for plants growing on the south-west slope of the post-flotation tailings dump. Among the species analysed, rowan (*Sorbus aucuparia*) accumulated smaller amounts of metals than the other plants, while silver birch (*Betula pendula*) showed the capacity for hyperaccumulation of zinc, which confirms that it is a suitable species for reclamation processes.

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GROUNDWATER VULNERABILITY GIS MODELS IN THE CARPATHIAN MOUNTAINS UNDER CLIMATE AND LAND COVER CHANGES

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Abstract. Water resources are facing nowadays with two main problems: climate change and land cover variation. Their influences on environment and water resources have been evidenced worldwide. In this work, we have applied a complex methodology based on Geographical Information System (GIS) to combine the spatial information of several parameters that allow to obtain the groundwater vulnerability under climate and land cover modifications. The spatial analysis performed in this paper includes the aquifers, water availability, load pollution index, and infiltration map raster grids data of Carpathian Mountains area, from Central Europe. The analysis presented in this study follow three periods, which include 30 years climate data models of 1961-1990 (1990s), 2011-2040 (2020s), and 2041-2070 (2050s). Land cover projections forecast future changes in artificial areas, agriculture areas, and forest areas for 2020s and 2050s. For both periods (2020s and 2050s), the very low vulnerability class area is reduced while the high class appears on a large area. The worst scenario is forecasted for 2050s (high vulnerability class increase up to 2.41% of the whole study area) and is mainly due to agriculture. These findings evidence the negative impact of land cover and climate changes on the groundwater resources in the Carpathians Mountains area. The original maps carried out in this work together with the concise methodology integrated in GIS may be a useful tool for the water resources management and future strategies plans of this region.
Keywords: *climatic models, aquifers, pollution load index, infiltration map, water availability, spatial analysis*

Abbreviations: GIS – Geographical Information Systems; K_c – crop coefficients; ET_c – crop evapotranspiration; ET_0 – potential evapotranspiration; AETc – actual crop evapotranspiration; PLI – pollution load index; PIC – potential infiltration coefficient; DEM – digital elevation model; asl – above sea level; Dfb – fully humid climate and warm summers; Dfc – fully humid precipitation conditions and cool summers; AOGCM – Atmosphere Ocean Global Climate Model; CMIP5 – Coupled Model Intercomparison Project phase 5; CanESM2 – Canadian Earth System Model 2; CCSM4 – Community Climate System Model version 4; INM-CM4 – Institute of Numerical Mathematics Climate Model version 4; ACCESS1.0 – Australian Community Climate and Earth System Simulator; HadGEM2-ES – Hadley Centre Global Environment Model version 2; MRI-CGCM3 – Meteorological Research Institute Coupled Global Climate Model version 3; IPSL-CM5A-MR Institut Pierre Simon Laplace Climate Model 5A Medium resolution; CNRM-CM5 – Centre National de Recherches Météorologiques Climate Model version 5; MIROC-ESM – Model for Interdisciplinary Research on Climate Earth System Models; MIROC5 – Model for Interdisciplinary Research on Climate version 5; CSIRO Mk 3.6 – Commonwealth Scientific and Industrial Research Organisation model version 3.6; CESM1-CAM5 – Community Earth System Model version 1 Community Atmosphere Model version 5; MPI-ESM-LR – Max-Planck-Institut für Meteorologie Earth System Model on Low Resolution; GFDL-CM3 – Geophysical Fluid Dynamics Laboratory Climate Model version 3; GISS-E2R – Goddard Institute for Space Studies ModelE 2 Russell ocean model

Introduction

Groundwater represents the most important freshwater resources for human purposes. Several authors highlighted the influence of climate and land cover changes occurred in the last decades on groundwater, both in term of quality and quantity. In particular, severe impact took place in Mediterranean countries and South-central Europe. Galleani et al. (2011), Čenčur Curk et al. (2014), Nistor et al. (2015) signalled the negative effect of climate change on groundwater. Kløve et al. (2014) illustrated the negative impact of climate changes on the related ecosystems. The recent climate warming (Haeberli et al., 1999) indicates negative effects on the natural and semi-natural places (Nistor and Mîndrescu, 2017). Due to climate change, in the next decades the mean annual temperatures are expected to increase (IPCC, 2001) and several models indicate rise of temperature during the 21st century (IPCC Assessment Report 5, 2013; Stocks et al., 1998; Shaver et al., 2000; Stavig et al., 2005; The Canadian Centre for Climate Modelling, 2014). Aguilera and Murillo (2009), Jiménez Cisneros et al. (2014), Kløve et al. (2014) signalled several problems that appeared in the biodiversity and surface waters depletion related to climate change. In the temperate zone of South Europe and Turkey, the precipitation amount will reduce (Čenčur Curk et al., 2014; Cheval et al., 2017; Nistor and Mîndrescu, 2017). The dynamics of the land cover and the human activities at continental scale are going to affect the water resources, which are sensitive to urbanization, agricultural lands' exploitations, and improper forest management, e.g. deforestation, chaotic building in the forest proximity. With respect to the decrease of precipitation, the groundwater recharge is influenced from quantity point of view (Nistor et al., 2015). On other hand, the possibility of rainfall intensity increase will affect directly the infiltration rate on short-term period.

The temperature pattern together with the land cover contribute at the evapotranspiration processes taking place on the recharge areas. In the central and eastern Europe, the Carpathian Mountains area represents a precious reservoir of water resources, especially for many establishments of the region, but also for the large cities located out of the Carpathians, such as Bucharest and Cluj-Napoca. Moreover, the studies show that the crop evapotranspiration (ETc) increase from the past period to 2050s (Nistor et al., 2016) and the deforestation of the several sectors in the Carpathian Mountain area could damage the regime of watershed basins.

The goal of this work is to define the most sensitive layers for groundwater at climate change and cover modifications on long-term and to generate the spatial analysis for groundwater vulnerability in the Carpathian Mountains area. We used high-resolution climate models of mean annual air temperature, mean annual precipitation, and annual ET0 for 1990s, 2020s, and 2050s periods and the CORINE land cover dated from 2010 (World Land Cover 30m) and three projections for 2050s. In addition, the aquifers layer of the study area and the digital elevation model (DEM) were integrated in the methodology of the paper. Thus, the determination of groundwater vulnerability in the study area is based on a spatial analysis by weights using ArcGIS environment.

Following the method of Nistor et al. (2015), tested in a small area of the Beliş district from Apuseni Mountains, our approach and obtained results are reliable at large scale, being important instruments for the panning of this large mountain area.

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Materials and methods

Study area

The Carpathian Mountains area extends from 44°28’ to 49°51’ N and from 18°00’ to 26°46’ E (*Fig. 1*). The geographical position in the central part of Europe and the large natural sectors of these mountains makes the Carpathians to be the most important ecosystem from this side of the continent (Nistor et al., 2016). Here, we simply defined the Western Carpathians in the northwestern side of the study area, the eastern Carpathians mainly in Romania and Ukraine, and Southern Carparthians. In addition, we highlighted the Curvatures Carpathians and Apuseni Mountains as two units used in the paper for descriptions and toponymy points of view. Moreover, the analysed area represents a significant source of drinking water with valuable aquifers (e.g. highly productivity fissured aquifers, highly productive porous aquifers). In base of the geomorphology present in the region, the orography of the Carpathians is much diversified and could be reflected in the runoff pattern and infiltration processes. The elevations ranges mainly from 300 m in depression and valleys to 2642 m in Gerlachovský Peak from Slovakia. The geology of the territory is complex and could be easier reflected into the aquifers composition. The main geological formations are represented by gneisses, mica schists and amphibolites in the Southern Carpathians and by marlstones, limestones, clays, marls, sandstones and sands in the Curvatures Carpathians and Eastern Carpathians. Pyroclastic rocks, volcanic rocks, marlstones, dolomitic limestones, gravels and clays are presented in the Western Carpathians and Apuseni Mountains. *Figure A1* in the *Appendix* (BGR and UNESCO, 2013) shows the geological formations of the Carpathian Mountains area. In base of the BGR and UNESCO (2013) map, the aquifers types are divided in six categories of productivity: highly productive fissured aquifers, highly productive porous aquifers, low and moderately productive fissured aquifers, low and moderately productive porous aquifers, locally aquiferous rocks - porous or fissured, practically non-aquiferous - porous or fissured. *Table 1* indicates the aquifers types in the Carpathian region area.

The climate of the study area has oceanic influences in the West and North-West, Baltic influence in the North and Mediterranean influence in the South-West. According to the Köppen–Geiger climate classification, the Carpathian Mountains area has Dfb climate in the main part pf territory, with fully humid climate and warm summers (Kottek et al., 2006). In the eastern sectors of the Romanian and Ukrainian Carpathians, in the central parts of the Slovakian Carpathians and in South Poland, the study area have a Dfc climate type, characterized by fully humid precipitation conditions and cool summers (Kottek et al., 2006). During the 1990s, the mean annual temperature in the study area ranged from –3.1 to 11.4 °C (*Fig. 2*). The precipitation pattern shows values between 546 mm to 1695 mm year^{-1} during 1990s (*Fig. 2b*). The annual ET0 in the Carpathian Mountains area ranged from 279 mm up to 548 mm during 1990s (*Fig. 2c*).

The land cover of the Carpathian Mountains area is composed at high elevations (over 1000 m asl) by forest and pasture (coniferous, mixed and broad-leaved forests), the valleys and depressions are covered by agricultural cultivated lands, villages and also cities. The cities and rural localities compose the artificial areas in the Carpathian mountain region. For groundwater vulnerability determination, the land cover has a quantitative role with respect to the evapotranspiration and water availability and a qualitatively role due to the phosphorous and load pollution present in each type of land cover.

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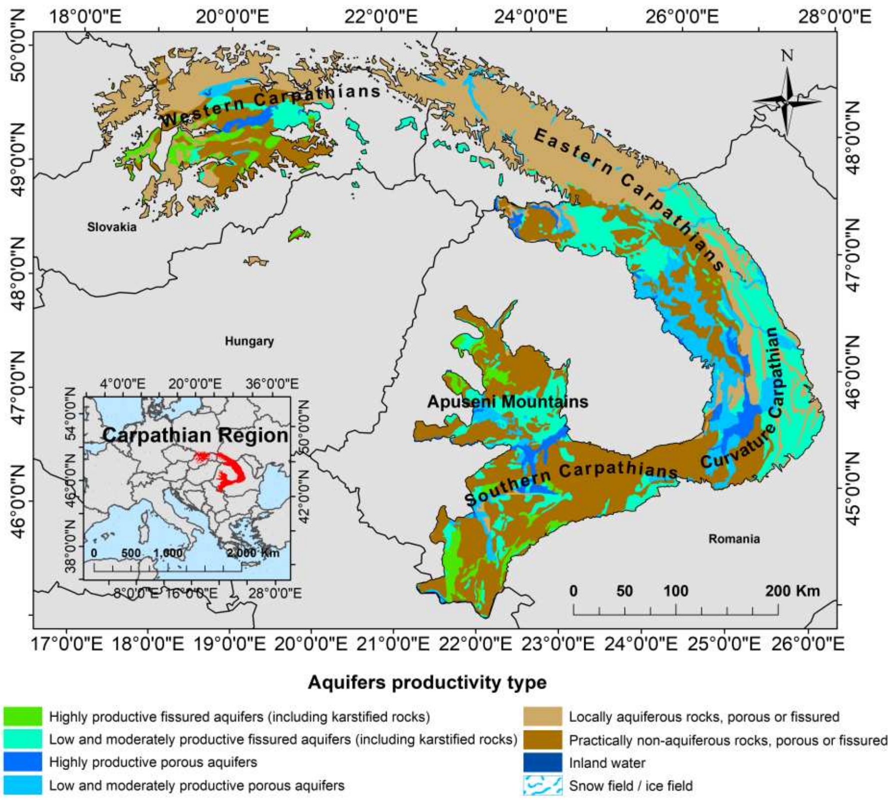

Figure 1. *Location of the Carpathian Mountains region on the South-central map and the main aquifers productivity type in the region*

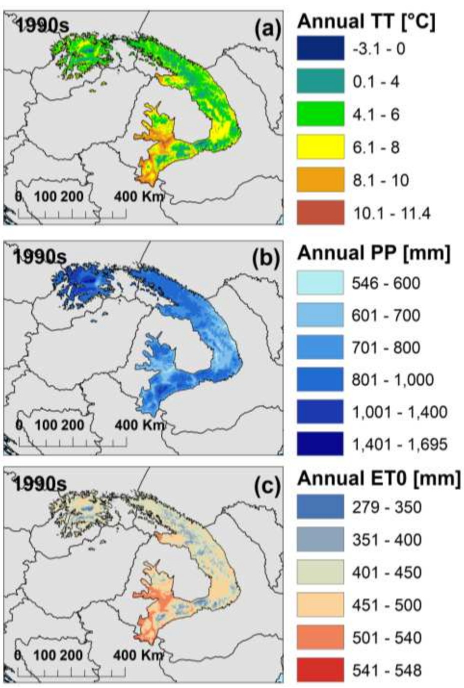

Figure 2. *Spatial distribution of temperature, precipitation, and ET0 in the Carpathian Mountains region. (a) The average of mean annual air temperature between 1961 and 1990 (1990s). (b) The average of mean annual precipitation between 1961 and 1990 (1990s). (c) The average of mean annual precipitation between 2011 and 2040 (2020s)*

Table 1. *Summary of the climate models used in the application of groundwater vulnerability assessment for the Carpathian region. (Sources: Scherrer, 2011; Watanabe et al, 2011; Hamann et al., 2013; IPCC Assessment Report 5, 2013; Knutti et al., 2013; Ohgaito et al., 2013; Wang et al., 2016)*

CMIP5 multi-model dataset which compose the ensemble average of 15 AOGCMs		
CanESM2	CCSM4	INM-CM4
ACCESS1.0	HadGEM2-ES	MRI-CGCM3
IPSL-CM5A-MR	CNRM-CM5	MIROC-ESM
MIROC5	CSIRO Mk 3.6	CESM1-CAM5
MPI-ESM-LR	GFDL-CM3	GISS-E2R

Climate data

High spatial-resolution data (1 km^2) in three periods (past – 1961-1990, present – 2011-2040, and future – 2041-2070) of monthly mean precipitation, monthly ET0, and annual ET0 were used in this study to carry out the water availability. The climate models of temperature and precipitation were developed and validated for whole Europe (Hamann et al., 2013).

Regarding the source of base dataset and type of meteorological stations, as well as the number of stations included in the normal period (1961-1990), we would like to mention that the data used in this paper is online available (http://www.cru.uea.ac.uk/) and it belong to the CRU TS 2.1 project (Mitchell and Jones, 2005). The dataset contains mean monthly precipitation and mean, minimum, and maximum monthly temperature and it were quality controlled and checked for inhomogeneities (New et al., 1998). For Europe, gridded climatological data, at finer spatial resolution of 0.5° lat X 0.5° long, were constructed using surface observations from about 1644 stations for temperature (344 for mean monthly temperature, 647 for minimum monthly temperature, and 653 for maximum monthly temperature). For the precipitation gridded data were used about 1333 meteorological stations.

The models performed by Hamann et al. (2013) were done in base of Parameter Regression of Independent Slopes Model (PRISM) for precipitation and ANUSplin for temperature, considering the Representative Concentration Pathway (RCP) of 4.5 for emission, which means a moderate climate changes projection, based on a globally predicts of +1.4 °C (±0.5). The ANUSplin interpolation procedure is in line with Mitchell and Jones (2005). In the climate models creation ClimateEU v4.63 software was used, available on the website (http://tinyurl.com/ClimateEU). The base-dataset is in principle described by Wang et al. (2016) in their study about historical and future climate in North America (https://sites.ualberta.ca/~ahamann/publications/pdfs/Wang_et_al_2016.pdf).

The climate models represent an ensemble average of 15 AOGCMs, consider the CMIP5 multi-model dataset accordingly IPCC Assessment Report 5 (2013). To represent the main clusters of similar AOGCMs, the individual models were selected (Knutti et al., 2013). The chosen of these models was based on the high validation statistics in the CMIP3 equivalents (Scherrer, 2011). The models which compose the ensemble average of AOGCMs are reported in the *Table 1*.

The bilinear interpolation was used to correct the artefacts in the AOGCM grid cells, for the adjacent areas. A Change Factor (CF) method for the GCM results have been

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applied instead the bias correction. The methodology of the climate models is in line with Daly (2006). Moreover, the models were validated and described by Hamann and Wang (2005), Mbogga et al. (2009), Wang et al. (2016), Dezsi et al. (2018). At the continental and regional scales, the evapotranspiration studies were completed by Nistor (2018a, b) and Nistor et al. (2018).

Aquifers data

The Carpathian Mountains area has a diversified geology, composed by clays, conglomerates, metamorphic, limestones, carbonated, and plutonic rocks (*Fig. A1*). Accordingly to the geological compositions, the International Hydrogeological Map of Europe (IHME), dating from 2013 at 1:1,500,000 scale (BGR and UNESCO, 2013) reported six types of aquifer's productivity (*Fig. 1*). In base of the productivity of aquifers, the vulnerability factor for each aquifer was assigned into the GIS database (*Table 2*) and further at spatial scale of Carpathian Mountains region (*Fig. 3*). For each media that compose the aquifer, the potential infiltration coefficient (PIC) was assigned accordingly to the hydrogeological specific literature (Civita, 2005). The importance of infiltration coefficients for groundwater highlights the aquifers sensitivity in term of quality (Čenčur Curk et al., 2014). *Figure A2* in the *Appendix* shows the PIC spatial distribution in the Carpathian Mountains area.

Table 2. *Aquifers productivity in Carpathian Mountains region. (Source: IHME, 2013; Čenčur Curk et al, 2014)*

Aquifers type	Vulnerability factor
Highly productive fissured aquifers (including karstified rocks)	0.8
Low and moderately productive fissured aquifers (including karstified rocks)	0.4
Highly productive porous aquifers	0.7
Low and moderately productive porous aquifers	0.3
Locally aquiferous rocks, porous or fissured	0.1
Practically non-aquiferous rocks, porous or fissured	0.05
Snow field/ice field	0

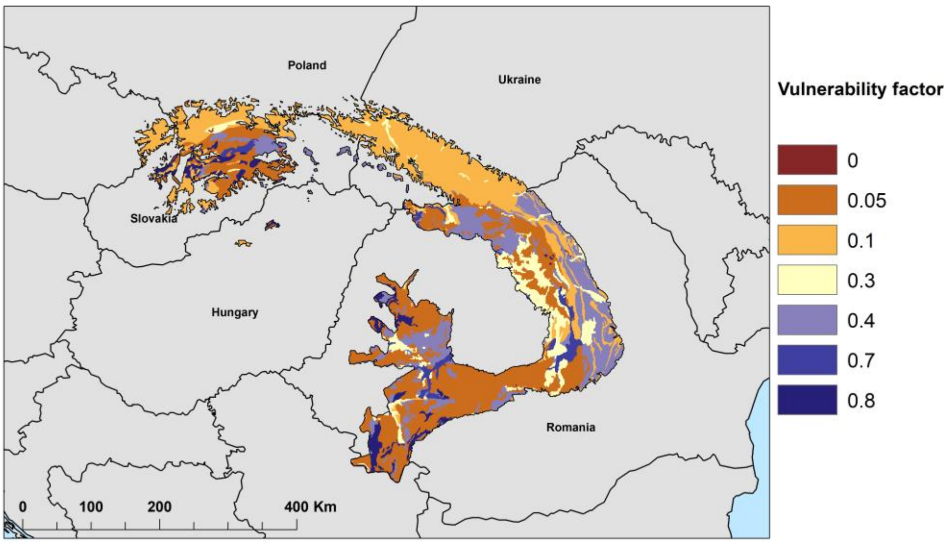

Figure 3. *Vulnerability factor distribution at spatial scale of the Carpathian Mountains region*

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Terrain data and infiltration map

The infiltration process is mainly controlled by the terrain configuration and lithology. In base of the Nistor et al. (2015) approach, the infiltration map is calculated as the ratio between PIC and slope angle. Thus, the digital elevation model (DEM) of the Carpathians region have been used to generate the slope angle and from the geological map it was processed the PIC. This reasoning considers that where the PIC is higher and the slope angle is lower, the infiltration values will be higher. The calculations were performed in ArcGIS software using normalized values (0 to 1) of the PIC and slope angle raster grid data. *Figure 4* depicts the infiltration map of the Carpathian Mountains area used in the groundwater vulnerability mapping.

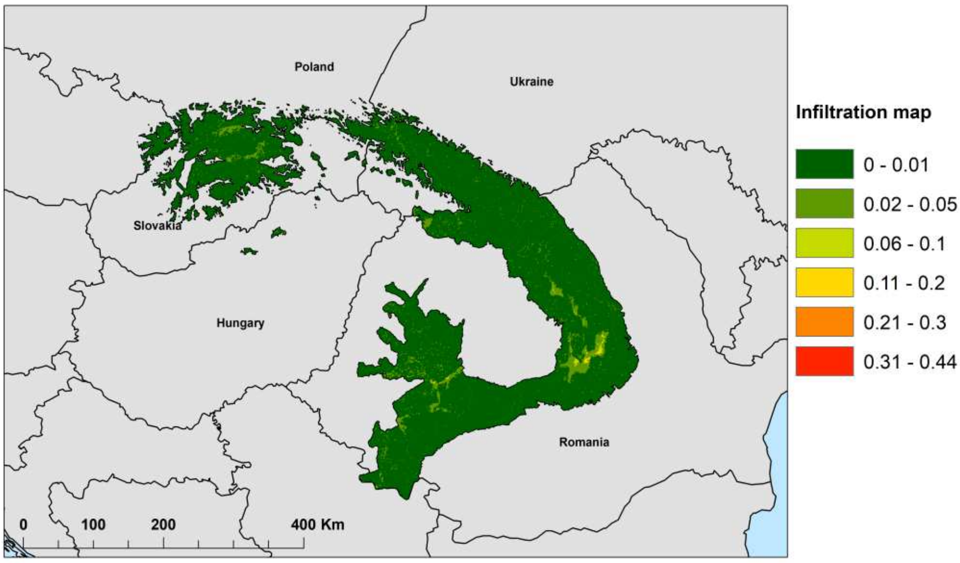

Figure 4. *Infiltration map of the Carpathian Mountains region*

Land cover data

The vegetation layer, bare soil, water bodies, and artificial areas over Carpathian Mountains area were collected from the World Land Cover database elaborated by China in collaboration with the United Nations. This land cover is 30 × 30 m in spatial resolution and cover entire study area indicating 10 classes of cover type. The future projections of the land cover were done accordingly to Nistor et al. (2015) only for the artificial, agricultural, and forest areas. All transformations have been processed on the vector layer of and cover, applying a linear buffer to the vector feature of artificial areas by 23 m, to agriculture by 41 m, and to forest by 80 m. Thus, the resulted surfaces were clipped and integrated in the original land cover vector layer for present and future (*Fig. A3*). *Table 3* reports the values of pollution load index (PLI) used in the present study. The resolution of the PLI at spatial scale (*Fig. 5*) were set at 1 km^2, to be in line with the climatological data.

Potential evapotranspiration (ET0)

Thornthwaite (1948) method (*Eq. 1*) was adopted for the long-term calculations of monthly ET0 during the three set time periods: 1961-1990 (1990s), 2011-2040 (2020s),

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and 2041-2070 (2050s). Even if old, this approach is still used in the regional studies of climate, hydrology, and agriculture (Zhao et al., 2013). The monthly and annual ET0 and water availability have been computed for whole Europe by Dezsi et al. (2018) and the gridded data are available through open access web site (https://doi.org/10.5281/zenodo.1044306). In base of monthly ET0 we have computed the annual ET0 and further annual ETc and AETc raster maps.

$$ET_0 = 16\left(\frac{10T_i}{I}\right)^{\alpha} \quad \text{(Eq.1)}$$

where:

ET_0 – monthly potential evapotranspiration [mm]
T_i – average monthly temperature [°C], $ET_0 = 0$ if mean temperature < 0
I – heat index (*Eq. 2*)
α – complex function of heat index (*Eq. 3*)

$$I = \sum_{i=1}^{12} \left(\frac{T_i}{5}\right)^{1.514} \quad \text{(Eq.2)}$$

where:

T_i – monthly air temperature

$$\alpha = 6.75 \times 10^{-7} I^3 - 7.71 \times 10^{-5} I^2 + 1.7912 \times 10^{-2} I + 0.49239 \quad \text{(Eq.3)}$$

where:

I – annual heat index

Table 3. *Corine Land Cover classes and relative pollution load index applied the Carpathian region. (Source: Wochna, 2011; Čenčur Curk et al., 2014; Nistor et al., 2015)*

CLC code 2012	CLC description	Pollution load index	Normalized values
133	Artificial surface	7	0.35
211	Non-irrigated arable land	12	0.80
313	Forest	2.8	0.18
321	Grasslands	2.5	0.16
324	Shrubland	2.6	0.17
332	Bareland	5	0.33
335	Permanent snow and ice	0.1	0.007
411	Wetland	2.3	0.15
512	Water bodies	3	0.20

ETc, AETc, and water availability

In base of crop coefficients (Kc) assigned to each land cover type we have carried out the ETc. Allen et al. (1998) present the standard Kc for various types of crops. Moreover, Grimmond and Oke (1999) calculated the Kc in the urban areas indicating the specific Kc for several cities in the United States. In South Europe, Nistor and Porumb-Ghiurco (2015) set a methodology for the ETc mapping at spatial scale.

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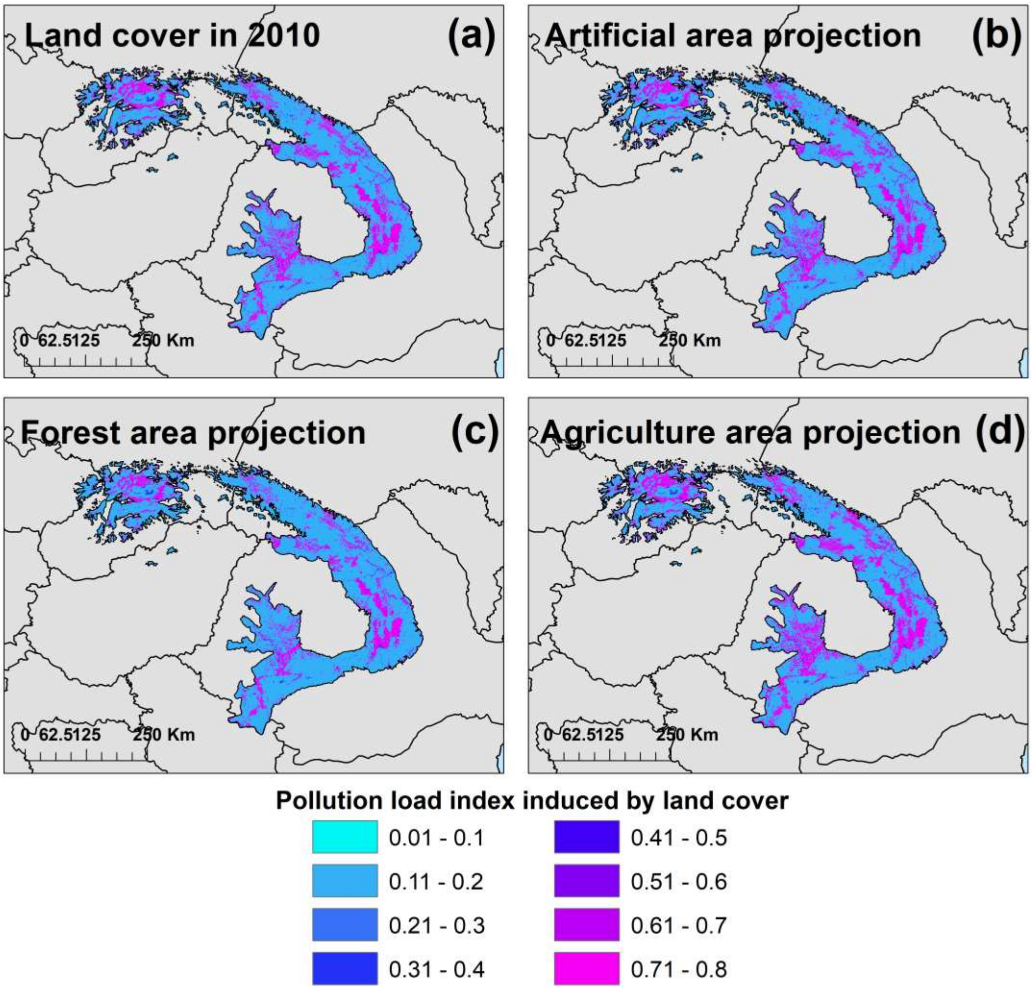

Figure 5. *Pollution load index calculated in base of land cover in the Carpathian Mountains region. (a) Pollution load index pattern related to land cover 2010. (b) Pollution load index pattern related to artificial area projection. (c) Pollution load index pattern related to forest area projection. (d) Pollution load index pattern related to agricultural area projection*

The calculation of ETc is the product of the ET0 and Kc (*Eq. 4*). We prefer to incorporate the land cover in the evapotranspiration calculation because the aquifers recharge is in base of effective precipitations, which are not included in the water demand for crops. In this work, we have used the standard annual Kc values (Nistor and Mîndrescu, 2017) together with the annual ET0 to determinate the annual ETc for the past, present, and future. The annual Kc values, for the land cover classes related to Carpathian Mountains are presented in the *Table 4*.

Budyko approach (1974) was adopted to carry out the AETc calculation. This method is often used in the hydrology and related studies which required the water balance determination (Gerrits et al., 2009).

$$\text{Annual ETc [mm]} = \text{annual ET0 [mm]} \times \text{annual Kc [mm]} \qquad \text{(Eq.4)}$$

From the difference between precipitation and AETc, we have found the water availability. Budyko (1974) formula is expressed in *Equation 5*. The water availability

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(*Eq. 7*) for 1990s, 2020s, and 2050s was subtracted from the annual AETc and annual precipitation. All raster grids operations were performed in ArcGIS environment.

$$\frac{AETc}{PP} = \left[\left(\varphi \tan\frac{1}{\varphi}\right)\left(1 - \exp^{-\varphi}\right)\right]^{0.5} \quad \text{(Eq.5)}$$

where:

AETc – actual land cover evapotranspiration [mm]
PP – total annual precipitation [mm]
Φ – aridity index (*Eq. 6*)

$$\varphi = \frac{ETc}{PP} \quad \text{(Eq.6)}$$

$$\text{Effective precipitation [mm]} = \text{annual precipitation[mm]} - \text{annual AETc[mm]} \quad \text{(Eq.7)}$$

Table 4. *Corine Land Cover classes and appropriate annual Kc used for ETc in Carpathian Mountains region. (Source: From Allen et al., 1998; Nistor and Mîndrescu, 2017)*

Corine land cover		**Kc annual**				
CLC code 2012	CLC description	Kc	Ks	Ku	Kw	Kclc
133	Artificial surface	-	-	0.26	-	0.26
211	Non-irrigated arable land	1.14	-	-	-	1.14
313	Forest	1.35	-	-	-	1.35
321	Grasslands	0.95	-	-	-	0.95
324	Shrubland	0.9	-	-	-	0.9
332	Bareland	-	0.2	-	-	0.2
335	Permanent snow and ice	-	-	-	0.51	0.51
411	Wetland	-	-	-	0.45	0.45
512	Water bodies	-	-	-	0.64	0.64

Kc – crop coefficient for plants, Ks – evaporation coefficient for bare soils, Ku – crop coefficient for urban areas, Kw – evaporation coefficient for open water, Kclc – crop coefficient for land cover

Groundwater vulnerability assessment using Spatial Analyst Tools

The groundwater vulnerability mapping was determinate by multi-layers analysis using Spatial Analyst Tools from ArcGIS. The method adopted here is based on the methodology applied by Nistor et al. (2015). In European regions, Čenčur Curk et al. (2014) performed the groundwater vulnerability in the South East Europe using weights. In the works of Stempvoort et al. (1993), Daly et al. (2002) and Dixon (2005), the appropriate weights for each layer were found.

We consider that the water availability represents one of the most important layers related to the water recharge quantity, which is driven by climate. Secondly, the land cover variability and implication for PLI and ecosystems play also an important role of the groundwater quality. Due to these reasons, we agree that water availability and ecosystems may have the same weights of 30%. For the aquifers vulnerability factor

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and infiltration map were set the equal weights of 20%. All layers were pondered using weights like in *Equation 8*. These weights represent the relative importance of the parameter in the group. The water availability and ecosystems have a balancing factor that shows the relative importance to the maximal deviations of the respective layer and its limitation to substitute another layer.

All layers were normalized under the standard method between 0 and 1. Final vulnerability map was divided in classes as follow: 0–0.2 for very low, 0.21–0.4 for low, 0.41–0.6 for medium, 0.61–0.8 for high, and 0.81–1 for very high. The groundwater vulnerability map was divided in four classes of vulnerability: very low, low, medium, and high.

$$GW\ V = (1\text{-}WA)^{1.5}\ X\ 0.3 + AV\ X\ 0.2 + IM\ X\ 0.2 + ES^{1.5}\ X\ 0.3 \qquad \text{(Eq.8)}$$

where:

GW V = Groundwater Vulnerability
WA = Water Availability
AV = Aquifer Vulnerability
IM = Infiltration Map
ES = Ecosystem Services

Results and discussion

The climate change in Europe and in the Carpathian Mountains area indicates significant variation in the mean air temperature and evapotranspiration values for 2020s and 2050s, but also slightly modifications in the precipitation extremes. In base of the climate models observations, the mean annual temperature is expected to increase from –0.8 to 13.5 °C during the 2020s (*Fig. 6a*) in the Carpathian Mountains area. The future projections indicate values of the mean air temperature from 0.2 to 14.4 °C during the 2050s period (*Fig. 6b*) in the study area. The precipitation pattern shows values between 460 mm and 1667 mm year-1 for the 2020s (*Fig. 6c*) and between 478 mm to 1730 mm year^{-1} for 2050s (*Fig. 6d*). The annual ET0 in the Carpathian Mountains area is expected to range from 312 mm to 674 mm during 2020s and from 327 mm to 713 mm during 2050s (*Fig. 6e* and *f*).

Climate change effects on the Carpathian Mountains are reflected into the annual ETc. During 1990s, the annual ETc ranges 56 mm to 740 mm while in the future periods the ETc is expected to increase up to 910 mm (2020s), respective 963 mm (2050s). *Figure A4* depicts the spatial variation of annual ETc in the study area, highlighting the high values (over 700 mm) in the Apuseni ad Eastern Carpathians, especially during the 2020s and 2050s periods. The large area with high values is represented by the forest projection scenario (*Fig. A3d*) while in the artificial and agricultural scenarios for 2050s the ETc pattern do not differ too much (*Fig. A3c* and *d*).

The implication of the annual ETc (*Fig. A4*) in the groundwater vulnerability is reflected through AETc and water availability. Thus, the annual AETc varies in the past period from 55 mm to 525 mm showing the maximum values in the Western Carpathians. During 2020s period, the annual AETc ranges from 63 mm to 575 mm indicating high values (over 500 mm) in the Western and Southern Carpathians. For the 2050s period, the annual AETc ranges from 66 mm to 596 mm, depicting the high values (over 500 mm) in western, southern, and eastern sides of the study area. In the

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future years of 2050s, a larger area with high values of the annual AETc occupies the territory in comparison with the 1990s and 2020s. *Figure A5* illustrates the spatial distribution of annual AETc in Carpathian Mountains area.

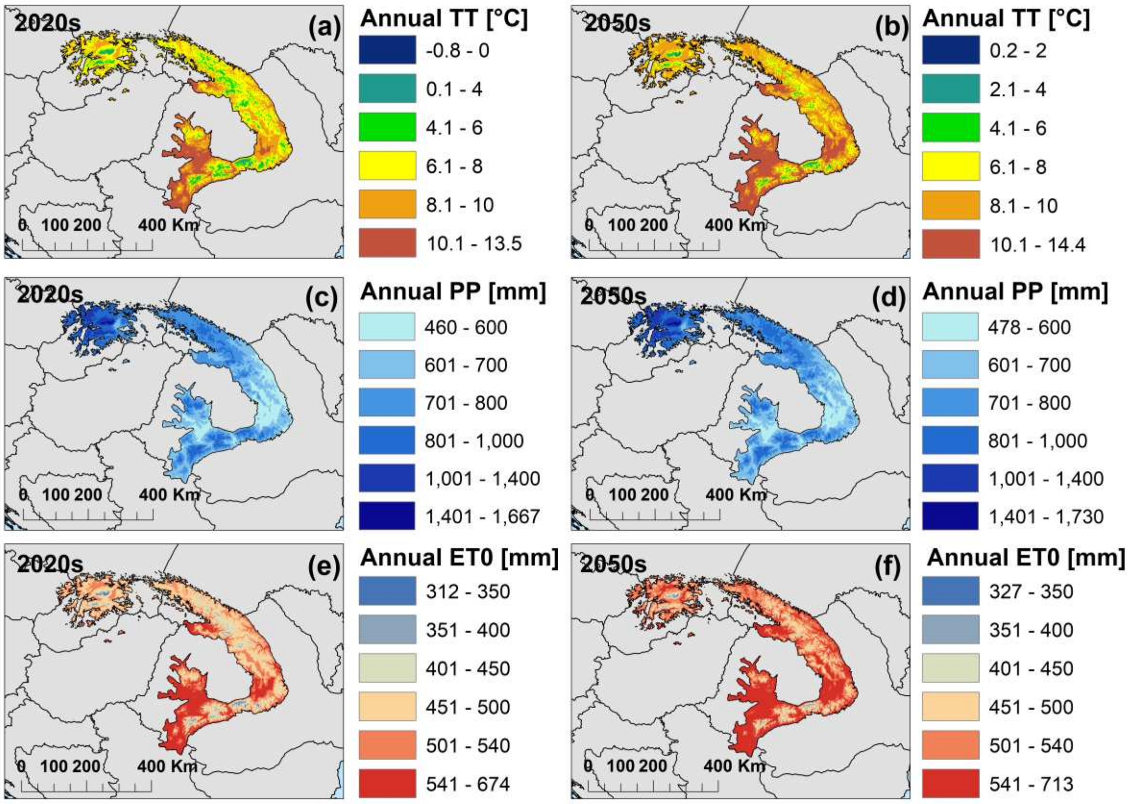

Figure 6. *Models projections of temperature, precipitation, and ET0 in the Carpathian Mountains region. (a) The average of mean annual air temperature between 2011 and 2040 (2020s). (b) The average of mean annual air temperature between 2041 and 2070 (2050s). (c) The average of mean annual precipitation between 2011 and 2040 (2020s). (d) The average of mean annual precipitation between 2041 and 2070 (2050s). (e) The average of annual ET0 between 2011 and 2040 (2020s). (f) The average of annual ET0 between 2041 and 2070 (2050s)*

Considering the annual AETc and precipitation patterns, the annual water availability was carried out for the 1990s, 2020s, and 2050s periods. In the Carpathian Mountains area, the water availability ranges during 1990s from 129 mm to 1635 mm (*Fig. 6a*), indicating the lower values (below 200 mm) in the South of Apuseni Mountains, in the western sides of the Curvatures Carpathians, eastern side of the Eastern Carpathains. The high values (over 800 mm) of the water availability spread in the Southern Carpathians and Western Carpathians, but also in the North of Eastern Carpathians. During the 2020s period, the water availability ranges from 66 mm to 1603 mm illustrating a large area with low water availability (below 200 mm) in the Southern and Eastern Carpathians. The annual water availability ranges in the 2050s from 69 mm to 1663 mm depicting low values of water availability in the Apuseni Mountains, Southern and Eastern Carpathians. *Figure 7* shows the water availability variation in the Carpathian Mountains area at spatial scale.

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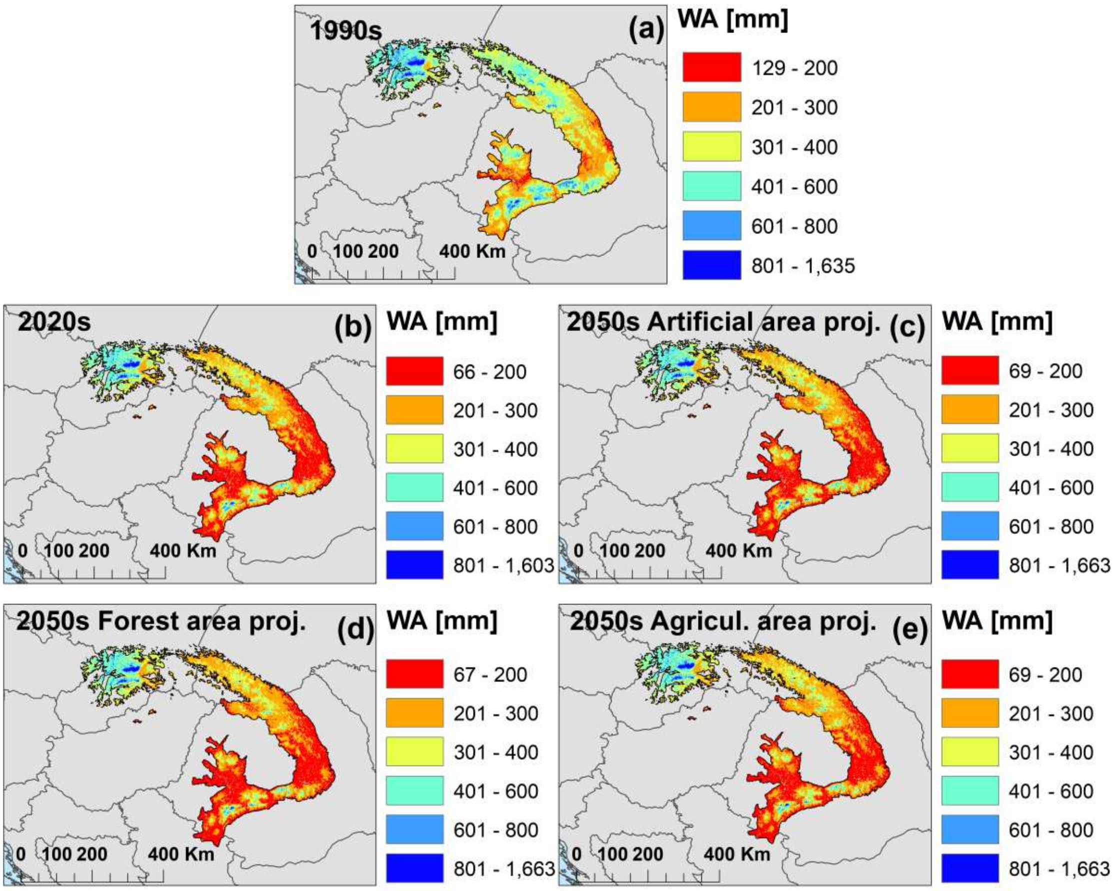

Figure 7. *Spatial distribution of water availability (WA) in the Carpathian Mountains region. (a) WA related to the past period (1990s). (b) WA related to the present period (2020s). (c) WA related to the future period (2050s) artificial area projection. (d) WA related to the future period (2050s) forest area projection. (e) WA related to the future period (2050s) agricultural area projection*

Groundwater vulnerability at spatial scale in the Carpathian Mountains area was calculated using spatial analysis by weights in the ArcGIS environment. A complex methodology was applied using climate models, geological data, DEM, and land cover of the study area. *Figure 7a* depicts the groundwater vulnerability map of Carpathian Mountains area during 1990s. The groundwater vulnerability in the 1990s shows major part of the territory as low vulnerable at climate change and land cover. The very low class was depicted in the Western and Southern Carpathians only in the elevated areas (above 2000 m). The high vulnerability areas were found in the Curvatures Carpathians and South of Apuseni Mountains. During the 2020s years the groundwater vulnerability map (*Fig. 8b*) indicates a large area of high vulnerability class, especially in the Curvatures Carpathians, while the very low vulnerability reduced at spatial scale. The future scenarios (Fig. *8c–e*) show decreasing in area of very low vulnerability class and increase of the high vulnerability class. The forest projection indicate the maps of worst scenario with 2.41% from the total area with high vulnerability, followed by the artificial area projection (*Table 5*).

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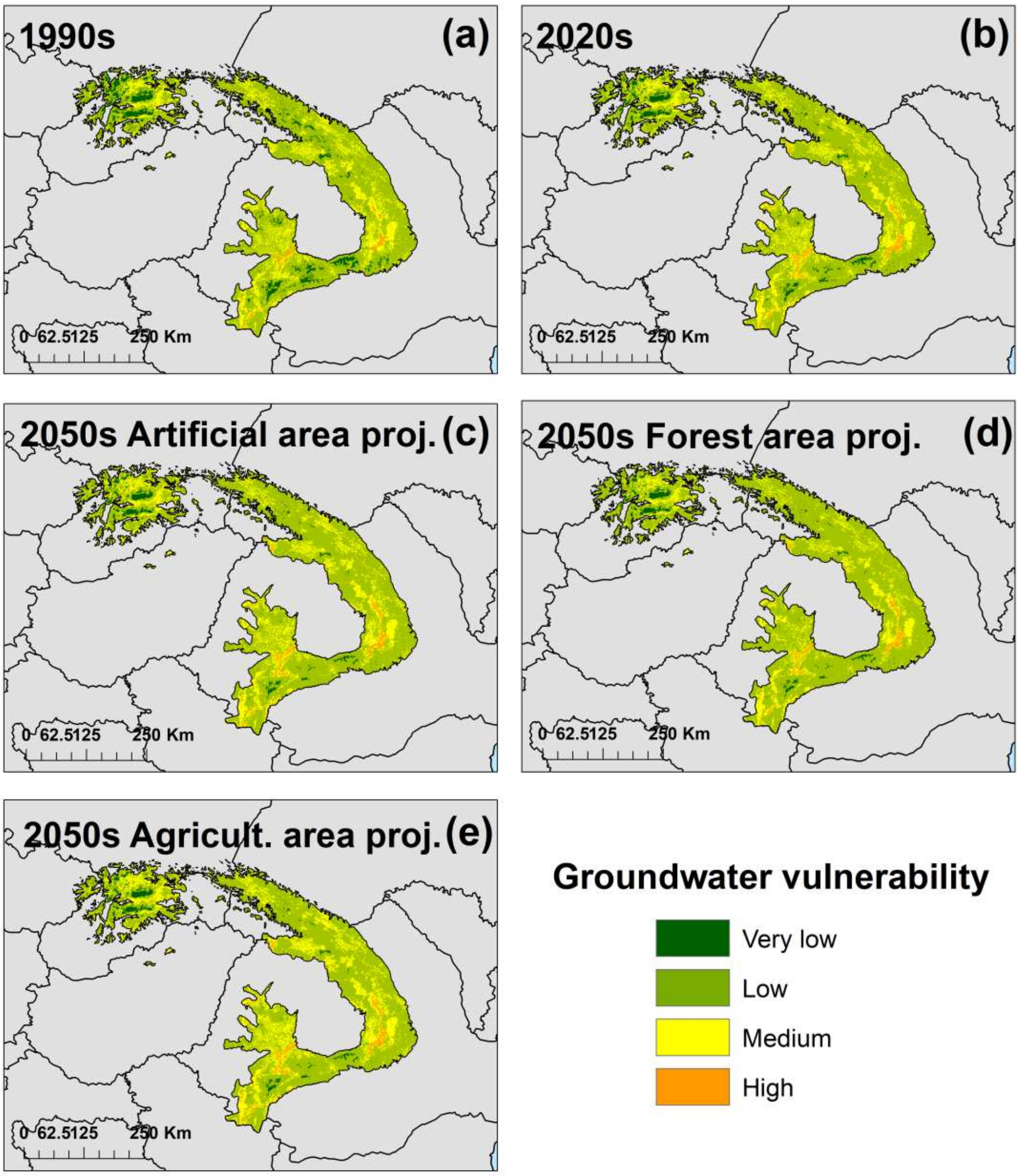

Figure 8. *Groundwater vulnerability map of the Carpathian Mountains region. (a) Groundwater vulnerability map related to the past period (1990s). (b) Groundwater vulnerability map related to the present period (2020s). (c) Groundwater vulnerability map related to the future period (2050s) artificial area projection. (d) Groundwater vulnerability map related to the future period (2050s) forest area projection. (e) Groundwater vulnerability map related to the future period (2050s) agricultural area projection*

The main aim of this work was to calculate the groundwater vulnerability at spatial scale of Carpathian region area. The analysis of multi-layers data in ArcGIS was focused on the climate models and land cover projections. Under the climate change, the southern areas of Carpathians are negatively affected in terms of groundwater vulnerability increases. These modifications are related to the increase of territory with low water availability (below 200 mm) for the 2020s and 2050s, in all scenarios. In this sense, the groundwater vulnerability increase from the quantitative point of view

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because the recharge of aquifers might a reduction. The quality of the groundwater was mainly controlled in our analysis by aquifers potential infiltration coefficient (PIC) (*Fig. A2*) and the pollution load index (PLI) (*Fig. 5*). As a first driver of groundwater quality, the higher PIC varies in the Carpathian Mountains region overlapping with the existing aquifers in limestones, sandstones, sandy, and gravels. These aquifers are defined as highly productive porous aquifers located mainly in the intermountain depressions. In these types of aquifers the hydraulic conductivity in relatively higher than in the aquifers formed by schists and gneisses or plutonic rocks. Secondly, the PLI factor follow the land cover distribution (*Fig. A3*). Thus, the artificial areas and agricultural lands influence the increases of groundwater vulnerability, while the forest and grass form the ecosystems, which provide an advantageous media for groundwater quality. For this reason, the low vulnerability extends on larger area (around 77.73%) in the forest projection model.

Table 5. *Percentage area of groundwater vulnerability calculated in Carpathian Mountains area*

Vulnerability classes	GW vulnerability 1990s (area %)	GW vulnerability 2020s (area %)	GW vulnerability 2050s, artificial projection (area %)	GW vulnerability 2050s, forest projection (area %)	GW vulnerability 2050s, agriculture projection (area %)
Very low	6.19	3.50	2.75	2.49	2.67
Low	70.45	71.73	72.29	77.73	68.66
Medium	22.16	22.64	22.83	17.87	26.25
High	1.19	2.13	2.13	1.91	2.41

The methodology of groundwater calculation and land cover projection applied in this survey is close to the procedure followed by Nistor et al. (2015). They used the appropriate data to determine the groundwater vulnerability on a district from Apuseni Mountains, in Romanian Carpathians. Here, we extrapolated the methodology to entire area of Carpathians Mountains using spatial analysis in ArcGIS. With few changes regarding the utilization of high-resolution climate models and geology implication, our results indicate appropriate pattern of groundwater vulnerability with the studies carried out by Čenčur Curk et al. (2014), Nistor et al. (2015). The limitations of this study may be improved using in-situ monitoring of spring's groundwater to observe the climate change effects on seasonal periods. In this study, we have focused on long-term period analysis, so the runoff and water availability variables on short-term period were neglected. Using additional methods, such as VESPA index (Galleani et al., 2011),) or spring variability in base of hydrographs, the groundwater vulnerability could be performed in specific locations and further to create various models at spatial scale. At this level, GIS techniques were the best solutions in sense of long-term period, land cover projections, and layer analysis.

Conclusions

Spatial analysis by weights in ArcGIS environment contributed to determine the groundwater vulnerability in the Carpathian Mountains area. A complex methodology including the climate models in three periods, aquifers and geology, morphometric

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terrain data, and the dynamic land use layers indicates a quarter part of the Carpathian Mountains area territory suffers high and very high groundwater vulnerability under climate change. Moreover, the future scenarios highlight almost a third part of the region in the high and very high vulnerability class. These results evidence that the territory of the Carpathian Mountains area is faced with the actual global threats that may occur also at regional scale. These problems are mainly related to both a reduction of precipitation amount and an increase in the mean air temperature. These findings can help the policymakers and the administrative staff of the region to take the counterbalance measures against the natural changes that are expected during the 21st century. The maps carried out in this study may represent strategic tools for the landscaping and delineating the areas with more restrictive actions.

We presented a long-term analysis which is based on spatial data and empirical formulas. Considering the complexity of the data used here, the errors may be neglected for the long period. However, the methodology was applied for other study cases from Europe and we assume that our results are valid. To be more accurate, the future work could be conducted at site scale to assess the groundwater vulnerability in situ (i.e. springs, check quality of groundwater in wells). The groundwater quality in the agricultural areas and different flow models of groundwater may improve the results at local scale.

Acknowledgements. The authors would like to thank Andreas Hamann from Alberta University for the climate models data and support during the revision process.

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APPENDIX

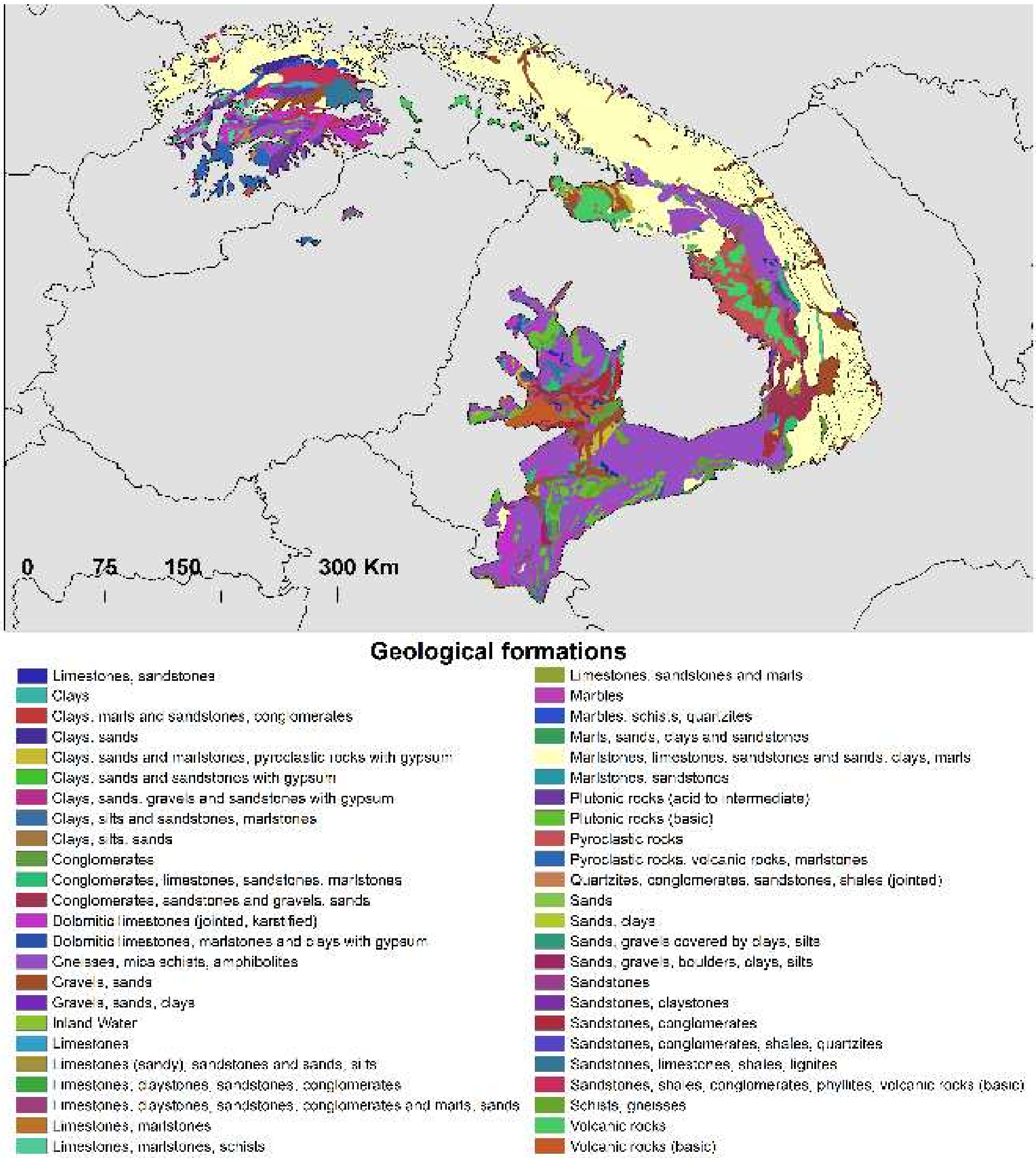

Figure A1. *Geological formations of the Carpathian Mountains region*

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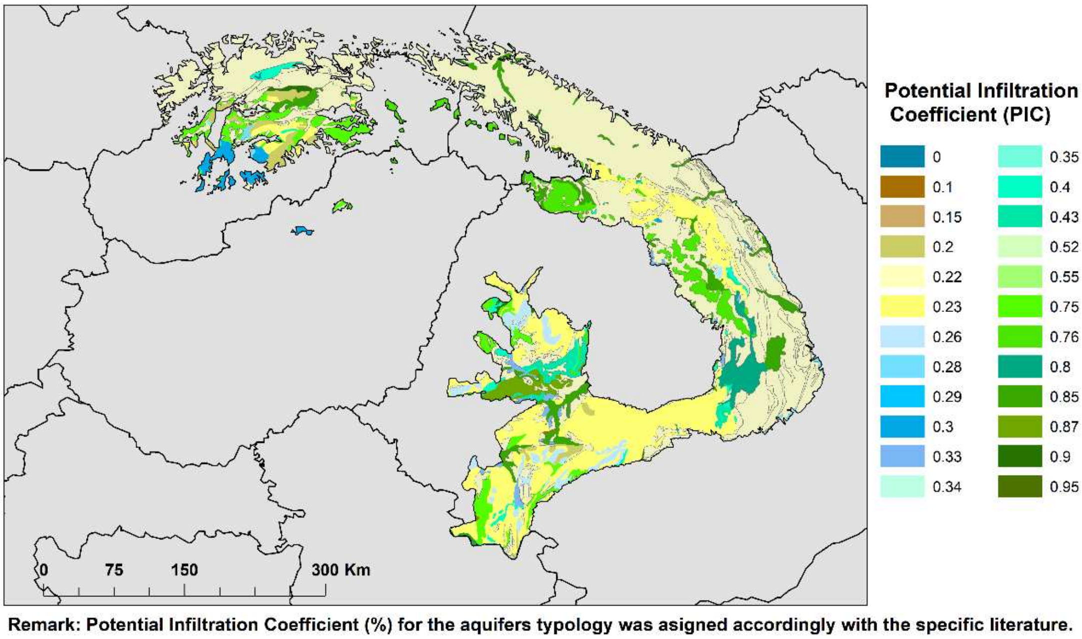

Figure A2. *Potential infiltration coefficient assigned to each type of aquifer in the Carpathian Mountains region*

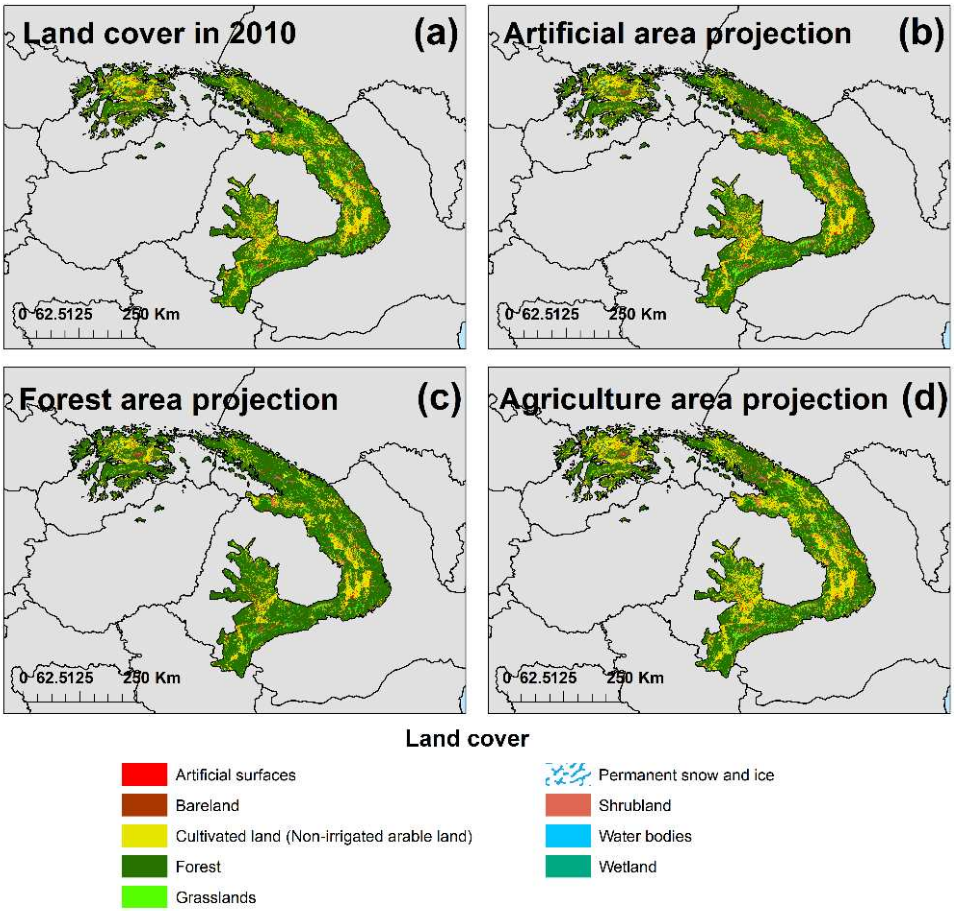

Figure A3. *Land cover of the Carpathian Mountains region in 2010 and future projections*

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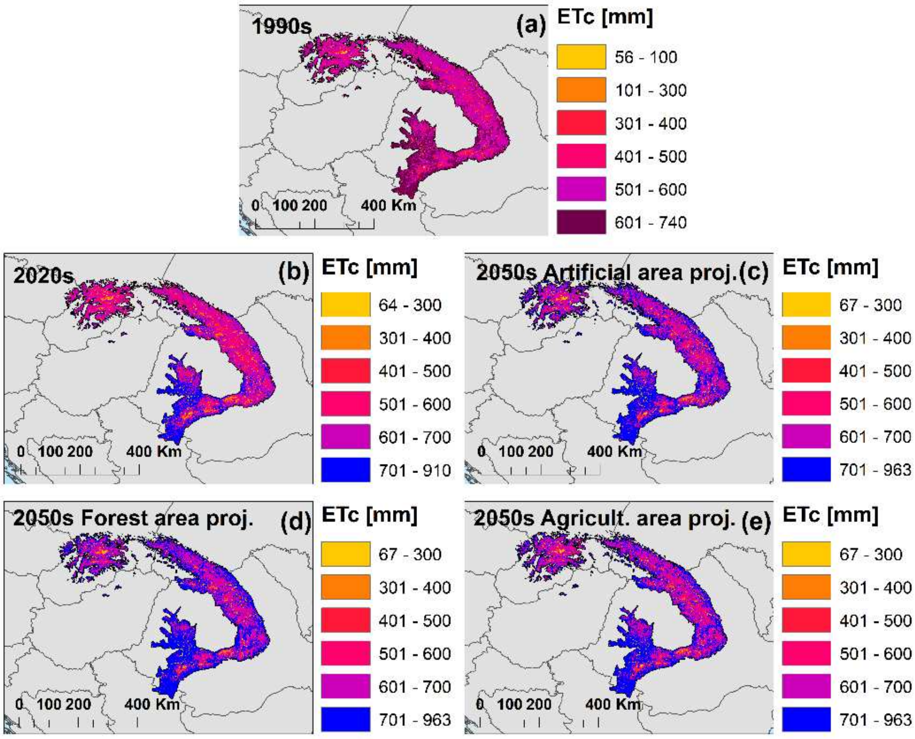

Figure A4. *Spatial distribution of annual crop evapotranspiration (ETc) in the Carpathian Mountains region and future projection under land cover and climate models*

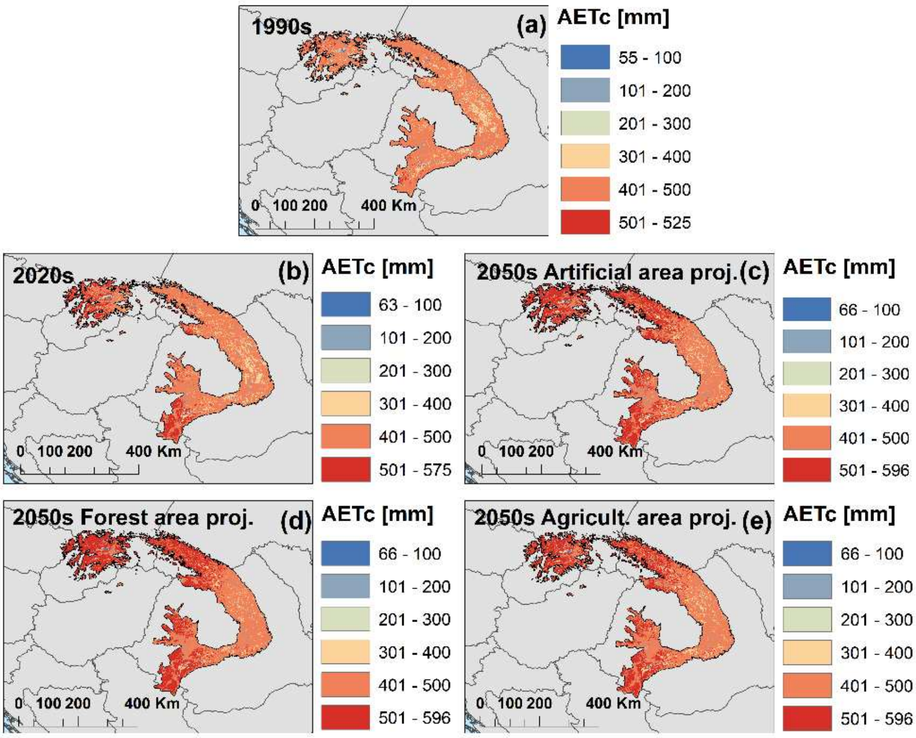

Figure A5. *Spatial distribution of annual actual crop evapotranspiration (AETc) in the Carpathian Mountains region and future projection under land cover and climate models*

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ROLE OF NEW HERBICIDES IN DODDER (*CUSCUTA CAMPESTRIS*) CONTROL IN SUGAR BEET (*BETA VULGARIS*) FIELDS

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Abstract. Dodder *(Cuscuta campestris)* control is a challenge and dodder is one of the factors limiting the cultivation of sugar beet in many regions of Iran. New herbicides can be useful in chemical control of dodder. This study was conducted to evaluate the effect of new mixture of herbicides on dodder control in sugar beet field. Results showed that the Propyzamide (3 L ha^{-1}) had the highest effect on dodder weed weight loss% and this treatment led to 99.59% reduction in its fresh and dry weight. In addition, herbicide treatments were significant at 0.01 significance level within 15 and 30 days; after this time application of Propyzamide 3 L ha^{-1} led to 90.63 and 100% reduction in dodder weed, respectively. The Propyzamide 3 L ha^{-1} led to 80.63% increase in sugar beet foliage dry weight within 30 Days After Herbicide Application (DAHA). In addition, between treatments the highest increase percentage of sugar beet yield was obtained by Ethofumesate (2 L ha^{-1}) along with Gallant Supper (1 L ha^{-1}) and the removal of broad leaf about 86.55% compared to control. Totally, Propyzamide (1.9 L ha^{-1}) along with Gallant Supper (0.75 L ha^{-1}) showed the best results with the lowest dosages.
Keywords: *chemical control, Ethofumesate, mixture herbicides, parasitic weed, Propyzamide*

Introduction

It is believed that parasitic weeds have emerged as a serious challenge in agricultural production globally. Research show that roughly 20 families (3,000–5,000 species) of higher plants are parasitic in the plant kingdom. They are likely to inflict production losses of 30–80% in main food and industrial crops around the world. Contrary to other weeds, conventional methods cannot control the parasitic weeds because of their life style. They mixed thoroughly with the host and their metabolic feature is strikingly similar to the host, which cannot be distinguished by treatments. Some of the parasites locate closely to the host root, which is hidden and cannot be detected until final are observed. Various approaches have been introduced including cultural, mechanical, chemical, use of resistant varieties, and biological to curb the negative effects of parasitic weeds; but most of them failed to achieve this aim.

One of the parasitic plant is field dodder, which is found on the stems and leaves of broadleaf plants, including weeds, field crops, vegetables, and ornamentals around the world. It is not easy to control field dodder because of close intimacy and interaction between the host and the parasite; thus, there must be herbicide that attacks the parasite without inflicting damage on the host (Sarić-Krsmanović and Vrbničanin, 2017).

Cuscuta campestris, also known as field dodder, has emerged as a widespread weed in several continents including Africa, Asia, Europe, Australia, and South America (Parker and Riches, 1993). All species of the genus *Cuscuta* receive their required mineral, water, and

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carbohydrates from their host plants because of the lake roots and leaves; besides some of the species of this genus are not able in terms of photosynthesis, including forage crops (especially alfalfa) and vegetables, some tree crops (grapevine, coffee), and ornamentals plants (Albert et al., 2008).

Dodder affects the growth and yield of the infected plants and it causes losses, which range from slight to complete destruction of the crop (Agrios, 1978 and Saric-Krsmanovic et al., 2017). Estimated yields sugar beet losses ranges are reduced by 3.5–4 t ha^{-1} (Aly et al., 2003). Sarić-Krsmanović et al. (2015) conducted a study to investigate to see how glyphosate, propyzamide, imazethapyr, and diquat herbicides are able to curb field dodder in alfalfa of Pot and field trials in Novi Sad (location Rimski Šančevi) and in the field at Popovići (in the vicinity of Mladenovac). The highest effectiveness of 95% and 97.5%, was reported by two glyphosate application rates (288 and 360 g a.i. ha) respectively.

If weed control is neglected, because of widely spaced rows and slow crop development in the early growing stages of sugar beet, up to 95% yield disappears (Petersen, 2004). The reduced use of herbicides has become a necessity to limit environmental pollution and safeguard human health. Consequently, in the last years, the general trend was to achieve a gradual reduction in doses applied in pre and post-emergence using several blends of products to assess their synergic effects. The reduced use of herbicides can be obtained by replacing herbicide treatments with revaluation of agronomic techniques and minimizing the dose rate of herbicides (Cioni and Maines, 2010). Chemical control plays an important role in weed control in sugar beet production, until sugar beets become established; they are very susceptible to competition from weeds. That is one reason why many growers like to use pre-plant or pre-emergence herbicides. Early poste-mergence herbicide applications also help reduce competition from weeds while the sugar beets are small (Morishita, 2003). The most important herbicide mixtures contain the following active ingredients: metamitron, phenmedipham, desmedipham and ethofumesate (Vasel et al., 2012). Several studies have been conducted to evaluate the effects of herbicides on weeds. Longden (1989) reported that Weed beet did not affect the concentration of sugar (sucrose), potassium, sodium, α amino nitrogen, or invert sugar in the crop beets. Root and sugar yields were progressively reduced by increasing densities of weed beet.

The results of the study by Khaksar et al. (2017) showed that weed management had significant effect on root yield and sugar yield. Mean compared results illustrated that plots treated with chemical control and hand weeding were superior to control in terms of root yield and sugar yield with less impurity. Mekki (2016) evaluated treatments such as unweeding, one-hand hoeing, two-hand hoeing and chemical herbicide application (Acetochlor) at a rate of 0.750 L/feddan as pre-emergence, (one feddan = 0.42 ha) on weed control of sugar beet. Twice hand-hoeing resulted in a sharp decrease in total fresh and dry weights of weeds at 75, 90 and 105 days after planting (DAP) and recording the highest root yield and its components in comparison with the other treatments. However, quality parameters were less affected using one or two-hand hoeing. Sharifi Ziveh et al. (2013) mentioned that consideration of the environmental negative effects of propyzamide and low environmental impact of ethofumesate herbicide is recommended for control dodder in the beet fields. The results of the study by Weinberg et al. (2003) showed that bleaching symptoms were observed in field dodder stems following the Flurochloridone, sulcotrione, and mesotrione treatments. Flurochloridone exerted its effects quickly; bleaching was observed in the stem 2 days after treatment (DAT) containing only 2% β-carotene and having a considerable accumulation of phytoene in comparison with the control. Nevertheless, stem elongation was not prevented by Flurochloridone treatment. Full recovery of pigment composition at newly elongated stems

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was recorded 6 DAT; sulcotrione and mesotrione exerted similar effects Flurochloridone made the recovery take more time with less speed.

Many factors are responsible for reduced yield in sugar beet in Iran from which the most important factor is weed (Mansourian et al., 2016). Parasite weeds are competitive with the sugar beet crop for light, nutrients and water resources (Zimdahl, 1980). In addition, sugar beet seedlings are not able to compete well enough with weeds (Draycott, 2008). The aim of this research was to evaluate the effect of tank mixture of herbicides on Cuscuta spp in sugar beet field.

Materials and methods

Study site and experimental design

This research was conducted at the Research Farm of Iranian Research Institute of Plant Protection, in Meshkin-Dasht-e-Karaj, Alborz province, Iran. Geographical parameters of the site are the followings: latitude of 35 ° and 41 min, longitude of 51 ° and 50 min east, height of 1200 m above sea level and its climate belongs to the semi-arid region in 2015.

This experiment was conducted in a randomized completed block design with 16 treatments and four replications (*Table 1*).

Table 1. *Experimental treatments and rates*

	The trial treatments
1	Propyzamide (2 L ha^{-1})
2	Propyzamide (2.5 L ha^{-1})
3	Propyzamide (3 L ha^{-1})
4	Ethofumesate (1.5 L ha^{-1})
5	Ethofumesate (2 L ha^{-1})
6	Ethofumesate (2.5 L ha^{-1})
7	Propyzamide (2.5 L ha^{-1}) + Betanal progress OF (3 L ha^{-1}) + the removal of grass leaf
8	Propyzamide (2.5 L ha^{-1}) + Gallant Supper (1 L ha^{-1}) + the removal of broad leaf
9	Ethofumesate (2 L ha^{-1}) + Betanal progress OF (3 L ha^{-1}) + the removal of grass leaf
10	Ethofumesate (2 L ha^{-1}) + Gallant Supper (1 L ha^{-1}) + the removal of broad leaf
11	Propyzamide (1.9 L ha^{-1}) + Betanal progress OF (2.25 L ha^{-1}) + the removal of grass leaf
12	Propyzamide (1.9 L ha^{-1}) + Gallant Supper (0.75 L ha^{-1}) + the removal of broad leaf
13	Ethofumesate (1.5 L ha^{-1}) + Betanal progress OF (2.25 L ha^{-1}) + the removal of grass leaf
14	Ethofumesate (1.5 L ha^{-1}) + Gallant Supper (0.75 L ha^{-1}) + the removal of broad leaf
15	Betanal progress OF (3 L ha^{-1}) + the removal of grass leaf
16	Gallant Supper (1 L ha^{-1}) + the removal of broad leaf

The experimental plots were set to consist of 12 m^2 (2 m in width and 6 m in length) including four planting lines (50 cm width). Seed was cultivated with an appropriate depth of 2 cm.

Each experimental plot was divided to two parts, in which 4 m^2 from top side of each plot was considered as witness (without herbicides application). It should be mentioned that herbicides application were applied only in 8 m^2 bottom down each experimental plot. Herbicides were applied when sugar beet was in two to four leaves stage (at the time of the complete attachment of the dodder to the host in each plot). Finally, the

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treatment assessments were in 15 and 30 day intervals after the application of herbicides.

For determination of dodder dry weight 30 days after treatment application, also to determine the total dry weight of weeds at the end of the growth season, they were collected by a 1 × 1 m^2 quadrature and placed in an oven for a period of one week at 75 °C after transferring to the laboratory until their drying. After drying, they were weighed by digital scales. In addition, eye scoring according to the EWRC (European Weed Research Council), scale was performed to determine the effect of herbicide treatments. At the end of the growth period, the roots of sugar beet were harvested in each experimental plot; after washing and cutting off the limbs, weighed to determine the yield.

Statistical analysis

Analysis of variance and comparisons were conducted using SAS (Version 9.1) software. The mean comparison was carried out by using Duncan's multiple range tests. Differences were accepted as significant at (P = 0. 05). Reporting of data as tables was conducted using Microsoft Excel 2016.

Result and discussion

Dodder weight loss%

The results showed that the effect of treatments on weight of dodder weed was significant at 1% significance level (*Table 2*). According to the results obtained after 30 days of herbicides application, Propyzamide (3 L ha^{-1}) had the highest effect on dodder weed fresh weight and this treatment led to 99.59% reduction in comparison with control (*Table 3*). Also, Propyzamide (2.5 L ha^{-1}), Propyzamide (2 L ha^{-1}), Propyzamide (2.5 L ha^{-1}) along with Betanal progress OF (3 L ha^{-1}) led to 99.57 98.47 and 97.08.14% reduction in dodder weed fresh weight. Different herbicides in dodder control have been studied. Sohrabi et al. (2001) reported three herbicides of Trifluoralin, Ethofumesate and Propyzamide with 0.8, 1.5 and 1.6 kg ha^{-1} respectively, Propizamide herbicide (1.6 kg ha^{-1}) reduced dodder density by 53% compared with control. Meighani et al. (2017) reported that Propizamide 2.5 kg ha^{-1} with 57% reduction in dry weight of dodder was the best treatment compared to the control and there was no significant difference between Ethofumesate 2, and 2.5 L ha^{-1} and Propyzamide 2 and 2.5 L ha^{-1}.

Table 2. *Analysis of variance (MS) for the studied traits*

Changed source	D.F	Dodder fresh weight loss (%) (30DAHA)	Dodder dry weight Loss (%) (30DAHA)	Dodder visual weed control rating (15DAHA)	Dodder visual weed control rating (30DAHA)	Total dry weight of weeds loss (%) (30DAHA)	The increase of sugar beet foliage weight (%) (30DAHA)	The increase of sugar beet yield (%)
Block	3	1172.17**	220.94^{ns}	358.07^{ns}	1432.47**	1090.59^{ns}	156.29^{ns}	548.29**
Treatments	15	1902.24**	5186.18**	981.77**	3441.22**	1234.06**	148.21*	3392.02**
Error	45	122.93	195.23	259.11	147.14	155.78	55.58	27.81
C.V		13.75	20.45	22.64	16.69	15.06	8.44	8.97

Ns, *, ** show non-significant, significant effects at 5% statistically level and, significant effects at 1% statistically level, respectively

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Results of Dodder dry weight loss% (30DAHA) showed that the highest losses (99.59%) obtained by Propyzamide 3 L ha^{-1} and this treatment showed no significant differences with Propyzamide 2.5 L ha^{-1}, Propyzamide 2 L ha^{-1}, Propyzamide (2.5 L ha^{-1}) along with Betanal progress OF (3 L ha^{-1}), Propyzamide (2.5 L ha^{-1}) along with Gallant Supper (1 L ha^{-1}), Ethoefumesate 2.5 L ha^{-1}, Ethofumesate 2 L ha^{-1}, Ethofumesate 1.5 L ha^{-1}, Ethofumesate (2 L ha^{-1}) along with Betanal progress OF (3 L ha^{-1}), Propyzamide (1.9 L ha^{-1}) along with Betanal progress OF (2.25 L ha^{-1}), Propyzamide (1.9 L ha^{-1}) along with Gallant Supper (0.75 L ha^{-1}) (*Table 5*).

Dodder visual weed control rating according to the EWRC

The results indicated that there is a significant difference between herbicide treatments in comparison with the control within 15 and 30 days after spraying ($P < 0.01$). After 15 days from spraying, the application of Propyzamide 3 L ha^{-1}, Propyzamide 2.5 L ha^{-1}, Propyzamide 2 L ha^{-1}, Propyzamide (2.5 L ha^{-1}) along with Gallant Supper 1 L ha^{-1} and the removal of broad leaf, Propyzamide 2.5 L ha^{-1} along with Betanal progress OF L ha^{-1} and the removal of grass leaf, Propyzamide 1.9 L ha^{-1} along with Gallant Supper 0.75 L ha^{-1} and the removal of broad leaf led to 90.63, 87.5, 87.5, 87.5 and 84.38% reduction in dodder weed in comparison with the control (*Tables 3* and *4*). In the follow-up, after 30 days application of Propyzamide 3 L ha^{-1}, led to 100% reduction in dodder weed in comparison with control (*Table 5*); also, other treatments decreased the dodder weed and it was founded that Propyzamide 2.5 L ha^{-1} caused 98.82% reduction.

Table 3. *Herbicide efficacy of dodder control based on EWRC scaling method*

Weed reaction		
Rating	**Weed control (%)**	**Description (Wilkinson, 1971)**
1	100	Completely destroyed
2	99-96.5	Very good control
3	96.5-93	Good control
4	93-87.5	Satisfactory control
5	87.5-80	Just satisfactory control
6	80-70	Unsatisfactory control
7	70-50	Poor control
8	50-1	Very poor control
9	0	As untreated

Table 4.1. *Visual evaluation for herbicides efficacy on dodder weed control*

Herbicide treatments	Visual weed control rating after 15 days	Visual weed control rating after 30 days
Propyzamide 3 L ha^{-1}	+ + + +	+ + + +
Propyzamide 2.5 L ha^{-1}	+ + + +	+ + + +
Propyzamide 2 L ha^{-1}	+ + + +	+ + + +
Ethofumesate 2.5 L ha^{-1}	+ + +	+ + + +
Ethofumesate 1.5 L ha^{-1}	+ +	+ + +
Ethofumesate 2 L ha^{-1}	+ +	+ + +
Betanal progress OF (3 L ha^{-1})	+ +	-
Gallant Supper (1 L ha^{-1})	+ +	-

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Table 4.2. *Visual evaluation for mixture herbicides efficacy on dodder weed control*

Herbicide treatments	Visual weed control rating after 15 days	Visual weed control rating after 30 days
Propyzamide (2.5 L ha^{-1}) + Gallant Supper (1 L ha^{-1})	+ + + +	+ + + +
Propyzamide (2.5 L ha^{-1}) + Betanal progress OF (3 L ha^{-1})	+ + + +	+ + + +
Propyzamide (1.9 L ha^{-1}) + Gallant Supper (0.75 L ha^{-1})	+ + +	+ + + +
Propyzamide (1.9 L ha^{-1}) + Betanal progress OF (2.25 L ha^{-1})	+ + +	+ + + +
Ethofumesate (2 L ha^{-1}) + Gallant Supper (1 L ha^{-1})	+ +	+ +
Ethofumesate (2 L ha^{-1}) + Betanal progress OF (3 L ha^{-1})	+ +	+ +
Ethofumesate (1.5 L ha^{-1}) + Gallant Supper (0.75 L ha^{-1})	+ +	+
Ethofumesate (1.5 L ha^{-1}) + Betanal progress OF (2.25 L ha^{-1})	+ +	+

Controlled more than 85%: + + + +
Controlled 70 to 85%: + + +
Controlled 50 to 70%: + +
Controlled 30 to 50%: +
Controlled below 30%: -

Total dry weight of weed loss (%)

According to results Ethofumesate (2 L ha^{-1}) along with Betanal progress OF (3 L ha^{-1}) and Ethofumesate (2 L ha^{-1}) along with Gallant Supper (1 L ha^{-1}) led to 76.25 and 76.33% reduction in herbs dry weight, respectively. In addition, it was found that Propyzamide 2 L ha^{-1} treatment had the lowest effect on herbs dry weight control. Sarić-Krsmanović et al. (2015) reported that using propyzamide (1500 and 2000 g a.i. ha) was not effective enough (85% and 87%, respectively).

The increase of sugar beet root yield and sugar beet foliage weight according to the results of this study, herbicide treatments had significant effect on root yield at 1% probability level (*Table 2*), between treatments the highest yield mean was obtained by Ethofumesate (2 L ha^{-1}) along with Gallant Supper (1 L ha^{-1}) and the removal of broad leaf, which means about 88.55% increase compared to control. Also other treatments increased root yield through weed control, Ethofumesate (2.5 L ha^{-1}) along with Gallant Supper (1 L ha^{-1}) and the removal of broad leaf, Propyzamide (2.5 L ha^{-1}) along with Gallant Supper (1 L ha^{-1}), Gallant Supper (1 L ha^{-1}), Betanal progress OF (3 L ha^{-1}), Propyzamide (1.9 L ha^{-1}) along with Gallant Supper (0.75 L ha^{-1}), Ethofumesate (1.5 L ha^{-1}) along with Gallant Supper (0.75 L ha^{-1}) and Ethofumesate (2 L ha^{-1}) along with Betanal progress OF (3 L ha^{-1}) resulted in 85.66%, 84.95%, 84.79%, 84.67%, 78.59% and 78.03%, respectively in comparison with the control. The lowest increase in yield was obtained by Propyzamide 2 L ha^{-1} by 18.2% in comparison with control (*Table 5*). Also, it was founded that Propyzamide (2.5 L ha^{-1}) along with Gallant Supper (1 L ha^{-1}) and Propyzamide (2.5 L ha^{-1}) along with Betanal progress OF (3 L ha^{-1}) treatments led to 93.96 and 93.97% increase of sugar beet leaf weight as the highest effects, respectively. In this regard, the effect of some herbicide treatments were studied on dodder fresh and dry weight and sugar beet yield by Meighani et al. (2017); their results indicated Propyzamide Burst 2.5 L ha^{-1} as the best herbicide treatment and causing the highest decrease in dodder fresh and dry weight (93 to 99%, compared to dodder infested control). Based on field studies of these researchers, Propyzamide Burst 2 to 2.5 L ha^{-1} and ethofumesate 2.5 L ha^{-1} were the best treatments. Since SC formulation is

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safer and easier to apply than WP formulation, Propyzamide Burst 2 to 2.5 L ha^{-1} and ethofumesate 2.5 L ha^{-1} are recommended for dodder control in sugar beet.

Table 5.1. *The effect of herbicide treatments on the percentage means of dodder fresh and dry weight loss, increasing sugar beet foliage dry weight and sugar beet increasing yield compared to the control with dodder*

Treatments	Dodder fresh weight loss (%) (30DAHA)	Dodder dry weight loss (%) (30DAHA)	Dodder visual weed control rating (15DAHA)	Dodder visual weed control rating (30DAHA)	Total dry weight of weeds loss (%) (30DAHA)	The increase of sugar beet foliage dry weight (%) (30DAHA)	The increase of sugar beet yield (%)
Propyzamide 3 L ha^{-1}	99.59a	99.59a	90.63a	100a	40.74c	80.63b	26.11de
Propyzamide 2.5 L ha^{-1}	99.57a	98.65a	87.5a	98.82a	38.83c	80.36b	25.62de
Propyzamide 2 L ha^{-1}	98.47a	97.5a	87.5a	98.82a	38.49c	80.29b	18.2e
Ethofumesate 2.5 L ha^{-1}	95.2ab	80.14ab	71.88abc	92.13a	43.66c	81.43b	27.8d
Ethofumesate 2 L ha^{-1}	94.01ab	89.5ab	65.63abc	79.8ab	41.69c	81.1b	20.72de
Ethofumesate 1.5 L ha^{-1}	92.11ab	89.3ab	68.75abc	83.21ab	41.17c	80.98b	19.02e
Betanal progress OF (3 L ha^{-1})	49.64de	9.05d	50c	21.8c	75.49a	92.65ab	84.79ab
Gallant Supper (1 L ha^{-1})	34.32e	9.32d	50c	23.61c	75.91a	91.74ab	84.95ab

Means, in each column, followed by at least one letter in common are not significantly different at the 1% probability level using Duncan's multiple-range test

Table 5.2. *The effect of mixture herbicide treatments on the percentage means of dodder fresh and dry weight loss, increasing sugar beet foliage dry weight and sugar beet increasing yield compared to the control with dodder*

Treatments	Dodder fresh weight loss (%) (30DAHA)	Dodder dry weight loss (%) (30DAHA)	Dodder visual weed control rating (15DAHA)	Dodder visual weed control rating (30DAHA)	Total dry weight of weeds loss (%) (30DAHA)	The increase of sugar beet foliage dry weight (%) (30DAHA)	The increase of sugar beet yield (%)
Propyzamide (2.5 L ha^{-1}) + Betanal progress OF (3 L ha^{-1})	97.08a	93.85a	87.5a	98.82a	51.07bc	93.73a	76.86bc
Propyzamide (2.5 L ha^{-1}) + Gallant Supper (1 L ha^{-1})	95.82ab	96.56a	87.5a	91.44a	76.26a	93.96a	85.66a
Ethofumesate (2 L ha^{-1}) + Betanal progress OF (3 L ha^{-1})	78.59bc	69.39ab	65.25abc	65.62b	76.25a	93.86a	78.03abc
Ethofumesate (2 L ha^{-1}) + Gallant Supper (1 L ha^{-1})	72.68c	56.49c	65.63abc	65.43b	76.33a	94.18a	86.55a
Propyzamide (1.9 L ha^{-1}) + Betanal progress OF (2.25 L ha^{-1})	84.05ab	84.23ab	81.25ab	90.27a	51.42bc	91.71ab	71.35c
Propyzamide (1.9 L ha^{-1}) + Gallant Supper (0.75 L ha^{-1})	95.02ab	93.64a	84.37a	91.01a	56.41abc	91.21ab	84.67ab
Ethofumesate (1.5 L ha^{-1}) + Betanal progress OF (2.25 L ha^{-1})	50.37de	14.82d	50c	31.49c	68.66ab	91.56ab	70.84c
Ethofumesate (1.5 L ha^{-1}) + Gallant Supper (0.75 L ha^{-1})	52.97abc	10.62d	53.13c	30.53c	72.03ab	93.7a	78.59abc

Means, in each column, followed by at least one letter in common are not significantly different at the 1% probability level using Duncan's multiple-range test

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Conclusion

According to the results of the present study, Propyzamide (3 L ha^{-1}) had the highest effect on dodder weed weight. After 15 and 30 days from spraying, the application of Propyzamide 3 L ha^{-1} led to 90.63 and 100% reduction in dodder weed, respectively in comparison with control. Accordingly, Propyzamide (2.5 L ha^{-1}) along with Gallant Supper (1 L ha^{-1}) and Propyzamide (2.5 L ha^{-1}) along with Betanal progress OF (3 L ha^{-1}) treatments demonstrated the best visual weed control ratings after both periods. In addition, between treatments the highest yield mean was obtained by Ethofumesate (2 L ha^{-1}) along with Gallant Supper (1 L ha^{-1}) and the removal of broad leaf. Gallant Supper treatment illustrated the highest effect on sugar beet yield (84.95%) compared to Betanal progress. Although different dosages of Propyzamide (2, 2.5, and 3 L ha^{-1}) showed better control on dodder fresh and dry weight loss, but sugar beet yield is not affected by specific weed and vast range of weeds are effective on sugar beet yield. Therefore, it sounds that tank mixture of herbicides such as Propyzamide along with Gallant Supper led to suitable control of most weeds and increase of sugar beet yield. Totally, according to the aims of this research, which was reduced use of herbicides by tank mixture of herbicides together, Propyzamide (1.9 L ha^{-1}) along with Gallant Supper (0.75 L ha^{-1}) showed the best results with the lowest dosages.

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GLYPHOSATE AND AMINOMETHYLPHOSPHONIC ACID IN POPULATION OF AGRICULTURAL FIELDS: HEALTH RISK ASSESSMENT OVERVIEW

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Abstract. Glyphosate is the most used herbicide in the world. In 2015, it was declared as probably carcinogenic to humans by the International Agency for Research on Cancer. In Valle del Mayo, Sonora, México, more than 20000 L of this herbicide are sprayed per year. Therefore, the objective of this work was to assess human health risk associated with exposure of water contaminated with glyphosate and aminomethylphosphonic acid (AMPA) on a population in agricultural fields of Valle del Mayo communities. A cohort study was performed among the exposed populations; glyphosate and AMPA concentrations were measured in water drains, private wells, and groundwater by high-performance liquid chromatography (HPLC). Health risk was subsequently assessed obtaining a hazard ratio. Concentrations for glyphosate were lower than 5 µg L^{-1} while those for AMPA were 15-342.5 µg L^{-1} or µg g^{-1}. The results showed a statistical correlation among people consuming water from private wells with diabetes ($p \leq 0.03$) and hypertension ($p \leq 0.004$). The resulting risk assessment hazard ratio was 0.22 for agricultural laborers and 0.39 for brick makers when an acceptable daily dose of 0.03 mg kg^{-1} d^{-1} was taken into account, indicating a potential health risk.
Keywords: *herbicide exposure, occupational exposure, HPLC, AMPA*

Introduction

The yield has been reduced in agricultural practices all over the world due to weeds, thus the reason for using herbicides. Glyphosate (N-Phosphonomethylglycine) is a systemic non-selective post-emergent herbicide, whose average soil life, fluctuates from 2 to 197 days, resisting the environment for up to three years (Chufan et al., 2014; Quarles, 2012). Its use has been recorded in at least 130 countries, commercially known as Faena®, Roundup®, Dicamba, among others (Dill et al., 2010; Cattani et al., 2014) even more 750 products contain glyphosate as active ingredient (NPIC, 2015). Glyphosate is an herbicide for residential, urban and agricultural everyday use around the world, its formulations are the most sold at world level, of which 6.1 thousand millions of kg of glyphosate have been applied only in the last decade (Benbrook, 2016). The mechanism of glyphosate on plants is by the shikimate metabolic pathway. It is a chemical highly water-soluble; in the soil it degrades rapidly to sarcosine acid and aminomethylphosphonic acid (AMPA), the last one is the main metabolic intermediary (Amrhein et al., 1980).

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International disagreements exist with respect to the classification of glyphosate as carcinogenic. According to the International Agency for Cancer Research (IARC), glyphosate is “probably carcinogenic for human beings” while for the United States Environmental Protection Agency (USEPA) “it is probably not carcinogenic”. However, exists scientific research with evidence that glyphosate produces: infertility, kidney problems (USEPA, 2015), endocrine disruption (Gasnier et al., 2009), apoptosis, cytotoxicity, and neurotoxic oxidative effects (Chaufan et al., 2014; Cattani et al., 2014; Ma and Li, 2015). It has also been related to minor illnesses, such as autism, Alzheimer, Parkinson, anxiety disorders, osteoporosis, inflammatory intestinal disease, osteomalacia, cholestasis and thyroid dysfunction (Samsel and Seneff, 2015). Also, has been proved that AMPA causes genotoxicity (Mañas et al., 2009; IARC, 2016).

Exposure to glyphosate and its degrading products in air, soil, and water is unavoidable, mainly in populations close to agricultural fields. In these places, aerial and mechanical pesticide applications are constant (up to 45 times per year). Despite people are not exposed directly to these chemical agents, they could be in contact with glyphosate or AMPA either by occupation or diet, which could be regarded as a human health risk. Occupational exposure is considered of a greater risk (Nawaz et al., 2014; Parrón et al., 2014; Angeli et al., 2015).

Valle del Mayo is one of the largest agricultural producers in northwestern Mexico where an extensive irrigation system provides water to more than 93000 ha of cultivation (INEGI, 2017). The most widely used herbicide is glyphosate at 1% in canals while a mixture of glyphosate with Tordon is used at 1.5% in drains. In total, more than 20000 L of glyphosate is sprayed in this region per year just to eliminate grass in irrigation canals and drains. In Mexico, no regulations exist for glyphosate while a regulation project proposed a permissible concentration of 100 $\mu g\ L^{-1}$ (PROY NOM-250-SSA1-2014); although, it is not officially approved yet.

Most of the agricultural laborers of Valle del Mayo live next to the irrigation drains for generations. They do not have a public water supply in their homes and municipality authorities sends trucks to deliver water to these places; however, delivery delays. Some houses have private wells built a few meters from the irrigation drains, which might imply infiltration of contaminants. Additionally, in most of the housing units, kitchens are found outdoors, which could cause food contamination and skin exposure to the applied herbicides. Due to this problem, the objective of this work was to assess health risk associated to exposure of contaminated water with glyphosate and AMPA in populations of agricultural fields in the communities of Valle del Mayo, Sonora, Mexico.

Materials and methods

Geographical location of the study

It is located in Valle del Mayo (26° 31’ 54” N 109° 38’ 02” W) in the municipalities of Navojoa, Etchojoa, and Huatabampo in the southern part of the State of Sonora in northwestern Mexico. The communities close to irrigation canals were selected by cartographic research related to the Irrigation District of Rio Mayo. The following criterions were considered for the selection: (1) Housing of participants less than 50 m from an irrigation canal or drain; (2) Drains or canals with weed problems; (3) Herbicide applied at least twice a year; (4) Difficult access to drinking water; (5) Communities with private wells; (6) People working close to drains and canals.

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Assessment of health risk by glyphosate and aminomethylphosphonic acid

Hazard identification

Environmental samples collection. The points were selected in areas were herbicide formulated with 1% of N-Phosphonomethylglycine at 99% and 0.001% of adherent in canals and a mix of 1.5% of N-Phosphonomethylglycine at 99% and tordon, and 0.001% of adherent in drains. The sample collection was carried out in irrigation drains or canals close to communities with private wells were the application of glyphosate is constant. GPS coordinates are the follow: Tres Carlos 26.919385, -109.512457, Saucobe 26.958041, -109.498360, Colonia Soto 26.907112, -109.602550, Sebampo 26.878628, -109.571211, La Esquina 26.804662, -109.716644, Ramal 26.792826, -109.767667, Moroncarit 26.734392, -109.614932, Entronque 26.860334, -109.636900 and Huatabampo 26.826744, -109.650587 (*Fig. 1*).

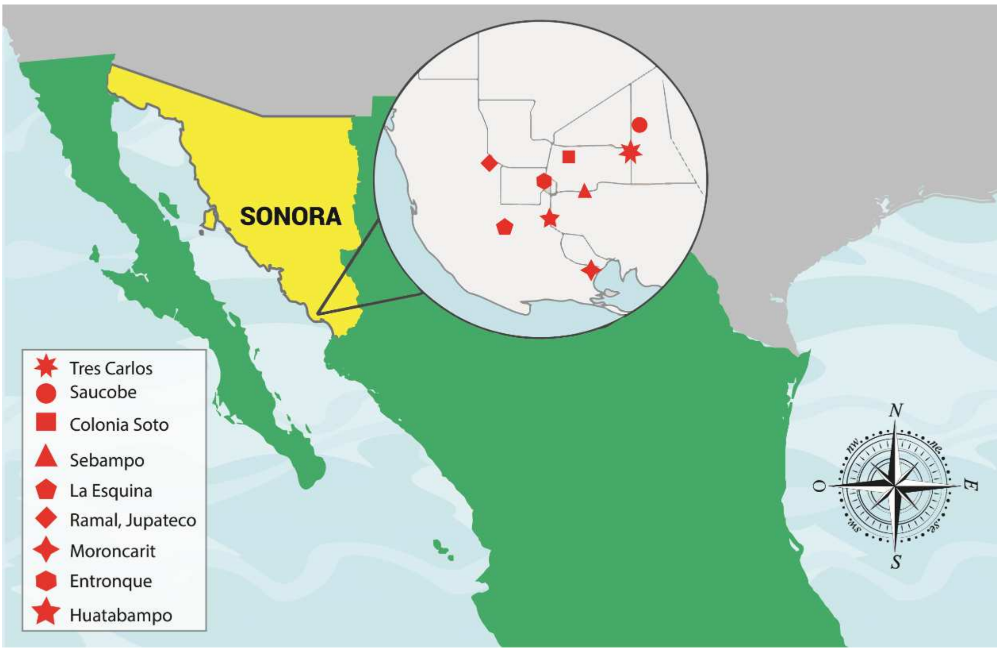

Figure 1. *Location of the selected communities for the risk assessment study of glyphosate and AMPA in Valle del Mayo, Sonora, Mexico*

The samples were collected in sterile 100 mL polypropylene wide-mouth flasks. Drain and/or canal water was taken during glyphosate application (when the brushwood was grown and the application was necessary); is for this reason the samples per place are different. In the case of soil, sediment or drain/bottom canal was also sampled where water was previously collected with a difference of one month, collecting approximately 200 g following standard methods. Well water samples were taken approximately every two months and always after herbicide application. Both were collected in the selected points from February 2016 to May 2017. All samples were transported in a cooler to the laboratory and preserved at -80 °C until analyses were performed.

HPLC analytic method. The method was adapted and modified for water and soil (Olivo et al., 2015; Peruzzo et al., 2008). Water and soil samples were purified by filtration through a cellulose acetate membrane of 0.45 µm (MF-Millipore^{TM}). Soil samples purification was performed by duplicate extraction shaking 15 g of the sample

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with 25 mL of KH_2PO_4 0.1 M for 15 min and subsequently filtered through Whatman^{TM} paper (Sigma-Aldrich, MO. U.S.A.) and centrifuged at 3500 rpm for 10 min. The obtained extract was filtered following the protocol for water samples (Peruzzo et al., 2008).

Derivatization was performed mixing 3-mL purified sample and 2-mL FMOC-Cl (0.005 M) (23186 Sigma-Aldrich, MO, U.S.A.) prepared with chloroform (650498 Sigma-Aldrich, MO, U.S.A.). It was maintained for 45 min avoiding radiation. After the reaction, 3-mL methyl chloride (270997 Sigma-Aldrich, MO, U.S.A.) were added to remove excess of FMOC-CL; subsequently, the supernatant was filtered using SPE polymeric columns (Strata-X^{TM}, Torrance, CA, U.S.A.) (Olivo et al., 2015). The collected filtered solution was taken for high-performance liquid chromatography (HPLC) (Agilent 1200 series, Santa Clara, CA, U.S.A.).

Modifications to chromatographic conditions were established as follows: Column Agilent C18 250 x 4.6 mm, the injection volume of 20 µL, water flux mobile phase 1 Ml min^{-1}, UV 240 nm and FL excitation 266 emission 315. Total analysis time per each sample was 30 minutes.

Method validation. The calibration curves were performed using one blank run and different aqueous concentration patterns of glyphosate and AMPA. The concentrations tested were from 5-25 and 15-90 µg L^{-1} for glyphosate and AMPA respectively.

Glyphosate and aminomethylphosphonic acid concentrations in vegetables washed with contaminated water with herbicides. It is possible to estimate the risk in vegetables based on the risk developed for water (Haas et al., 1999; Shuval et al., 2007; Mota et al., 2009). Taking into account that the population in the Mayo Valley uses irrigation water from drains for domestic activities, in this case, wash vegetables before eating them; the volume of water retained by the most consumed vegetables was calculated applying *Equation 1:*

$$CV = CxVr \tag{Eq.1}$$

where *CV* is the concentration in vegetables (mg g^{-1}); *C,* is the concentration of herbicide in water, and *Vr,* is the volume of water retained in the vegetable. Considered rough-texture vegetables, which have been reported to retain approximately 0.108 mL g^{-1} and 0.0036 mL g^{-1} the smooth ones (Mota et al., 2009).

Exposure evaluation

Cohort/population study. An agricultural health study was developed, which included individuals located in areas close to drains and who made use of water for domestic activities and consumption.

Population size. A total of 586 persons lived at ≤ 50 m from irrigation drains in Valle del Mayo, which was estimated visiting populations. Population size was estimated with a margin of error of 10% and confidence level of 90% using the statistical software Epi Info 7.2 (CDC, 2017).

Exposure estimation. Socioeconomic, consumption, health and symptomatology surveys were applied to the sampling populations, estimating water and food consumption patterns (portion size and frequency). The population was characterized in age, sex, occupation, schooling, socioeconomic condition, feeding, and water consumption groups.

Three exposure scenarios were established for the analysis: (a) Participants drink water from private wells close to irrigation drains (always/never); (b) Days accumulated to

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which they are exposed to (if well water is used some days or always); (c) If participants are present during herbicide application (aerial exposure).

The levels of intensity were estimated using data from the questionnaires. Aliments with higher frequency were assessed and associated with glyphosate exposure.

Toxicity assessment

The dose of a potential toxin for the human body by contact with contaminants (water, soil) should indicate the amount of the chemical substance ingested per kilogram of corporal weight per day (mg kg^{-1} day^{-1}). To calculate the dose for each source, *Equation 2* (USEPA, 1989; CIDA, 2009) was used:

$$\text{Dose (soil, water, food ingestion)} = C \times I \times AF \times Dh \times Dd \times Dw / BW \times 16 \quad \text{(Eq.2)}$$

Where C (mg kg^{-1} or L^{-1}), is the concentration of soil or water contaminants; I (kg day^{-1}), is water or soil intake in children or adults; AF, is the absorption factor in the gastrointestinal tract wherein agreement with oral exposures the value of 1 is used (HC, 2014); Dh, hours of exposure per day (0-16); Dd, days of exposure in a week (0-7); Dw, weeks of exposure in a year (0-52); BW (kg) corporal individual weight.

Risk characterization

The risks by exposure to soil and water were estimated by integrating the results of the toxicity assessment in each source and comparing them with tolerable daily intake (TDI) (HC, 1995) or also named reference dose (RfD) (USEPA, 2017) to obtain the hazard quotient, which is defined with *Equation 3:*

$$HQ = \frac{\sum Doses}{RfD} \quad \text{(Eq.3)}$$

The following RfD doses were compared in this research: 0.03 mg kg^{-1} day^{-1} (HC, 1995), 0.1 mg kg^{-1} day^{-1} (USEPA, 2017), 0.5 mg kg^{-1} day^{-1} (EFSA, 2015).

It is considered to be a potential human health risk when HQ is greater than 0.2.

Statistical methods

Concentrations and calibration curves were processed using calculus sheets of Microsoft Excel, 2016. The dose and hazard quotient was performed with the software Risk Calculation Tools (CIDA, 2009). In addition for exposure assessment were calculated significant differences to associate the consumption of contaminated water with herbicides and the suffering from diseases were analyzed using StatCalc tables 2 x 2 x N from statistical software Epi Info 7.2, 2017.

Results and discussion

The selected communities are shown in *Figure 1.* In method validation by calibration curves, a correlation coefficient (R^2) of 0.994 was obtained for glyphosate whereas that for AMPA was 0.9917. Retention times were 8.3 min and 26.5 min for glyphosate and AMPA, respectively.

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Hazard identification

During the sampling period, herbicide was applied 3 times in Saucobe and in La Esquina, and 2 times in Colonia Soto and in Sebampo; in the other sampling points no weed problems were presented and only one application was recorded. A total of 90 results of glyphosate and AMPA from 45 samples (15 for irrigation water, 15 for well water and 15 from soil); where, 70% of the samples were positive for AMPA; these results are in agreement with IARC monograph (2016) which mention that are mayor percentage of positive samples of AMPA in superficial waters. For glyphosate, the samples showed concentrations lower than 5 µg L^{-1} whereas those for AMPA were from 15-342.5 µg L^{-1} or µg g^{-1} (*Table 1*).

Table 1. *Glyphosate and AMPA concentrations in water and soil samples in different communities of Valle del Mayo, Sonora, Mexico*

Date	Valle del Mayo Community	Herbicide	Concentrations in environmental samples		
			Superficial water (µg L^{-1})	Well water (µg L^{-1})	Soil (µg L^{-1})
February-March	3 Carlos	Glyphosate	-	-	-
		AMPA	+	-	35.6
April-May	Huatabampo	Glyphosate	-	-	-
		AMPA	18	+	36.76
	Colonia Soto	Glyphosate	-	-	-
		AMPA	+	-	22.5
June-July	Ramal	Glyphosate	-	-	-
		AMPA	+	+	-
	La Esquina	Glyphosate	-	-	-
		AMPA	35.7	+	342.75
August-September	Saucobe	Glyphosate	-	-	-
		AMPA	36.8	+	279.1
	Colonia Soto	Glyphosate	-	-	-
		AMPA	+	+	89
	Moroncarit	Glyphosate	-	-	-
		AMPA	-	-	+
October-November	Sebampo	Glyphosate	+	+	-
		AMPA	+	+	56.7
	Colonia Soto	Glyphosate	-	-	-
		AMPA	-	-	42
	La Esquina	Glyphosate	+	-	-
		AMPA	-	+	298
December-January	Saucobe	Glyphosate	+	-	-
		AMPA	-	-	56.3
February-March	La Esquina	Glyphosate	-	-	-
		AMPA	+	+	-
	Sebampo	Glyphosate	-	-	-
		AMPA	+	-	47
April-May	Saucobe	Glyphosate	-	-	-
		AMPA	-	+	159

*Symbol (+) refers to having a signal ≤ 5 µg L^{-1} for glyphosate and ≤ 15 µg L^{-1} for AMPA. Symbol (-) indicates that no signal was produced in the retention time

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The positive samples (60%) for AMPA in the private wells maintained the following characteristics: built scarcely a few meters (6-8 m) from drains or irrigation canals covered by stone, brick or cement and excavated from 3-6 m in depth, which infers infiltration (*Fig. 2*). The results proved that glyphosate degrades rapidly although its presence in soil could reach groundwater by lixiviation (IARC, 2016).

Figure 2. *Photographs of private wells in the selected sites of Valle del Mayo, Sonora, Mexico (right: La Esquina private well, left: Saucobe private well)*

Exposure to glyphosate at a dose of 300 mg kg^{-1} causes severe toxicity signs; a dose from 1000 to 5000 mg kg^{-1} causes death. The maximum permissible glyphosate concentration is different in drinking water depending on the country (700 µg L^{-1} in the U.S.A; 280 µg L^{-1} in Canada; 10 µg L^{-1} in Australia) (Laubli et al., 2016). With respect to the glyphosate concentration found (≤ 5 µg L^{-1}), the detection limit of the method used in this study could be improved.

Exposure evaluation

Among the participants of the cohort study (n = 64) (*Fig. 3*), 40% of those surveyed came from the Mayo indigenous ethnic group. Age range oscillated from 15 to 81 years old; the maximum level of studies registered were secondary school and 43% of the participants were low-average socioeconomic status. As to occupation, 18.75% of the workers surveyed were brick makers, 15.63% agricultural laborers and the rest were housewives and students. As for water use, 23.81% used drain water for domestic activities; 10.94% drain water for drinking; 53.7% private well water for domestic activities; and 37.5% private well water for drinking. Brick makers used irrigation drain water for making the bricks. Housewives (22%) mentioned their children used drain and canal water for recreational activities.

The surveyed population declared (we verify with the prescriptions of the participants) having diabetes, obesity, hypertension; and dermatological, gastrointestinal and respiratory problems, among others (*Fig. 4*). Individuals, who expressed water consumption from private wells, recorded greater frequency in getting sick ($p \leq 0.01$). With respect to the correlation between water consumption from private wells and frequent diseases, statistical significance was found with diabetes ($p \leq 0.03$) and hypertension ($p \leq 0.004$). Concerning these results is necessary to take into account that the place where these people live is agricultural and they may be exposed to a large number of agrochemicals, which are used in this area. Arrebola et al. (2015) have demonstrated that individuals with obesity, exposure to high levels of certain persistent organic contaminants, were associated with the risk of developing hypertension,

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independently of other factors, such as age, tobacco or alcohol consumption. Moreover, the high risks of these contaminants doubled this risk. The glyphosate applied in the drains of the Valley is a mix with tordon at 1.5% of the total formulated; mixtures can be more harmful to human health (Hernández et al., 2013).

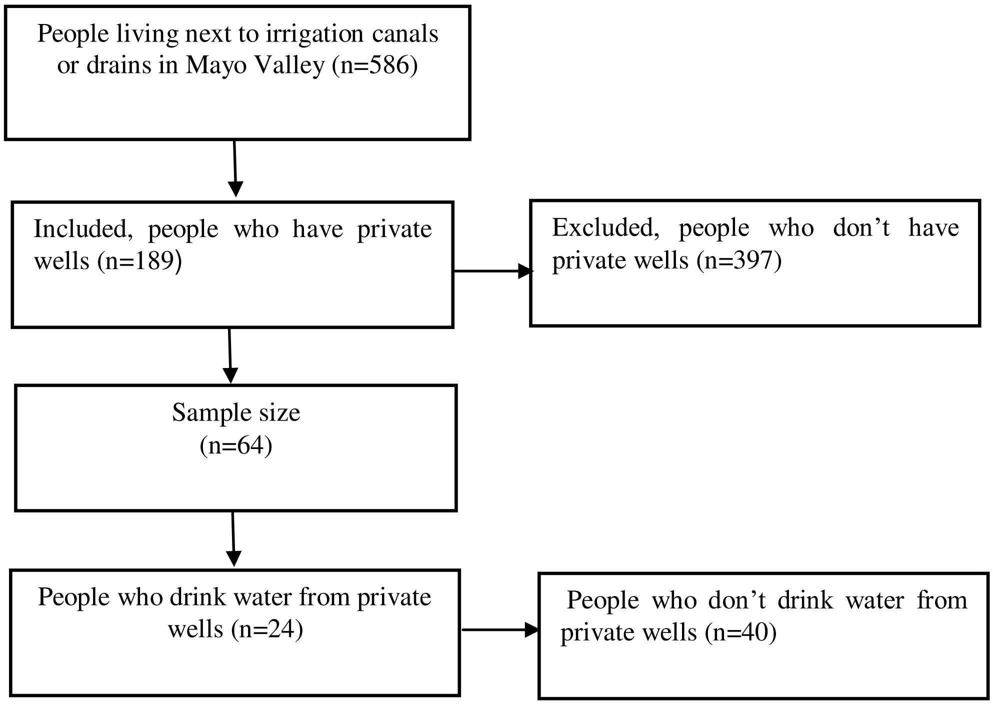

Figure 3. *Systematic selection of the inclusion and exclusion criteria*

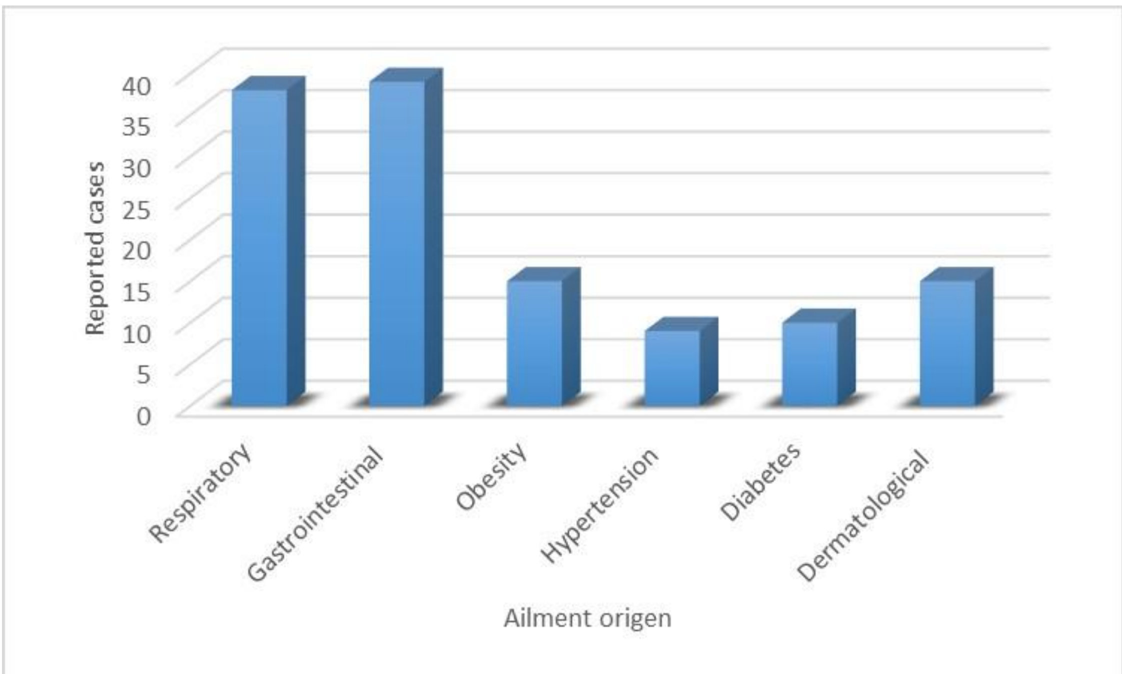

Figure 4. *Most common diseases between the population of Valle del Mayo, Sonora, Mexico*

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Swanson et al. (2014) made a correlation between glyphosate application and chronic diseases. The diseases with statistical significance were autism, thyroid cancer, senile dementia; significance was also observed with incidence and prevalence of diabetes, obesity, pancreas and kidney cancer and hypertension with a correlation lower than 90%, different from the present research where hypertension recorded a high correlation.

Among the fresh vegetables most consumed by the individuals in the communities, were lettuce, squash, carrot, cucumber, chard or purslane.

Toxicity assessment

The gastrointestinal absorption is the quotient of the fraction of chemical absorbed orally between the fraction absorbed in principal study. Different parameters, obtained from the survey, were considered to assess health risk by glyphosate and AMPA exposure, in agricultural laborers, brick makers, housewives, and children from 2 to 16 years old and toddlers. For each one of the different receptors, the following was taken into account: housewives washed clothes with well water, which they also used it to wash food before ingesting, implying oral and dermal contact. Children in the communities usually took baths and swim into drain water, and finally, agricultural laborers and brick makers drank water from the well and consumed food washed with well water. With respect to soil, intake was considered accidental, as well as dermal contact with soil and drain water (*Table 2*).

Risk characterization

This study obtained a hazard quotient of 0.39 and 0.22 for brick makers and agricultural laborers respectively using, according to the formula, an RfD of 0.03 mg kg^{-1} day^{-1}, which determined the potential health risk for AMPA in Valle del Mayo populations.

In the past, different countries used to have standardized ADIs of 0.03 mg kg^{-1} day^{-1} (HC, 1995), 1.75 mg kg^{-1} day^{-1} (USEPA, 2009), 0.1 mg kg^{-1} day^{-1} for glyphosate and AMPA (FAO and WHO, 2011). Currently, laws regulate different ADIs: 0.3 mg kg^{-1} day^{-1} (APVM, 2017), 0.5 mg kg^{-1} day^{-1} (EFSA, 2015). The hazard quotients obtained using EFSA regulation are shown in *Table 3.* These results indicate that no health risk hazard exists on the population of Valle del Mayo.

One of the concerns is that no sufficient regulations exist for AMPA. It the past was reported that there are not enough concentrations in the environment to consider it a health problem (HC, 1995). However, other studies in the literature, as well as the present, found AMPA concentrations in well water greater than those of glyphosate (Kolpin et al., 2006; Battaglin et al., 2014).

The regulations of different countries agreed to a wide range of acceptable daily intake (ADI) for glyphosate and AMPA of 0-1 mg kg^{-1} day^{-1} (FAO and WHO, 2016). Controversially, several regulations and organizations have emphasized that the glyphosate studies, which IARC have based on, were badly designed, so they cannot be used as evidence to prove its carcinogenic effects. Other agencies (USEPA and EFSA) clarified there was not enough proof to support glyphosate as probably carcinogenic, so they establish the herbicide as “probably does not cause cancer”. Despite before the IARC publication, all these regulatory agencies had graded glyphosate as currently the most harmful considering a lower ADI (EFSA, 2016; USEPA, 2017; APVM, 2017).

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Table 2. *Parameters considered in the different receptors exposed to glyphosate and AMPA*

Parameters	**Exposed population**				
	Brick makers	**Agricultural laborer**	**Housewife**	**Toddler**	**Children**
Maximum glyphosate concentration in private well water, drain and soil water (mg L^{-1})*1	$0.5x10^{-2}$				
Maximum AMPA concentration in private well water (mg L^{-1})*1	$1.5x10^{-2}$				
Maximum AMPA concentration in agricultural drain water (mg L^{-1})*1	$3.7x10^{-2}$				
Maximum AMPA concentration in soil (mg g^{-1})*1	$34.3x10^{-2}$				
Maximum glyphosate concentration in vegetables (mg g^{-1})*2	$5.4x10^{-7}$				
Maximum AMPA concentration in vegetables (mg g^{-1})*2	$1.62x10^{-6}$				
Intake water rate for adults and children (L day^{-1})*3	2.9	2.4	1.8	0.6	1
Soil intake rate for adults and children (g day^{-1})*3	0.02	0.02	0.02	0.05	0.05
Dermal contact with water (cm^2)*4	17640	3390	890	890	10140
Dermal contact with soil (g cm^{2-1} $event^{-1}$)*4	$1.1x10^{-4}$		$1.0x10^{-4}$	$1.1x10^{-4}$	
Vegetable intake (g day^{-1})*4	137			67	98
Absorption factor for the gastrointestinal tract*4	1				
No. of days in one week of exposure*5	7				
No. of weeks in one year of exposure*5	40	52			
Years of exposure*6	42	40	45	0.58-4	5 to 15
Corporal weight of receptor (kg)*7	70			16.5	32.97

*1Maximum concentration found of the herbicide determined by High-Performance Liquid Chromatography
*2Concentration depending on the private well water retained in leafy vegetables
*3Exposure factors handbook (USEPA 2011)
*4Health Canada's PQRA model 2004 (HC 2012)
*5Data provided by DDRRM (2017)
*6Average number of years the receptor has been living in the site
*7Average weight expressed in the surveys

With the assurance of the majority of the regulatory agencies worldwide that glyphosate is not hazardous to health, the Food and Agriculture Association (FAO) and the World Health Organization (WHO) agreed that due to its low toxicity, it is not necessary to establish a reference dose for glyphosate in food residues (FAO and WHO, 2016). This new regulation allows glyphosate-resistant genetically modified cultivations, which represent approximately 56% of glyphosate use worldwide (Benbrook, 2016). It is important to mention FAO reported that 795 millions of individuals in the world are undernourished, of which 780 million live in underdeveloped regions (FAO, 2015), which is why both the use of herbicides and the

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convenience of eradicating famine should weigh up improvement in agriculture production and possible health problems.

The position of glyphosate as probable carcinogenic was assessed by IARC with enough background evidence in animal experiments to grade it as such for humans. The monograph published by the IARC is a thorough global compilation of the main scientific research on glyphosate, which clearly showed that glyphosate was harmful to flora and fauna and could cause cancer to humans (IARC, 2015).

Table 3. *Risk quotient per occupational exposure to glyphosate and AMPA*

Exposure matrix	**Brick makers**		**Agricultural laborer**		**Housewife**		**Toddler**		**Children**	
	Gly*[1]	**AMPA**	**Gly**	**AMPA**	**Gly**	**AMPA**	**Gly**	**AMPA**	**Gly**	**AMPA**
Soil	$1.09x10^{-6}$	$7.5x10^{-5}$	$1.09x10^{-6}$	$7.5x10^{-5}$	$1.09x10^{-6}$	$7.5x10^{-5}$	$1.5x10^{-5}$	$1.03x10^{-3}$	$7.5x10^{-6}$	$5.18x10^{-5}$
Water	$2x10^{-4}$	$1.52x10^{-3}$	$1.7x10^{-4}$	$1.2x10^{-3}$	$1.28x10^{-4}$	$3.8x10^{-4}$	$1.8x10^{-4}$	$5.4x10^{-4}$	$5.8x10^{-5}$	$4.5x10^{-4}$
Food	$1.02x10^{-9}$	$3.17x10^{-9}$	$1.02x10^{-9}$	$3.17x10^{-9}$	$1.06x10^{-9}$	$3.17x10^{-9}$	$2.11x10^{-9}$	$6.58x10^{-9}$	$1.55x10^{-9}$	$4.82x10^{-9}$
Dermal	$1.48x10^{-4}$	$1.02x10^{-2}$	$7.9x10^{-5}$	$5.4x10^{-3}$	$6.7x10^{-6}$	$4.6x10^{-5}$	$1.18x10^{-5}$	$8.1x10^{-4}$	$6.7x10^{-5}$	$4.6x10^{-3}$
TDI*[2]	$3.5x10^{-4}$	$1.1x10^{-2}$	$7.8x10^{-5}$	$6.7x10^{-3}$	$1.4x10^{-4}$	$9.2x10^{-4}$	$2x10^{-4}$	$2.3x10^{-3}$	$2.1x10^{-6}$	$1.58x10^{-4}$
Bathing in drains									$1.4x10^{-4}$	$5.6x10^{-3}$
ADI*[3]	**0.5 mg kg^{-1}**									
HQ*[4]	$7.1x10^{-4}$	$2.3x10^{-2}$	$1.57x10^{-4}$	$1.34x10^{-2}$	$2.7x10^{-4}$	$1.85x10^{-3}$	$4.16x10^{-4}$	$4.78x10^{-3}$	$2.7x10^{-4}$	$1.1x10^{-2}$

*[1]Glyphosate, is the dose (mg kg^{-1}) per day that ingests the exposure population
*[2]TDI, is the sum of all doses by exposure matrix type
*[3]Acceptable Daily Intake, is the amount of glyphosate or AMPA that can be ingested without health risk
*[4]Hazard Quotient, is according to USEPA (2017), the ratio of the potential exposure to the substance and the level at which no adverse effects are expected

Conclusions

Currently, ADIs regulation for glyphosate and AMPA to compare the present quotients, unlikely health risk exists on the population of Valle del Mayo, Mexico. Therefore, these results using an ADI of 0.03 mg kg^{-1} day^{-1} suggest a potential health risk due to glyphosate and AMPA in the agricultural laborers and brick makers of this region. The permissible reference dose of glyphosate and AMPA has a very large margin (0-1 mg kg^{-1} day^{-1}) even traces in food are permissible according to current regulations. In addition, we determinate that water consumption from the private well near to irrigation canals correlated statistically with diabetes ($p \leq 0.03$) and hypertension ($p \leq 0.004$). It is necessary to develop more toxicity studies that indicate an accurate value to establish that the herbicide and its metabolite do not cause harm to the human beings in a short and long-term.

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IN VITRO SYMBIOTIC GERMINATION POTENTIALS OF SOME *ANACAMPTIS, DACTYLORHIZA, ORCHIS* AND *OPHRYS* TERRESTRIAL ORCHID SPECIES

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Abstract. Germination and propagation of terrestrial orchids is highly challenging due to their small size seeds and absence of endosperms. Some orchid species are extremely dependent on fungi for germination of their seeds. Although germination occurs symbiotically between the seed and the fungus, it is not coincidental that fungus species are specific to orchid species. Aim of this study is to show the impact of the fungi, isolated from the tubers of ten naturally grown species of orchids, collected from mountainous area of Van province, Turkey, on in vitro symbiotic germination of the seeds. The collected orchids species were *Dactylorhiza romana* (Seb.) Soo subsp. *georgica* (Klınge) Soo ex Renz & Taub., *Orchis pinetorum* Boissier & Kotschy, *Orchis spitzelii* Saut. ex W.D.J. Koch, *Orchis coriophora* L., *Orchis collina* Banks & Solander, *Orchis anatolica* Boiss., *Orchis simia* Lamarck, *Ophrys straussii* H. Fleischmann & Bornmüller, *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski, *Anacamptis pyramidalis* (L.) L. C. Rich. In the study from orchid tubers, *Rhizoctonia* sp., *Aspergillus* sp., *Alternaria* sp., *Penicillium* sp., *Trichoderma* sp. and *Fusarium* sp. fungi species are isolated. Firstly, orchid seeds and fungal isolates placed in oat culture medium were transferred to Van Waes & Debergh culture medium in subculture. The highest germination rates obtained in the seeds are 71.19% in *Dactylorhiza romana* subsp. *georgica* species with *Rhizoctonia* sp.; 78.26% in *Orchis pinetorum* species with *Penicillium* sp.; 27.77% in *Orchis spitzelii* species with *Fusarium* sp.; 83.07% in *Orchis coriophora* species with *Rhizoctonia* sp.; 75.00% in *Orchis collina* species with *Fusarium* sp.; 38.88% in *Orchis anatolica* species with *Alternaria* sp.; 73.91% in *Orchis simia* species with *Fusarium* sp.; 91.60% in *Ophrys straussii* species with *Fusarium* sp.; 93.75% in *Dactylorhiza umbrosa* species with *Fusarium* sp. and 56.00% in *Anacamptis pyramidalis* species with *Rhizoctonia* sp. At the end of the study, it was understood that the fungi isolated from their own or other tubers had different effects on the germination of each orchid species.
Keywords: *fungal isolates, in vitro, mycorrhiza effect, symbiotic germination, terrestrial orchid*

Introduction

The most distinctive features of the orchid seeds are their very small size, absence of endosperm and undeveloped embryo. These seeds have a length of 0.25-1.2 mm, width of 0.09-0.27 mm and weight of 0.3-1.4 mg (Arditti, 1967). The orchids grown at high altitudes occasionally fail to grow and set seed due to low temperatures or the ones grown under dense forests grow slowly and cannot reach to a desired level of development due to lack of light (Sezik, 1984). Thus, in addition to appropriate temperature, light, oxygen, moisture and soil conditions required in the microclimate of the environment in which the seed fall to germinate, an appropriate symbiotic relationship should be established with a mycorrhizal fungus for germination of the

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seeds (Sezik, 1984). As the orchid seeds do not contain nutrient reserves, successful germination cannot be achieved unless a carbohydrate source, such as glucose, is provided (Ingold and Hudson, 1993).

The symbiotic capacity of a fungal isolate depends primarily on the nature of the isolate and then the orchid species with which it coexists. The results obtained on the existence of a specificity between orchid species and mycorrhizal fungi indicate that there is no strict specificity at the species-species level, and that, on the other hand, orchid-fungus relationships are not entirely random. According to Batty et al. (2001), terrestrial orchids may have narrow or broad potential specificity (Warcup, 1981; Alexander and Hadley, 1983; Muir, 1989), but the specificity of their associations with endophytes in natural habitats is still poorly understood. The specificity of the orchid–mycorrhiza association is variable between species (Steinfort et al., 2010). An orchid may form mycorrhizal associations with more than one fungal species, and a fungal species might associate with more than one orchid under in situ conditions (Bonnardeaux et al., 2007; Otero et al., 2004).

When these relations existing in the nature are moved to the laboratory conditions; the fungal isolates are isolated from the orchid tubers and roots and inoculated into the medium. In this study, the impacts of the fungal isolates isolated from orchid tubers, collected from the nature and inoculated into nutrient medium, on germination of the seeds are determined.

Material and methods

The material of the research consists of orchid seeds and fungi obtained from the tubers of the plants. The plants used in the research are naturally grown in Van province, Turkey, collected from Gevaş District Altınsaç neighbourhood in July-2014 when the plants formed capsules, and identified according to Davis (1984).

Orchid species

The orchid species examined in the study are *Dactylorhiza romana* (Seb.) Soo subsp. *georgica (*Klınge) Soo ex Renz & Taub., *Orchis pinetorum* Boissier & Kotschy, *Orchis spitzelii* Saut. ex W.D.J. Koch, *Orchis coriophora* L., *Orchis collina* Banks & Solander, *Orchis anatolica* Boiss., *Orchis simia* Lamarck, *Ophrys straussii* H. Fleischmann & Bornmüller, *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski, *Anacamptis pyramidalis* (L.) L. C. Rich.

Dactylorhiza romana subsp. *georgica, Orchis pinetorum* ve *Orchis spitzelii* species were determined in the same location (N 38°23'30.2" and E 42°53'43.8") while *Orchis coriophora* and *Dactylorhiza umbrosa* species were obtained in the same location (N 38°24'16.2" and E 42°53'76.6"). *Anacamptis pyramidalis, Orchis collina, Orchis simia,* and *Ophrys straussii* species were collected from N 38°23'61.7" and E 42°55'03.2" coordinates while *Orchis anatolica* species was collected from N 38°23'59.3" and E 42°54'98.1" coordinate *(Table 1* and *Fig. 1).*

Fungi

Fungi obtained from isolations in tuber samples, collected during seed collection, are used.

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Table 1. *GPS coordinates where orchid species are collected*

No	Orchid species	GPS Coordinates		Altitute (m)
1	*Dactylorhiza romana* subsp. *georgica*	N 38°23'30.2"	E 42°53'43.8"	1864
2	*Orchis pinetorum*	N 38°23'30.2"	E 42°53'43.8"	1864
3	*Orchis spitzelii*	N 38°23'30.2"	E 42°53'43.8"	1864
4	*Orchis coriophora*	N 38°24'16.2"	E 42°53'76.6"	1631
5	*Dactylorhiza umbrosa*	N 38°24'16.2"	E 42°53'76.6"	1631
6	*Anacamptis pyramidalis*	N 38°23'61.7"	E 42°55'03.2"	1724
7	*Orchis collina*	N 38°23'61.7"	E 42°55'03.2"	1724
8	*Orchis simia*	N 38°23'61.7"	E 42°55'03.2"	1724
9	*Ophrys straussii*	N 38°23'61.7"	E 42°55'03.2"	1724
10	*Orchis anatolica*	N 38°23'59.3"	E 42°54'98.1"	1724

Figure 1. *Locations where orchid species are collected (Google Earth, 2018)*

Surface sterilization of the seeds

Orchid seeds are sterilized in small envelopes made from coarse filter papers as they are very small and have a dusty structure. Seeds of each species weighting 100 mg are enveloped and used for experiments. For each experiment, 100 mg of the seeds were planted in total of 9 petri dishes containing Potato Dextrose Agar (PDA) media in 3 replicates, 3 pots in each replicate.

The seeds were shaken for 5 min with 2% sulfuric acid, sterilized with 1-2 drops of Tween-20 and 10% commercial bleach for 12 min and then rinsed 3 times with sterile distilled water before sowing to Çığ and Yılmaz (2017).

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Fungus isolation from tubers

The orchid tubers, washed from the soil in the tap water, were shaken in the sterilized cabinet for 3 min in 3% bleach solution and then rinsed 3 times with sterile distilled water and wiped with sterilized drying papers. 2-3 pieces of tuber, cut into sections with 0.5-1 cm length with scalpel, were planted in PDA petri dishes and incubated for 3-4 days at 24 °C. The PDA medium was prepared with 39 g/l potato dextrose agar dose (Zettler et al., 2001; Sharma et al., 2003) and sterilized for 20 min in an autoclave operating at 121 °C and 1.2 atmosphere pressure.

Developed fungal hyphae were identified on light microscope after being transferred to water agar (WA, 15 g/l) petri dishes and stored in refrigerator in glass tubes. Fungus isolation procedures and identification were done in Mycology Laboratory of the Department of Plant Protection, Faculty of Agriculture at Yüzüncü Yıl University.

Identification of isolates

Morphological and microscopic features of the fungi incubated in PDA and WA for 7 days at 25 °C were used in identification of the isolates obtained in the study (Barnett, 1965; Booth, 1971; Burgess et al., 1994; Domsch et al., 1980; Gilman, 1959; Singh et al., 1991; Ogoshi, 1975). Identification of *Trichoderma* species was done by using the interactive key http://nt.arsgrin.gov/taxadescriptions/keys/TrichodermaIndex.cfm (Samuel et al., 2006).

Composition of the oat medium (OM)

In order to create the oat medium described by Clements and Ellyard (1979), 2.5 g milled and powdered oat was boiled in 1000 ml of distilled water for one hour and drained from the gauze. The pH of the cooled medium was adjusted to 5.5 and it was autoclaved by adding 7 g/l agar as in the PDA medium.

Seed sowing in symbiotic culture medium

The fungi used in the study were planted in petri dishes containing PDA in advance and 0.5 cm discs were taken with cork borer when they were 10 days old. Each disk was placed on one side of the petri dish containing OM, which is a symbiotic medium. Petri dishes were incubated in the dark at 24 °C for a couple of days. Seed sowing was done on the other side of the petri dishes where there is no fungus. The petri dishes were kept at 23 ± 1 °C in the dark during germination. The seeds were taken to Van Waes & Debergh (VWD) (1986) culture medium while sub-culturing.

Statistically analysis

The data were analyzed using the statistical software package SPSS. The means were grouped using the Duncan multiple comparison test (Düzgüneş et al., 1987).

Results and discussions

Fungus isolation and identification

Six orchid tuber samples/orchid species were used for fungal isolation. The identification of the fungi was done by the references after isolations from tuber

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samples. *Rhizoctonia* sp., *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Trichoderma* sp., and *Alternaria* sp. were isolated from orchid tubers. The identification was based on morphological and microscopic features (*Table 2*).

Table 2. *Morphological and microscopic features of the fungi isolated from tuber samples*

Fungi	Morphological and microscopic features		References
	Colony color in PDA	**Shape of conidia**	
Rhizoctonia sp.	White/brown	No conidia	Ogoshi, 1975
Aspergillus sp.	Black/green	Spherical	Singh et al., 1991; Barnett, 1965
Fusarium sp.	Pink/orange/purple	Ovoid to elongated (macroconidia and microconidia)	Booth, 1971; Burgess et al., 1994
Penicillium sp.	Green	Ellipsoidal	Domsch et al., 1980; Gilman, 1959; Singh et al., 1991
Trichoderma sp.	Green	Globose, subglobose/ellipsoidal	Samuels et al., 2006
Alternaria sp.	Brown/black	Ovoid/ ellipsoidal	Domsch et al., 1980; Gilman, 1959

Rhizoctonia sp. and *Aspergillus* sp. fungi from *Dactylorhiza romana* subsp. *georgica* (1) species*;* *Fusarium* sp. and *Penicillium* sp. fungi from *Orchis pinetorum* (2) species*;* *Rhizoctonia* sp. and *Fusarium* sp. fungi from *Orchis spitzelii* (3) species*;* *Trichoderma* sp.*,* *Fusarium* sp. and *Penicillium* sp. fungi from *Orchis coriophora* (4) species*;* *Alternaria* sp. fungus from *Orchis collina* (5) species*;* *Fusarium* sp. fungus from *Orchis simia* (7) species*;* *Fusarium* sp., *Aspergillus* sp. and *Penicillium* sp. fungi from *Ophrys straussii* (8) species,*;* *Rhizoctonia* sp., *Fusarium* sp. and *Alternaria* sp. fungi from *Dactylorhiza umbrosa* (9) species (3 isolates); *Fusarium* sp.*,* *Alternaria* sp., *Penicillium* sp. and *Aspergillus* sp. fungi from *Anacamptis pyramidalis* (10) species were isolated, while no fungus was isolated from *Orchis anatolica* (6) species (*Table 3*).

Fungi were infected not only in the orchid species they were isolated from, but also into the cultures where the seeds of other species were planted. Photographs of symbiotic germination were taken with OLYMPUS SZ61 binocular microscope dp20 camera, 10X x 1.2 software (*Fig. 2*).

Table 3. *Orchid species and isolated fungi*

Species	Fungi					
	***Rhizoctonia* sp.**	***Aspergillus* sp.**	***Fusarium* sp.**	***Penicillium* sp.**	***Trichoderma* sp.**	***Alternaria* sp.**
Dactylorhiza romana subsp. *georgica* (1)*	R	As	-	-	-	-
Orchis pinetorum (2)	-	-	F	P	-	-
Orchis spitzelii (3)	R	-	Fo	-	-	-
Orchis coriophora (4)	-	-	Fe	P	T	-
Orchis collina (5)	-	-	-	-	-	Alt
Orchis anatolica (6)	-	-	-	-	-	-
Orchis simia (7)	-	-	F	-	-	-
Ophrys straussii (8)	-	As	Fs	P	-	-
Dactylorhiza umbrosa (9)	R, R1, R2	-	F	-	-	Alt
Anacamptis pyramidalis (10)	-	As	F	P	-	Alt

*Each plant species was coded with a number

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Symbiotic germination

Isolated fungal isolates were shown like *Rhizoctonia* (R, R1, R2), *Aspergillus* (As), *Fusarium* (F, Fo, Fs), *Penicillium* (P), *Trichoderma* (T) and *Alternaria* (Alt) codes.

Table 4. *The effects of isolated fungi on the germination of orchid seeds*

Fungi	Germination percentage (%)									
	Species 1	**Species 2**	**Species 3**	**Species 4**	**Species 5**	**Species 6**	**Species 7**	**Species 8**	**Species 9**	**Species 10**
1As							18.18 h		16.66 l	
1R	71.19 a*			45.23 f	20.20 i	6.66 d	34.78 d		53.84 h	18.91 kl
2F				39.47 g	64.00 c		73.91 a	91.60 a	63.79 e	37.23 e
2P				33.33 h				15.38 k	70.86 d	44.44 cd
3Fo	40.00 e		27.77 a	61.53 d	42.64 f			56.25 e	54.85 gh	22.22 hi
3R			16.00 c	48.38 e	41.66 f			42.85 g	54.54 gh	56.00 a
4Fe	26.47 f			41.46 g	15.38 j		35.00 d	83.33 b	60.37 f	
4P		31.57 c			33.33 g		31.25 e		56.49 g	
4T	17.64 g			65.86 c	68.18 b	29.72 b	66.66 b	18.18 j	69.54 d	20.00 jk
5Alt					48.59 e	38.88 a		79.78 c	52.94 h	
7F	55.80 c			27.58 i		14.28 c	25.98 f	57.14 e	79.16 b	28.57 g
8As				36.12 h	27.97 h		37.50 c		37.40 k	42.85 d
8Fs	45.68 d			29.62 i				15.38 k	41.17 j	
8P		78.26 a		60.00 d	62.74 c			20.83 i	50.00 i	
9Alt	37.63 e			66.66 c	66.17 b		37.50 c	15.00 k	68.75 d	53.33 b
9F				80.95 a	27.94 h		25.00 f		17.14 l	44.76 c
9R	45.78 d		20.83 b	83.07 a	18.75 i	38.29 a		30.00 h	61.29 f	17.56 l
9R1				77.77 b	50.00 e			65.38 d	17.64 l	21.05 ij
9R2				66.66 c	58.33 d			11.42 l	76.77 c	
10Alt	63.21 b			59.21 d	15.00 j		22.36 g		74.67 c	7.93 n
10As		48.27 b		13.33 j	10.63 k			9.19 m	41.30 j	23.07 h
10F	57.69 c			67.85 c	75.00 a		12.82 i		93.75 a	34.04 f
10P		34.32 c		40.00 g	16.00 j			50.00 f		12.50 m

*Means followed by the same letter in the line indicate no statistical difference at 5% level
Orchid species 1: *Dactylorhiza romana* subsp. *georgica*, 2: *Orchis pinetorum*, 3: *Orchis spitzelii,* 4: *Orchis coriophora*, 5: *Orchis collina,* 6: *Orchis anatolica*, 7: *Orchis simia*, 8: *Ophrys straussii*, 9: *Dactylorhiza umbrosa,* and 10: *Anacamptis pyramidalis*
Fungal isolates: *Aspergillus* (As), *Alternaria* (Alt), *Fusarium* (F, Fo, Fs), *Penicillium* (P), *Rhizoctonia* (R, R1, R2), and *Trichoderma* (T)

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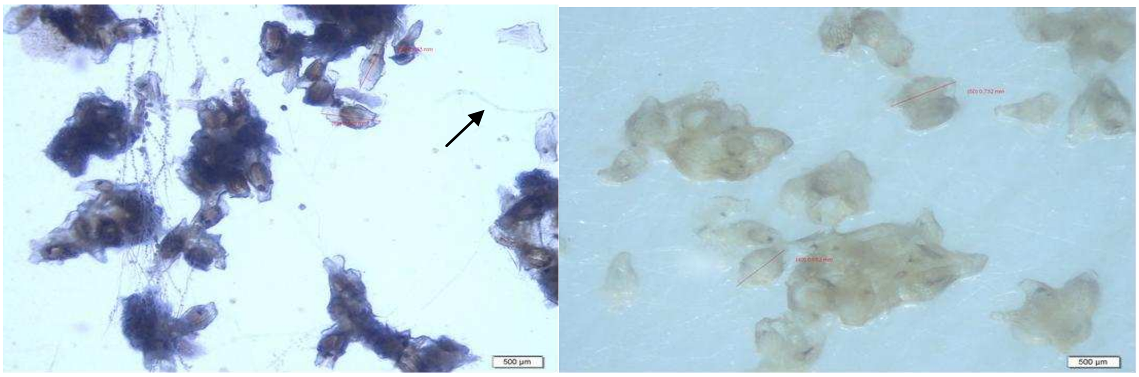

***Figure 2.** Symbiotic germination*

The effects of the isolated fungi on the germination of orchid seeds were found statistically significant ($p < 0.05$) (*Table 4*). 71.19% germination rate is observed in *Rhizoctonia* (R) fungus obtained from the tuber of *D. romana* subsp. *georgica* (species number 1) (*Fig. 3*). No germination is observed with the fungi obtained from tubers of species number 2 *O. pinetorum* (*Fig. 4*), while the impact of R and Fo isolated from the tubers of species number 3 *O. spitzelii* on germination is found as 16% and 27.77%, respectively (*Fig. 5*). T isolate, isolated from the tubers of species number 4 *O. coriophora,* provided 65.86% germination rate (*Fig. 6*). From the species number 5 *O. collina,* only sub-culturing is done and the impact of its own seeds on germination was determined as 48.59% (*Fig. 7*). As no fungus was isolated from the tubers of species number 6 *O. anatolica*, germination is obtained using fungi isolated from tubers of other orchid species (*Fig. 8*). In the symbiotic germination between the seeds of species number 7 *O. simia* and F fungus isolated from its tubers, a success rate of 25.98% is achieved (*Fig. 9*). Fs and P fungi isolated from the tubers of species number 8 *O. straussii* had impacts of 15.38% and 20.83% respectively on germination of its own seeds (*Fig. 10*). R, R1, R2, Alt and F fungi isolated from the tubers of species number 9 *D. umbrosa* had impacts of 61.29%, 17.64%, 76.77%, 68.75% and 17.14% on germination of its own seeds, respectively (*Fig. 11*). F, Alt, As and P fungi isolated from the tubers of species number 10 *A. pyramidalis* had impacts of 34.04%, 7.93%, 23.07% and 12.5% respectively on germination of its own seeds (*Fig. 12*).

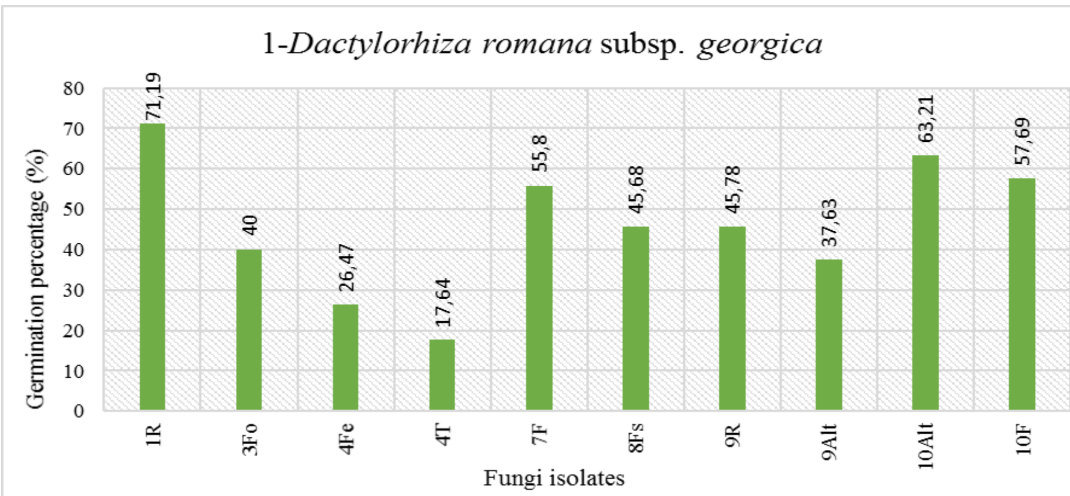

***Figure 3.** Effects on the germination rates of fungi on D. romana subsp. georgica germination*

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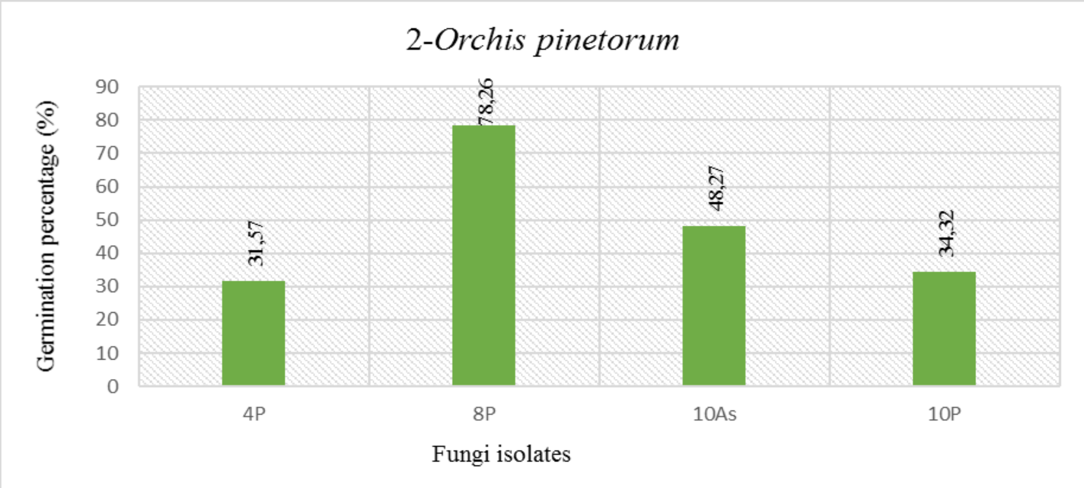

Figure 4. *Effects on the germination rates of fungi on O. pinetorum germination*

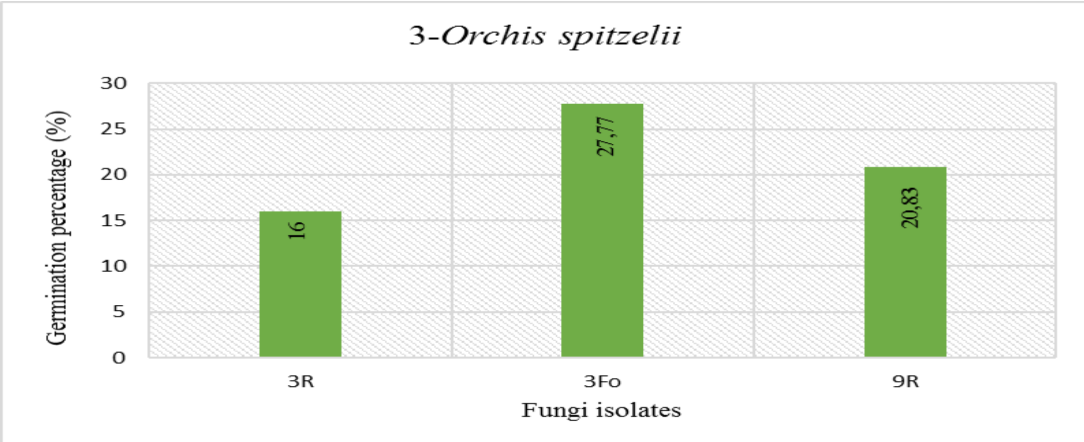

Figure 5. *Effects on the germination rates of fungi on O. spitzelii germination*

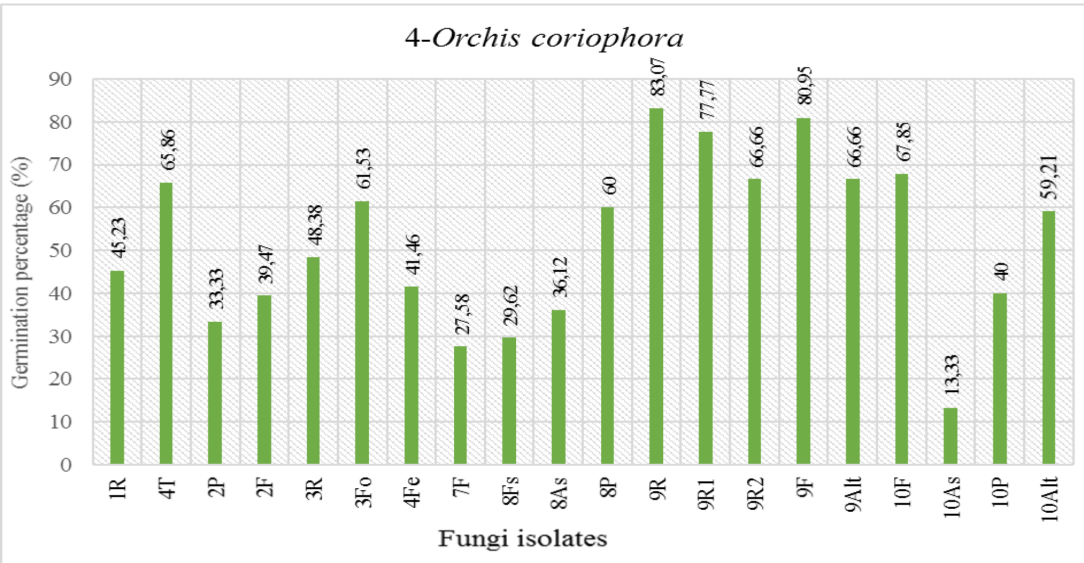

Figure 6. *Effects on the germination rates of fungi on O. coriophora germination*

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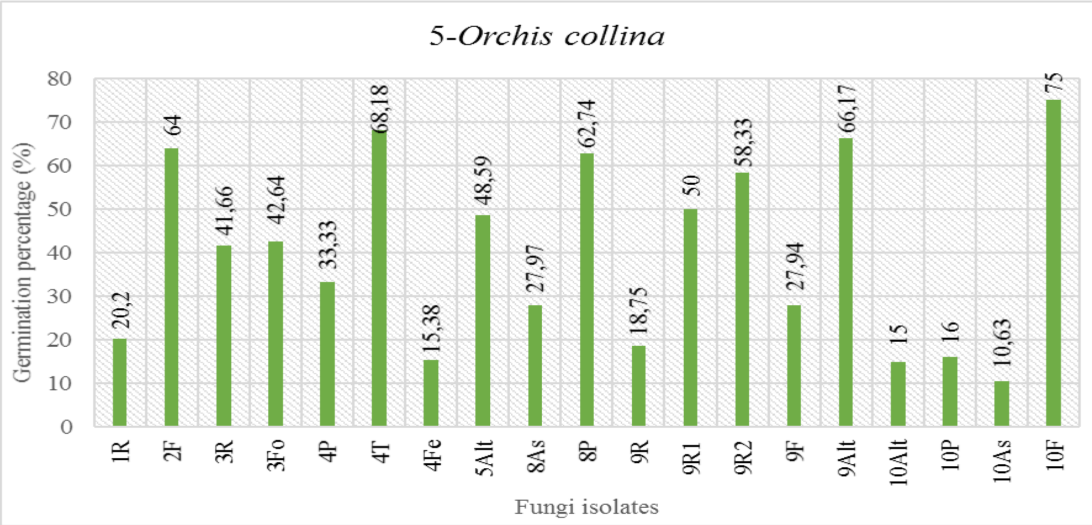

Figure 7. *Effects on the germination rates of fungi on O. collina germination*

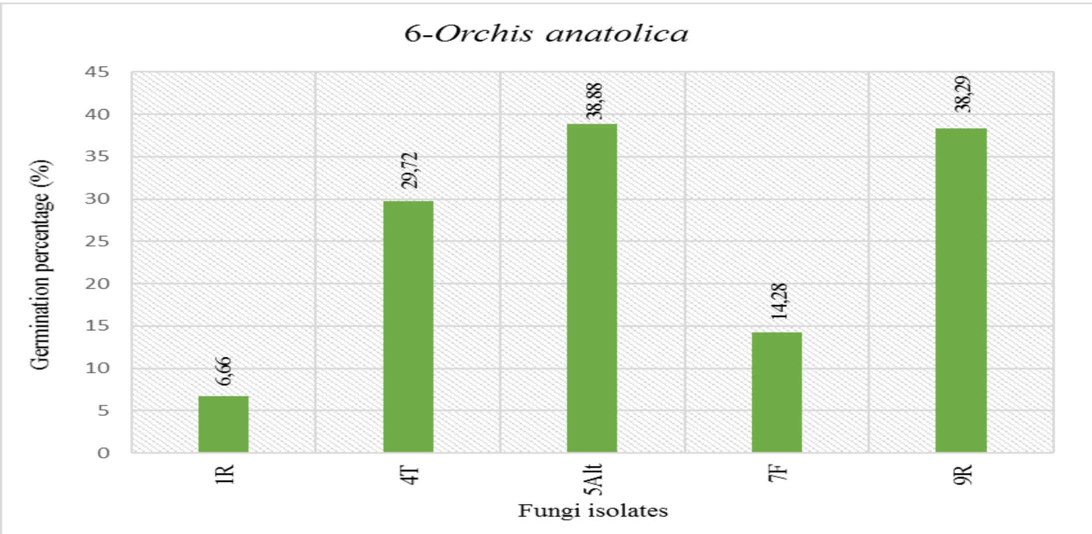

Figure 8. *Effects on the germination rates of fungi on O. anatolica germination*

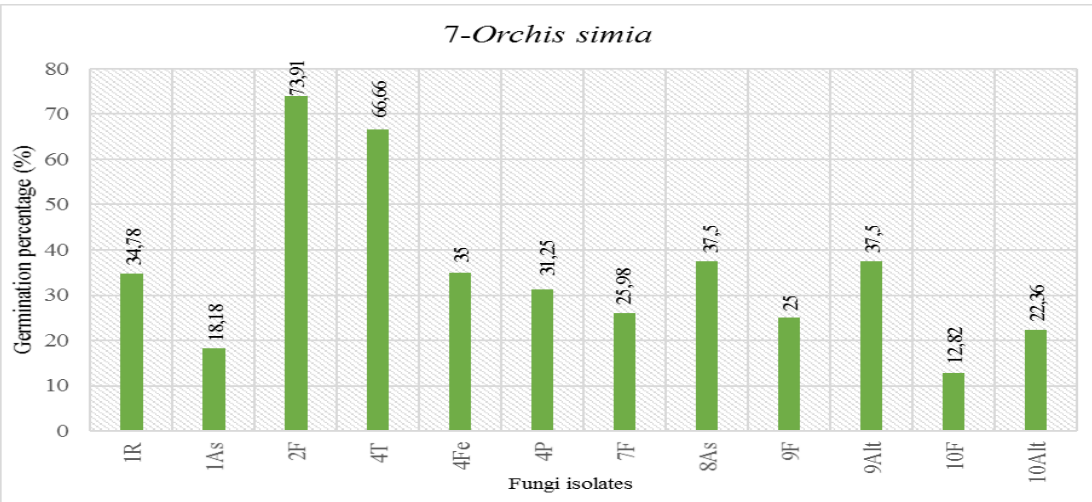

Figure 9. *Effects on the germination rates of fungi on O. simia germination*

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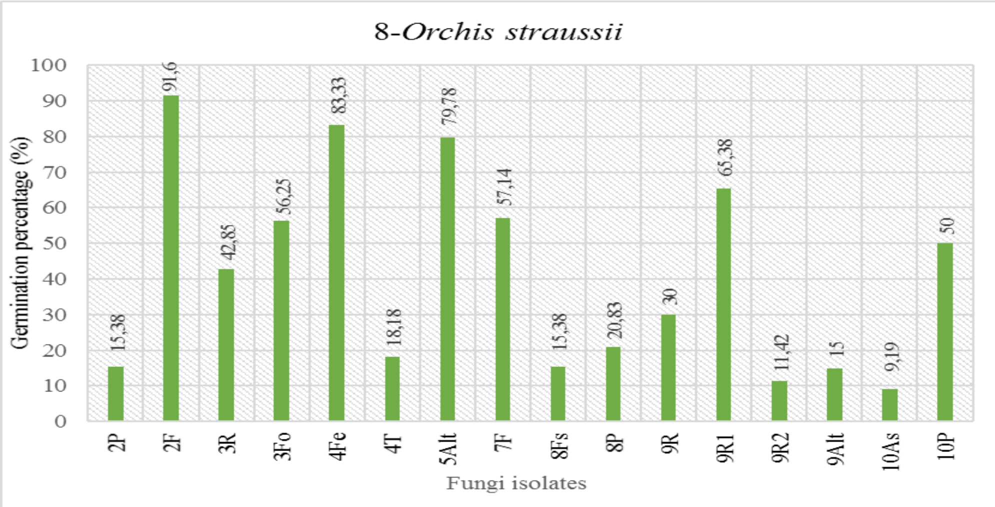

Figure 10. *Effects on the germination rates of fungi on O. straussii germination*

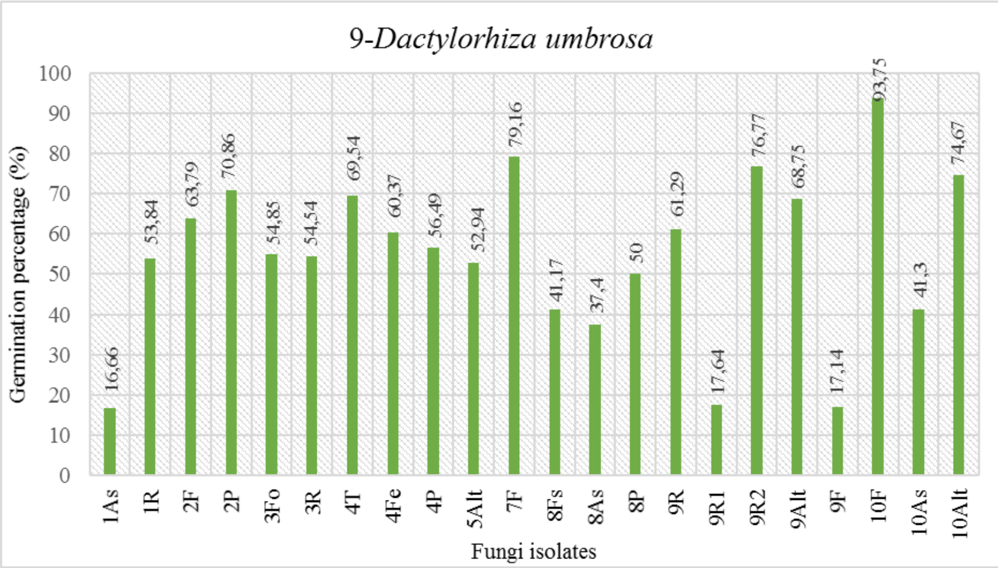

Figure 11. *Effects on the germination rates of fungi on D. umbrosa germination*

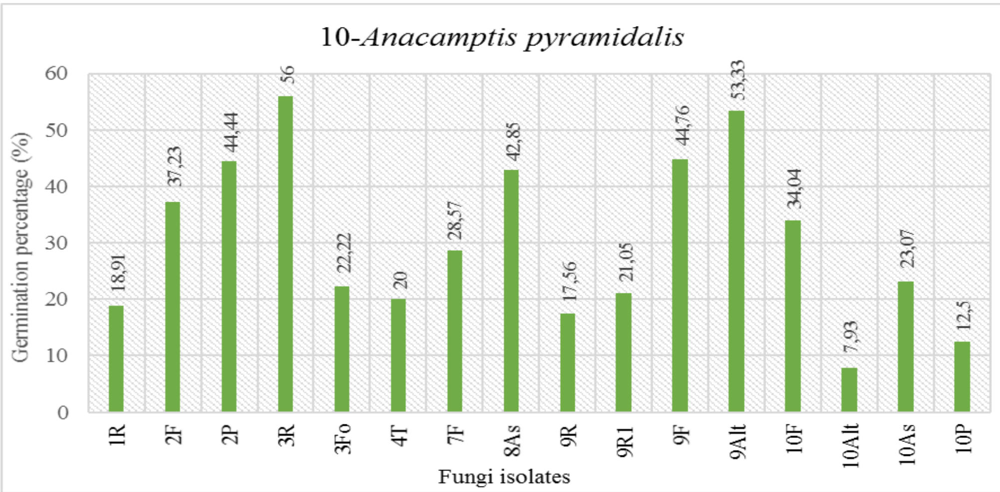

Figure 12. *Effects on the germination rates of fungi on A. pyramidalis germination*

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Bernard (1909) stated that the symbiotic relationship between the orchid and the fungus is specific to species, while other researchers reported no such specific relation between a mycorrhizal fungus and an orchid species exists (Burgeff, 1936; Curtis, 1939). In another study, it was determined that fungi isolated from orchid tubers were *Rhizoctonia, Corticium, Armillaria, Fomes, Hymenochaeta* species, while *Aspergillus, Penicillium, Phytophtora* and *Trichoderma* were reported to be initiating and promoting germination (Arditti, 1967).

Clements and Ellyard (1979) reported that the effect of the nutrient medium content on symbiotic culture experiments was important for in vitro germination of orchid seeds and suggested primarily to obtain the most effective *Rhizoctonia* isolate and to prepare the optimal medium for the isolate. In line with this, it has been reported that some fungi have no or little effect on orchid seed germination and the most suitable nutrient medium for controlling parasitic condition is oat medium with additives (Hadley, 1983; Tsutsui and Tomita, 1986). Clements et al. (1986) reported in their study on symbiotic germination of the temperate zone orchids in Europe that *Ceratobasidium*-like fungi are affective on *Dactylorhiza* species and *Tulasnella*-like fungi are affective on *Orchis, Ophrys* and *Serapias* orchid species. Researches show that although ground oat added to the symbiotic culture medium used in the studies on *Rhizoctonia* sp. fungus and orchid seed combination has a stimuli effect on germination, amounts above a certain concentration reduces the degree of stimulation. Smreciu and Currah (1989) have shown that the fungal isolates obtained from an orchid species are symbiotic in germination of another orchid species, and even can act in different development stages of the orchid plant. From 19 fungal isolates isolated from different orchid species collected between March and May in the Eastern Mediterranean Region, 8 pieces of *Fusarium* sp., 2 pieces of *R. solani*, 2 pieces of *Macrophomina* sp., 2 pieces of *Trichoderma* sp., 2 pieces of *Pythiaceous* sp. and 2 pieces of *Absidia* sp. fungi are identified (Vakkasoğlu, 1995). 44 out of 47 fungal isolates in a study, in which mycorrhiza forming fungi were isolated from the roots and tubers of various orchid species collected in the Aegean and the Mediterranean Region in April, were identified as *Fusarium*, while 2 were identifies as *Rhizoctonia* and 1 was identified as *Papulaspora* species (Gezgin, 2004). Another study, in which *Fusarium* sp. was isolated in greater proportions than others, was conducted using *Serapias vomeracea* subsp. *laxiflora* and it was determined that they are contaminant (Özkoç, 1991). *Alternaria, Aspergillus, Fusarium, Macrophomina, Rhizoctonia, Trichoderma* and *Verticillium* fungi species were isolated from eleven orchid species belonging to *Anacamptis, Cephalanthera, Dactylorhiza* and *Orchis* genus collected in Van province (Çığ and Yılmaz, 2014). According to the literature, the fungi isolated from orchid tubers in our study stimulate seed germination and plant growth. This is also evident from the results we have obtained. The germination rate varied with species. Not all the orchid species germinated with all fungi. On the contrary, it is observed that one fungal isolate yielding germination success in one species did not achieve the same effect in another one. In some cases, the fungus has not succeeded in germinating the seeds or did not have a high germination rate with the plant it was isolated from. This can be explained by not achieving 100% of the ecological conditions in the aseptic environment and by the special relationship between the orchid species and the fungus. Özkoç and Dalcı (1993) reported that not all the isolates had the same effect on *Orchis laxiflora* seeds cultivated in oat medium (OM) and modified oat medium (MOM) in the presence of 11 fungal isolates brought from different countries and found that two Turkish isolates were

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ineffective in germination. In the germination experiment with 11 fungal isolates in *Serapias vomeracea* subsp. *laxiflora* and *Orchis laxiflora* species, it was observed that not all the isolates did have the same effect (Özkoç, 1991). According to the results obtained from our study, the lowest germination rate in species number 1 was 17.64% with T isolate; and the highest germination rate was obtained with R isolate with 71.19%. In species number 2, the lowest and highest germination rates were found as 31.57% and 78.26% respectively with P isolate. In other species, the lowest and highest germination percentages and fungi isolates in which they are symbiotic were obtained as follows: for species number 3 as 16% (R) - 27.77% (Fo); for species number 4 as 27.58% (F) - 83.07% (R); for species number 5 as 10.63% (As) - 75% (F); for species number 6 as 6.66% (R) - 38.88% (Alt); for species number 7 as 18.18% (As) - 73.91% (F); for species number 8 as 9.19% (As) - 91.60% (F); for species number 9 as 16.66% (As) - 93.75% (F) and for species number 10 as 7.93% (Alt) - 56% (R).

Successful germination of *Dactylorhiza iberica* (Bieb. ex Willd.) Soó, *Dactylorhiza umbrosa* (Kar. et. Kir.) Soó and *Orchis palustris* Jacquin species with binucleus *Rhizoctonia* and *Rhizoctonia solani* isolates in the oat medium and the modified oat medium is achieved (Çığ and Yılmaz, 2017). The overall expected effect of *Rhizoctonia* isolate in orchid germination studies is success, however, as can be seen in our study, *Rhizoctonia* sp. fungus, which plays a role in seed germination of many species, did not have proportionally the highest average germination figure. When we look at the success rates of fungal isolates along with the ones that cannot stimulate germination, the highest average is provided by *Fusarium* isolate by 93.75%. In this case, it is clear that fungal isolates and species give different germination success rates together. According to Salifah et al. (2011), a total of 31 different species of fungus was isolated and inoculated onto *Grammatophyllum speciosum* seed on oat meal agar. The result obtained from the test demonstrated that seed germination rates were best when co-cultured with *Fusarium* sp. number 3. Initial seed germination rates were best when co-cultured with *Fusarium* sp. 3 and *Trichoderma* sp. 2, yielding an increment in 63.3% and 55.7%, respectively when compared to the seeds' original size.

The relationship between orchid species and fungal isolates may vary from symbiosis to parasitism, as some researchers have found that parasitic properties of the fungus can increase and unbalance the relationship between the orchid and fungus by depletion of nutrients in the culture medium and fungus might have a parasitic effect in symbiotic environments with high nutrient content (Tomita and Tsutsui, 1988; Özkoç, 1991). Seeds germinated in oat medium were sub-cultured in VWD culture medium. During this process, fungal isolates have developed to such an extent that encapsulates the whole petri dish due to high nutrient concentration and had parasitic effect on germinating seeds. On the other hand, germinated seeds did not have enough required nutrients to develop in oat medium.

Environmental factors such as relative humidity and temperature were reported to have a profound influence on the infectiveness of a variety of fungi (Ibrahim et al., 2011). According to the researches, growth rate of fungi varies depending on temperature and relative humidity. The optimum growth temperatures for the majority of fungi studied was found to fall from 25 to 30 °C (Sharma and Razak, 2003). For example, the growth of *Fusarium oxysporium* was found to reach its maximum at 30 °C after 7 days of incubation which was drastically reduced below 15 °C and above 35 °C (Farooq et al., 2005). As can be seen, for a successful orchid germination protocol, both fungi and seeds enter into symbiotic life together with the common satisfaction of them.

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Conclusions

As a result of the study, different germination success rates were achieved in each orchid species in the presence of fungal isolates. The idea of using a more diluted media during sub-culturing or even using temperature applications to control fungal growth will be given in the methodology section of subsequent new studies to be undertaken with consideration of culture media in which germination took place. But the most important step that must be taken before anything else is to make a molecular identity in order to reveal the true identity of the isolated fungi.

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WATER QUALITY ASSESSMENT OF TEKALA RIVER, SELANGOR, MALAYSIA

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Abstract. A study on water quality status of Tekala River, Selangor, Malaysia was conducted at two sampling times. A total of six sampling stations were selected along the river representing the upstream (S1 and S2), the middle stream (S3 and S4) and the downstream (S5 and S6) of the Tekala River. In this study, in-situ and ex-situ analyses were conducted to determine the quality of Tekala River. Physical, chemical and biological parameters included biochemical oxygen demand (BOD), chemical oxygen demand (SOD), ammoniacal nitrogen, total suspended solids (TSS), dissolved oxygen (DO), pH, temperature, total dissolved solid (TDS), salinity, conductivity, *Escherichia coli*, coliform and macroinvertebrate. According to the result obtained from this study, Tekala River is classified under Class I and Class II based on water quality index and National water quality standard. Two-way ANOVA showed a significant difference between parameters (ammoniacal nitrogen, BOD, pH, temperature, conductivity, DO and TDS) of sampling station. Significant difference was found between ammoniacal nitrogen, pH and temperature with sampling time. The correlation test revealed that there is relationship between *Escherichia coli* with ammoniacal nitrogen, temperature and DO. There is also relationship found between coliform with pH and BOD.
Keywords: *water quality, biomonitoring, water pollution, water management, biological parameters*

Introduction

Over the centuries, river has been very important to the human society. River has also provided water for irrigation, industrial and domestic uses. Additionally, river plays an important role in assimilating municipal and industrial effluents as well as runoffs from agricultural land and the surrounding area in a watershed (Al-Badaii et al., 2013). The importance of water quality has become a serious matter especially when it involves the assessment of producing the biologically-accepted water system as a whole (Ashraf and Hanafiah, 2017; Muhammad Mansoor et al., 2018). The demand for safe and clean drinking water has increased by leaps and bounds in developing countries that have deteriorated environment (Gelover et al., 2006). The target of the Millennium Development Goal (MDG) which is the 'access to safe drinking water' has set its track globally with about 6.1 billion people in 2010 using improved drinking water sources, an increase of over 2 billion since 1990 (MDG, 2012).

Selangor state can be divided into several districts which are Klang, Kuala Langat, Gombak, Kuala Selangor, Hulu Langat, Hulu Selangor, Petaling, Sabak Bernam and Sepang. Selangor is one of the most populated and industrialized state in Peninsular Malaysia. As the most populated state, rivers in Selangor are facing serious

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contamination due to anthropogenic activities. On the other hand, study by Al-Badaii et al. (2013) found that Semenyih River, Selangor was contaminated with NH_3-N, TSS, COD and NO_3 and highly polluted with PO_4 and faecal coliform. The sources of contamination were originated from industrial, agricultural, livestock farming and erosion. Mohamad Ali (2010) stated that the main pollution in Selangor River is from poultry farms, wet market activities and industrial wastewater.

Tekala River is a recreational river that has potential to be contaminated with faecal pathogens (such as bacteria and viruses) from human sewage and animal manure. Contaminated recreational water can cause diseases. Therefore, this study aims to measure the water quality status of Tekala River based on the physico-chemical and biological parameters. In view of the above facts, Tekala River has been chosen due to its importance as recreational area. If this river is classified as not safe then further action should be taken to maintain and improve its quality.

Materials and methods

Study area

This study was conducted at Tekala River, Selangor, Malaysia (*Fig. 1a*) within Tekala River Recreational Park (TRRP) that was established since 1982. The highest level of the river leads up to a sparkling waterfall which cascades into a natural rock pool. TRRP is located 13 km from Semenyih via Jalan Semenyih – Hulu Langat and 50 km from Kuala Lumpur.

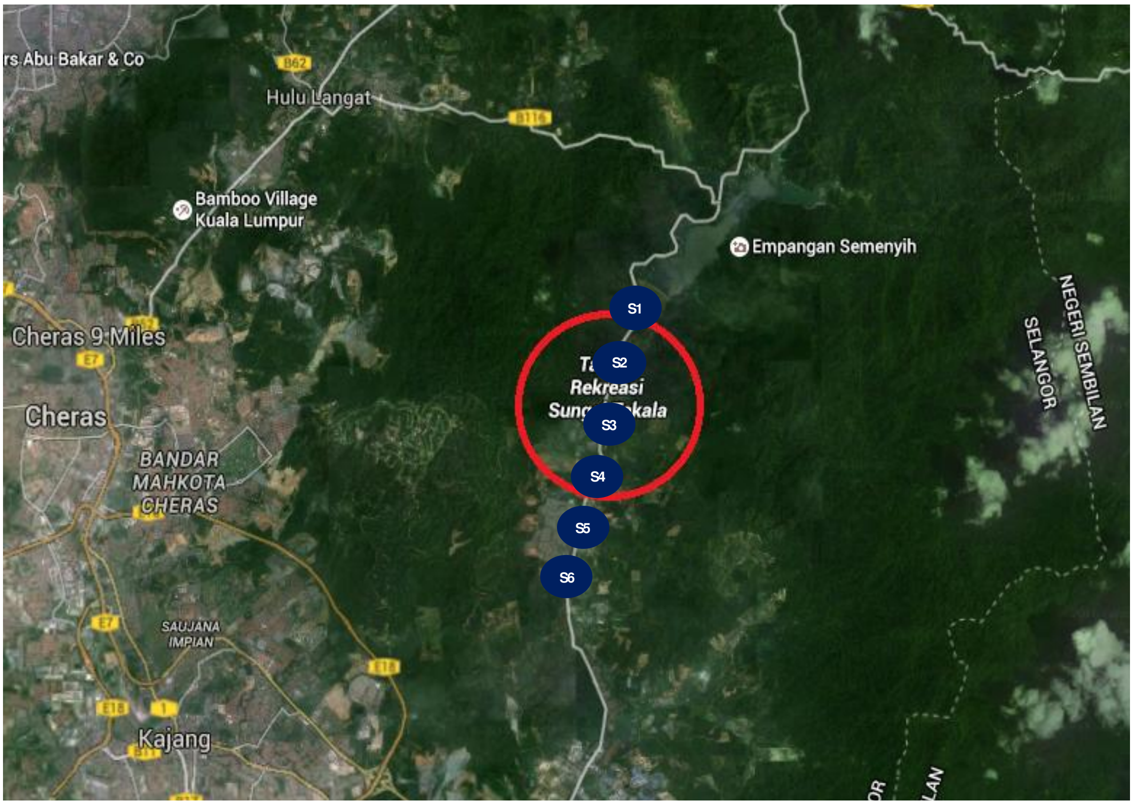

Figure 1. *Map of Tekala River and locations of six sampling points*

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Measurement of ex-situ parameters

The water quality index was determined based on the physico-chemical and biological parameters. The parameters include biochemical oxygen demand (BOD), chemical oxygen demand (COD), ammoniacal-nitrogen (NH_3^-N), total suspended solids (TSS), dissolved oxygen (DO), pH, water temperature, total dissolved solid (TDS), salinity, conductivity, *Escherichia coli (E. coli)*, coliform and macroinvertebrate.

Samples collection and preservation

Water samples were collected from the selected river and have been collected for two visits in May 2017 and October 2017. A total of 18 water samples were collected from 6 sampling stations and the samples were kept in polyethylene bottles. For BOD sample, another 18 water samples were collected in glass bottle wrapped with aluminium foil. This is to avoid sunlight from penetrating the bottles and react with sample and cause changes in the BOD. During sampling for BOD, no air bubble was observed as it can influence the oxygen content of the sample. All bottles were preserved at 4–10 °C in an ice box before transported to the laboratory. Preservation of samples need to be done to maintain the actual composition of water samples and can minimize changes in chemical composition during transportation to the laboratory.

DO, water temperature, TDS, salinity, conductivity and pH values were measured in-situ. The COD, TSS, BOD, ammoniacal-nitrogen, *E. coli* and coliform in the water were analysed in the laboratory using standard procedure of HACH. For macroinvertebrate, samples were collected by using Surber net at 6 stations based on different habitats. All collected samples were transferred into plastic. Then, samples were preserved by using 70% ethanol in the laboratory for further identification.

Water was kept in dark condition and in temperature around 4–5 °C to reduce biological activity, to avoid changes and to preserve the actual condition of sample as it is before conducting any analysis. BOD samples were kept in glass bottle preserved at 30 °C for about 5 days prior to measurement. The use of glass bottle can avoid any changes in activity and microorganism metabolism. Samples for *E. coli* and coliform test were kept at 4–5 °C and analysis was conducted at least 4–8 h after sampling activity.

Biological Oxygen Demand (BOD) was determined by using conventional method by incubating the water samples for a standard time period in incubator at 20 °C and the oxygen consumption was determined at the end of the incubation period. The first measurement of DO values (DO_1) was carried out within 24 h after sampling by using YSI model 5000. The reading was recorded on a data sheet. The second reading of DO values was determined after 5 days of incubation (DO_5). BOD values were obtained from the difference in DO values of the first reading (DO_1) and the second reading (DO_5). The BOD value is expressed in milligrams per liter using *Equation 1:*

$$\text{BOD (mg/l)} = DO_{initial} - DO_{final} \qquad \text{(Eq.1)}$$

Chemical oxygen demand

COD was measured by using Reactor Digestion Method approved by USEPA for reporting wastewater analysis. Samples were first homogenized to dissolve containing solids for better representative samples. The homogenized water samples were then added into glass vials containing strong oxidizing agent, potassium dichromate. This mixture was heated for 2 h at 150 °C to allow them to react. Colorimetric determination of COD

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with high range using HACH DR/2010 was used to measure COD value in the water samples.

Total suspended solid

Whatmann filter paper with pore size of 0.45 µm was put in petri dish each and dried in the drying oven at the temperature of 105 °C for 1 h. After an hour, the petri dish was taken out and cooled for 30 min in the desiccator. Before performing filtration of the samples, the weighed filter paper was obtained. This was the initial weight of the filter paper (A). This filter paper then was used in filtration process. For the filtration process, the filter paper was inserted between the Buchner funnel and the suction flask. The water sample in the bottle was shaken and about 200 ml of sample was used in the filtration. After that, the filter paper was put back into petri dish and was dried in the drying oven at 105 °C and the final weight of the filter paper was recorded (B).

For the calculation (*Eq. 2*),

$$x\,mg/L = \frac{(B-A)\ X\ 1000}{\text{Volume of sample used (ml)}} \qquad \text{(Eq.2)}$$

where,

A = initial weight of the filter paper (mg)
B = final weight of the filter paper + soluble solid (mg)

Ammoniacal-nitrogen

Ammoniacal-nitrogen was measured by using HACH DR 2700 based on Nessler Method. 25 ml of sample and 25 ml of deionized water (blank) were transferred into glass cell each and then three drops of mineral stabilizer, three drops of polyvinyl alcohol and 1.0 ml of Nessler reagent were added to each transferred sample. The mixture was measured by using spectrometer at 425 nm. Deionized water was used as blank and the reading was recorded for each sample.

Measurement for biological parameter

Escherichia coli and coliform

100 ml of water sample was used for determination of *E. coli* and coliform. Water samples were filtered in cellulose membrane filter. After filtration, Eosin Methylene Blue (EMB) agar was added with no air bubbles observed on the agar. The agar was preserved for 22 ± 2 h at 35 °C. Organisms that produce a colony with golden-green metallic within 24 h of incubation on the agar were considered members of *E. coli* group. While organisms that produce colony with light purple were classified as coliform group.

Quantitative data was obtained by counting the number of each colony formed by assumption that each colony represents one bacterium. Colony of coliform and *E. coli* that grow was counted and expressed in unit of cfu (colony forming unit) (APHA, 1980) and calculated as below (*Eq. 3*):

$$E.coli/100\,ml = \frac{100\ X\ Number\ of\ colony}{\text{Volume of sample filtered (ml)}} \qquad \text{(Eq.3)}$$

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Macroinvertebrate

All the samples from the river were taken to the laboratory for identification process. Sample from different stations were put in different bottles. Each sample was analyzed by identifying specific characteristics for each species such as head shape, body segment and type of wings. Samples that had already been identified were preserved in 70% ethanol.

Water quality classification

Classification in water quality index (WQI) was determined based on the water quality index as below (*Eq. 4*):

$$\begin{aligned} \text{WQI} = &\left(0.22 \text{ x SIDO}\right) + \left(0.19 \text{ x SIBOD}\right) + \left(0.16 \text{ x SICOD}\right) \\ &+ \left(0.15 \text{ x SIAN}\right) + \left(0.16 \text{ x SITSS}\right) + \left(0.12 \text{ x SIpH}\right) \end{aligned} \quad \text{(Eq.4)}$$

where:
SIDO = Sublndex DO (% saturation)
SIBOD = Sublndex BOD
SICOD = Sublndex COD
SIAN = Sublndex NH_3-N
SISS = Sublndex SS
SipH = Sublndex pH
Statistical analysis was conducted by using IBM SPSS Statistics 20. Two-way ANOVA was conducted for comparing water quality parameter at different stations and sampling times. Correlation analysis was also conducted to determine the relationship between physico-chemical and biological parameters.

Results

Physico-chemical parameters

Mean value for total suspended solid (TSS) for two sampling times is shown in the *Figure 2a*. TSS ranged from 1.67 mg/L to 8.17 mg/L. The average value of TSS is 4.15 ± 0.48 mg/L and this value is categorized under Class I of NWQS. Based on the Two-way analysis of variance (ANOVA) result, there is no significantly different between TSS versus station and sampling time ($P > 0.05$, $P = 0.746$).

Figure 2b presents the mean value for ammoniacal nitrogen (NH_3^-N) for both sampling times. It was found that NH_3^-N were ranged from 0.03 mg/L to 0.31 mg/L with an average value of 0.18 ± 0.04 mg/L and falls under Class I of NWQS. Two-way analysis of variance (ANOVA) shows that there is significantly different between NH_3^-N and station ($P < 0.05$, $P = 0.000$). There is also significant difference between NH_3^-N and sampling times ($P < 0.05$, $P = 0.038$). Significantly different was also found between the effect of different stations and different sampling times on NH_3N ($P < 0.05$, $P = 0.037$).

Mean value for biological oxygen demand (BOD) for two sampling times is shown in *Figure 3a* is ranged from 0.04 mg/L to 1.42 mg/L. The average value of BOD is 0.78 ± 0.14 mg/L, categorized under Class I of NWQS. There is no significantly

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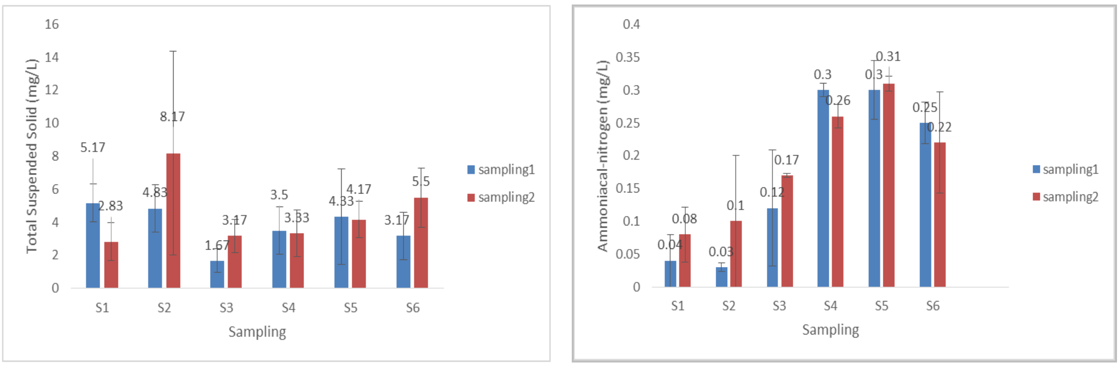
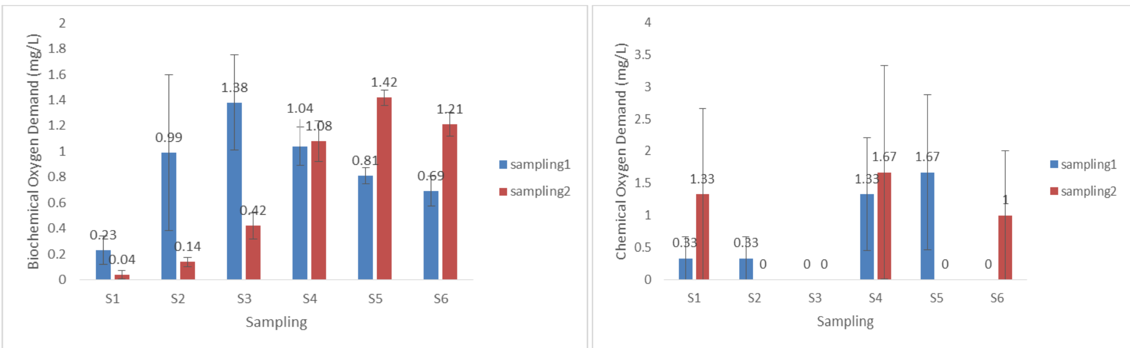
different between BOD and sampling times (P > 0.05, P = 0.189). However, there is significant difference between BOD and station (P < 0.05, P = 0.000).

Figure 3b shows the mean value for chemical oxygen demand (COD) that were ranged from 0.33 mg/L to 1.67 mg/L, with an average value of .64 ± 0.22 mg/L (Class I). Two-way analysis of variance (ANOVA) shows that there is no significant difference between COD with station and sampling time (P > 0.05, P = 0.656).



a b

Figure 2. *(a) TSS values for six sampling stations; (b) Ammoniacal nitrogen values for six sampling stations, where; the upstream (S1 and S2), the middle stream (S3 and S4) and the downstream (S5 and S6)*



a b

Figure 3. *(a) Biochemical oxygen demand for six sampling stations; (b) Chemical oxygen demand for six sampling stations, where; the upstream (S1 and S2), the middle stream (S3 and S4) and the downstream (S5 and S6)*

pH was ranged between 5.00 to 6.8 with an average of 5.60 ± 0.67, classified under Class III and IV for the first and second samplings, respectively (*Fig. 4a*). A significantly different was observed between pH and sampling times and station (P < 0.05, P = 0.025) and (P < 0.05, P = 0.000), respectively. Mean value for temperature for two sampling times is shown in *Figure 4b* ranged from 20.76 to 21.49 °C. There is significant difference between temperature and sampling times (P < 0.05, P = 0.000) as well as between temperature and station (P < 0.05, P = 0.000).

It was found that the conductivity for both sampling times were ranged from 0.013 mS/cm to 0.017 mS/cm (*Fig. 5a*). The average conductivity is 0.0014 ± 0.0003 mS/cm and classified in Class I and IIA. Based on the result, there is no significant difference

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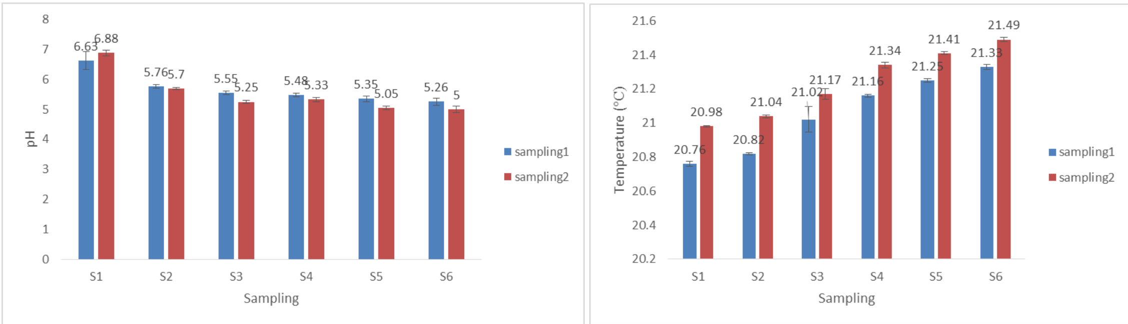
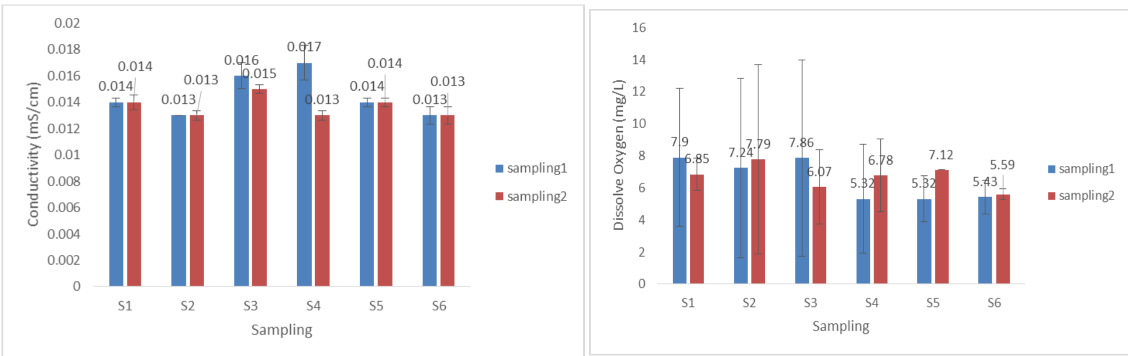
between conductivity and sampling times (P > 0.05, P = 0.292), whereas there is significant difference between conductivity and station (P < 0.05, P = 0.006). For dissolve oxygen, the mean value for both sampling times were ranged from 5.32 mg/L to 7.86 mg/L as shown in *Figure 5b*. The average DO is 6.60 ± 0.29 mg/L (Class IIA and IIB). There is no significantly different between DO and sampling times (P > 0.05, P = 0.078). However, there is significant difference between DO and station (P < 0.05, P = 0.000).



a **b**

Figure 4. *(a) pH values for six sampling stations; (b) Temperature values for six sampling stations, where; the upstream (S1 and S2), the middle stream (S3 and S4) and the downstream (S5 and S6)*



a **b**

Figure 5. *(a) Conductivity values for six sampling stations; (b) Dissolved oxygen values for six sampling stations, where; the upstream (S1 and S2), the middle stream (S3 and S4) and the downstream (S5 and S6)*

Figure 6(a) shows the mean value for salinity for two times sampling ranged from 0.003 to 0.01 with an average of 0.007 ± 0.001 (Class I). No significantly different between salinity with station and sampling time was observed (P > 0.05, P = 0.813). The mean value for total dissolved solid ranged from 0.009 g/l to 0.011 g/l (*Fig. 6b*). The average of TDS is 0.009 ± 0.0001 and classified under Class I. There is no significant difference between TDS and sampling times (P > 0.05, P = 0.423), while significantly different was found between TDS and station (P < 0.05, P = 0.032).

Water Quality Index (WQI) for all stations times is shown in *Figure 7*. WQI has been considered as one criterion for surface water classifications, based on the use of

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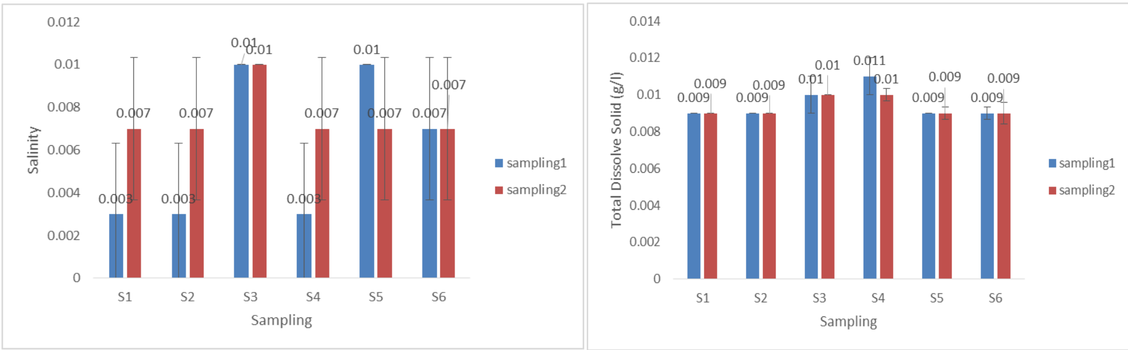
standard parameters for water characterization. This index is a numeric expression used to transform large quantities of water characterization data into a single number, which represents the water quality level (Mohamad Ali, 2010). The WQI values recorded were ranged from 85.45 to 94.86 with the highest WQI recorded at station 2, while the lowest WQI was measured at station 4. The results show that WQI obtained from this study fall under Class I (clean category). Overall, the WQI value of Tekala River was classified under Class II which indicated as clean.



a b

Figure 6. *(a) Salinity values for six sampling stations; (b) Total dissolved solid values for six sampling stations, where; the upstream (S1 and S2), the middle stream (S3 and S4) and the downstream (S5 and S6)*

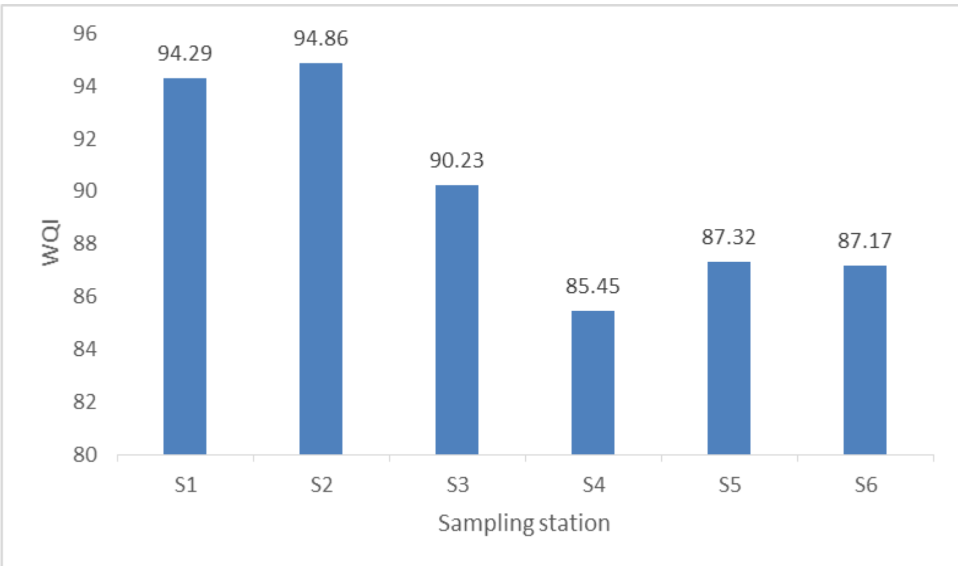


Figure 7. *WQI value for six sampling stations*

Biological parameter

Mean value for *E. coli* and total coliform for two sampling times are shown in *Figures 8a* and *8b*. *E. coli* and total coliform values were ranged from 1 to 16 cfu/ml and 3 to 48 cfu/ml, respectively. The average value for *E. coli* and total coliform are 8 ± 2 cfu/ml and 21 ± 4 cfu/ml, respectively and both are classified under Class I. Pearson's

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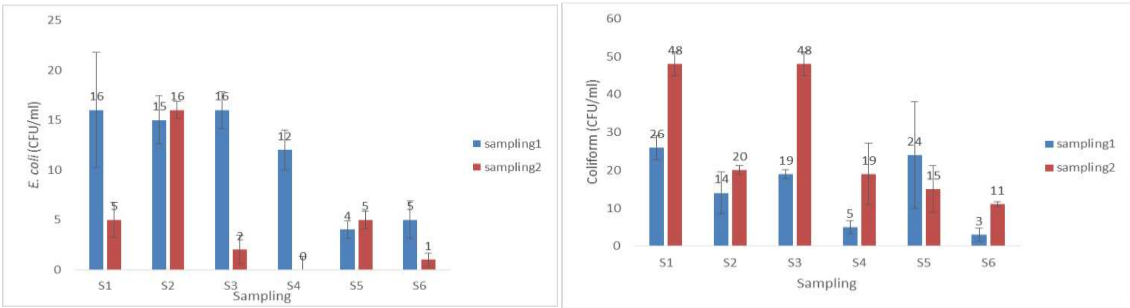
correlation was conducted to examine relationship between *E. coli* and coliform with the physico-chemical parameters (*Table 1*). Based on the test, there is relationship between *E. coli* with NH_3^-N, temperature and DO. For coliform, there is relationship between coliform with pH and BOD.

Macroinvertebrate

Total value for macroinvertebrate for two sampling times is shown in *Figure 9*. The abundance of macroinvertebrate was ranged from 10 to 68 (*Fig. 9*). Pearson's correlation was conducted to examine the relationship between abundance of macroinvertebrate with the physico-chemical parameters (*Table 2*). Based on the test, there is relationship between abundance of macroinvertebrate with temperature of the river.



a b

Figure 8. *(a) E. coli values for six sampling stations; (b) Coliform values for six sampling stations, where; the upstream (S1 and S2), the middle stream (S3 and S4) and the downstream (S5 and S6)*

Table 1. *Pearson's correlation between E. coli and coliform with physcio-chemical parameters*

PARAMETER	E.coli	Coliform	TSS	BOD	NH3N	COD	pH	Temperature	Conductivity	DO	Salinity	TDS
E.coli	1											
Coliform	-0.19	1										
TSS	0.15	-0.12	1									
BOD	-0.03	-0.47**	-0.094	1								
NH3N	-0.43**	-0.102	0.111	0.256	1							
COD	-0.25	0.206	-0.229	0.089	0.118	1						
pH	0.29	0.477**	-0.019	-0.56**	-0.287	-0.06	1					
Temperature	-0.67**	-0.297	-0.022	0.377*	0.591**	0.139	-0.75**	1				
Conductivity	0.02	0.021	-0.206	0.179	0.111	-0.03	-0.083	0.108	1			
DO	0.53**	0.222	-0.053	-0.157	-0.49**	-0.05	0.371*	-0.550**	-0.230	1		
Salinity	-0.21	0.086	-0.043**	0.112	-0.003	0.040	-0.231	0.190	0.014	-0.08	1	
TDS	0.02	-0.134	-0.114	0.111	0.067	0.110	-0.164	0.149	0.748**	-0.31	0.022	1

**Correlation is significant at 0.01 level (2-tailed)
*Correlation is significant at 0.05 level (2-tailed)

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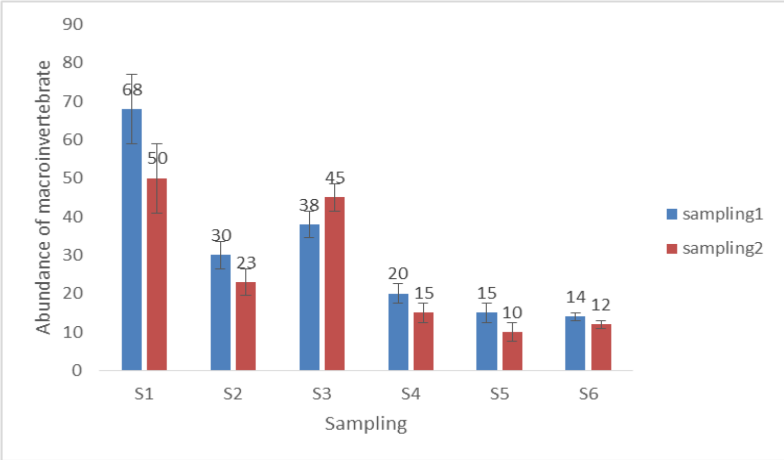

Figure 9. *Abundance of macroinvertebrate for six sampling stations, where; the upstream (S1 and S2), the middle stream (S3 and S4) and the downstream (S5 and S6)*

Table 2. *Pearson's correlation between abundance of macroinvertebrate with physico-chemical parameters*

PARAMETER	X	TSS	NH3N	COD	pH	Temperature	Conductivity	DO	Salinity	TDS	BOD
X	1										
TSS	0.054	1									
NH_3N	-0.523	-0.405	1								
COD	-0.310	-0.207	0.404	1							
pH	0.441	-0.183	-0.322	-0.045	1						
Temperature	-0.633*	-0.098	0..692*	0.250	-0.807*	1					
Conductivity	-0.062	-0.437	0.168	-0.149	0.017	-0.058	1				
DO	0.141	0.162	-0.639*	-0.198	-0.447	-0.518	-0.122	1			
Salinity	-0.440	-0.269	0.299	-0.007	-0.369	0.509	0.033	-0.121	1		
TDS	-0.302	-0.356	0.202	0.292	-0.214	-0.120	0.786**	-0.221	-0.039	1	
BOD	-0.217	-0.158	0.244	0.147	-0.692*	-0.587*	0.202	-0.140	-0.084	0.089	1

**Correlation is significant at 0.01 level (2-tailed)
*Correlation is significant at 0.05 level (2-tailed)

Discussion

Physico-chemical parameters

Total Suspended Solids (TSS) are solids in water include a wide variety of material, such as silt, decaying plant and animal matter, industrial wastes, and sewage. High concentrations of suspended solids can cause many problems for stream health and

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aquatic life (Bilotta and Brazier, 2008). According to Wood and Armitage (1997), suspended solid in the river may be due to bank erosion, sediment load and anthropogenic activities or due to runoff from surrounding areas. In second sampling, station 2 surrounded by dense vegetation, thus recorded the highest TSS. Vegetation can reduce the erosion and increase sediment entrapment. However, there is no significant different between TSS with station and sampling time. The maximum threshold limit of TSS for Malaysian rivers which support aquatic life is 150 mg/l (Al-Badaii et al., 2013). TSS value recorded in this study are within the range of the limit.

Ammoniacal nitrogen (NH_3^-N) is a common pollutant in freshwater ecosystem and it is frequently found associated with organic compound or sometimes from industrial effluents (Alabaster and Lloyd, 1982; Magdalena et al., 2015). Abdel-Raouf (2012) stated that the presence of NH_3^-N in the water is mainly originated from the domestic sewage and waste water from certain types of industries. Ammoniacal nitrogen indicates nutrient status, organic enrichment and health of water body (Radojevic et al., 2007). Higher NH_3^-N values can be toxic to fish, but in small concentrations, it could serve as nutrients for excessive growth of algae (Al-Badaii et al., 2013). There is significant different between NH_3N with station and sampling times implying NH_3N is influenced by sampling location and time of sampling. The maximum threshold level of NH_3N for Malaysian rivers which support aquatic life is 0.9 mg/l. The value of NH_3N obtained from this study is still within the range.

Biochemical Oxygen Demand (BOD) is a measurement of the amount of dissolved oxygen used by aerobic microorganisms when decomposing organic matter in water. It is a measure of the quantity of oxygen used by microorganisms in the oxidation of organic matter. Microorganisms such as bacteria are responsible for decomposing organic waste. When organic matter such as dead plants, leaves, grass clippings, manure, sewage or even food waste is present in a water supply, the bacteria will begin the process of breaking down this waste. When this happens, much of the available dissolved oxygen is consumed by aerobic bacteria, affecting oxygen level needed for other aquatic organisms (USGS, 2016).

If there is a large quantity of organic waste in the water supply, there will also be a lot of bacteria present working to decompose this waste. In this case, the demand for oxygen will be high so the BOD level will be higher. When BOD levels are high, dissolved oxygen levels decrease because the oxygen that is available in the water is being consumed by the bacteria (Al- Badaii et al., 2013). Since less dissolved oxygen is available in the water, fish and other aquatic organisms may not survive. If there is no organic waste present in the water, there will be fewer bacteria present to decompose it and thus the BOD will tend to be lower and the DO level will be higher.

Chemical Oxygen Demand (COD) is a measurement of the oxygen required to oxidize soluble and particulate organic matter in water (Boyd, 1973; Kunlasak et al., 2013). It is used to measure the total quantity of oxygen-consuming substances in the complete chemical breakdown of organic substances in water. It does not differentiate between biologically available and inert organic matter. COD measurements can be made in a few hours while BOD measurements take five days (Othman et al., 2012). Generally, the lower COD level indicates a low level of pollution, while the high level of COD points out the high level of pollution of water in the study area (Al-Badaii et al., 2013). The COD value obtained in this study is low indicating this river has low level of pollution.

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pH of a water sample is a measure of the concentration of hydrogen ions (Tank and Chippa, 2013). If the pH of water is too high or too low, most of the aquatic organisms will not be able to adapt to the extreme condition. pH can also affect the solubility and toxicity of chemicals and heavy metals in the water. Various concentration of solution, solid or gaseous that enters into river can influence pH of the river due to reaction of dissociation which may produce H+ or OH- ions (Chang et al., 1983; Lelis et al., 2016). Based on the result, pH ranged from 5.00 to 6.88. There is significance different between pH with station and sampling times indicating pH is influenced by sampling location and time of sampling. pH slightly decreased from station 1 to station 6. pH of water body can be affected by several factors. One of the factors is the amount of plant growth and organic material within a body of water. Carbon dioxide is released when this material is decomposed. The carbon dioxide combines with water to form carbonic acid. Although this is a weak acid, large amounts of it will lower the pH. Decaying vegetation produces organic acids, thus change the pH of the river (Gasim et al., 2007). Station 1 to station 6 were surrounded by vegetation. Different station may have different rate of decaying vegetation. This might explain the decreased of pH from station 1 to station 6. Rainfall could also affect pH of water body due to the interaction with carbon dioxide molecules in the atmosphere. This creates H_2CO_3 in the raindrops, lowering the pH value of rainwater. A pH level of 5.65, though acidic, is not considered acid rain. Acid rain requires a pH below 5.0. If rain falls on a poorly buffered water source, it can decrease the pH of nearby water through runoff.

Water temperature is one of the most important characteristics of an aquatic system. It affects dissolved oxygen levels, species composition, chemical and biological processes (Tank and Chippa, 2013). A change in water temperature can affect the general health of the aquatic organisms, thus changing the quality of the stream. There is significant difference between temperature with sampling times and station. The temperature of surface water is usually between 0 ºC and 30 ºC. The lowest temperature was recorded at station 1 and the highest at station 6 for both samplings. There is slightly increased of temperatures from station 1 to station 6. Usually there is an increase in temperature from the first station to the last station (Bordalo et al., 2001). Water temperature varies along the length of a river with latitude and elevation. The geographical location of sampling stations are differ on elevation and environment. Station 1 and station 2 were located in upstream, station 3 and station 4 in the middle meanwhile stations 5 and 6 in the downstream. Vegetation cover may influence water surface temperature. Area from station 1 to station 6 were covered by vegetation. However, the vegetation in the upstream was denser than middle and downstream. The vegetation acts as protection to surface water from directly being heated by solar radiation. The forest canopies will shield the water surface from the emission of long wave radiation from the sun. Denser vegetation gives more protection to the surface water thus this might explain the reason why station 1 had low temperature compared to other stations. Human activities in the surrounding area might also influence the water temperature. The middle and downstream had a lot of human contact as the river is a recreational area. Downstream area vegetation had some disruption as a lot of facilities being built in this area. Water temperature can also be influenced by variation of precipitation. According to Shuhaimi-Othman et al. (2008) large volume of water inputs and higher flow rate were responsible for cooling down the river temperature. There is only little difference of temperature between first and second samplings.

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Conductivity can be defined as an ability of the aquatic system to transmit electric current based on the level of dissolved ions in a water body. Conductivity in water is affected by the presence of inorganic dissolved solids such as chloride, nitrate, sulfate and phosphate anions or sodium, magnesium, calcium, iron, and aluminum cations. Conductivity normally coinciding with the level of dissolved salt in water and has a strong correlation with salinity. An increasing in level of dissolved salt would result an increased in conductivity. There is slightly difference of salinity based on times and location of the sampling in this study. Based on the result, there is no significance different between conductivity and sampling times but there is significant different between conductivity and different station which means conductivity is influenced by sampling location. Conductivity in streams and rivers is affected primarily by the geology of the area through which the water flows. Streams that run through areas with granite bedrock tend to have lower conductivity because granite is composed of more inert materials that do not ionize when washed into the water. On the other hand, streams that run through areas with clay soils tend to have higher conductivity because of the presence of materials that ionize when washed into the water. Soil and rocks release dissolved solids into the waters that flow through or over them. Therefore, the geology of a certain area will determine the conductivity. Most streams have a fairly constant range of conductivity under normal circumstances. Therefore, significant changes in conductivity can be an indicator that a discharge or some other source of pollution has entered the water. This might explain the reason why conductivity for different sampling times is constant and the difference can be seen mostly based on the sampling location. The conductivity was ranged from 0.013 mS/cm to 0.017 mS/cm. This is considered low and does not affect the water quality (Suki et al., 1988; Rashid et al., 2013).

Dissolved oxygen (DO) measures the amount of gaseous oxygen dissolved in an aqueous solution. Oxygen gets into water by diffusion from the surrounding air, by aeration and as a waste product of photosynthesis from phytoplankton, algae, seaweed and other aquatic plants (Othman et al., 2012). The amount of dissolved oxygen varies depending on temperature, pressure and salinity. Solubility of oxygen decreases as temperature increases. This is due to the fact that warmer surface water requires less dissolved oxygen to reach 100% air saturation compared to cooler water. There is negative correlation of DO and temperature which means higher temperature will reduce DO level. Temperature slightly increased from station 1 to station 6 and the DO level recorded is slightly reduced from station 1 to station 6. Dissolved oxygen decreases exponentially as salt levels increase. There is significance difference between DO and station. In freshwater systems such as lakes, rivers and streams, dissolved oxygen concentrations will vary by season, location and water depth and it will decrease with higher temperature, salinity and elevation (Beale et al., 2000). Oxygen concentrations vary with the volume and velocity of water flowing in a stream. Faster flowing water areas, such as in a mountain stream or large river tend to be more oxygen rich because more oxygen enters the water from the atmosphere in those areas than in slower and stagnant areas. Oxygen is more easily dissolved into water at low altitudes that at high altitudes. Oxygen is also more easily dissolved into water with low levels of dissolved or suspended solids. Removal of riparian vegetation may lower oxygen concentrations due to increased water temperature resulting from a lack of canopy shade and increased suspended solids resulting from erosion of bare soil. Upstream area has

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faster flowing water compared to lower area thus this might explain why level of DO is higher in upstream area.

Salinity is the total concentration of all dissolved salts in water. Freshwater contains few salts and thus has low salinity. Most of natural freshwater do not show much different on level of salinity unless there are additional discharged from surrounding environment into the river. The salinity value recorded from this study is low thus it would not have huge impact in the environment.

Total dissolved solid (TDS) is a measure of the amount of material dissolved in a water sample. This material includes dissolved minerals and organic matter but can also include contaminants. There is significant difference found between TDS with station. TDS is influenced by sampling location. TDS can vary spatially and temporally due to natural and anthropogenic factors such as climate, soil type, relief, land use and human activities (Augustijn et al., 2011). TDS in water supplies originated from natural sources, sewage, urban and agricultural run-off and industrial wastewater. Water containing TDS concentrations below 1,000 mg/l is usually acceptable but water with extremely low concentrations of TDS may also be unacceptable to consumers because of its flat, insipid taste to consumers. No recent data on health effects associated with the ingestion of TDS in drinking-water appear to exist (WHO, 2004).

Water quality index (WQI) provides relative indication of the quality of water. A river with WQI value in the ranges 0-59 is considered polluted, 60-80 is considered slightly polluted and 81-100 is considered the water body is clean. A higher value indicates a better quality of water (Suki et al., 1988; Rashid et al., 2013). From this study, the average WQI calculated at each sampling station for both sampling times are described as follows: Station S1 (94.29: Class I), S2 (94.86: Class I), S3 (90.23: Class II), S4 (85.45: Class II), S5 (87.32: Class II) and S6 (87.17: Class II) (*Fig. 4*). The results show that WQI obtained from this study fall under Class I (clean category). Overall, the WQI value of Tekala River was classified under Class II which indicated as clean. Generally, WQI tends to decrease from upstream to downstream of river due to increasing of pollutant. WQI recorded slightly reduced from station 1 to station 6.

Biological parameter

E. coli is mainly originated from the feces of warm blooded organism. This type of bacteria usually entered into water surface by run-off from agricultural and residential areas (Pearson et al., 1987; Inatsuka et al., 2010). From the first sampling, there is a decrease of *E. coli* from station 1 to station 6. Second sampling recorded less number of *E. coli*. There is correlation relationship found between *E. coli* and NH_3^-N, temperature and DO. It is reported that fecal coliform levels were lower at higher temperature, high dissolved oxygen and high pH implying that one of the fecal coliform sources could be related to human recreational activities (Al-Badaii et al., 2013). This might explain the downtrend of bacteria from first station to the last station. Since this river is a recreational area, it probably potentially exposed to fecal contamination sources from human activities (swimming, camping) that cannot be detected by measuring physico-chemical parameters alone.

From the first and second samplings, there is a decrease of coliform from station 1 to station 6. Second sampling recorded less number of coliform. There is correlation relationship found between coliform with pH and BOD. Water with lower pH value helps in maintaining the survival of indicator bacteria (Parhad et al., 1974; Mara, 2013). A study done by Šolić and Krstulović (1992) also suggested that the optimal pH value

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for coliform survival is between pH 6 and pH 7 and other pH value above or below these range can cause rapid decline in fecal coliform population. The bacteria population is influenced by favourable condition and food sources provided by the sediments for the bacteria (Davies et al., 1995). Lower temperature of surface water can cause rapid decline in coliform and *E. coli* population in the water (McDaniels et al., 1985; Pope et al., 2003).

Macroinvertebrate

Macroinvertebrates and water quality are interrelated to each other, as macroinvertebrates are a potential biological indicator of water quality. They are most frequently used in biomonitoring studies (Harikumar et al., 2014). Often many species within a community with varying sensitivities to stresses and relatively quick reaction times, resulting in a spectrum of graded, recognisable responses to environmental perturbation. The responses to different types of pollution have been established for many common species (Ollis et al., 2006). Stream communities are shaped by environmental influences at multiple spatial scales. Their distribution, interaction and adaptation can be influenced by abiotic factors which vary in space and time. Climate is one of the factors that influence population dynamics of aquatic insects (Nor Zaiha et al., 2015). In this study, the number of macroinvertebrate found is low. Most of the macroinvertebrate found belong to the same family. A study on surber net was conducted at the forested stream site by Wan Mohd Hafezul et al. (2016). They found very low species abundance was recorded by the surber net. Its small size makes it difficult to set on rough substrates in deep water and often results in the loss of large organisms that are fast enough to crawl out of the front of the sampler. Usually area disturbed within the surber net is exposed, providing an opportunity for aquatic fauna to escape (Wan Mohd Hafezul et al., 2016).

The most dominant insect found in all stations is water strider from family Gerridae. Based on the correlation test, there is relationship between abundance of macroinvertebrate and temperature. Water striders most prefer waters around 25 °C. Large numbers can indicate moderate, or slightly polluted water quality. In the upstream area, a few mayfly from order Ephemeroptera was caught in station 4. Ephemeroptera is a group of aquatic insects which is sensitive to environmental interference and can only survive in clean and oxygen-rich waters. Therefore, they are often considered as good biological indicators of water quality (Nor Zaiha et al., 2015). The most significant environmental factor affecting life-history patterns, especially growth rates and the seasonal timing of aquatic insects, is water temperature. Mayfly is widely distributed in peninsular Malaysia. It inhabits upstream rivers of peninsular Malaysia. Low water temperature seemed to favor mayfly abundance. Predators, particularly fish, play a role in the mayfly community (Suhaila et al., 2016). In station 4, there is no fish found in fast flowing water thus mayfly might prefer the area. From station 3 to 6, there is no more mayfly found. Station 3 has fish community, thus this area might not be preferred by mayfly. There is less human contact from station 1 to station 3, where visitor usually stay in area station 4 to station 6.

There are several other organisms that were found in the river such as freshwater shrimp, freshwater crabs and water penny. Freshwater crabs are one of the most ecologically important macro-invertebrate groups in tropical inland waters worldwide. These strictly freshwater decapods are found in almost all clean freshwater bodies in the tropics from moist lowland forests to rugged mountains. Crabs live in rivers, streams,

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waterfalls, wetlands, karsts, caves and many are semi-terrestrial. Almost all require pristine water conditions to survive and are excellent indicators of good water quality (Cumberlidge et al., 2009). Generally, it can be concluded that more abundant macroinvertebrate communities exist in the littoral zone than in the deeper river bed. The individual numbers are usually very low in the deep water zone which is dominated by continually shifting sand (Csányi et al., 2012). Type of food sources and physical properties of river determine the distribution of aquatic organisms (Cummins and Merritt, 1996).

Conclusions

From this study, physico-chemical parameters and biomonitoring analysis have been conducted to determine the status of water quality at Tekala River, Selangor. Water quality index for Tekala River were ranged from 85.45 to 94.86, thus indicated that the water quality status for both sampling times and six sampling stations are classified under Class I and Class II. It was found that there is significance different between ammoniacal nitrogen, pH and temperature. Correlation was also found between several physico-chemical parameters with biological parameter. From the Pearson's correlation test, there is relationship between *E. coli* with ammoniacal nitrogen, temperature and DO. For coliform, there is relationship between coliform with pH and BOD. The data from this study was also compared with the National water quality standard (NWQS) and most parameters were classified under Class I to Class IIB, implying that this river is safe to be used for recreational purposes. It is recommended that further research on other parameters on this river to be conducted in order to get better assessment on the water quality.

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THE IMPACT OF HERBICIDES AND THEIR MIXTURES ON THE CONTENT OF POLYPHENOLS IN EDIBLE POTATO TUBERS

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Abstract. The aim of the study was to determine the influence of applied herbicides and their mixtures on the polyphenol content in tubers of three edible potato varieties. The field experiment was conducted in the years of 2008-2010 in the Agricultural Experimental Station Zawady (52°03'N and 22°33'E) belonging to the University of Natural Sciences and Humanities in Siedlce, Poland. The experiment was established as two-factor in the split-plot system in three repetitions. The studied factors included: I – three varieties: Cekin, Satina and Tajfun. II – five ways of care: 1. mechanical care – control object, 2. mechanical-chemical care - mechanical care + Command 480 EC (chlomazon 480 g·l), 3. mechanical-chemical care - mechanical care + Command 480 EC (chlomazon 480 g·l) + Dispersion Afalon 450 SC (linuron 450 g·l), 4. mechanical-chemical care - mechanical care + Stomp 400 SC (pendimethalin 400 g·l), 5. mechanical-chemical care - mechanical care + Stomp 400 SC (pendimethalin 400 g·l) + Dispersion Afalon 450 SC (linuron 450 g·l). Polyphenols were determined in a fresh mass of potato tubers using the Swain and Hillis method using the Folin-Ciocalteu reagent. The results of the research were statistically analysed using the analysis of variance. The conducted own studies showed that the polyphenol content in potato tubers was on the level from 155.8 to 162.5 $mg \cdot kg^{-1}$ of fresh weight and depended significantly on the variety cultivated. Also the herbicides and their mixtures used in the experiment had a significant influence on the concentration of polyphenols in tubers, causing them to increase (from 2.2 to 6.0 $mg \cdot kg^{-1}$ of fresh weight) in relation to the control object that was cultivated only mechanically. In addition, meteorological conditions significantly modified the accumulation of polyphenols. Their smallest (on average-50.5 $mg \cdot kg^{-1}$) content in potato tubers was obtained in 2009 with the smallest sum of precipitation in the examined three-year period and the average air temperature of 15.1 °C.
Keywords: *potato, phenols, methods of care, variety, antioxidants*

Introduction

For many years, it was believed that polyphenols are anti-nutritious substances, however, currently the importance of this group of compounds as active food components is significantly increasing, due to the recent studies on their pro-health effects on the human body (Gumul et al., 2005).

Polyphenols occur in various plant species, have a diversified structure, molecular weight, physical, biological and chemical properties and have a multidirectional effect on food, on the one hand, they shape the taste and colour, and on the other, they show

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antioxidant activity, stabilizing fats and other labile food components (Jaszka et al., 2010; Perla et al., 2012; Rytel et al., 2014).

Polyphenols are one of the largest groups of antioxidants. They include over 8000 identified substances and are considered the most numerous antioxidants in our diet (Ross and Kasum, 2002; Manach et al., 2004).

The studies of many authors (Chun et al., 2005; Mattila and Hellström, 2007; Navarre et al., 2009; Ezekiel et al., 2013) show that potatoes are the most important source of polyphenols, after apples and oranges, which contain on average - 160 $mg \cdot kg^{-1}$ of fresh weight.

Many studies show that the chemical composition of potato tubers is modified by genotype, agrotechnical measures and atmospheric conditions during the growing season. Therefore, the purpose of these studies was to determine the effect of the herbicides used and their mixtures on the polyphenol content in tubers of three edible potato varieties.

Material and methods

The field experiment was conducted in the years of 2008-2010 in the Agricultural Experimental Station Zawady (52°03'N and 22°33'E) belonging to the University of Natural Sciences and Humanities in Siedlce, Poland. The experiment was established as two-factor in the split-plot system in three repetitions.

The studied factors included:

I – three varieties: Cekin, Satina and Tajfun.

II – five ways of care:

1. mechanical care – control object,
2. mechanical-chemical care, i.e., until the emergence hilling combined with harrowing, and about 7 days before the emergence the herbicide Command 480 EC (chlomazon 480 $g \cdot l$) in a dose 0.2 $l \cdot ha^{-1}$,
3. mechanical-chemical care, i.e. until the emergence hilling combined with harrowing, and about 7 days before the emergence spraying with a mixture of herbicides Command 480 EC (chlomazon 480 $g \cdot l$) in a dose 0.2 $l \cdot ha^{-1}$ + Dispersion Afalon 450 SC (linuron 450 $g \cdot l$) in a dose 1.0 $l \cdot ha^{-1}$,
4. mechanical-chemical care, i.e., until the emergence hilling combined with hailing, and about 7 days before the emergence herbicide Stomp 400 SC (pendimethalin 400 $g \cdot l$) in a dose 3.5 $l \cdot ha^{-1}$,
5. mechanical-chemical care, i.e., until the emergence hilling combined with hailing, and about 7 days before the emergence spraying with a mixture of herbicides Stomp 400 SC (pendimethalin 400 $g \cdot l$) in a dose 3.5 $l \cdot ha^{-1}$ + Dispersion Afalon 450 SC (linuron 450 $g \cdot l$) in a dose 1.0 $l \cdot ha^{-1}$.

The field experiment was established on soil classified to the department – autogenous soils, order – brown-ground soils, type – Luvisols made of light clay sands and strong loamy sands, the gradin class of IVa and IVb in terms of agricultural suitability classified as a very good rye complex. This soil was characterised by a slightly acid reaction, very high abundance of phosphorus, high abundance of potassium and the average wealth of magnesium. A potato was grown in the position after winter cereals. The experiment used the permanent organic fertilisation with manure 25 $t \cdot ha^{-1}$ and the mineral one in quantities: 100 $kg \cdot ha^{-1}$ N, 100 $kg \cdot ha^{-1}$ $P_2 0_5$ and 150 $kg \cdot ha^{-1}$ $K_2 0$.

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Samples of potato tubers (50 tubers) were taken from plots during harvest and stored at 10-12 °C. Polyphenols were determined in a fresh mass of potato tubers using the Swain and Hillis methods with the Folin-Ciocalteu reagent (Swain and Hillis, 1959). The results of the research were statistically analysed during the analysis of variance. The significance of the sources of variation was tested by the "F" Fischer-Snedecor test, and the significance assessment of differences at the level of significance of p = 0.05 between the compared averages, using the multiple Tukey intervals.

Particular growing seasons during the studies were characterised by variable weather conditions (*Table 1*). The highest amount of rainfall was noted in the growing season in 2010 – 459.7 mm and the average air temperature was higher by 0.9 °C compared to the long-term average. The smallest amount of rainfall – 354.4 mm was noted in 2009, the average temperature was higher by 0.4 °C. The growing season 2008 was characterised by the rainfall at the level of 371.4 mm and the air temperature did not differ significantly from the long-term average 14.9 °C. According to the calculated hydrothermal coefficient of Sielianinow, the growing seasons of 2008, 2009 and 2010 were characterised by the lack of drought, however, the alternating months occurred with extreme conditions, from severe drought to the lack of drought.

Table 1. *Characteristic of weather conditions in the years 2008-2010 (Zawady Meteorological Station)*

Years	Months						
	IV	**V**	**VI**	**VII**	**VIII**	**IX**	**IV-IX**
	Rainfalls (mm)						**Sum**
2008	28.2	85.6	49.0	69.8	75.4	63.4	371.4
2009	8.1	68.9	145.2	26.4	80.9	24.9	354.4
2010	10.7	93.2	62.6	77.0	106.3	109.9	459.7
Multiyear sum (1987-2000)	38.6	44.1	52.4	49.8	43.0	47.3	275.2
	Air temperature (°C)						**Mean**
2008	9.1	12.7	17.4	18.4	18.5	12.2	14.7
2009	10.3	12.9	15.7	19.4	17.7	14.6	15.1
2010	8.9	14.0	17.4	21.6	19.8	11.8	15.6
Multiyear mean (1987-2000)	7.8	12.5	17.2	19.2	18.5	13.1	14.7
	Sielianinow's hydrothermic coefficients*						**Mean**
2008	1.04	2.18	0.94	1.25	1.36	1.73	1.39
2009	0.26	1.72	3.08	0.44	1.48	0.57	1.28
2010	0.40	2.14	1.20	1.15	1.74	3.10	1.61

*Value of coefficients Sielianinovs (Bac et al., 1998): <0.5 strong drought; 0.51-0.69 mild; 0.70-0.99 weak pure drought; ≥1 fault drought

Results and discussion

A study by many authors (Al-Saikhan et al., 1995; Reddivari et al., 2007a; Hamouz et al., 2013; Murniece et al., 2013) shows that the polyphenol content in potato tubers varies in a wide range from 53.0 to 1098.0 mg$\cdot$kg^{-1} of fresh weight and depends on both the genotype, agrotechnical factors and climatic conditions.

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The conducted own studies showed that the polyphenol content in potato tubers was on the level from 155.8 to 162.5 mg·kg^{-1} of fresh weight and depended significantly on the cultivar (*Table 2*). The highest amount of the discussed component was accumulated by the Satina variety – an average of 162.5 mg·kg^{-1}, significantly less in Tajfun – an average of 158.1 mg·kg^{-1}, which was confirmed in the studies of Reddivari et al. (2007b), Hamouza et al. (2010); Wierzbicka et al. (2015) and in the earlier studies by Gugała et al. (2017). In their opinion, the content of phenolic compounds in tubers depends mainly on the variety.

Table 2. *Content of polyphenols in potato tubers depending on the cultivar (mg·kg^{-1} fresh matter)*

Experimental factors	Cultivars			Mean
	Cekin	Satina	Tajfun	
1. Mechanical care – control object	152.5	160.3	153.2	155.3
2. Mechanical care + Command 480 EC (chlomazon 480 g·l)	154.7	161.2	156.4	157.5
3. Mechanical care + Command 480 EC (chlomazon 480 g·l) + Dispersion Afalon 450 SC (linuron 450 g·l)	156.8	164.2	160.2	160.3
4. Mechanical care + Stomp 400 SC (pendimethalin 400 g·l)	156.8	162.9	158.8	159.5
5. Mechanical care + Stomp 400 SC (pendimethalin 400 g·l) + Dispersion Afalon 450 SC (linuron 450 g·l)	158.3	163.8	161.9	161.3
Mean	155.9	162.5	158.1	-

$LSD_{0,05}$ - for: cultivars – 1.5; weed control methods – 2.1; interaction: cultivars x weed control methods - non-significant

The herbicides and their mixtures used in the experiment significantly influenced the concentration of polyphenols in tubers, causing them to increase (from 2.2 to 6.0 mg·kg^{-1} of fresh weight) in relation to the control object that was cultivated only mechanically (*Tables 2* and *3*). The highest concentration of the discussed component on average – 161.3 mg·kg^{-1} of fresh weight was noted on object 5, where the mixture of Stomp 400 SC (pendimethalin 400 g·l) + Dispersion Afalon 450 SC (linuron 450 g·l) herbicides was used and 3. (on average – 160.3 mg·kg^{-1} d.w.), sprayed with the mixture of Command 480 EC (chlomazon 480 g·l) + Dispersion Afalon 450 SC (linuron 450 g·l) herbicides. Also, in their earlier studies, Zarzecka and Gugała (2011) showed that under the influence of herbicides and their mixtures (Plateen 41.5, WG Plateen 41.5 WG + Fusilade Forte, Barox 400 SL, Barox 400 SL + Fusilade Forte), the discussed component increased. Similar changes were noted by Mickovski et al. (1984) using the Patoran herbicide with other preparations in tobacco cultivation. Hamouz et al. (2013) cultivating potatoes according to ecological and conventional methods obtained ambiguous results regarding the content of polyphenols. While Chauhan et al. (2013) after the application of the imidacloprid insecticide in potatoes noted a reduction in polyphenol content.

Meteorological conditions in the years of conducting the research significantly modified the accumulation of polyphenols (*Table 3*). It was found that their smallest (on average 150.5 mg·kg^{-1}) content in potato tubers was obtained in 2009 with the smallest sum of precipitation in the examined three year period and the average air temperature of 15.1 °C. While the highest content, on average – 163.5 mg·kg^{-1} of fresh weight was obtained in the growing season of 2010, which was characterized by the highest rainfall

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and the highest air temperature. The influence of climatic conditions on this feature was also observed in own studied by Reddivari et al. (2007a, b), Zarzecka and Gugała (2011) and Hamouz et al. (2013).

Table 3. *Content of polyphenols in potato tubers depending on years of research [mg·kg^{-1} fresh matter]*

Experimental factors	**Years**			**Mean**
	2008	**2009**	**2010**	
1. Mechanical care – control object	159.0	147.6	159.4	155.3
2. Mechanical care + Command 480 EC (chlomazon 480 g·l)	161.2	148.6	162.6	157.5
3. Mechanical care + Command 480 EC (chlomazon 480 g·l) + Dispersion Afalon 450 SC (linuron 450 g·l)	164.3	150.4	166.6	160.3
4. Mechanical care + Stomp 400 SC (pendimethalin 400 g·l)	162.9	152.1	163.4	159.5
5. Mechanical care + Stomp 400 SC (pendimethalin 400 g·l) + Dispersion Afalon 450 SC (linuron 450 g·l)	164.4	153.7	165.9	161.3
Mean	162.4	150.5	163.5	-

$LSD_{0,05}$ - for: years – 1.5; weed control methods – 2.1; interaction: years x weed control methods - non-significant

No interaction was found between the years and methods of using herbicides and the varieties and ways of applying herbicides on the polyphenol content in potato tubers.

Conclusions

1. The cultivars accumulated a different amount of phenolic compounds in tubers. The Satina variety has accumulated the most of this component, while Tajfun the least.
2. The herbicides and their mixtures used in the study significantly increased the content of polyphenols in potato tubers.
3. The content of polyphenols in potato tubers depended on the weather conditions during the potato growing season.

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EVOLUTION OF COMMON BEANS COLLECTED FROM LAKE VAN BASIN FOR THEIR RESISTANCE TO THE COMMON BACTERIAL BLIGHT (*XANTHOMONAS AXONOPODIS* PV. *PHASEOLI*)

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Abstract. The present study determined the reactions of common bean landraces grown in Lake Van Basin of Turkey against common bacterial blight disease (CBB) caused by *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*). For this purpose 83 bean landraces collected from the basin and two resistant (HR-45, HR-67) and one susceptible (Dresden) lines were evaluated for their reaction to *Xap*. The experiments were conducted in randomized experimental design with three replications in a growth chamber having 23 ± 2 °C temperature and 16 h light-8 h dark period. Bean seeds were sown in the pots having 2:1 mixture of peat:perlite and *Xap* inoculated by spraying when the seedlings reached two trifoliate leaves stage. Disease severity was assessed three weeks after Xph inoculation using 1-5 scale. In light of the findings, it was detected that there was a variation with regard to tolerance to the disease among common bean landraces. While fourteen landraces were assigned as resistant against CBB, 49 landraces were found to be moderately susceptible to CBB. The present study demonstrated the existence of resistance sources against CBB within Lake Van Basin bean landraces that could potentially be used for breeding resistant cultivars.
Keywords: *disease, Phaseolus vulgaris L., landraces, Xap, artificial inoculation*

Introduction

Common bean is among the most widely grown species in the world and have been grown widespread in Turkey due to economical and nutritional aspects. It is originated in South and Central America (including Mexica, Guatemala, Colombia, and Peru) and has been cultivated since 5000 BC. Common bean has a wide distribution area from sea level to 3000 m altitude (Şalk et al., 2008; Koutsika-Sotiriou and Traka-Mavrona, 2008). While China is the first fresh bean producer in world, Turkey ranks fourth with 651.094 tons meeting about 2.75% of the world production (FAO, 2016). There has been a large variability in common bean having a widespread distribution in Turkey (Erdinc et al., 2013, 2017a, b). Common bean ranks third in the world in terms of importance in *Fabaceae* (Blair et al., 2009). It reported that besides common bean supplies 30% need of protein, it helps to fight many diseases because of the antioxidant

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compounds it contains. Additionally common bean has been used cosmetic and paint industry (Singh et al., 2007).

Many abiotic and biotic stress factors have caused problems in common bean. Common bacterial blight (CBB) is one of the major seed-borne diseases which give rise to yield and quality losses and is caused by *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*). Pathogen shows its detrimental effects in tropical, subtropical and temperate regions (Vandemark et al., 2008). Seed is a very significant factor in the spread of Xph because the viability of the pathogen can be maintained 30 years on the seed (Dursun et al., 2002; Shi et al., 2011a), sometimes leads to yield losses exceeding 40% (Vandemark et al., 2008). There is no satisfactory chemical control for CBB and usage of the resistant cultivars is one of the most effective and environmentalist approaches (Opio et al., 1996; Park et al., 1999).

It is indicated that CBB is a common threat today on all continents where grown common bean. In Turkey, it was determined first time by Sönmezalp in 1966. *Xap* was also identified in Nigde, Kayseri and Yozgat provinces and in some areas of East Anatolia (Bozkurt, 2009).

The climate of the Lake Van Basin differs from the cold Eastern Turkey where it is located. The basin has a microclimate which allows for production vegetable including common bean etc. (Erdinc et al., 2008). The province of Van is located between 37°55′ and 39°24′ north longitude and 42°05′ and 44°22′ east latitude and at an altitude of 1720 m above sea level. It has a continental climate. Comparatively rich genetic diversity was found among the Lake Van Basin common bean population by phenotypic and molecular markers (Ekincialp, 2012).

Many researchers reported that the resistance to *Xap* was managed by a dominant gene (Drijfhout and Blok, 1987; Silva et al., 1989; Urrea et al., 1999; Zapata et al., 2011). Resistance to CBB in common bean has been reported as a quantitative trait with low to medium heritability (Silva et al., 1989), conditioned by 1-5 genes with additive gene action (Tar'an et al., 2001). Therefore, screening of genetic resources of the region and providing the resistant or tolerant landraces in breeding programs by determining reactions against the disease of genetic material is important in development of resistant cultivars.

Material and methods

Plant materials and pathogen

Eighty two landraces of common bean and one runner bean genotype (G30) collected from Lake Van Basin of Turkey (Ekincialp, 2012), two resistant (HR-45 and HR-67) (Yu et al., 2004; Gillard et al., 2009; Shi et al., 2011a, b, 2012) and one susceptible lines (Dresden) (Shi et al., 2011a, 2012) from Dr. Ali Reza NAVABİ was used in the present study (*Table 1*). *Xap* isolate was obtained from Prof. Dr. Hüseyin BASIM (Akdeniz University, Agriculture Faculty, Department of Plant Protection).

Pathogenicity assays

In the first artificial inoculation trial, the eighty-three bean landraces and control lines (HR-45, HR-67 and Dresden) were tested against *Xap*, then based on the results, twelve landraces with control lines were selected for the second trial. Common bean seeds was sown to the pots including 2:1 mixture of peat:perlit in a randomized

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experimental design with three replications in a chamber growth having 23 ± 2 °C temperature and 16 h light-8 h dark period.

Table 1. *Passport information of the genotype used in the study*

Accession	Location	Grow habit	Accession	Location	Grow habit
G1	Van-Merkez	Indeterminate	G46	Van-Gevaş	Indeterminate
G2	Van-Merkez	Indeterminate	G47	Van-Gevaş	Indeterminate
G3	Van-Merkez	Indeterminate	G48	Van-Gevaş	Indeterminate
G4	Van-Merkez	Indeterminate	G49	Van-Gevaş	Indeterminate
G5	Bitlis-Tatvan	Indeterminate	G-50	Van-Gevaş	Indeterminate
G6	Bitlis-Tatvan	Indeterminate	G-51	Van-Gevaş	Indeterminate
G7	Bitlis-Tatvan-Gevar	Indeterminate	G-52	Van-Gevaş	Indeterminate
G8	Bitlis-Tatvan	Indeterminate	G-53	Van-Gevaş	Indeterminate
G9	Bitlis-Hizan	Indeterminate	G-55	Van-Gevaş	Indeterminate
G10	Bitlis-Tatvan-Gevar	Indeterminate	G-57	Van-Gevaş	Indeterminate
G11	Bitlis-Hizan	Determinate	G-58	Van-Gevaş	Indeterminate
G12	Bitlis-Tatvan	Indeterminate	G-59	Van-Gevaş	Determinate
G13	Bitlis-Tatvan	Indeterminate	G-60	Van-Gevaş	Indeterminate
G14	Bitlis-Tatvan	Indeterminate	G-61	Van-Gevaş	Indeterminate
G15	Bitlis-Tatvan	Indeterminate	G-62	Van-Gevaş	Determinate
G16	Bitlis-Tatvan	Indeterminate	G-63	Bitlis-Adilcevaz	Indeterminate
G17	Bitlis-Tatvan	Indeterminate	G-64	Bitlis-Adilcevaz	Indeterminate
G18	Van-Erciş-Purmak	Indeterminate	G-65	Bitlis- Adilcevaz	Indeterminate
G19	Van-Erciş-Çelebibağı	Indeterminate	G-66	Bitlis- Adilcevaz	Indeterminate
G20	Van-Erciş	Indeterminate	G-67	Bitlis- Adilcevaz	Indeterminate
G21	Van-Erciş-Tekevler	Indeterminate	G-68	Bitlis- Adilcevaz	Indeterminate
G22	Van-Erciş-Tekevler	Indeterminate	G-70	Bitlis- Adilcevaz	Indeterminate
G23	Van-Erciş-Tekevler	Indeterminate	G-71	Bitlis- Adilcevaz	Determinate
G24	Van-Erciş-Tekevler	Indeterminate	G-72	Bitlis- Adilcevaz	Indeterminate
G25	Van-Erciş-Tekevler	Indeterminate	G-73	Bitlis-Adilcevaz	Indeterminate
G26	Van-Erciş	Indeterminate	G-74	Bitlis-Adilcevaz	Indeterminate
G27	Van-Erciş	Indeterminate	G-75	Bitlis-Adilcevaz	Indeterminate
G28	Van-Erciş	Indeterminate	G-76	Bitlis-Adilcevaz	Determinate
G29	Van-Gevaş-G.konak	Determinate	G-77	Bitlis-Adilcevaz	Determinate
G30	Van-Gevaş (*P. coccineus*)	Indeterminate	G-78	Bitlis-Adilcevaz	Determinate
G31	Van-Gevaş	Indeterminate	G-90	Van-Edremit	Indeterminate
G32	Van-Gevaş	Indeterminate	G-91	Van-Edremit	Indeterminate
G33	Van-Gevaş	Indeterminate	G-92	Van-Edremit	Indeterminate
G34	Van-Gevaş	Indeterminate	G-93	Van-Edremit	Indeterminate
G35	Van-Gevaş	Indeterminate	G-94	Van-Edremit	Indeterminate
G36	Van-Gevaş	Indeterminate	G-95	Van-Bahçesaray	Determinate
G37	Van-Gevaş	Indeterminate	G-96	Van-Bahçesaray	Indeterminate
G39	Van-Gevaş	Indeterminate	G-97	Van-Bahçesaray	Indeterminate
G40	Van-Gevaş	Indeterminate	G-98	Van-Bahçesaray	Indeterminate
G41	Van-Gevaş	Indeterminate	G-99	Van-Bahçesaray	Indeterminate
G42	Van-Gevaş	Indeterminate	HR-45	Canada	Determinate
G43	Van-Gevaş	Indeterminate	HR-67	Canada	Determinate
G44	Van-Gevaş	Indeterminate	Dresden	Canada	Determinate

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Xap isolate was inoculated to the bean seedlings with two trifoliate leaves. In this stage, Xph grown on KB medium King B (pepton 20 g/L, gliserol 10 ml/L, K_2HPO_4 1.5 g/L, $MgSO_4$./H_2O 1.5 g/L, agar 18 g/L) at 28 °C for 48 h. (King and Raney, 1954) and then the grown Xph colonies were suspended in distilled water and adjusted to 10^8 cfu ml^{-1} (OD = 0.13) (Osdaghi et al., 2009). The bacterial mixture was spread onto 15 days old common bean seedlings with fully expanded trifoliate leaves. The benches with pots were covered with polyethylene sheet for providing moisture to favor development of *CBB*. After 48 h, the covers were opened. Plants were irrigated weekly including Hoagland nutrient solution. In twenty-first days after inoculation, CBB symptoms in the plants were assessed using 1-5 scale (*Table 2*) which was modified for this study and the disease severity was determined with Townsend–Heuberger equation (Townsend and Heuberger, 1943).

The disease severity was calculated using the following formula:

$$Disease\ severity = \frac{\Sigma\ (Rating\ number\ x\ Number\ of\ leaves\ in\ the\ rating)}{Total\ number\ of\ leaves\ x\ Highest\ rating}\ x100$$

Table 2. *The values of 1-5 scale that was used to determine the disease severity of Xap in the bean landraces*

Scale value	Statement
1	No visible symptom
2	Necrosis in 1-5% of leaf or single spots
3	Necrosis in 6-25% of leaf
4	Necrosis in 26-50% of leaf
5	Necrosis in leaf more than 50% or plant death

As a result of calculating of the scale values, the landraces were assigned as <2: resistant, <3: moderately susceptible, <4: susceptible, <5: extreme susceptible (Dursun et al., 2002; Osdaghi et al., 2009). In the both artificial inoculation trials, the data were analyzed using the statistical software package SPSS. The means were grouped using the Duncan multiple comparison test (Düzgüneş et al., 1987)

Results and discussion

First artificial inoculation trial

The results of the first artificial inoculation are given in *Table 3*. In the result of the analysis of variance, it was found that the differences between the landraces were significant ($p<0.05$). According to the results obtained, 14 of the landraces were scaled to 1-2 and rated as resistant to CBB and the genotype G99 was the most resistant genotype with a scale value of 1.42. The great majority of landraces (49 landraces) was ranged from 2 to 3 and was found to be moderately susceptible to CBB. It was also determined that 19 landraces were susceptible and their disease severity scales were varied from 3 to 4. The genotype G37 was the most susceptible one with a scale value of 4.32. Two resistant (HR-45 and HR-67) control lines were found to have lower scale values than the studied bean landraces and the susceptible line, Dresden, was found to

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be in the group of sensitive landraces with a score of 3.29 (*Table 3*). When frequency distribution according to disease scale values were examined, 18.60% of the landraces including control lines were found to be resistant, 63.96% of them were moderately susceptible, 16.28% of them were susceptible and 1.17% of them were very susceptible (*Fig. 1*).

Table 3. *The disease severity values of first artificial inoculation in the bean landraces calculated according to Townsend–Heuberger equation*

Accession #	Scale value	Disease response	Accession #	Scale value	Disease response	Accession #	Scale value	Disease response
G1	1.86 C-J*	R	G30	1.73 D-J	R	G63	1.84 C-J	R
G2	2.18 B-J	MS	G31	2.69 A-J	MS	G64	3.07 A-I	S
G3	1.75 D-J	R	G32	1.00 J	R	G65	2.47 A-J	MS
G4	2.07 B-J	MS	G33	2.17 B-J	MS	G66	3.25 A-H	S
G5	1.65 F-J	R	G34	1.75 D-J	R	G67	2.19 B-J	MS
G6	2.79 A-J	MS	G35	1.82 C-J	R	G68	2.58 A-J	MS
G7	2.36 B-J	MS	G36	3.41 A-F	S	G70	2.55 A-J	MS
G8	1.72 D-J	R	G37	4.32 A	HS	G71	2.27 B-J	MS
G9	2.16 B-J	MS	G39	2.42 B-J	MS	G72	3.02 A-I	S
G10	2.12 B-J	MS	G40	3.49 A-F	S	G73	3.01 A-I	S
G11	2.07 B-J	MS	G41	2.84 A-J	MS	G74	2.38 B-J	MS
G12	1.69 E-J	R	G42	3.10 A-I	S	G75	3.90 AB	S
G13	2.41 B-J	MS	G43	2.35 B-J	MS	G76	2.38 B-J	MS
G14	2.28 B-J	MS	G44	2.80 A-J	MS	G77	3.19 A-I	S
G15	2.26 B-J	MS	G46	2.81 A-J	MS	G78	3.01 A-I	S
G16	2.22 B-J	MS	G47	3.01 A-I	S	G90	2.29 B-J	MS
G17	2.45 B-J	MS	G48	2.44 B-J	MS	G91	3.49 A-F	S
G18	2.45 B-J	MS	G49	2.73 A-J	MS	G92	2.48 A-J	MS
G19	2.62 A-J	MS	G-50	2.56 A-J	MS	G93	2.40 B-J	MS
G20	1.95 C-J	R	G-51	2.35 B-J	MS	G94	3.53 A-E	S
G21	2.72 A-J	MS	G-52	2.91 A-I	MS	G95	2.47 A-J	MS
G22	2.47 A-J	MS	G-53	3.89 AB	S	G96	3.67 A-C	S
G23	3.01 A-I	S	G-55	3.10 A-I	S	G97	2.09 B-J	MS
G24	3.59 A-D	S	G-57	2.87 A-J	MS	G98	2.25 B-J	MS
G25	1.97 C-J	R	G-58	3.02 A-I	S	G99	1.42 G-J	R
G26	2.26 B-J	MS	G-59	2.49 A-J	MS	HR-45	1.40 H-J	R
G27	1.93 C-J	R	G-60	2.29 B-J	MS	HR-67	1.34 IJ	R
G28	2.23 B-J	MS	G-61	2.09 B-J	MS	Dresden	3.29 A-G	S
G29	2.94 A-I	MS	G-62	2.05 B-J	MS			

*There were significant differences among the different letter(s) at $P < 0.05$ level (according to Duncan's multiple comparison test). R: Resistant, MS: Moderately susceptible, S: Susceptible, HS: High susceptible

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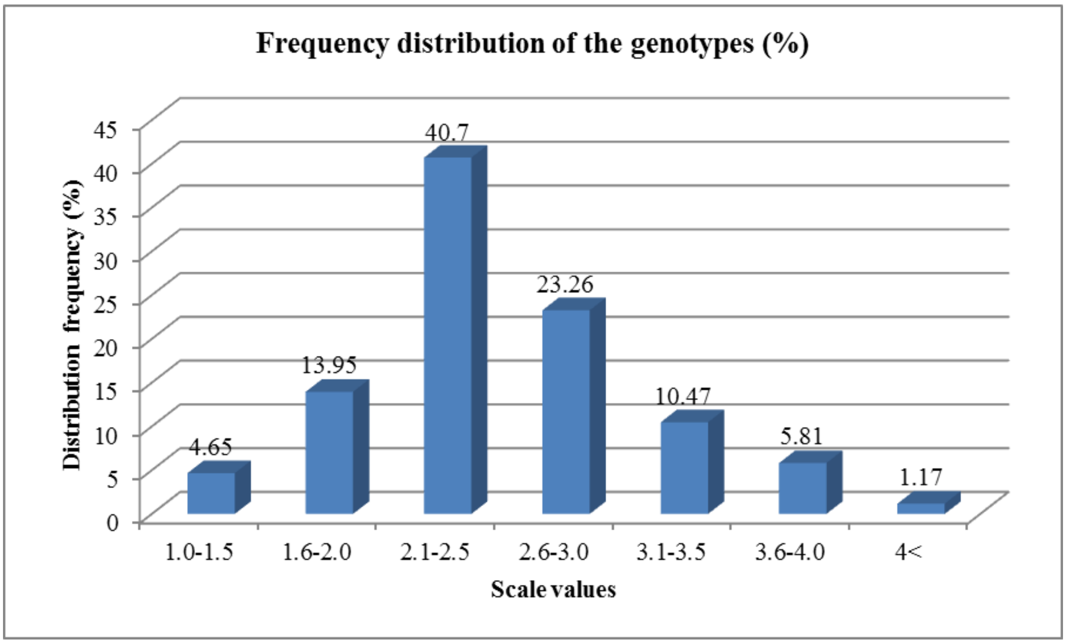

Figure 1. *Distribution frequency (%) of reaction to CBB of the bean landraces according to scale values*

Second artificial inoculation trial

Based on the first artificial inoculation trial, twelve landraces resistant to CBB with sufficient number of seeds (the landraces G12 and G32 were excluded) were artificially re-inoculated. Resistant (HR-45 and HR-67) and sensitive (Dresden) lines were also used for control in the second trial. When the disease severity values obtained at the end of the trial were examined, it was determined that the landraces had a scale value of 1-2 and showed resistance. It was determined that the lowest value of the landraces was 1.27 (in the genotype G27), while the highest value was found in the landraces G35 and G63 with 1.83. It was also found that the HR-45 and HR-67 lines reached lower scale values than the other landraces (scale values of 1.11 and 1.00, respectively). As a result of the analysis of variance, it was found that the differences between the landraces were statistically significant ($p<0.05$). Disease severity reactions of the resistant landraces have generally been observed to be similar to each other in both studies (*Fig. 2*).

Xap, among the most important seed-borne bacterial pathogens in bean (Bastas and Sahin, 2017), is also among the serious bacterial diseases causing yield and quality loses in Turkey (Demir and Gündogdu, 1994; Donmez, 2004). In surveys conducted in the Central Anatolia Region, Xap was reported to be among the most common bacterial pathogens with 11.11% of presence (Bastas and Sahin, 2017).

In current study, the severity of disease in artificial inoculation was evaluated after 21 days in both studies. As a matter of fact, Marquez et al. (2007) reported that even if an aggressive isolate is used, classifications that would have been made by previous evaluations on 14 days might be erroneous.

It has been determined that there is a variation in the reactions of the bean landraces to BCC. As a matter of fact, according to Ekincialp (2012), there is a high genetic variation in the morphological and molecular characterization studies carried out among these landraces. The landraces G30, G37, and G53 which are morphologically and

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genetically most distant from each other, have also been found to differ in their CBB disease reactions, while G30 is resistant and G53 is susceptible and G37 is highly susceptible. It has also been reported that the G30 genotype (*P. coccineus*) is differentiated from other landraces in terms of general habitus and morphological characteristics (number of flower buds in the cluster, seed width, seed length, seed height and seed weight) (Ekincialp, 2012).

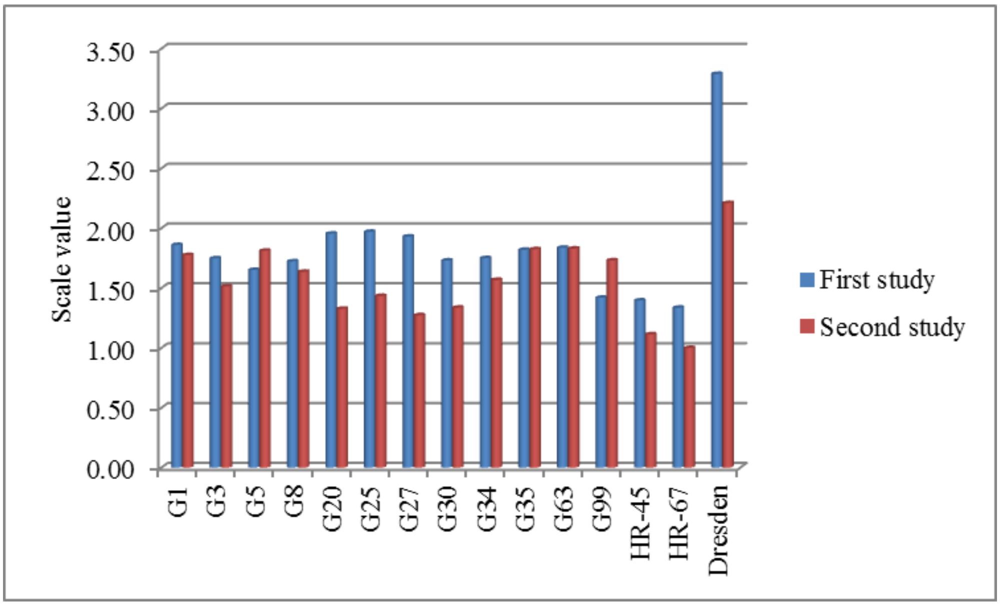

***Figure 2.** Reactions of the bean landraces were classified as resistant based on 1-5 scale in artificial inoculation method for CBB in first and second trials*

It was noticed that the landraces G12 and G30 that were resistant to bean anthracnose (Ekincialp and Sensoy, 2018) were also resistant to CBB in the present study. Moreover, all landraces susceptible to CBB have been found to be susceptible to anthracnose disease. It was observed that the G30 genotype belonging to *P. coccineus* was present in a group of resistant landraces in both studies. It is generally stated that *P. coccineus* (runner bean) has similar or higher resistance level as *P. vulgaris*, P. *acutifolius* (tepary bean) shows the highest level of resistance (Drijfhout and Blok, 1987; Singh and Munoz, 1999).

The results of the landraces used for control purposes during pathogenicity tests are shown in *Table 3* and *4*. As seen in the tables, the reactions of these landraces to the disease were determined as resistant (R) in HR-45 and HR-67 sensitive (S) in Dresden. It was also reported in the literature that HR-45 and HR-67 were resistant to *Xap* (Yu et al., 2004; Gillard et al., 2009; Shi et al., 2011 a, b, 2012) and Dresden was susceptible to XAP Shi et al., 2011a, 2012).

In the present study, a large number of bean landraces were screened and approximately 17% of them was found to be resistant to *Xap* (*Table 3*; *Fig. 3*). Halo blight and common blight screening studies were conducted in beans in different regions of Turkey; it was demonstrated that some landraces were resistance to halo blight (Benlioglu et al., 1994; Dursun et al., 2002) and only one variety was resistant to common blight (Dursun et al., 2002). It was determined that 50% of the landraces used in the mentioned studies were highly susceptible. Osdaghi et al. (2009) determined that

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two of the 30 cultivars/lines were resistant to CBB. On the contrary, Fininsa and Tefera (2006) reported that 117 out of 201 landraces (approximately 58%) in Ethiopia were resistant to CBB.

Table 4. *Disease severity of second artificial inoculation in the bean landraces*

Accessions #	Scale value
G1	1.78 B*
G3	1.51 B-E
G5	1.81 AB
G8	1.64 B-D
G20	1.33 C-F
G25	1.44 B-E
G27	1.27 D-F
G30	1.34 C-F
G34	1.57 B-D
G35	1.83 AB
G63	1.83 AB
G99	1.73 BC
HR-45	1.11 EF
HR-67	1.00 F
Dresden	2.21 A
p value	0.001

*There were significant differences among the different letter(s) at P < 0.05 level (according to Duncan's multiple comparison test)

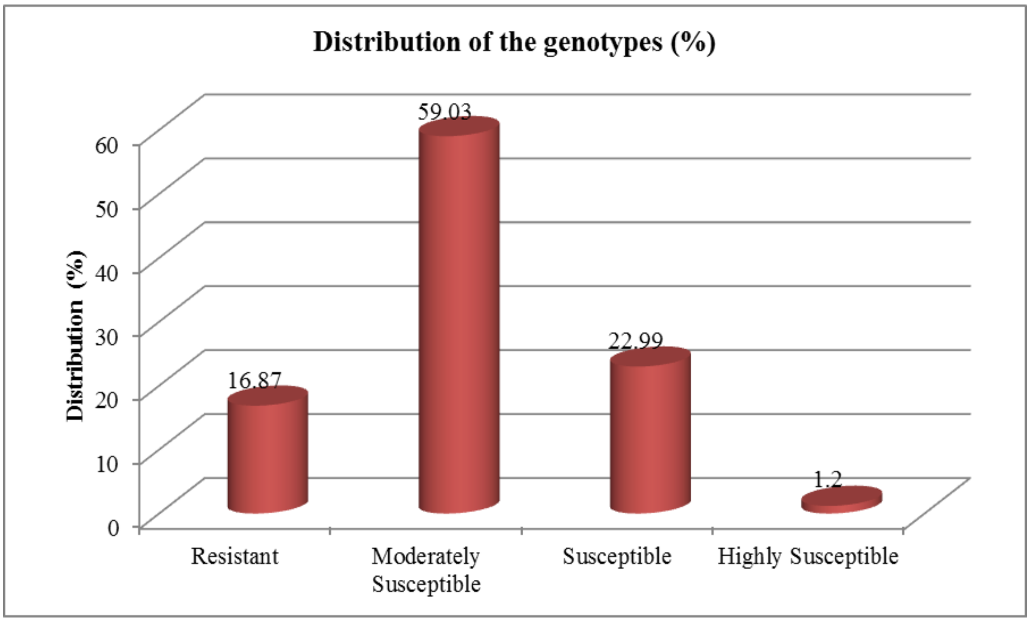

Figure 3. *Distribution of the landraces excluding control lines according to disease response*

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Conclusions

Turkey is an important country in the production of green beans and meets 3% of the world production. Maintaining or advancing the current position will be through the development of highly productive, tolerant varieties that can meet consumer and producer demands. In the present study, the resistance status of bean landraces collected from Lake Van Basin, which is an important potential for vegetable production in the region, is revealed. From this point of view, it is noticed that there is a source of resistance. Resistant landraces are expected to be used as parents in bean improvement programs in the future. Considering the efforts of countries in recent years to protect their local gene resources, this situation is better understood. This is the first study and report of resistance against CBB in Lake Van Basin bean landraces. The present study demonstrated the existence of resistance sources against CBB within them that could potentially be used for breeding resistant cultivars.

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SPECIES DIVERSITY AND VEGETATION STRUCTURE FROM DIFFERENT CLIMATIC ZONES OF TEHSIL HARIGHEL, BAGH, AZAD KASMIR, PAKISTAN ANALYSED THROUGH MULTIVARIATE TECHNIQUES

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Abstract. The phytosociological survey of western Himalayan subtropical and moist temperate forests of Tehsil Harighel, District Bagh Azad Jammu and Kashmir, Pakistan was carried out during 2015-2017. Sampling was done by using random stratified sampling technique at 12 different sites. Based on importance value twelve plant communities were recognized. They were merged into three plant associations using cluster analysis and Detrended correspondence analysis viz. *Olea -Dodonea – Micromeria* association, *Pinus- Oxalis-Dactylis* association and *Pinus-Diospyros -Myrsine* association. The average number of species per site varied between 17-48, Species richness 3.00 to7.01; Shannon Diversity 2.49 to 3.82; Evenness 0.46 to 0.72 and Equitability 0.73 to 0.91. Canonical Correspondence Analysis (CCA) revealed that altitude and edaphic characteristics like potassium, organic matter, saturation, electrical conductivity and pH play a significant role in controlling the distribution pattern of plant species. Low value of species diversity and associated components clearly reflect that forest structure is deteriorated in the investigated area with poor regeneration potential. Therefore, immediate conservation measures by integrating local populations' perceptions are urgently recommended.
Keywords: *environmental gradient, cluster analysis, richness, DCA, CCA*

Introduction

Vegetation structure, species composition, diversity and richness pattern, maturity value are the important ecological characteristics that are highly correlated with anthropogenic and environmental variables (Gairola et al., 2008; Shaheen et al., 2011a; Ahmad et al., 2013; Amjad et al., 2014a, b; Ilyas et al., 2015). Vegetation is the distinct physiognomic unit whose structure can be clearly differentiated from other such unit (Hussain and Illahi, 1991). Vegetation, soil and climate are interrelated with each other. The variation in any one of these components may bring change in the associated component (Kent, 2012; Amjad et al., 2014b; Ilyas et al., 2015). Geographical or topographical factors like altitude and aspect play critical role in structuring vegetation (Ellenberg, 1996; Gallardo-Cruz et al., 2009; Scherrer and Korner, 2010; Shaheen et al., 2011b). Vegetation is the chief component of environment in majority of habitat which

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facilitates the ecosystem services and biodiversity (Gardner et al., 2009). Therefore assessment of vegetation structure is the main indicator in conservation and ecosystem management.

Species diversity is a function of the number of species present in a given area and reflects the productivity and health of ecosystem (Ruiz et al., 2008). The most easily interpretable indicator of diversity is species richness which is controlled by a complex of environmental variables (Whittaker, 1977; Shrestha and Jha, 2009) mainly altitude, aspect and moisture (Vetaas, 2000; Schuster and Diekmann, 2005) The precise diversity measurement is helpful in understanding the various phenomena involved in organization and development of plant communities (Shoukat et al., 1978; Malik and Malik, 2012). The anthropogenic pressure in the form of deforestation, overgrazing and fuel wood extraction can be clearly reflected from low species diversity and richness in the area. Thus vegetation structure information along with species biodiversity assessment is a prerequisite for biodiversity conservation and ecosystem management (Willoughby and Alexander, 2000, 2005).

The computer-based multivariate statistical techniques helps ecologists in structuring large data sets and analyzing impact of environmental factors on distribution pattern of plant species (Bergmeier, 2002; Anderson et al., 2006). Such techniques reduce the complexity of data by classifying vegetation and relating the results to abiotc (environmental) components (Dufrene and Legendre, 1997; McCune and Mefford, 1999; Ter Braak and Prentice, 1988). Various multivariate techniques such as Two way Indicator species analysis, Cluster analysis, Canonical correspondence analysis and Detrended correspondence analysis have been extensively employed by ecologists recognize the environmental gradient among vegetation structure and species diversity in mountainous ecosystems (Daubenmire, 1968; Vetaas and Grytnes, 2002; Tavili and Jafari, 2009).

Azad Jammu & Kashmir, Pakistan harboured diverse climate, soil type and rich diversity. Various studies have been conducted previously to analyze vegetation in different part of Azad Jammu & Kashmir (Siddiqui et al., 2010; Shaheen et al., 2011a; Malik and Malik, 2012; Amjad et al., 2014a,b; Bokhari et al., 2016; Amjad et al., 2017). However many potentially diverse remote areas like Harigal were not still explored by the ecologist particularly using advanced phytosociological techniques. Moreover previous studies are restricted to floristic inventory and quantitative attributes and lack novelty in term of multivariate analysis. Therefore present study was designed to analyze the variation among species diversity and vegetation structure along the environmental gradient using multivariate approach. The findings of current research work will be helpful for designing effective conservation strategies for sustainable management of plant resources of area particular for minimizing anthropogenic pressure.

Materials and methods

Study area

Tehsil Harighel of District Bagh, Azad Jammu & Kashmir, Pakistan is located in western Himalayan foot hills of Pir-Panjal range between latitude 33°54'-34°08' N and longitude 73°01'-73°38' E longitude. The altitude of area varies between 980 m to 2052 m. Total area of District Bagh is 1368 km^2 with 54.78% under forest cover. Population is 0.434 million (Anonymous 2007). The climate of area varies from

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subtropical humid to temperate type at various elevations with mean annual precipitation of 1500 mm. The average annual temperature is 21 °C with maximum up to 40 °C during July and minimum up to 2 °C during January (Anonymous, 2009; Shaheen et al., 2011a).

Collection and identification of plant specimen

Three plant specimens of each species were collected. The specimens were carefully dried and mounted on herbarium sheet. Different floras and monograph of various areas of Pakistan were used for Identification of specimens. The identified specimens were confirmed at AJ&K Medicinal and Aromatic Plant Herbarium PARC, Pakistan. International Plant Name Index (IPNI) was used to obtain correct botanical name. The identified specimens were deposited in Herbarium of Women University of Azad Jammu & Kashmir Bagh.

Field sampling

Field surveys were carried out during 2014-2016 following specific locality procedure. Twelve sites were selected based on altitude, aspect and physiognomy (*Fig. 1*). The altitude and geographical coordinates of each site was determined by using GPS and aspect by using Suunto Tandem survey master compass. Detailed stratified sampling was done using quadrat method. The quadrat size was 1 ×1 m^2, 5 ×5 $m^2$and 10 × 10 $m^2$for herbs, shrubs and trees respectively. Composition and abundance data of plant species were recorded from each quadrat on prepared Excel data sheets.

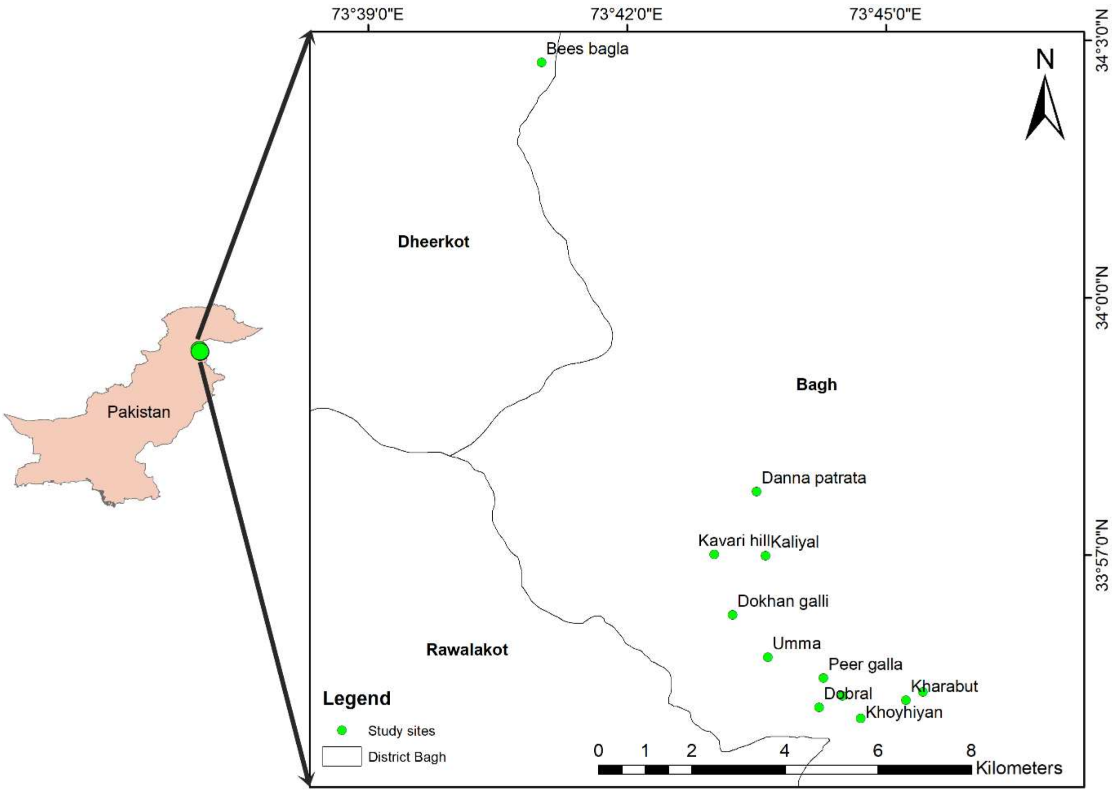

Figure 1. *Map showing location of different study sites*

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Three soil samples were collected from each site at depth of 15 cm and mixed to make composite. Different physico-chemical properties of soil like texture, pH, organic matter, electrical conductivity, saturation, % of phosphorous, potassium and calcium carbonate were determined in Pakistan Soil and Water Testing Laboratory Rawalpindi using standard methods (Jackson, 1962; Hussain, 1989).

Data storage and analysis

The importance value of each plant species was recorded following Curtis and McIntosh (1950) by using *Equation 1:*

$$IVI = R.D + R.F + R.C. \tag{Eq.1}$$

where IVI = importance value index, R.D = relative density, R.F = relative frequency, and R.C. = relative cover.

Species diversity was calculated by following Shannon (1949) by using *Equation 2:*

$$H = \sum\left(\frac{n}{N} \times \frac{\ln (n)}{\ln (N)}\right) \tag{Eq.2}$$

where H = species diversity, n = number of individual of its species, N = total number of individual of all species.

Species richness was calculated by following Margalef (1958) using *Equation 3:*

$$R = \frac{S-1}{\ln (N)} \tag{Eq.3}$$

where R = species richness, N = total number of individual of all species.

Equitability was calculated by following Sheldon (1969) using *Equation 4:*

$$E = \frac{H}{\ln (S)} \tag{Eq.4}$$

where H = Diversity, S = total number of species.

Evenness was calculated by following Gibson’s evenness index using *Equation 5:*

$$G = \frac{eH}{S} \tag{Eq.5}$$

where, H = Diversity, S = total number of species.

Species maturity was calculated by following Pichi-Sermollis (1948) using *Equation 6:*

$$M = \frac{F}{S} \tag{Eq.6}$$

where, M= Species maturity, F = total frequency, S = total species number.

Species composition and abundance data of 158 species and 7 environmental variables from 390 quadrats at twelve different sampling sites (390 quadrats) were processed in MS Excel in accordance with the PCORD V.5 and CANACOO V.5 requirements. The grouping of plant species was carried out by using cluster analysis in PC ORD software (McCune and Grace, 2002; McCune and Mefford, 2005). Wards

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method was used as linkage method to group the same stands. These associations were documented at three level of division on the bases of dendrogram. The vegetation and environmental data was further subjected to ordination analysis using CANACOO version 5.00 (TerBraak and Smilauer, 2002). DCA and CCA analysis was carried out to verify the faithfulness of grouping/association, check the ecological gradient of plant association and to find the vegetation environmental relation. The explanatory power (strength) of different environmental factors was checked by Monte Carlo Permutation test (reduced model, 4999 permutations).

Results

Based on cluster analysis and DCA, 158 plant species and 12 sites were grouped into three associations (*Fig. 2*) which are as follows:

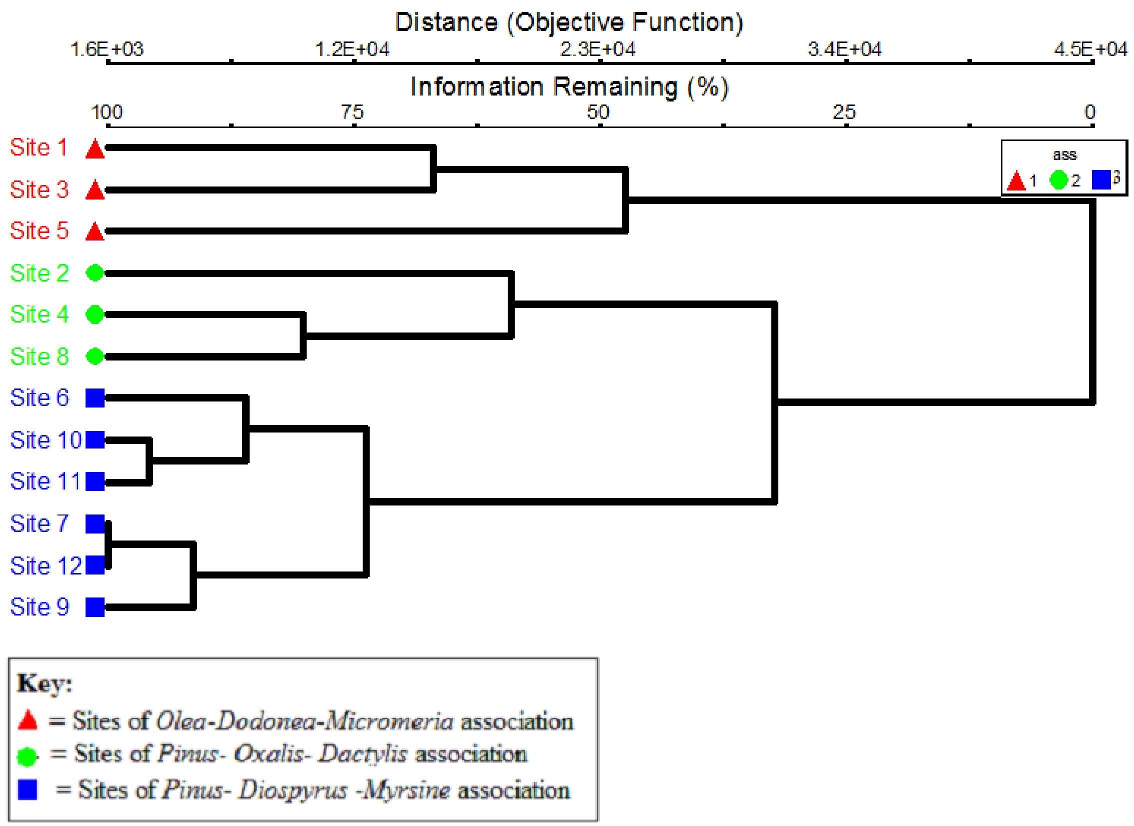

Figure 2. *Cluster analysis dendrogram representing three plant associations*

Olea-Dodonea-Micromeria (A) association

This association harboured at an altitudinal range of 980-1165 m. The association comprised of three communities having 51 species which include 3 trees, 15 shrubs and 33 herbs. The dominant species were *Olea ferruginea* (I.V = 60.02), *Dodoneae viscosa* (IV = 27.21) and *Micromeria biflora* (I9.92). Whereas *Berberis lycium*, *Maytenus nemorosa* and *Ficus palmata* were associated plant species (*Table 1*). Soil was loamy with average pH of 7.36. Soil saturation was 40%; electrical conductivity 0.83 µs/cm; organic matter 0.73%; phosphorus 3.66 mgKg^{-1} and potassium 106.66 mgKg^{-1}.

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Pinus-Oxalis-Dactylis (B) association

This association lies in between 1079 and 1658 m and consisted of 3 plant communities having 79 Plant species which include 8 trees, 15 shrubs and 56 herbs. *Pinus roxburghii* (I. V = 33.65), *Oxalis spiralis* (I. V = 12.64) and *Dactylis glomerata* (I. V = 11.81) were dominant. Whereas *Myrsine Africana* and *Indigofera linifolia* were associated plant species (*Table 1*). Soil texture was loamy with average pH 7.17. Soil saturation 39.66%; electrical conductivity 0.70 µs/cm; organic matter 0.76%; phosphorus 4.4 mg kg^{-1} and potassium 120 mg kg^{-1}.

Pinus-Diospyrus-Myrsine (C) association

This association was recorded at an altitude of 1450-20520 m having 6 Plant communities having 110 species. *Pinus wallichiana* (I. V = 40.13), *Diospyrus lotus* (I. V = 11.84) and *Myrsine Africana* (I. V = 9.78) were dominant. *Quercus dilitata*, *Berberis lycium*, *Sarcococca saligna* and *Vibernum grandiflorum* were the associated plant species (*Table 1*). Soil was loamy with average pH of 7.23. Soil saturation was 40.05%; electrical conductivity 0.64 µs/cm; organic matter 0.81; phosphorus 5.33 $mgKg^{-1}$ and potassium 116.66 mg kg^{-1}.

Table 1. *Mean relative importance value of species in three associations recorded by normal cluster analysis during monsoon 2016 from Tehsil Harighel*

Species name	Abbrev.	Association A	Association B	Association C
Olea ferruginea Royle.	Ole fer	60.02	9.01	3.55
Pinus roxburghii Sarg.	Pin rox	9.71	33.65	2.32
Acacia nilotica (L.) Wild. ex Delile	Aca nil	2.47	0	1.47
Pyrus pashia Buch.-Ham. ex D. Don	Pyr pas	0	6.21	4.97
Diospyros lotus L.	Dio lot	0	5.16	11.84
Pinus wallichiana L.	Pin wal	0	8.22	40.13
*Ailanthus altissima (*Mill.) Swingle	Ail alt	0	4.09	1.04
Quercus dilatata Lindl.	Que dil	0	0	9.52
Dalbergia sissoo Roxb. ex DC	Dal sis	0	0	0.39
Broussonetia papyrifera (L.) L Her. Ex Vent.	Bro pap	0	0	0.88
Juglans regia L.	Jug reg	0	0	1.56
Aesculus indica (Wall. ex Cambess.) Hook.	Aes ind	0	0	1.00
Mimosa pudica L.	Mim pud	0	0	0.64
Quercus incana Roxb.	Que ina	0	1.90	4.63
Salix nigra Marshall	Sal nig	0	2.40	0
Bauhinia variegate L.	Bau var	0	0	1.08
Dodonaea viscosa (L.) Jacq.	Dod vis	27.21	0	0
Maytenus nemorosa Marais	May nem	10.83	0	0
Myrsine africana L.	Myr afr	8.55	11.09	9.78
Berberis lycium Royle.	Ber lyc	11.80	5.98	9.23
Cotoneaster racemiflorae Pojark.	Cot rac	4.32	2.68	1.65
Zanthozylum alatum Roxb.	Zan ala	5.12	4.07	2.43

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Nerium oleander L.	Ner ole	6.46	0	0.77
Rubus fruticosus L.	Rub fru	0	0	0.72
*Indigofera linifolia (*L. f.) Retz	Ind lin	0	10.12	3.76
Viburnum grandiflorum Wall. ex DC.	Vib gra	0	0	8.70
Rosa brunonii Lindl.	Ros bru	1.30	5.42	4.64
Ricinus communis L.	Ric com	0	2.03	0
Wikstroemia canescens Wall. ex Meisn.	Wik can	1.61	6.53	5.90
Debregearsia salcifolia	Deb sal	0	6.38	0.75
Sarcococca saligna (D. Don) Müll. Arg.	Sar sal	0	3.20	8.18
Ziziphus mauritiana Lam*s*.	Ziz mau	2.69	0	0
Adhatoda zeylanica Medik.	Adh zey	0	0	0.81
Desmodium elegans Schltdl.	Des ele	0	0	0.49
Petrorhagia saxifraga (L.) Link	Pet sax	0	0	0.40
Rubus niveus Wall. ex G. Don	Rub niv	0	0	1.14
Jasminum grandiflorum L.	Jas gra	0	0	1.28
Ficus palmata Forssk.	Fic pal	10.24	4.59	3.74
Punica granatum L	Pun gra	4.98	2.49	2.44
Rubus ellipticus Sm.	Rub ell	0	8.70	1.28
Elaeagnus umbellate	Ela umb	0	0	2.14
Oteostagia limbata (Benth.) Boiss.	Ote lim	3.83	0	0
Rumex hastatus D. Don	Rum has	1.10	2.61	0.97
Rhus catinus Scop.	Rhu cat	1.66	2.03	1.07
Machilus odoratissimus Nees	Mac odo	0	2.52	2.41
Jasminum humile L.	Jas hum	0	0	0.49
Asplenium adiantum-nigrum L.	Asp adi	0	2.31	0.89
Lespedeza juncea (L. f.) Pers.	Les jun	4.36	3.05	0
Senecio amplectens A. Gray	Sen amp	0	2.33	0.47
Adiantum caudatum L.	Adi cau	0	1.08	2.90
Clinopodium vulgare L.	Cli vul	0	0.90	0
Scutellaria ovata Hill	Scu ova	5.83	0.96	0
Eulaliopsis binata Retz.	Eul bin	5.48	1.18	0
Adiantum incisum Forssk.	Adi inc	0	0.30	0
Oenothera rosea L Her. ex Aiton	Oen ros	0	0.47	6.70
Thalictrum Spp.	Tal spp	0	1.06	0.73
Androsace rotundifolia Hardw.	And rot	1.21	0.51	0.78
*Micromeria biflora (*Buch.-Ham. ex D. Don) Benth.	Mic bif	19.92	7.48	7.11
Poa pratensis L	Poa pra	0	0	1.26
Ajuga parviflora Benth.	Aju par	1.38	0	1.64
Setaria pumila (Poir.) Roem. & Schult.	Set pum	5.47	0	0
Lepidium ruderale L**.**	Lep rud	0	5.57	0
Oxalis spiralis Ruiz & Pav. ex G. Don	Oxa spi	0	12.64	1.77
Fragaria visca L.	Fra vis	0	0.62	4.60
Sonchus arvensis L.	Son arv	0	0.94	1.29

Clinopodium umbrosum (M. Bieb.) K. Koch	Cli umb	0	2.05	0
Oxalis corniculata L.	Oxa cor	0	7.43	0
Pteris cretica L.	Pte cre	0	4.83	2.80
Origanum vulgare L.	Ori vul	0	0	0.23
Dryopteris filix-mas (L.) Schott	Dry fil	7.03	2.78	0
Dactylis glomerata L.	Dac glo	0	11.81	0.54
Bidens biternata (Lour.) Merr. & Sherff	Bid bit	4.16	0	0
Themeda anathera (Nees ex Steud.) Hack	The ana	6.78	8.47	0
*Conyza canadensis (*L.) Cronquist	Con can	1.49	0	1.07
Ipomoea purpurea (L.) Lam.	Ipo pur	4.21	0	0.73
Taraxacum officinale L.	Tar ofi	2.73	0.28	2.40
Veronica laxa (Benth.)	Ver lax	2.89	4.85	0.87
Carpesium abrotanoides L.	Car abr	2.25	0	0
Crotalaria juncea L.	Cro jun	4.95	0	0
Agrostis stolonifera L.	Agr sto	3.40	0	0.58
*Gerbera gossypina (*Royle) Beauverd	Ger gos	0	4.78	1.81
Verbascum thapsun L.	Ver tha	0.07	0.33	0
Geranium ocellatum Cambess	Ger oce	0	0.62	0
Cynoglosum lanceolatum Forssk	Cyn lan	0	0.04	0.45
Galium aparine L.	Gal apa	0	2.25	6.19
Vicia sativa Guss.	Vic sat	0	0.74	1.69
*Cynodon dactylon (*L.) Pers.	Cyn dac	0	2.85	2.94
Pteris umbrosa R. Br.	Pte umb	0.73	0	0
Cichorium intybus L.	Cic int	0	0.97	0
Prunella vulgaris L.	Pru vul	0	0.99	1.95
Viola canescens Wall.	Vio can	0	1.44	0
Euphorbia helioscopia L.	Eup hel	0	6.98	0
Arundo donax L.	Aru don	0	0.87	0
Onopordum acanthium L.	Ono aca	0	1.65	0
Mentha arvensis L.	Men arv	0	2.54	1.23
Mentha longifolia L.	Men lon	0	1.49	1.69
Epipactis helleborine (L.) Crantz	Epi hel	0	3.57	0
Cyprus niveus	Cyp niv	0	1.53	1.46
*Silybum marianum (*L.) Gaertn.	Sil mar	0.40	0	0.43
Euphorbia hirta	Eup hir	0.07	0	1.77
Aegopodium podagraria L.	Aeg pod	12.08	0	0
Valerianella szovitsiana Fisch. & C.A. Mey.	Val szo	3.38	0	0
Linum spp.	Lin spp	0	0	1.69
Trichodesma indicum (L.) Lehm	Tri ind	6.45	0	0
Astragalus leucocephalus Graham ex Benth.	Ast leu	1.64	0	0
Salvia lanata Roxb.	Sal lan	2.21	0	0
Trifolium repens L.	Tri rep	0	0	5.16
Polygala abyssinica R. Br. ex Fresen	Pol aby	3.88	0	0

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Spirace canescans	Spi can	2.56	0	0
Plantago major L.	Pla maj	0	0	4.77
Lactuca floridana (*L.*) Gaertn.	Lac flo	0	0	0.53
Adiantum capillus-veneris L.	Adi cap	0	0	1.98
Ranunculus arvensis L	Ran arv	0	2.11	2.13
Ranunculus sceleratus L.	Ran sce	0	0	4.65
Hedera nepalensis K. Koch	Hed nep	3.00	0	2.98
Pennisetum orientale Rich.	Pen ori	12.48	1.17	1.01
Cannabis sativa L.	Can sat	0	4.32	0
Plantago lanceolata L.	Pla lan	0	3.44	2.72
Plectranthus spp	Ple spp	0	1.58	0
Solanum nigrum L.	Sol nig	0	2.43	0
Lathyrus aphaca L.	Lat aph	0	1.25	0
Juncus articulates	Jun art	0	0	1.03
*Cirsium vulgare (*Savi) Ten	Cir vul	0	0	0.41
Dicliptera bupleuroides Nees	Dic bup	0	0	1.46
Phlomis bracteosa Royle ex Benth.	Phl bra	0	0	0.63
Polygonatum geminiflorum Decne.	Pol gem	0	0	1.77
Dryopterious odantoloma	Dry oda	0	0	0.95
Thymus linearis Benth.	Thy lin	0	0	1.79
Achillea millefolium L.	Ach mil	0	0	0.94
Vicia monantha Retz.	Vic mon	0	0	0.76
Artemisia vulgaris L.	Art vul	0	0	2.09
Astrogates condolleances	Ast con	0	0	0.56
Adiantum tenerum Sw.	Adi ten	0	0	0.93
Anaphalis triplinervis (Sims) C.B. Clarke	Ana tri	0	0	0.33
Onopordum acanthum	Ono aca	0	1.65	0
Urtica dioca L	Urt dio	0	0	0.85
Scutellaria chamaedrifolia Hedge & Paton	Scu cha	0	0	0.10
Serratula praealta L.	Ser pra	0	0	0.59
Clematis grata Wall.	Cle gra	0	0	0.64
Silene conoidea L.	Sil con	0	0	0.36
Convolvulus arvensis L.	Con arv	0	0	0.67
Anagalis arvensis L.	Ana arv	1.17	0	0.47
Barleria cristata L.	Bar cri	3.24	0	0
*Stellaria media (*L.) Cirillo	Ste med	0	0.83	0
Rumex dantatus L	Rum dan	2.29	0	0.23
Fragaria nubicola	Fra nub	0	0	4.61
Duchesnia indica (Andr.) Focke	Dus ind	0	2.86	0
Adiantum venustum D. Don	Adi ven	0	0	0.76
Hedera helix K.Koch	Hed hel	0	2.56	1.14
Brachiaria reptans (L.) C.A. Gardner & C.E. Hubb.	Bra rep	0	0	0.09
Pteris cheltron Andr.	Pte che	0	0	1.15

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Vincetoxicum hirundinaria Medik.	Vin hir	0	0	0.69
Galium asprellum Michx.	Gal asp	0	4.39	2.12
Galium asperuloides Edgew. S	Gal aspe	0	0	1.56
Galium elegan Blocki	Gal ele	0	0.79	0
*Clematis barbellate*Edgew.	Cle bar	0	0.90	0
Medicago polymorpha L.	Med pol	0	0	0.72

A = Olea-Dodonea-Micromeria association; B = Pinus-Oxalis-Dactylis association; C = Pinus-Diospyrus-Myrsine association

Detrended correspondence analysis

Detrended Correspondence analysis is the indirect gradient analysis which applied here to highlight the ecological gradients controlling the spatial variations among the plant species. Sum of all variance or eigen values was 3.93. The first Eigen value was proven to be quite high (0.57) which reflect strong gradient strength in species distribution patterning along this DCA axis. The DCA diagram explains altitudinal, aspect and environmental gradient. The sites and species of lower altitude, northern aspect and mesic habitat (*Pinus-Diospyros-Myrsine* association) were positioned on right side of diagram whereas site and species of higher altitude southern aspect and xeric habitat (*Olea -Dodonea –Micromeria* association) were positioned on left side of diagram (*Figs. 3* and *4*).

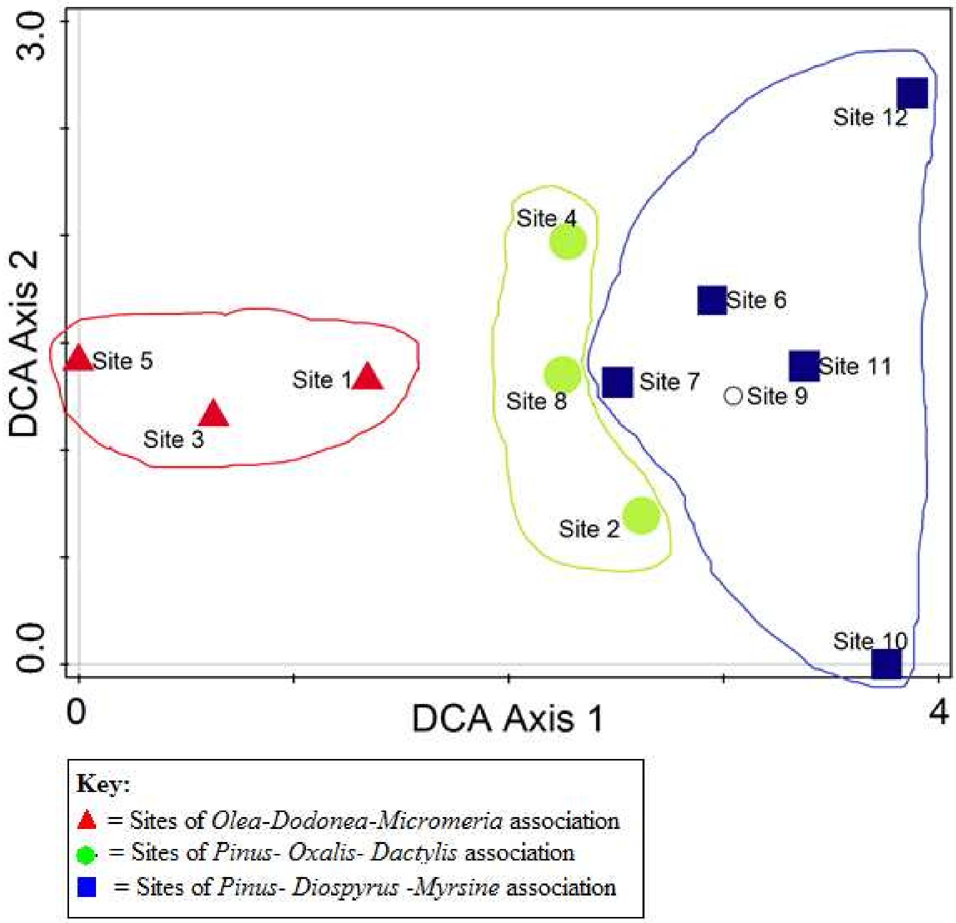

***Figure 3.** DCA analysis diagram representing distribution pattern of plant association*

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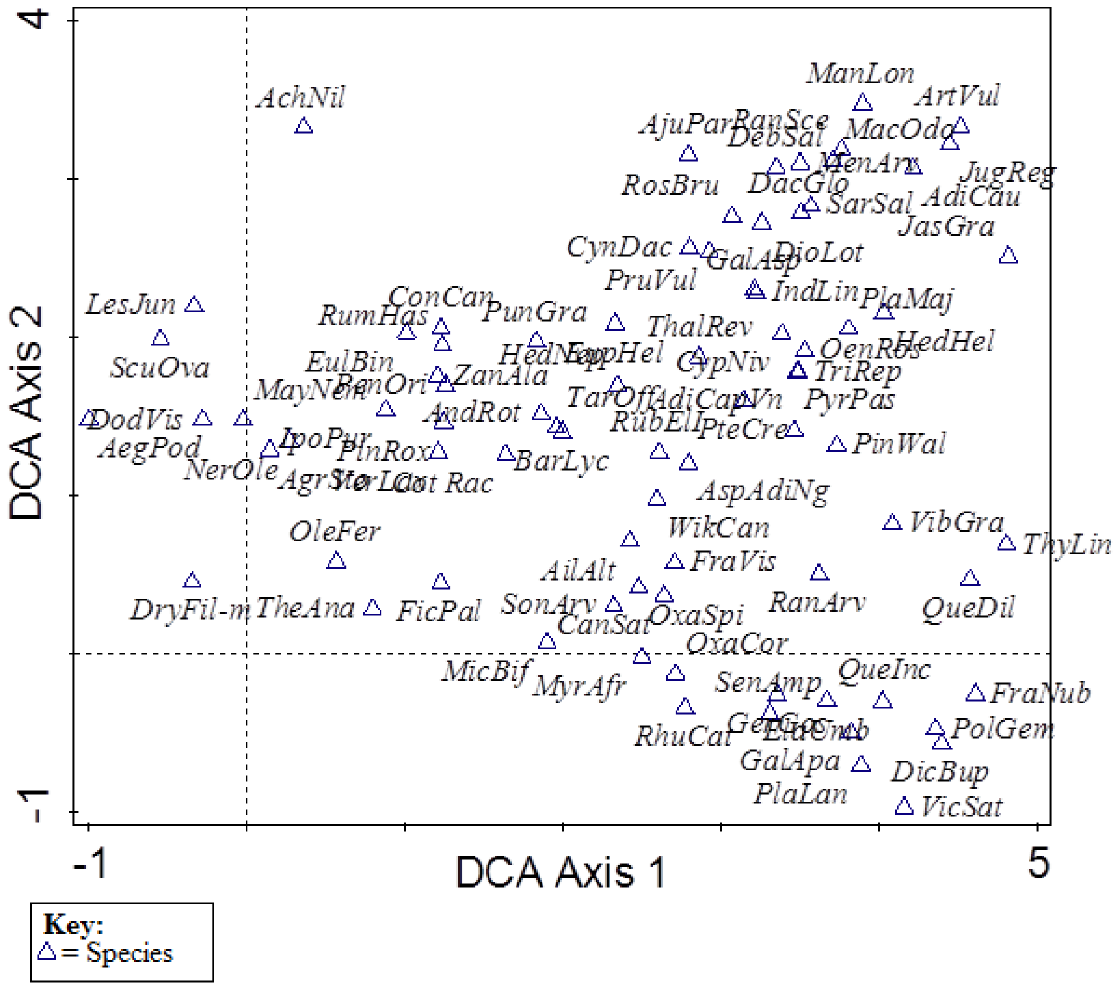

Figure 4. *DCA diagram showing distribution of plant species*

Canonical correspondence analysis

Canonical correspondence analysis (CCA) was carried out here to find the relationship between environmental factors and species distribution pattern in different zones of Tehsil Harighel. The high Eigen value of first axis explains high gradient strength along this axis. Nearly half of variation is explained by first two axes therefore remaining two axis were excluded. CCA revealed Altitude and soil physic-chemical properties particularly potassium content, organic matter, electric conductivity and pH play a significant role in the distribution of plant species while phosphorous and saturation play minor role. The communities harboured at site 1, site 6 and site 7 were under cumulative influence of pH and electric conductivity. The plant community of sites 2 and site 3 has significant correlation with electric conductivity. The plant communities of site 4 were under cumulative influence of electric conductivity and potassium. Whereas plant communities of site 9, site 11 and site 12 were under cumulative influence of altitude, potassium and saturation. Site 4 was outlier and not affected by any environmental variable (*Fig. 5*). Maximum number of species were not affected by any environmental variable as they are located in the center of diagram. E.C play a significant role in grouping of *Pinus roxburghii, Punica granatum, Indigofera*

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linifolia, Rubus ellipticus, *Rumex hastatus*. The pH showed strong correlation with *Olea ferruginea*, *Dodonea viscosa*, *Dryopteris filix-mas*, *Silybum marianum* and *Scutellariaovata*. Organic matter plays a significant role in distribution of *Jasminum grandiflorum*, *Dicliptera bupleuroides*, *Fragaria nubicola* and *Thymus linearis*. Saturation, phosphorus and potassium are important in distribution of *Machilus odoratissimus*, *Rosa brunonii*, *Cyprus niveus*, *Urticadioca*, *Scutellaria chamaedrifolia*, *Mentha arvensi* and *Mantha longifolia* (*Fig. 6*).

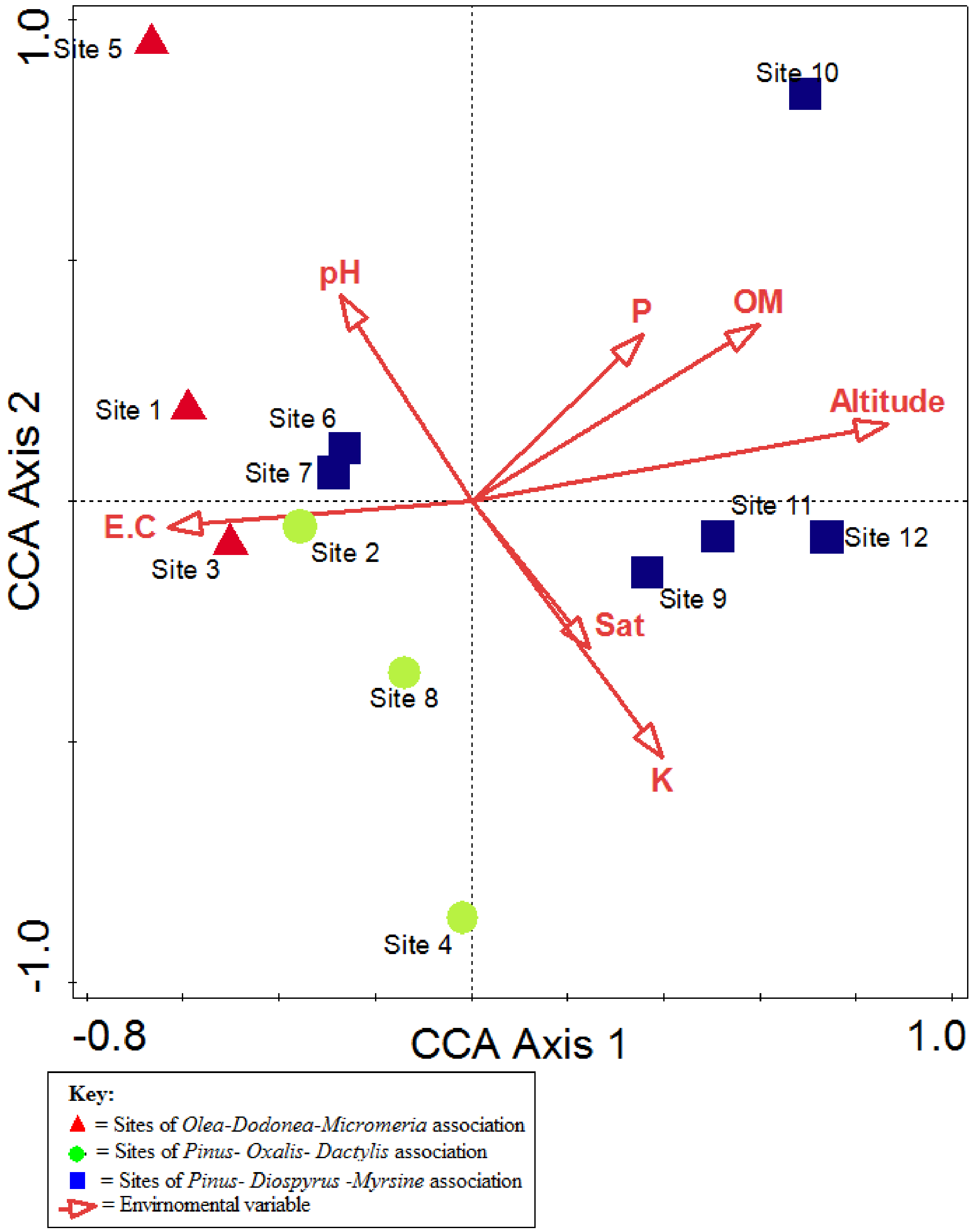

Figure 5. *CCA biplot diagram showing distribution of sites along the environmental variable*

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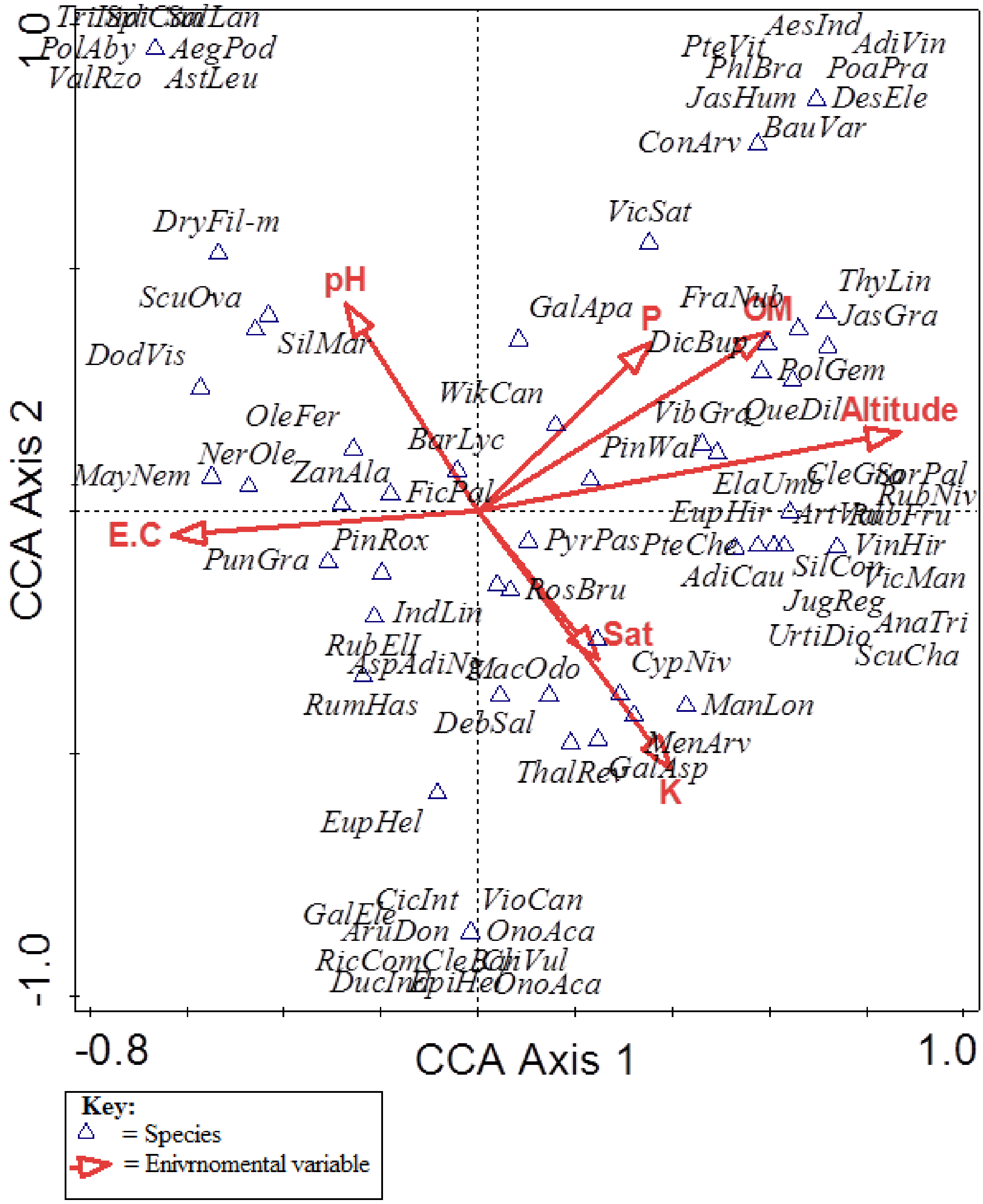

Figure 6. *CCA biplot diagram showing distribution of plant species along the environmental variable*

Species diversity and its associated components

The average Shannon diversity was 3.01 and ranged between 2.49 and 3.82. The average diversity was low (H = 2.55) in *Olea -Dodonea –Micromeria* association and high (H = 3.29) in *Pinus-Diospyrus–Myrsine*. The average species richness was 4.90 ranged between 3 and 7.01. The richness was low (R = 3.24) in *Olea-Dodonea-Micromeria* association and high (R = 5.50) in *Pinus-Oxalis-Dactylis* association. The altitude was significantly positively correlated with diversity and richness and explains

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21% variation in diversity and 19% in richness. Average Evenness was 0.63 ranges between 0.46-0.72. Average equitability was 0.86 and ranged between 0.93-0.91. All the forest stands was immature as the maturity value is less than 60% (*Table 2*).

Table 2. *Diversity and its components recorded from Tehsil Harighel*

Association	Communities	Altitude (m)	Species number	Diversity (H)	Richness (R)	Evenness (G)	Equitability (E)	Maturity (M)
Olea-Dodonea-Micromeria	*Olea-Dodonaea-Pinnisetum*	980	17	2.49	3.00	0.715	0.88	33.92
	Olea-Micromeria-Dodoneae	1165	23	2.62	3.55	0.63	0.85	31.39
	Olea-Aegopodium-Dryopteris	1340	21	2.54	3.17	0.60	0.83	37.39
				2.55	**3.24**	**0.64**	**0.85**	**34.23**
Pinus-Oxalis-Dactylis	*Dactylis-Pinus-Myrsine*	1079	34	2.74	4.93	0.47	0.78	30.44
	Pinus-Olea-Galium	1240	48	2.97	7.01	0.71	0.91	24.78
	Pinus-pinus-Wikstroemia	1658	28	2.97	4.56	0.72	0.90	35.92
				2.89	**5.50**	**0.63**	**0.86**	**30.38**
Pinus-Diospyrus-Myrsine	*Pinus-Oenethera-Trifolium*	1450	30	3.82	4.50	0.56	0.83	34.55
	Pinus-Fragaria-Sarcococca	1554	38	2.96	5.30	0.55	0.83	40.61
	Diospyros-Pinus	1760	40	3.19	5.32	0.67	0.88	41.05
	Pinus-Quercus-Galium	1864	35	3.20	5.64	0.65	0.881	32.95
	Pinus-Micromeria-Quercus	1964	36	3.33	6.23	0.64	0.88	41.42
	Pinus-Adiantum-Quercus	2052	43	3.27	5.61	0.65	0.88	34.49
				3.29	**5.43**	**0.62**	**0.87**	**37.52**

Discussion

Plant associations reflect the environmental condition under which they develop (Malik, 1986; Ilyas et al., 2015). The climate of Tehsil Harigal is of subtropical and

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temperate type (Shaheen et al., 2011a,b; Malik and Malik, 2012) but marked difference in microclimatic, topographic and edaphic factors lead to the establishment of three different plant association with respect to the floristic element and micro environmental conditions. The spatial distribution of plant species in these associations were controlled by different environmental factor like altitude, topography and physcio-chemical properties of soil. The micro gradient established due to variation and interaction among these factors result in different type of vegetation (Hanson and Churchill, 1965) DCA and CCA analysis clearly reflect that altitude and phsico-chemical properties of soil are the main governing factor in the distribution pattern of plant species. This was strongly supported by Ahmad et al. (2009); Shaheen et al. (2012), Amjad et al. (2014 b), Ilyas et al., (2015), Rahman et al. (2016) and Sadia et al. (2017). The large scale pattern in species distribution and physiognomy is mainly governed by climate but physico-chemical properties of soil also govern the distribution pattern on local or micro level (Bakkenes et al., 2002). Climate can be characterized by different variable mainly available moisture which mainly determines the distribution pattern (Leeman and Cramer, 1991). The moderate rainfall and high temperature of Tehsil Harigal result in the stratified forests. Soil is key factor that play important role in selection of plant by bringing evolutionary changes (Barbour et al., 1980). The vegetation of particular area has strong relationship with the soil (Ali et al., 2004). The physico- chemical properties are directly related to soil depth which play key role in establishment of plant association or communities (Khan et al., 2013) The Electrical conductivity of soil of *Olea-Dodonea-Micromeria* association was high whereas organic matter, pH, available moisture nutrient content as compared to *Pinus-Oxalis-Dactylis* association and *Pinus-Diospyros-Myrsine* association which result in establishment of different vegetation type. Slight differences in the available nutrients are positively correlated with variations in community structure (Noor and Khatoon, 2013).

Species diversity is reflection of the health and productivity of ecosystem. Diversity was low in the investigated area due to heavy grazing, deforestation, road construction and fuel wood extraction etc. (Ram et al., 2004; Kumar and Bhatt, 2006). The species diversity and richness was high in *Pinus-Diospyrus–Myrsine* association which might be due to number of coexisting and interacting plant species having overlapping niche (Saxena and Singh, 1982). Moreover the site of higher altitude (*Pinus-Diospyrus–Myrsine* association) are difficult to access by local inhabitant which also resulting high species diversity and richness. The species richness and diversity is positively correlated with altitude. Our findings are in accordance with Malik and Malik (2012) who reported positive correlation between diversity and altitude which might be due to increase in humidity. Differences in altitudes, aspects and slopes result in variation in species diversity and association types. The plant communities harboured at North facing slopes have high diversity with thick vegetation because of high moisture content on southern slope as compared to north facing slope. The same results were obtained by Khan et al. (2011); Amjad et al. (2014a) and and Haq et al. (2015). Plant growth and survival can be affected by amount of water runoff and infiltration which is dependent upon position and smoothness of slope. Aspect and steepness of slope affect the amount of solar radiation which in turn affect temperature on the ground surface (Sukopp and Werner, 1983) and the amount and type of soil accumulated (Monsen et al., 2004). The evenness and equitability is intermediate due to the long term stable climatic condition and uniform pattern of species distribution in majority of plant communities in Harigal. The maturity index reflects immature forest structure throughout investigated area because

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plant species were less adapted to microclimate. The natural balance of plant communities was disturbed due to high anthropogenic pressures which further enhance this pattern (Saxena and Singh, 1982; Shaheen et al., 2011a).

Conclusion

The current study suggests that environmental gradient has profound influence on the distribution of plant species in Tehsil Harighel and association of plant change due to change in environmental variable. Further low value of species diversity and associated indices reflects deteriorating forest structure due to immense anthropogenic pressure in the form of intense grazing, severe deforestation for fuel, fodder and construction purpose, trampling and road construction etc. Therefore immediate conservations measures are needed to preserve the rich plant species diversity of these degraded forests. Land use mapping and vegetation assessment using modern software like ERDAS, arc GIS and NDVI, and are also recommended.

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PLANT ANALYSIS APPLICATION FOR ENVIRONMENTALLY FRIENDLY FERTILIZATION OF WINTER BARLEY (*HORDEUM VULGARE* L.)

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Abstract. The aim of the work was to improve fertilisation recommendations for winter barley by investigating the effect of basic nitrogen (N) fertilisation (0, 40, 80, 120 kg ha^{-1} year^{-1}) on the nutritional status of this crop in a long-term mineral fertilisation experiment involving clearly distinct nutrient supply levels and by determining limiting values indicative of a satisfactory level of nutrition for nitrogen (N), phosphorus (P), potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg) concentrations. Rising N supply levels gradually increased the N content of the aboveground organs of barley, reaching a maximum (4.11–5.61 N%) at the 120 kg N ha^{-1} dose. On this soil, which was well supplied with phosphorus, higher N levels only resulted in a significant increase in the P content of barley in two years. The soil was also well supplied with potassium, N fertilisation enhanced the K concentrations of winter barley at tillering in most years, but generally had no influence on the Na, Ca and Mg content of barley. In the tillering phase the nutritional status of barley was satisfactory at the following concentrations: 3.70–5.80 N%, 0.45–0.75 P%, 3.50–5.02 K%, 0.20–0.60 Na%, 0.40–0.60 Ca% and 0.18–0.30 Mg%.
Keywords: *tillering, macroelements, microelements, nutrient concentration*

Introduction

Investigations on the nutritional status of winter barley and the determination of limiting values for nutrient supply levels for use in diagnostic plant analysis have been an important area of fertilisation research for many decades, but few papers have been published in this field in recent years.

Although applying more nitrogen (N) helps achieve the desired yield (West et al., 2016; Mazur and Mazur, 2015), but results in greater levels of environmental pollution (Wang et al., 2016). According to various authors (Dobermann, 2005; Sebilo et al., 2013) 30-65% of the applied N fertilizer is taken up by crops. In the opinion of Roberts (2008) nutrient loss to the environment is only a concern when fertilizers or manures are applied at rates above agronomic need. The rate of uptake and partition of N is largely determined by supply and demand during various stages of plant growth. The N is needed for early tiller development of barley to set up the crop for high yield potential (Shafi et al., 2011). The N fertilisation of winter barley should be based on soil and plant analysis. Soil analysis is generally carried out prior to sowing, but a combination of monitoring the mineral N content (N_{min}) during the vegetation period and plant

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analysis is rarely applied in Hungary. Plant analysis is of outstanding importance in checking the nutritional status of the crop, and its efficiency improves with the number of nutrients analysed (Németh et al., 2010). Plant analysis can play a major role when diagnosing mineral nutrition problems (Munson, 1998). Comparing soil and plant analysis results can greatly assist in the interpretation (Jones, 1998), but may have an adverse impact on the interpretation of plant analysis results (Munson, 1998). The dilution effect makes the interpretation of plant analysis results difficult, but it can be taken into account by relating plant data to a certain stage of growth (Roy et al., 2006). Mengel (1972) emphasised that if plant analysis is to be suitable for use in fertilisation recommendations, it is essential to determine the limiting concentrations representative of nutritional status, which is primarily the task of scientists (Németh et al., 2010; Izsáki, 2014). The optimum values for individual plant species are given, as the physiological requirements of the plant are similar in all regions and soils, only fluctuating over a narrow range (Kádár, 1992). It was suggested by Tarnawa et al. (2017) that reference values should be determined not only for each species but also for each cultivar, but this is complicated by the large choice of cultivars, the frequent changes in the cultivar grown and the time required to elaborate limiting values for nutrient supply levels (Izsáki, 2015). Various authors (Reuter and Robinson, 1997; Elek and Kádár, 1980) give a fairly wide range of limiting values (2.50–5.25%) for satisfactory N supplies to winter barley during the tillering phenophase. When judging the nutritional status of winter barley it is not sufficient to determine the N concentration; interactions between N and other nutrients should also be investigated, together with the various nutrient ratios. These were considered by Kádár and Lásztity (1981) to be the key components of plant analysis. In classical papers on plant analysis, nutrient content was regarded as the quantitative and nutrient ratios as the qualitative indicator of nutritional status (Németh et al., 2010; Kádár and Lásztity, 1981). Various interactions can be detected in the course of N nutrition, of which the N/phosphorus (P), N/potassium (K), N/magnesium (Mg), N/calcium (Ca) and N/copper (Cu) ratios are the most important (Németh et al., 2010; Armstrong, 1998; Debreczeniné and Sárdi, 1999; Usherwood and Segars, 2001). The interaction between N and other nutrients is still controversy among the reports available (Shah et al., 2017). Relatively few papers have been published on the N nutritional status and nutrient interactions of winter barley at tillering, and these cannot be regarded as up-to-date results due to the rapid genetic gain achieved by developments in breeding.

The aim of this paper was to describe the effect of N supply levels on the nutrient content of winter barley at tillering and on interactions between the nutrients, based on long-term mineral fertilisation experiments, and to determine limiting values for nutrient supply levels, which can be used to judge the nutritional status of winter barley in the tillering phase.

Materials and methods

Long-term mineral fertilisation experiment

The long-term mineral fertilisation experiment was set up at the Experimental Station of the Faculty of Economic, Agricultural and Health Sciences of Szent István University in Hungarian Great Plain, Szarvas, Hungary in 1989 (*Fig. 1*). The soil of the experimental station, a chernozem meadow soil calcareous in the deeper layers, had the following parameters: depth of the humus-containing layer 85–100 cm, pH potassium

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chloride (KCl) of the ploughed layer 5.0–5.2, humus content 2.8–3.2%, no calcium carbonate ($CaCO_3$) content, upper level of plasticity according to Arany (K_A) 50, dry matter content 32%. The groundwater level was at an average depth of 300–350 cm. Before the experiment was set up in autumn 1989 the ammonium (NH_4)-lactate AL-phosphorus pentoxide (P_2O_5) content averaged 156 mg kg^{-1} and the AL- potassium oxide (K_2O) content 322 mg kg^{-1}, and the nutrient supply level of the soil, based on the methods and limiting values officially recognised in Hungary (Buzás et al., 1979), was medium–good for nitrogen (N), good for phosphorus (P), potassium (K), copper (Cu) and zink (Zn), and very good for magnesium (Mg) and manganese (Mn). The experiment included every possible combination (4^3) of four levels each of three factors (N, P and K fertilisation), giving a total of 64 treatments arranged in a split-split plot design with three replications. *Within the three true replications, the N fertiliser treatments were present in 48 internal replications and the P treatments in 16.*

Figure 1. *Experimental site*

Experimental factors and treatments

Factor A (K fertilisation) involved the following treatments:
K_0 = without K fertilisation,
K_1 = 300 kg ha^{-1} $year^{-1}$ K_2O from 1989–1992, 100 kg ha^{-1} $year^{-1}$ from 1993 onwards,
K_2 = 600 kg ha^{-1} K_2O in 1989, 1000 kg ha^{-1} in 1993 and 600 kg ha^{-1} in 2001,
K_3 = 1200 kg ha^{-1} K_2O in 1989, 1500 kg ha^{-1} in 1993 and 1200 kg ha^{-1} in 2001.
Factor B (P fertilisation) involved the following treatments:
P_0 = without P fertilisation,
P_1 = 100 kg ha^{-1} $year^{-1}$ P_2O_5,
P_2 = 500 kg ha^{-1} P_2O_5 in 1989, 1993 and 2001,
P_3 = 1000 kg ha^{-1} P_2O_5 in 1989, 1993 and 2001.
Factor C (N fertilisation) involved the following treatments:
N_0 = without N fertilisation,
N_1 = 80 kg N ha^{-1} $year^{-1}$ (40 kg ha^{-1} basic fertiliser + 40 kg ha^{-1} top-dressing),

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N_2 = 160 kg N ha^{-1} $year^{-1}$ (80 kg ha^{-1} basic fertiliser + 80 kg ha^{-1} top-dressing),
N_3 = 240 kg N ha^{-1} $year^{-1}$ (120 kg ha^{-1} basic fertiliser + 120 kg ha^{-1} top-dressing).
N_{1B} = 40 kg N ha^{-1} $year^{-1}$ basic fertiliser
N_{2B} = 80 kg N ha^{-1} $year^{-1}$ basic fertiliser
N_{3B} = 120 N ha^{-1} $year^{-1}$ basic fertiliser
The results of the basic fertilizer treatments are included in the paper.

The aim of the periodic application of high rates of P and K fertiliser was to create clearly distinguishable supply levels in the soil in order to investigate different nutritional situations and to determine limiting values for soil nutrient supply levels. The N basic fertiliser and top-dressing was applied in the form of ammonium nitrate (34%), the P as superphosphate (18%) and the K as potassium chloride (40 or 60%).

Crop management

Each year four crops were sown in a full crop rotation on 4 × 192 plots, where the area of the main plots was 320 m², the subplots 80 m² and the sub-subplots 4 × 5 = 20 m². The forecrop of winter barley was fibre hemp (*Cannabis sativa*) in 2006 and 2007 and canary grass (*Phalaris canariensis* L.) in 2010 and 2011. The two-row winter barley cultivar GK Stramm was used in the experiment. The results recorded in the long-term experiment in the 17th–19th years (2005/2006, 2006/2007, 2007/2008) and in the 22nd (2010/2011) and 23rd (2011/2012) years are discussed in the present paper. In these years sowing took place between October 8th and November 3rd with a row distance of 12 cm and *5 million seed/ha*. No chemical weed control was required in the experiment. Other plant protection measures were carried out when necessary. Top-dressing was applied on a single occasion at the end of tillering after plant sampling. The winter barley was harvested at full maturity using a plot combine at the end of June.

Weather conditions

The weather conditions during the growing season are presented in *Table 1.*

Table 1. *Weather data of experiment during the period tested, (Szarvas, 2006-2008, 2011-2012)*

Year	Rainfall, mm			Average temperature, °C		
	Winter half year (X-III.)	Vegetation period (X-VI.)	Year total	Winter half year (X-III.)	Vegetation period (X-VI.)	Year total
Average 1901-1975	225	362	538	3.4	7.5	10.6
2005	-	-	721	-	-	10.2
2005/2006	204	379	537	2.2	6.9	10.7
2006/2007	159	293	548	6.5	10.2	12.2
2007/2008	200	370	464	4.2	8.4	11.7
2010/2011	282	435	373	3.6	8.2	11.5
2011/2012	121	230	363	3.1	7.9	11.5

In the 17th year of the long-term experiment (2005/2006) the rainfall quantity (379 mm) was slightly higher than the long-term mean (362 mm), while the mean temperature (6.9 °C) was lower than the long-term mean (7.5 °C). In the year with the least favourable weather (2006/2007) the total rainfall was 69 mm less than the long-term mean while the mean temperature was 2.7 °C higher. In 2007/2008 both the

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rainfall quantity and the mean temperature were close to the long-term mean (370 mm and 8.4 °C). The wettest growing season was the 22nd year of the experiment, with 73 mm more rain than the long-term mean and a mean temperature 0.7 °C higher than the mean. In the 2011/2012 growing season the temperature was similar to the long-term mean, while the rainfall quantity (230 mm) was well below the mean.

Soil contents

The K_2O and P_2O_5 contents of the soil were determined using the AL method (Hungarian standard MSZ 20135:1999). When evaluating the results, the K and P values recorded in the ploughed (30 cm) layer were used to represent the K and P supply levels in the soil. The K and P supply levels given for the various years are those recorded in the autumn of the previous year (*Table 2*).

Table 2. *P and K supplies of the soil, (Szarvas, 2006-2008, 2010-2011)*

Treatment	Years of experiment				
	2006	**2007**	**2008**	**2010**	**2011**
AL- K_2O in plought layer (mg kg^{-1})					
K_0	199	211	235	218	210
K_1	320	322	345	324	320
K_2	324	301	322	294	286
K_3	377	340	375	346	335
AL- P_2O_5 in plought layer (mg kg^{-1})					
P_0	150	141	118	133	118
P_1	217	222	181	206	224
P_2	186	193	158	194	186
P_3	282	273	269	251	233

Plant samples

Plant samples were taken from 2 × 1 m on each plot at tillering (growth stage 4–5 on the Feekes scale), prior to N top-dressing, to determine the nutrient content of winter barley. The nutrients N, P, K, sodium (Na), calcium (Ca) and magnesium (Mg) were analysed in five years (2006–2008 and 2011–2012) from dried, ground samples of the complete aboveground plant organs. The nutrient contents were determined using the inductively coupled plasma optical emission spectrometry method (ICP-OES) after digestion with first nitric acid and then hydrogen peroxide (*MSZ-08-1783-6:1983 3.1., MSZ-08-1783-26-34:1985, MSZ-08-1783-36:1985*). The data are given in terms of dry matter. In the present paper is discussed the effect of basic N fertilisation.

Statistical analysis

Statistical analysis was performed by means of analysis of variance followed by Student's t-test and least significant differences (LSD) at the 0.05 probability level, using the SPSS program, version 15.

Envelope curves

Correlation analysis on the extensive database was performed by plotting data pairs for N concentration and grain yield in a coordinate system and envelope curves were drawn for each set of points (Izsáki, 2014; Helget, 2016; Izsáki, 2017). Values located along the envelope curve represent cases when factors influencing the yield have optimum values and the yield is only influenced by the N concentration. Those located below the envelope curve indicate that one or more yield-influencing factors are not optimum, so the yield level does not depend only on the N nutritional status. The higher the yield level for which the optimum nutrient supply level is determined, the narrower the optimum range, while the range is wider for lower yield levels.

Results

Effect of N supply levels on the macroelement content of winter barley

The effect of N basic fertiliser (N_0 = 0, N_{1B} = 40, N_{2B} = 80, N_{3B} = 120 kg ha^{-1}) on the N, P and K contents of winter barley in the tillering phase, averaged over the P and K treatments, can be evaluated from the data presented in *Table 3*.

Table 3. *Effect of N supplies on the N-, P- and K-concentrations of winter barley at the tillering, (Szarvas, 2006-2008, 2011-2012)*

Year	Nutrient concentration (%)				$LSD_{5\%}$	Average
	N_0	N_{1B}	N_{2B}	N_{3B}		
			N%			
2006	3.04 ^{d}	3.60 ^{c}	4.39 ^{b}	4.89 ^{a}	0.20	3.98
2007	3.42 ^{a}	3.68 ^{a}	4.09 ^{a}	4.55^a	0.58	3.94
2008	4.07 ^{d}	4.70 ^{c}	5.28 ^{b}	5.61^a	0.26	4.91
2011	3.00 ^{b}	3.27 ^{a}	3.47 ^{a}	3.66^a	0.29	3.35
2012	2.94 ^{c}	3.67c	3.85^b	4.11^a	0.32	3.64
			P%			
2006	0.57 ^{c}	0.60^b	0.63^a	0.63^a	0.02	0.61
2007	0.44 ^{b}	0.48 ^{b}	0.58^a	0.57^a	0.05	0.52
2008	0.63	0.67	0.65	0.65	NS	0.65
2011	0.38	0.40	0.41	0.41	NS	0.40
2012	0.33	0.30	0.29	0.32	NS	0.31
			K%			
2006	3.93	4.02	4.02	4.03	NS	4.00
2007	3.53 ^{c}	4.00 ^{b}	4.49 ^{a}	4.69 ^{a}	0.38	4.18
2008	3.91 ^{a}	4.13 ^{a}	4.14^a	4.21^a	0.23	4.10
2011	2.81 ^{a}	3.00 ^{a}	3.07 ^{a}	3.07 ^{a}	0.25	2.99
2012	3.89	4.11	4.16	4.12	NS	4.07

NS: non-significant. Values in each row followed by the same letters are not significantly different at $p < 0.05$ according to Student's t-test

Without N fertilisation the N content of the total aboveground organs of barley ranged over a relatively narrow interval (2.94–3.42%) in four of the five years. In the year that had average but favourably distributed rainfall supplies the N concentration reached a level of around 4%. Regardless of the year, increasing rates of N fertiliser led to significant increases in the N concentration of barley compared with the control given no N fertiliser (N_0). The N content of the aboveground shoot reached a maximum concentration of 4.11–5.61% in the N_{3B} treatment (120 kg N ha^{-1} year^{-1}) except in 2011. The winter half of the 2010/2011 growing season was extremely wet, and the 188 mm precipitation recorded in November and December leached a considerable quantity of NO_3-N out of the soil of N-fertilised treatments, with the result that the N concentration

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of barley was the lowest in this year. The P concentration of barley at tillering was greatly influenced by the year and ranged from 0.32–0.65% in the N_3 treatment.

Even though the experiment was set up in 1989 the AL-K_2O content of the ploughed layer was 322 mg kg^{-1}, which dropped to 199 mg kg^{-1} by autumn 2006, in the 16th year of the experiment, without K fertilisation. The soil was also well supplied with K, and improvements in the N supply level increased the K content of barley at tillering in three of the five years. There was also a tendency for N fertilisation to increase the K concentration in the other two years. Except in the 2010/2011 growing season, the K concentrations exhibited little difference within each N supply level, with a value of 4.03–4.69% in the N_3 treatment.

The effect of basic N fertilisation on the Na, Ca and Mg contents of winter barley in the tillering phase, averaged over the P and K treatments, can be seen from the data in *Table 4*.

Table 4. *Effect of N supplies on the Na, Ca and Mg content of winter barley at the tillering, (Szarvas, 2006-2008, 2011-2012)*

Year	**Nutrient concentration (%)**				**LSD$_{5\%}$**	**Average**
	N_0	**N_{1B}**	**N_{2B}**	**N_{3B}**		
			Na%			
2006	0.23 ^{b}	0.27 ^{b}	0.35 ^{a}	0.39 ^{a}	0.06	0.31
2007	0.36	0.27	0.25	0.28	NS	0.29
2008	0.31 ^{a}	0.36 ^{a}	0.40 ^{a}	0.41 ^{a}	0.05	0.37
2011	0.16	0.15	0.18	0.19	NS	0.17
2012	0.21	0.19	0.23	0.22	NS	0.21
			Ca%			
2006	0.41 ^{b}	0.46 ^{a}	0.48 ^{a}	0.51 ^{a}	0.03	0.46
2007	0.43 ^{b}	0.48 ^{a}	0.50 ^{a}	0.52 ^{a}	0.04	0.48
2008	0.55	0.51	0.51	0.53	NS	0.52
2011	0.41 ^{a}	0.44 ^{a}	0.46 ^{a}	0.51 ^{a}	0.06	0.46
2012	0.54	0.57	0.57	0.50	NS	0.55
			Mg%			
2006	0.18 ^{a}	0.20 ^{a}	0.22 ^{a}	0.23 ^{a}	0.02	0.21
2007	0.21	0.21	0.22	0.23	NS	0.22
2008	0.23	0.24	0.25	0.26	NS	0.24
2011	0.17	0.17	0.18	0.19	NS	0.18
2012	0.22	0.20	0.22	0.23	NS	0.22

NS: non-significant. Values in each row followed by the same letters are not significantly different at $p < 0.05$ according to Student's t-test

For most field crops, Na is not one of the essential macroelements, but winter barley takes up almost as much Na as Mg, especially on solonetz soils well supplied with Na. In two of the five years N fertilisation was found to induce a significantly greater rate of Na incorporation compared with the N_0 treatment. The Na concentration in the tillering phase differed considerably in the different years, with values ranging from 0.19–0.41% in the N_{3B} (120 kg ha^{-1} N) treatment. The Ca content of barley exhibited a significant increase with better N supplies in three of the years. The Ca content was not greatly influenced by the year. On this soil, which is extremely well supplied with Mg, a slight but significant increase in Mg concentration as the result of N fertilisation could only be detected in one year. Depending on the year and the N supply level, the Mg content of barley at tillering ranged from 0.17–0.26%.

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Correlations between nutrient concentrations and the yield

The experimental data revealed clear differences in the N concentration of barley leaves at tillering in the various N treatments. Diagnostic plant analysis is only of practical interest if a correlation exists between the leaf N concentration and the yield, allowing limiting values to be determined for the N nutritional status of winter barley. Based on the correlation between the leaf N concentration of winter barley in the tillering phase and the grain yield (*Fig. 2*), the limiting value of satisfactory N supplies at a yield level of over 5.5 t ha^{-1} is 3.70–5.80 N%.

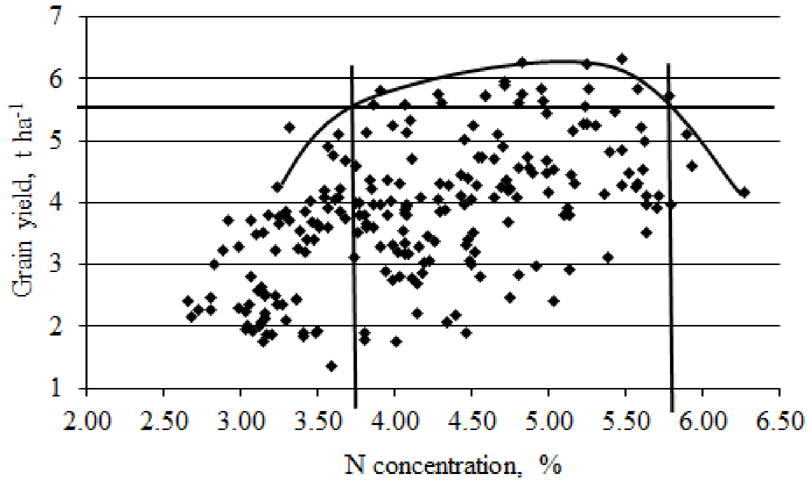

***Figure 2.** Relationship between the N-concentration of leaf at the tillering and the grain yield (Szarvas, 2006-2008, 2011-2012)*

The limiting values for satisfactory supplies of the other macroelements were determined by plotting the P, K, Na, Ca and Mg concentrations associated with grain yield in a coordinate system and then reading off the macroelement concentrations corresponded to a yield level that was at least 90% of the maximum value (*Figs. 3–7*).

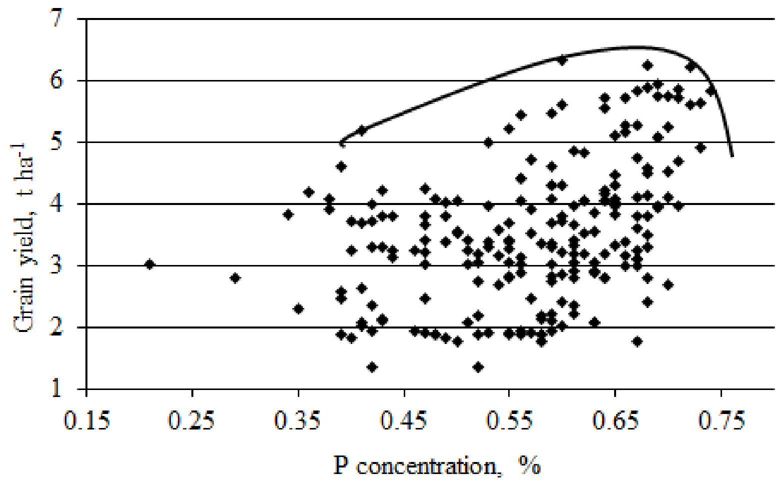

***Figure 3.** The limiting values for satisfactory P supply (Szarvas, 2006-2008, 2011-2012)*

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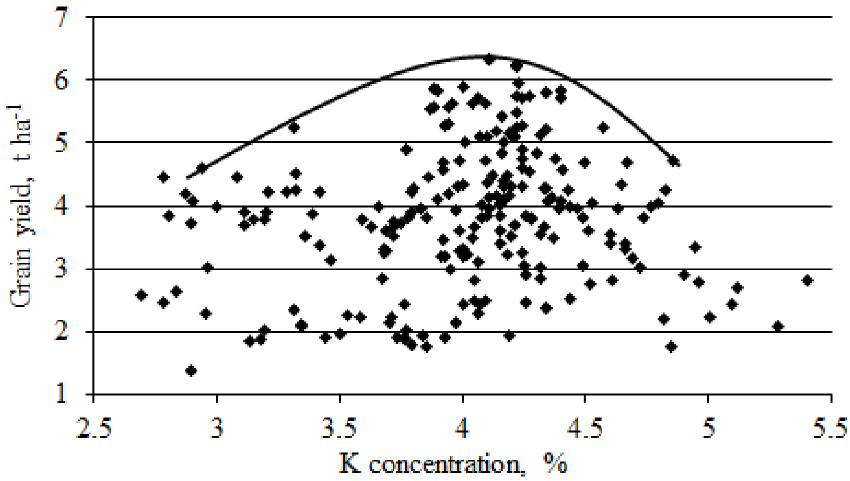

Figure 4. *The limiting values for satisfactory K supply (Szarvas, 2006-2008, 2011-2012)*

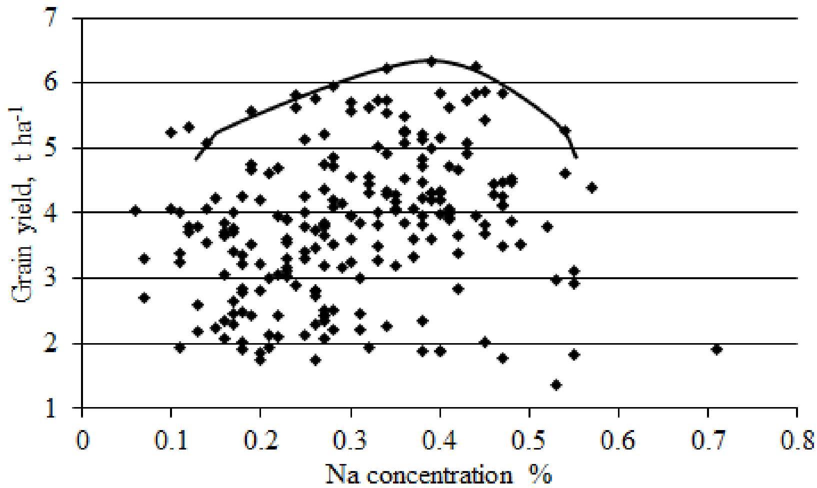

Figure 5. *The limiting values for satisfactory Na supply (Szarvas, 2006-2008, 2011-2012)*

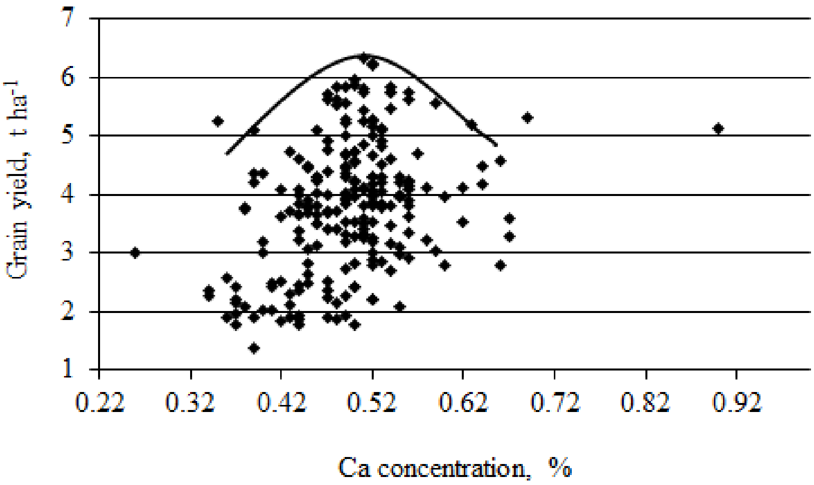

Figure 6. *The limiting values for satisfactory Ca supply (Szarvas, 2006-2008, 2011-2012)*

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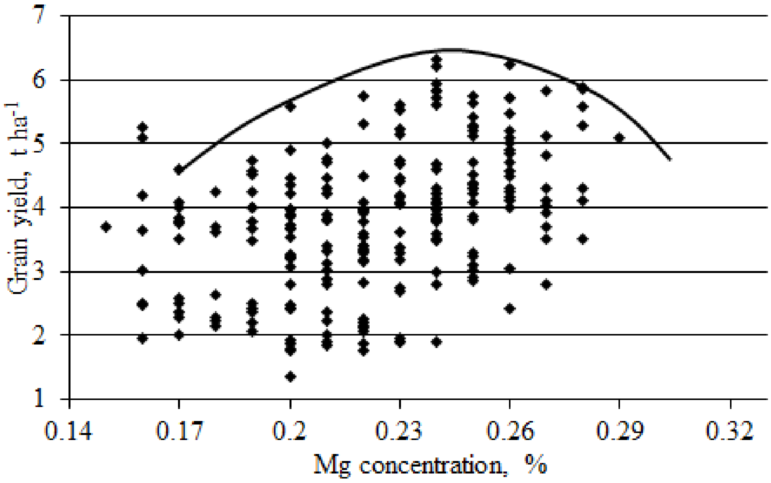

Figure 7. *The limiting values for satisfactory Mg supply (Szarvas, 2006-2008, 2011-2012)*

Discussion

In a pot experiment on winter barley in the tillering phenophase, Kostadinova (2014) found that the application of mineral fertiliser at rates of 200 mg kg^{-1} P_2O_5 and 200 mg kg^{-1} K_2O without N fertiliser resulted in leaf N concentrations of 2.15%, which rose by more than 50% as the N supplies increased. Kádár and Csathó (2015) examined the effect of N fertilisation on the nutrient content of winter barley at tillering in a soil containing 128 mg kg^{-1} AL-P_2O_5 and 243 mg kg^{-1} AL-K_2O and reported that without N fertilisation the leaf N content was 1.86%, which rose significantly to 3.03% at a dose of 200 kg ha^{-1} N, tending to decline again at the maximum dose of 300 kg ha^{-1} N. In the opinion of Mills and Jones (1996) ranges of sufficiency are most commonly used in assessment of plant nutrition. In the present work the satisfactory N concentration for winter barley at tillering was found to be 3.70–5.80%, which deviated somewhat from the optimum N concentrations given by other authors. Fageria (2014) determined a very narrow range of satisfactory N concentrations (4.7–5.1%), while Reuter and Robinson (1997) reported a fairly wide range (2.5–5.0%). Under Hungarian conditions Elek and Kádár (1980) established a value of 4.70–5.25 N%. On this soil, which is well supplied with P, rising N supplies only led to a significant increase in the P content of barley in two years. According to Kádár and Csathó (2015) better N supplies reduced the P concentration of winter barley in the tillering phase, while in the present work the maximum P concentration was recorded in the N_2 treatment. Sanchez (2007) considers the value above 0.4 P concentration satisfactory. Both Elek and Kádár (1980) and Kádár (2012) stated that a P concentration of 0.4–0.5% represented satisfactory P supplies in winter barley, while a value of 0.45–0.75% was found in the present work and Reuter and Robinson (1997) reported an optimum range of 0.3–0.6%. In agreement with the present results, Kádár (2012) and Kádár and Csathó (2015) reported that better N supplies stimulated the cation uptake of winter barley. A K concentration of 4.2–5.0% was found by Elek and Kádár (1980) to represent satisfactory K nutritional status in winter barley at tillering, while this was later modified by Kádár (2012) to 3–4%. Reuter and Robinson (1997) considered a wider range of values to be acceptable (3.5–5.0 K%), the same range as found in the present work. If the Na concentration of barley in the tillering phenophase was higher than 0.8%, this was judged by Reuter and Robinson (1997) to be toxic, while values below 0.50% were found to be satisfactory.

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The present results showed that the satisfactory Na content for winter barley at tillering was 0.20–0.60%. In the case of Ca and Mg the present results were in good agreement with the limiting values given in the literature. Barczak (2008) has demonstrated that higher N supplies generally increased the content of macroelements in winter barley biomass in all the plant growth stages assayed. Kádár and Lásztity (1981) emphasised that nutrient ratios only provide information on the relative quantities of two nutrients, not on the actual quantity of the individual nutrients, so the knowledge of ratios alone is insufficient to determine whether the nutrients are present in optimum, excessive or deficient quantities. The quantity of a given nutrient in the plant depends on the available supplies of other nutrients and on the interactions between them (Füleky et al., 1999). Elek and Kádár (1980) pointed out that nutrient ratios are more constant and less dependent on plant age than the concentrations. Between N and P nutrients are positive interaction, but their physiological effects are antagonists of each other. The present results suggest that a satisfactory N/P ratio is in the range of 7.7–8.2, which is similar to the value of 8.3 recommended by Reuter and Robinson (1997), but lower than the 11.9–12.0 reported by Elek and Kádár (1980). In the case of the N/K ratio, which is negative interaction, similar values were determined in the present work (1.1–1.2) to those given in the literature. According to Brar et al. (2011) the N/K interaction **is** dependent on the form of N supplied. The satisfactory N/Na ratio calculated from the present data (9.6–18.5) was higher than the 5–10 optimum reported by Reuter and Robinson (1997). Higher ratios were also found for the N/Ca and N/Mg ratios (9.2–9.6 and 19.3–20.5, respectively) than those given by the same authors (5.0–8.3 and 16.6–17.5, respectively), while the K/Mg and Ca/Mg ratios found in the present work were similar to those given by Reuter and Robinson (1997).

The limiting values for satisfactory macroelement concentrations and the associated nutrient ratios are presented for winter barley in the tillering phenophase in *Table 5*.

Table 5. *The limiting values for satisfactory nutrient supplies of winter barley at tillering (Feekes 4-5)*

Nutrient	Nutrient concentrations and ratios	
	Own investigation	***Based on the literature data**
N%	3.70-5.80	2.5-5.25
P%	0.45-0.75	0.20-0.60
K%	3.50-5.02	2.50-5.00
Na%	0.20-0.60	<0.50
Ca%	0.40-0.60	0.20-1.00
Mg%	0.18-0.30	0.14-0.30
N/P	7.7-8.2	6.4-8.8
N/K	1.1-1.2	0.7-1.1
N/Na	9.7-18.5	5.0-10.5
N/Ca	9.3-9.7	5.3-8.3
N/Mg	19.3-20.6	16.7-17.5
K/Na	8.3-17.5	7.0-10.0
K/Mg	16.7-19.4	16.7-23.3
Ca/Mg	2.0-2.2	2.0-3.3

*Reuter and Robinson, 1997; Elek and Kádár, 1980; Sanchez, 2007; Plank and Donohue, 2000

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Conclusions

1. Rising N supply levels gradually increased the N content of winter barley at the tillering phenophase, reaching a maximum (4.11–5.61 N%) at the 120 kg N ha^{-1} $year^{-1}$ dose.
2. The soil of the long-term fertilisation experiment is well supplied with phosphorus, the P concentration of barley at tillering was greatly influenced by the year, than N supply and ranged from 0.32–0.65% in the N_{3B} treatment.
3. On this soil, which was also well supplied with K, N fertilisation enhanced the K concentration of winter barley in most years.
4. N fertilisation generally had no influence on the Na, Ca and Mg concentrations of barley.
5. In the tillering phenophase the nutritional status of winter barley was satisfactory at the following concentrations: 3.70–5.80 N%, 0.45–0.75 P%, 3.50–5.02 K%, 0.20–0.60 Na%, 0.40–0.60 Ca% and 0.18–0.30 Mg%.
6. *The aim of experimental work was to develop fertilization consultancy with plant analysis, practical guidance for leaf fertilization.*

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RELATIVE HUMIDITY MODELING WITH ARTIFICIAL NEURAL NETWORKS

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Abstract. Air humidity has great importance for living beings, especially plants. Air humidity controls vaporisation on earth's surface and transpiration of plant leaves. Additionally, it prevents most of the radiation from the sun and reflected sun rays from reaching the ground and prevents excessive heating or cooling. The purpose of this study is to predict relative humidity as an important climate parameter, based on annual total precipitation, average ambient temperature, and altitude. Regression and artificial intelligence network models were developed by using monthly average temperature, total precipitation, and altitude parameters obtained from 177 meteorological stations in Turkey to predict relative humidity. When analysed, the model developed with the artificial neural network method had greater predictive power ($R^2 = 0.84$) than the model developed with multiple linear regression ($R^2 = 0.76$). In this study may be applicable in climate conditions that are similar to those in Turkey.
Keywords: *Turkey, multiple linear regression, ANNs, humidity, climate*

Introduction

The amount of water in the air is referred to as air humidity. Air humidity is important for the development of plants and prevents most of the radiation from the sun and reflected sun rays from reaching the ground, thus preventing excessive heating or cooling. When the relative humidity of air decreases, the transpiration rate of plants increases. This causes decreased turgor pressure in plant cells. The relative humidity ratio in ambient air should be greater than 65% for turgor pressure to be balanced. If the relative humidity of air constantly decreases, transpiration increases; if water absorbed by the roots fails to correspond to water lost via transpiration, plant stomas will close and decrease transpiration. In this case, the gas exchange necessary for photosynthesis and respiration will fail, and as a result plant growth will either slow or completely stop (Cox et al., 2016).

The transpiration rate of plants is affected by sunlight, wind, air humidity, air pressure, and the amount of water in soil. During winter months, temperature and light intensity are relatively low. Therefore, the transpiration rate decreases and plants need less water. Generally, in places where temperatures are at low levels during summer or winter, plants need less water, whereas in places where temperatures are higher, plants need more water (Rohli and Vega, 2013).

Additionally, air relative humidity has an important role in storage of products. If relative air humidity in a barn is high, the amount of water in stored products will increase. Increased water retention will lead to product losses due to excessive heating, decay, and rotting (Pixton, 1982).

Because air humidity has an effect on water loss in an ecosystem, it is important in terms of ecology. Therefore, ground humidity is considered as a criterion in determining climate type. For example, places with more than 80% relative humidity are characterised as rain forests, whereas places with less than 20% humidity are characterised as extremely dry climates (Clarke, 1954).

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Air humidity controls vaporisation on the earth's surface and transpiration of plant leaves. As vaporisation and transpiration increase, the amount of water decreases inside root sections and on the ground surface. Fog as air humidity is important for dry regions. Although there may be no precipitation events in deserts for years, fog in the air supports plant survival. However, higher relative humidity or foggy and cloudy weather can cause various types of fungus that cause different diseases in plants to grow and spread, thus decreasing agricultural yield in humid regions (Yurtseven, 1999; Kanber et al., 1992).

Air humidity thus has great importance for living beings, especially plants. Yet, in analysing previous studies regarding prediction of climate parameters, there were few studies on relative humidity prediction. The purpose of this study is to predict relative humidity as an important climate parameter for ecologic studies, based on certain measurements that are believed to be related to relative humidity: annual total precipitation, average ambient temperature, and altitude.

Prediction models developed in this purpose are based on classical regression analysis (Tokar and Johnson, 1999) and artificial neural networks (ANNs) (Al-Alawi et al., 1998; Luk et al., 2000; Kumar et al., 2002; Çelik et al., 2016) that are increasingly important in recent years. Multiple linear regression techniques are the most widely used statistical tools for discovering the relationships among variables. ANNs are considered nonlinear statistical data modeling tools where the complex relationships between inputs and outputs are modeled or patterns are found. ANNs have been very useful in many aspects of climatical research and modeling in recent years (Terzi and Keskin, 2010; Keskin et al., 2011).

Comparison of regression and ANN models have shown that the performance values of the ANN models are better than the regression models in general (Yadav and Chandel, 2014; Kumar et al., 2015).

Materials and methods

Turkey is located in the northern hemisphere, between 36°-42° N and 26°-45° E. More than half of the country has an altitude of more than 1,000 m. Approximately one third of the country is covered with medium-height plainlands, highlands, and mountains, and 10% is at low altitude. The highest and most mountainous areas are located on the eastern side of the country. The most rugged area in the north is the North Anatolian Mountains, and the roughness of south, east, and southeast regions is caused by the Toros Mountains. The highest mountain in the country is Mount Ağrı, at 5,166 m. The largest plains are Çukurova, Konya Plains, and Harran Plains. The longest river with a spring source and flowing into the sea is Kızılırmak with a length of 1,355 m. The largest natural lake is Van Lake with an area of 3,713 km². Atatürk Dam Lake is the largest artificial lake with a 817 km² area. The largest island is Gökçeada with a 279 km² surface area. Total land area is 770,760 km², and total water area is 9,820 km² (Şahin, 2006).

Turkey is located between a temperate zone and sub-tropical zone. Although Turkey is surrounded by seas on three sides, differences in mountain elongation and land forms cause different climate types with different properties. The coastal section of Turkey has a temperate climate because of the tempering effect of the sea. The North Anatolian Mountains and Toros Mountain range prevent the mild sea climate from penetrating to

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the inner regions. Therefore, the inner regions of the country have a continental climate (Atalay, 1997).

Due to climate differences by location and geographical properties, natural vegetation consists of various plant formations, including forests, shrubs or bushes, or weeds. These plant groups that show humid, semi-humid, or dry characteristics based on the effects of climate differ in geographical spread, morphology, ecology, and floristic properties. In Turkey, there are approximately 12,000 plant taxa (Günal, 2013).

This study attempts to predict relative humidity by using certain parameters (average annual total precipitation, average ambient temperature, and altitude) collected between 1987-2017 from 177 large climate stations in Turkey. The location of these stations is given in *Figure 1*.

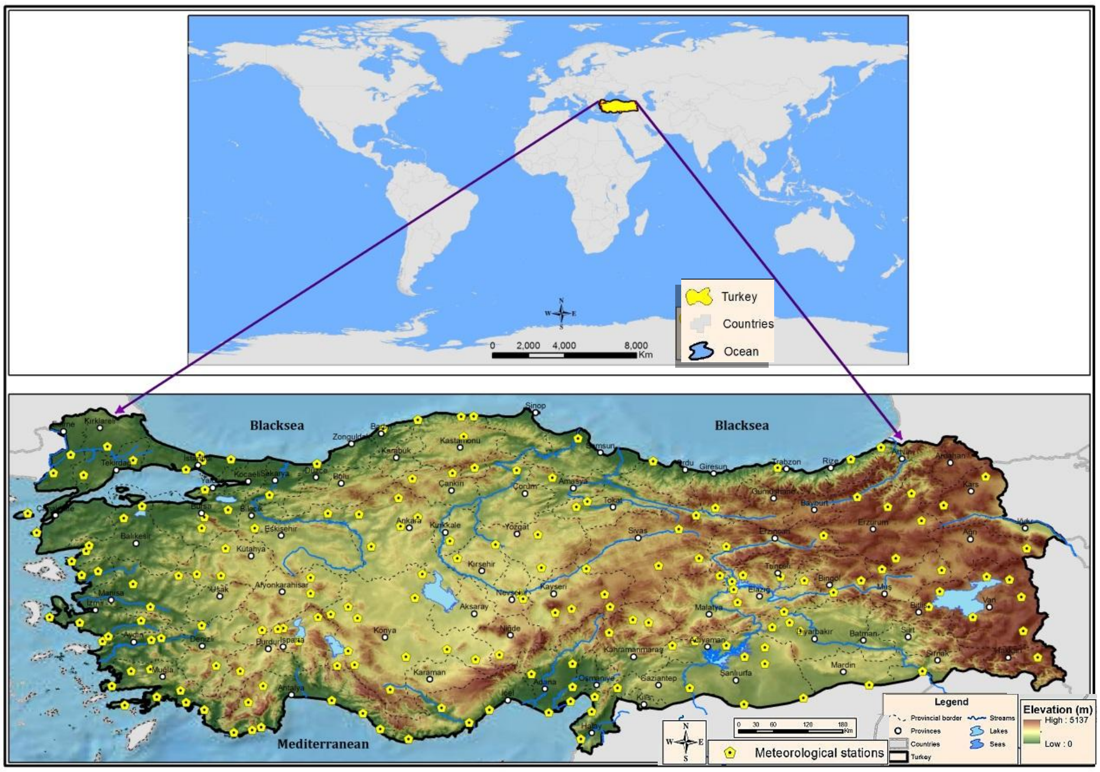

Figure 1. *Study area*

Regression analysis, which forms the basis of the multiple linear regression model adopted in the study, enables the development of a model of the relationship between dependent and independent variables; this model enables predictions (Kalıpsız, 1994).

The multiple linear regression model used in the analysis is presented in *Equation 1*.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + \varepsilon i \qquad \text{(Eq.1)}$$

In this model, Y is the dependent variable, X_1 is temperature, X_2 is precipitation, and X_3 is altitude, b_0 is where the regression curve intersects the y axis, b_1 is the regression coefficient for the first independent variable, b_2 is the regression coefficient for the

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second independent variable, b_3 is the regression coefficient for the third independent variable; εi is a random error variable for zero average, and σ2 is variance.

Today, artificial intelligence applications are used for predicting meteorological events. Successful results were found especially in applications with artificial neural networks (Keskin and Terzi, 2006; Kumar et al., 2002; Saplıoğlu and Çimen, 2010; Zealand et al., 1999).

The artificial neural network (ANN) adopted in this study was developed based on learning with trial and generalisation concepts of the human brain. The biologic neural system can be explained as a three-layered system that constantly receives information, interprets this information, and produces decisions for this interpretation. Receiver neurons turn a stimulus within the organism or from the outside world into electrical signals that transfer information. Reaction neurons turn electrical impulses created by the brain into appropriate reactions as organism output. *Figure 2* shows a block diagram of a neural cell (neuron) (Öztemel, 2003).

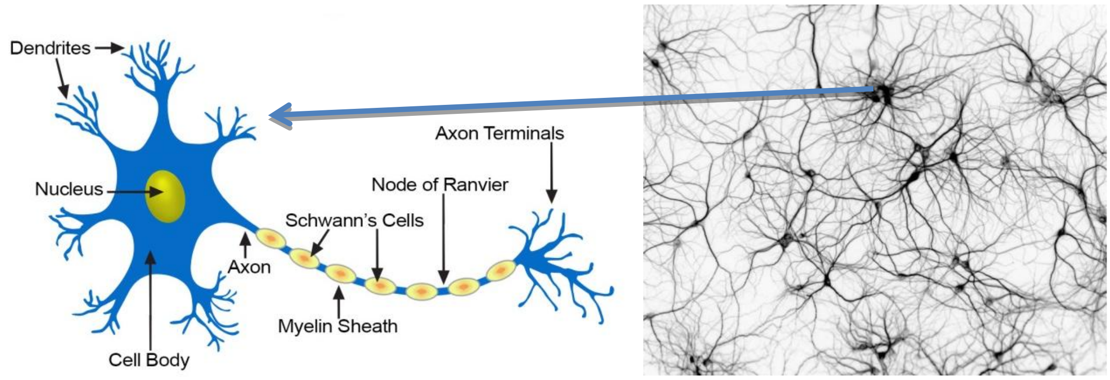

Figure 2. *Structure of neuron. (Anonymous, 2018a, b)*

An artificial neuron cell consists of five main components: inputs, weights, summation (uniting) function, activation (transfer) function, and output. Inputs are outside-world information to an artificial neuron cell. Information received from the outside world or a previous layer are sent to the artificial neuron cell as input. Weights indicate the importance of the information sent to an artificial cell and the effect on the neuron. These are coefficients determining the effect of inputs received by the artificial neuron. The summation function calculates net input to a cell. The value obtained from the summation function is processed by a linear or non-linear derivable activation function, and the output of operation elements is calculated. Activation functions enable curvilinear matching between input and output units. Out value is the value obtained after application of the activation function (Elmas, 2003; Öztemel, 2003).

Input and corresponding output information are fed into ANNs, and the neural network is trained to learn the relationship between input and output. ANNs can be grouped as feedforward and feedback or recurrent based on information flow type. In a feedforward network structure, information flows forward. Generally, in a feedforward network, there is one input layer, one or two hidden layers, and outputs layers. These networks are called multi-layered feedforward networks (Schalkoff, 1997).

Additionally, ANNs learn with supervised, unsupervised, and reinforcement learning. Back propagation algorithms in supervised learning have two types of connections. In the first form, corresponding outputs for certain inputs are computed in

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a forward connection and by using weights. In the second form, the connection is backwards; to minimise errors on output layers, weights are organised backwards. A feedforward operation transfers data on the input layer to the first hidden layer. In all these stages, the number of computing elements in each layer is important (Elmas, 2003).

Because there is no operation on the input layer of ANNs, the number of computing elements on input and output layers depends completely on the applied problem. The number of hidden layers and number of computing elements on hidden layers can be determined through trial and error, but the number of neurons on input and output layers is considered (Şen, 2004).

In ANNs, network input and output can be pre-processed to increase the efficiency of network training. This operation is called normalisation. In the literature, there are various data normalisation practices. This model adopted one of the most common methods, Min-Max, and data were reduced to between 0 and 1 (Jayalakshmi and Santhakumaran, 2011).

Results

In this study, regression and ANN models were developed to predict relative humidity in 177 regions in Turkey that have different geographical and climate conditions. Meteorological data of the past 30 years (1987-2017) in the 177 regions were obtained from Turkish State Meteorological Service (TSMS) and used for modelling (*Table 1*).

Table 1. *Descriptive statistics of sample plots*

	N	Minimum	Maximum	Mean	Std. Error	Std. Deviation	Variance
Altitude (m)	177	2	2400	722.93	45.062	599.516	359419.779
Precipitation (mm)	177	196.3	2279.5	627.146	20.6666	274.9511	75598.131
Temperature (°C)	177	3.7	20.2	13.169	0.2809	3.7367	13.963
Relative humidity (%)	177	46.7	82.0	63.338	0.4920	6.5455	42.843

Multiple regression analysis was used to determine the collective effect of dependent variables (temperature, precipitation, and altitude) on the independent variable (relative humidity). Based on multiple regression analysis, a mathematical equation with statistical significance of $p < 0.001$ and an R^2 value of 0.76 was computed (*Eq. 2*; *Fig. 3*).

$$RH(\%) = 105 - (2.477x\text{Temp.}) + (0.005xPrecip.) - (0.017xAltit.) \quad \text{(Eq.2)}$$

The Neural Network Toolbox plug-in for MATLAB was adopted to create the ANN model. In this ANN model, three input parameters (temperature, precipitation, altitude) and one output parameter (relative humidity) were predicted. When the model was structured, a 3-input, 1-output Levenberg-Marquardt (LM) algorithm was used for predicting relative humidity. First, on average 70% of data were used as “input” to train the network, 20% were used for testing the developed model, and 10% were used for validation purposes.

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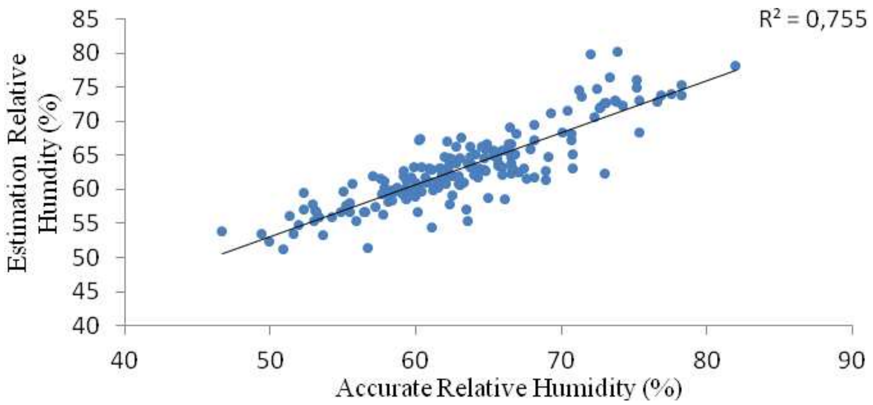

***Figure 3.** The relationship between accurate and estimation relative humidity (%)*

To determine the number of hidden layers and hidden neurons, various trials were conducted. The model with 1 hidden layer and 10 neurons provided the best results. Sigmoid activation function was used between layers. A maximum iteration number of 12 was obtained. In each execution, the algorithm was stopped after 6 epochs (*Fig. 4*). To determine the relationship between training data and output results obtained after training, regression analysis was applied. Analysis indicated an R^2 value of 0.84. Similarly, analysis was conducted on test data, validation data, and all data, with R^2 values of 0.83, 0.79, and 0.84, respectively (*Fig. 4*).

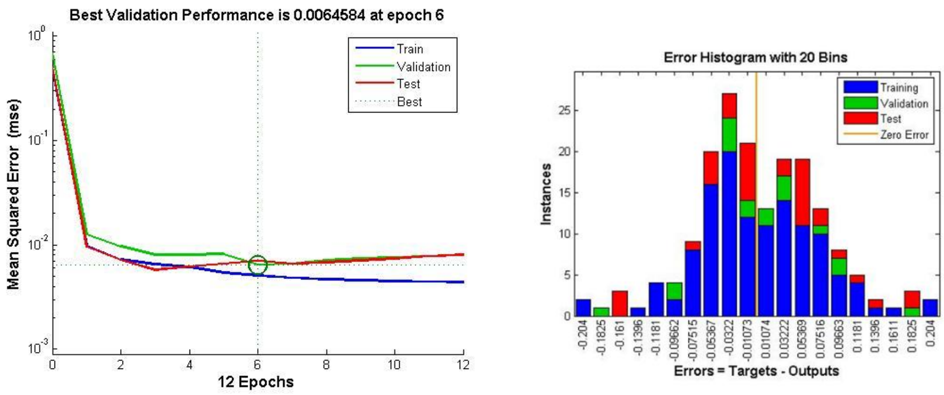

***Figure 4.** Best validation performance and error histogram of model*

To determine the relationship between training data and output results obtained after training, regression analysis was applied. Analysis result indicated R^2 value of 0.84. Similarly, analysis were conducted on test, validation, and on all data and R^2 values were found 0.83, 0.79, and 0.84 respectively (*Fig. 5*).

Discussion and conclusions

The purpose of this study was to predict certain parameters thought to related to relative humidity. To predict relative humidity, various models were developed by using temperature, precipitation, and altitude parameters. Calculated values for the model and

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real values were compared with statistical methods. Comparison results indicated that predicted and measured relative humidity values had good fit.

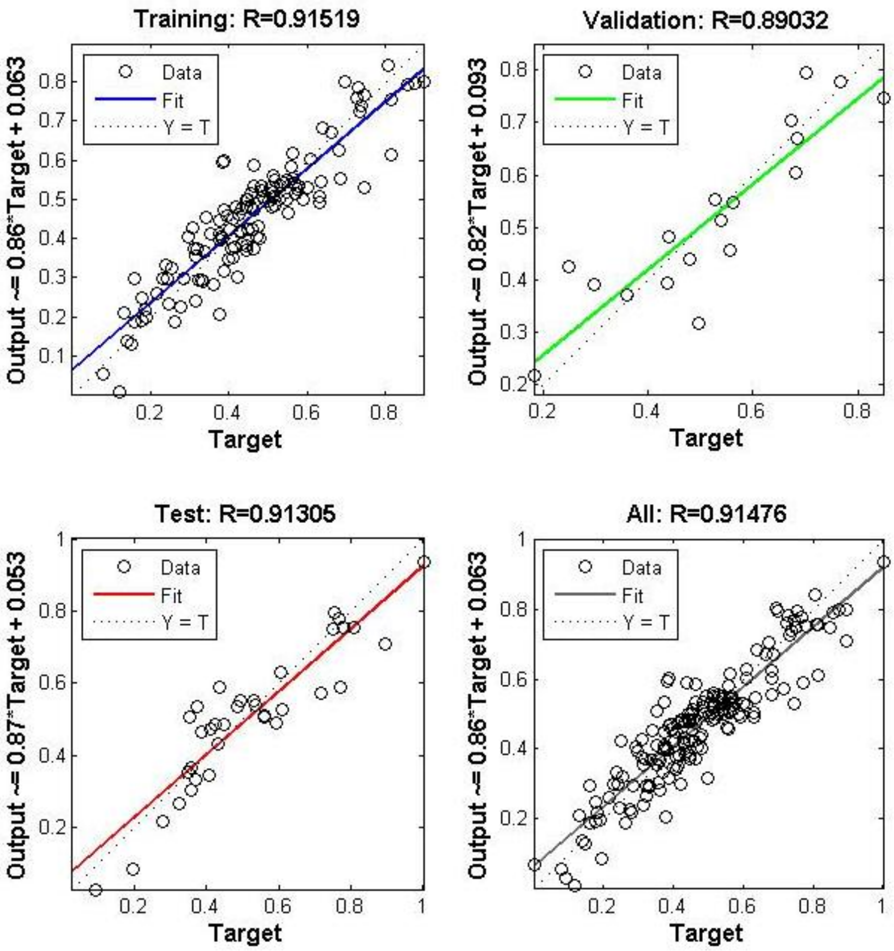

Figure 5. *Target and output relations of ANN model*

When analysed, the model developed with the ANN method had higher predictive power than the model developed with multiple linear regression. Similarly, Tokar and Johnson (1999) conducted a study to predict daily flow rate and used an ANN method with precipitation, temperature, and snow melting data. They compared results obtained from statistical regression and the ANN model and determined that the ANN model provided an extremely systematic approach. Kumar et al. (2015) predicted monthly average global solar radiations by using some parameters in ANNs and regression models. Comparison of ANNs and regression models have shown that the performance values of the artificial neural network models are better than the regression models as in this study. Yadav and Chandel (2014) predicted average global solar radiations by using some parameters in ANNs and regression models. ANNs had better performance than regression model too.

Similar studies were conducted by different researchers to predict other climate parameters. Erkaymaz and Yaşar (2011) predicted temperature by using water vapour pressure, relative humidity, wind, and air pressure parameters in an ANN method. In another study, Terzi (2006) predicted daily water temperatures of Eğirdir Lake by using

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daily air temperature, solar radiation, and relative humidity parameters in ANNs, Saplıoğlu and Çimen (2010) predicted missing daily precipitation of Portland by using measured daily precipitation parameters in ANNs.

Nastos (2014) predicted maximum daily precipitation for the next coming year of Athens (Greece) by using some parameters in ANNs. Correlation coefficient between the measured and predicted maximum daily precipitation were found to be 0.48. Deo and Şahin (2015) determined of feasibility of the ANNs for predicting the monthly Standardized Precipitation and Evapotranspiration Index (SPEI). In this purpose, a total of 30 ANN models were developed with 3-layer ANNs by them.

They ever that the ANNs was a useful data-driven tool for forecasting monthly SPEI based on performance evaluation measures. Ramasamy et al. (2015) predicted wind speeds with ANNs. Temperature, air pressure, solar radiation and altitude are taken as inputs for the ANNs to predict daily mean wind speeds. Correlation coefficient between the measured and predicted wind speeds was found to be 0.98.

Applying this study in Turkey under different geographic and climate conditions is important as these models can also be applied in climate conditions that are similar to those in Turkey. Additionally, when of the models was investigated, the strength of the model with ANN model was much higher than regression analysis; therefore, it is believed that the ANN model can be used for relative humidity prediction.

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MODIFIED ATMOSPHERE FOR THRIP DISINSECTION ON CUT LOTUS FLOWERS

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Abstract. In Thailand, lotus flowers are one of exportable products for flower trade industry. Common blossom thrip (*Frankliniella schultzei*) contamination on cut lotus flowers after harvest has been a major problem for export. *F. schultzei* is a quarantine insect pest. Modified atmospheres (MA) without oxygen content are considered an alternative to methyl bromide fumigation to control thrips. MA treatment has been used to control insect pests in agricultural product commodities. Different combinations of carbon dioxide, nitrogen, ozone levels, and treatment times were used to effect mortality of the common blossom thrips and postharvest quality of cut lotus flowers. Exposure to different concentrations of O_3 fumigation for 2 days could kill almost all *F. schultzei* (100% of larvae and ≥ 96% of adults). However, a concentration of 50-250 ppm of O_3 could not control *F. schultzei* on lotus flowers completely. O_3 fumigation caused color change in lotus flowers. High concentrations of O_3 (≥ 150 ppm) had a negative effect on the visual quality of lotus flowers. The results revealed that thrips mortality increased with increased CO_2 level and storage time. 100% CO_2 caused 100% mortality of both adults and larvae of *F. schultzei* when there was a 6-h exposure. MA were more effective in disinfecting of thrips on cut lotus flowers after 9 h fumigation to ≥ 50% CO_2 caused complete mortality to *F. schultzei*. There was not much difference in lotus color in response to atmosphere modified by a combination of CO_2 and N_2. Therefore, CO_2 disinfestation treatment has the potential to be developed commercially as an alternative postharvest control for common blossom thrips on lotus flowers.
Keywords: *CO_2 fumigation, color change of lotus flowers, Frankliniella schultzei, post-harvest treatment, visual quality*

Introduction

Sacred lotus (*Nulumbo nucifera* Gaerth) is an interesting plant. All parts of the lotus can be utilized and processed into a variety of products. Thailand has a suitable climate for this plant. Currently, Thailand has plenty of wetlands and often there are flooding problems; therefore it is suitable for lotus planting. Growers can generate an income throughout the year. *N. nucifera* has the potential to develop into a Thai economic crop for export. Major lotus flower markets in the country include Pak Klong Market, Four Corner Thai Market, and flower markets in each province. The major foreign markets include Hong Kong, Singapore, the Netherlands, Japan, and the USA.

Thrips are a major problem for lotus flowers before and after harvest. *Frankliniella schultzei* (Trybom) are found throughout the year and predominantly attack lotus flowers (Bumroongsook, 2018). Therefore, postharvest control is an important process to export cut lotus flower. Lotus flowers for export require consistent quality and phytosanitary certification to meet international standards. However, it is difficult to find effective and sustainable pest management both in pre-harvest and post-harvest. The lotus shape contributes to the habitat for thrips. In order to control the thrip population among lotus flowers, growers regularly spraying insecticides, which is

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ineffective because not all of the thrips are killed. Therefore, it is necessary to remove post-harvest thrips in accordance with the standard of agricultural material export to foreign countries. The Thai Department of Agriculture has set the criteria for the removal of insect pests for orchids and ornamentals, with the methyl bromide rate of 20-24 g/m^3 for 90 min or insecticide dips (Thai Department of Agriculture, 2003). However, some shipments of lotus flowers have been rejected over the past few years because of the presence of thrips on lotus flowers.

Fumigation has been the primary method of postharvest disinfection for insect pests of cut flowers and agricultural products to meet export requirements (Pupin et al., 2013; Williams and Muhunthan, 1998). At present, methyl bromide is the main method which exporters use for insect disinfestation of cut flowers prior to export. It is smokeless, colorless, odorless, and non-flammable. It is an ozone-depleting substance; therefore, safe alternatives must be used to protect life and the environment. Innovative insect disinfestation techniques should be assessed. Modified atmosphere (MA) is defined as altering the composition of atmosphere in the storage packaging in order to extend the shelf life of agricultural products (Jayas and Jeyamkondan, 2002; Liguori et al., 2015). MA treatments have been used to control insects in agricultural product commodities (Beaudry, 1999, 2000; Liu, 2008, 2013). MA includes ultra low oxygen, ozone, carbon dioxide, and a dynamic concentration of carbon dioxide and oxygen (Kerr et al., 2013; Pinhero et al., 2009; Raffo et al., 2009; Tiwari et al., 2010). Ozone causes mortality throughout all life stages of the bean thrips (Leesch et al., 2007).

Carbon dioxide (CO_2) has direct and indirect effects on the physiological response and mortality of insects (Grodzinski et al., 1999). It affects the knockdown and recovery of insects (Nilson et al., 2006). In addition, it induces toxicity at the cellular level of living organisms; it can cause damage to the respiratory system of insects and deplete air reserves and prevents the exchange of oxygen through the insect egg chorion (Lagunas-Solar et al., 2006). The rate of insect egg development depends on the CO_2 concentration (Kerr et al., 2013). High levels of CO_2 concentration are applied as an anesthetic for insect knockdown. Badre et al. (2005) showed that CO_2 blocked glutamate receptors. High CO_2 concentrations caused hearts of *D. melanogaster* larvae to stop working and interfered with synaptic transmission at the neuromuscular junction. Carbon dioxide exposure might affect insect longevity, mating success and growth, feeding, development, reproduction, and behavior (Bartholomew et al., 2015). With a 24-h exposure time at 25 °C, a lethal concentration of CO_2 for all bed bugs stages was approximately 30% (Wang et al., 2014). The use of high CO_2 concentrations in gastight large bags is a possible method for preventing the occurrence of post-harvest pests on agricultural commodities, including rice, cocoa, beans, and various dried herbs during storage (Pons et al., 2010).

Ozone (O_3) is the most common air pollutant. It is a product of nitrogen oxides and volatile organic compounds under sunlight. It has harmful effects on humans, plants, and insects (Kampa and Castanas, 2008; Díaz-de-Quijano et al., 2012; Farré-Armengol, 2016). Surface ozone decreases crop production, disables forest growth, and effects plant species composition. (Ashmore, 2015). At relatively low concentrations, tropospheric ozone is toxic to susceptible plant species and causes chlorotic mottle, pigmented stipple, and necrosis. Foliar injury of cutleaf coneflower growing near the edge of the Clingmans Dome trail was significantly greater than that in the Purchase Knob (70% vs. 40% ozone-injured plants, respectively), due to different ozone exposures (Szantoi et al., 2009). Injury were consistent within species, genetic variation

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among plants and diversification of physiological vigor (Brace et al., 1999). Ozone gas fumigation is a quarantine treatment to control stored pests and insect pests on fresh agricultural products (Hollingsworth and Armstrong, 2005). O_3 treatment can be used to control thrips and mealybugs on some selected agricultural products (Liu, 2013).

This research is undertaken to assess MA using dynamic concentrations of CO_2 balanced with N_2 and O_3 for thrip disinfestation and their impacts on the color of lotus flowers and quality.

Material and methods

Sample collection

Pesticide-free lotus farms that are situated at Ladkrabang District in Bangkok, Thailand were selected for sample collection. Standard buds of Roseum Plenum lotus flowers aged 9 to 10 days were collected from these lotus farms. Both adults and larvae of *F. schultzei* were obtained from blossom lotus flowers.

Experimental design

The experimental design was 6 × 7 factorial in completely randomized design to assess the effects of the combination of CO_2 and N_2 against *F. schultzei* on lotus flowers (30 replicates/group). Factor A is different composition of CO_2 balanced N_2 and factor B is exposure time.

Effects of O_3 against *F. schultzei* on lotus flowers was performed by using 7 × 7 factorial experiments in completely randomized design (30 flowers/group). Factor A is different composition of O_3 and factor B is exposure time.

The experiments were carried out at the entomological laboratory, King Mong's Institute of Technology Ladkrabang.

Effects of the combination of CO_2 and N_2 on F. schultzei

The experiment involved exposing MA to both adults and larvae of *F. schultzei*. *F. schultzei* were transferred to a young lotus petal and placed in a vial. Then, they were placed in a plastic chamber. The air was expelled and a gas mixture of CO_2 and N_2 was introduced into the air-tight plastic chamber. The experiments were conducted for various modified atmosphere packaging: 0, 25, 50, 75, 100% (v/v) CO_2 with remaining N_2, and stored for 1 to 3 days at 15 °C. After the treatments, insects were considered dead if they did not move. They were scored as alive if they moved. The dead insects were observed at 1, 2, and 3 days of exposure to the MA and storage temperature of 15 °C.

Effects of the combination of CO_2 and N_2 on F. schultzei on lotus flowers

A cut lotus flower aged 9 to 10 days was exposed to different combinations of CO_2 and N_2 and stored for 3-72 h. The ambient air was removed and the CO_2 and N_2 mixture was introduced into the polypropylene plastic package (10 × 15 inches) before being sealed by a gas filling vacuum packing machine (Hualiangthai model DZQ400/500). After the specified treatments, the package was opened to investigate dead or alive thrips after 1, 2, and 3 days of exposure to MA and a storage temperature of 15 °C.

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Effects of O_3 on F. schultzei

F. schultzei was placed on a young lotus petal, transferred to a vial, and kept in a gas-tight plastic chamber. The experimental procedure exposed different concentrations of O_3 (0, 50, 75, 100, 125, 150, 250, 500, 750, and 1,500 ppm) using an ozone generator (Enaly model OZX-300 AT). The observation times were 3, 6, 9, 12, 24, 48, and 72 h. The mortality of adult and larva of *F. schultzei* was assessed.

Effects of O_3 on F. schultzei on lotus flowers

A cut lotus flower aged 9 to 10 days was placed in a plastic chamber and exposed to different concentrations of O_3 (0, 50, 75, 100, 125, 150, 250 ppm) and stored for 3 to 72 h at 15 °C. The observation times were 3, 6, 9, 12, 24, 48, and 72 h. The mortality of *F. schultzei* was assessed. The color of petals at the outer curvature was measured with a miniscan (EZ4500, Hunter) and displayed color as three numerical values: L *, a *, and b *.

Data analysis

Analysis of variance was used to evaluate these treatment differences by comparing the mean of the control with the mean of each treatment followed by the Duncan multiple range test for mean separation ($p \leq 0.05$).

Results

Effects of the combination of CO_2 and N_2 on F. schultzei

The experiments evaluated the effects of a CO_2 and N_2 mixture on *F. schultzei*. Total mortality was achieved at ≥ 6 h using 100% CO_2 atmospheres (*Table 1*). At 25% CO_2, the mortality of thrip larvae during all exposure time was significantly lower ($p < 0.05$) when compared with any other CO_2 concentration. At 100% N_2, the percentages of dead insects were 63% at an exposure time of 24 h. Complete mortality occurred in 72 h. Within 48 h of exposure time, 100%N_2 was more effective in controlling thrip larvae than 25% CO_2 ($p < 0.05$). At 72 h exposure time, all treatments killed 100% of thrip larvae.

Table 1. *Effect of CO_2 and N_2 fumigation on mortality of F. schultzei larvae (N = 10)*

Treatment	Mortality percentage of thrips after (h) treatment[1]						
	3	6	9	12	24	48	72
Control	1.00c	3.70d	5.50d	10.00e	14.00e	53.00c	100.00a
100% N_2	4.60bc	16.00bc	34.00b	43.00c	63.00c	95.00a	100.00a
25% CO_2	1.00c	12.20c	21.00c	30.00d	42.00d	89.00b	100.00a
50% CO_2	3.70bc	19.00bc	38.00b	61.00b	86.00b	98.00a	100.00a
75% CO_2	7.40b	22.00b	39.00b	50.00c	85.00b	100.00a	100.00a
100% CO_2	21.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a

[1]Means followed by different letters indicate significant difference between concentrations

The mortality of *F. schultzei* adults under different MA conditions and storage times are illustrated in *Table 2*. *F. schultzei* adults showed almost the same response to different levels of CO_2 and N_2 mixtures as did larvae (*Tables 1* and *2*). Total mortality

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of adults occurred at ≥ 6 h using 100% CO_2 (*Table 2*). With the initial time of 48-h exposure, 100% N_2 was more effective in controlling *F. schultzei* adults than 25% CO_2 ($p < 0.05$). The results showed that complete mortality in all concentrations occurred 72 h after treatment.

Table 2. *Effect of CO_2 and N_2 fumigation on mortality of F. schultzei adult (N = 10)*

Treatment	**Mortality percentage of thrips after (h) treatment**[1]						
	3	**6**	**9**	**12**	**24**	**48**	**72**
Control	1.00c	3.70e	4.60e	9.20e	17.10d	60.00c	100.00a
100% N_2	4.60c	13.00cd	32.00bc	46.00bc	80.00b	96.00a	100.00a
25% CO_2	1.00c	9.30d	24.00d	40.00c	53.00c	93.00ab	100.00a
50% CO_2	1.90bc	15.00bc	29.00cd	32.00d	58.00c	86.00b	100.00a
75% CO_2	6.50b	19.00b	35.00b	51.00b	86.00b	100.00a	100.00a
100% CO_2	23.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a

[1]Means followed by different letters indicate significant difference between concentrations

Effects of the combination of CO_2 and N_2 on F. schultzei on lotus flowers

There was a significant effect of CO_2 balanced N_2 and exposure time on thrips mortality ($F_{5,1218} = 9457.12$, $p < 0.00001$; $F_{6,1218} = 27578.07$, $p < 0.00001$, respectively). Moreover, the significant interaction between treatments and exposure time on thrip mortality was observed ($F_{30,1218} = 1864.27$, $p < 0.00001$).

High mortality of thrips on lotus flowers packaged in plastic bags was observed under different MA and storage times (*Table 3*). The exposure time of 3 h and 100% CO_2 had the highest thrip mortality, followed by 100% N_2, 50% and 75% CO_2 having 46.29, 49.30, and 50.09% mortality, respectively whereby 25% CO_2 having the lowest mortality (39.21%). At 12 h of exposure time, the results showed no significant differences among treatments and no live thrips were observed with 100% N_2 and all concentrations of CO_2. Higher CO_2 concentrations and longer exposure time could increase mortality of mortality of *F. schultzei* (*Fig. 1*). All treatments killed 100% of *F. schultzei* kill after 48 h of exposure.

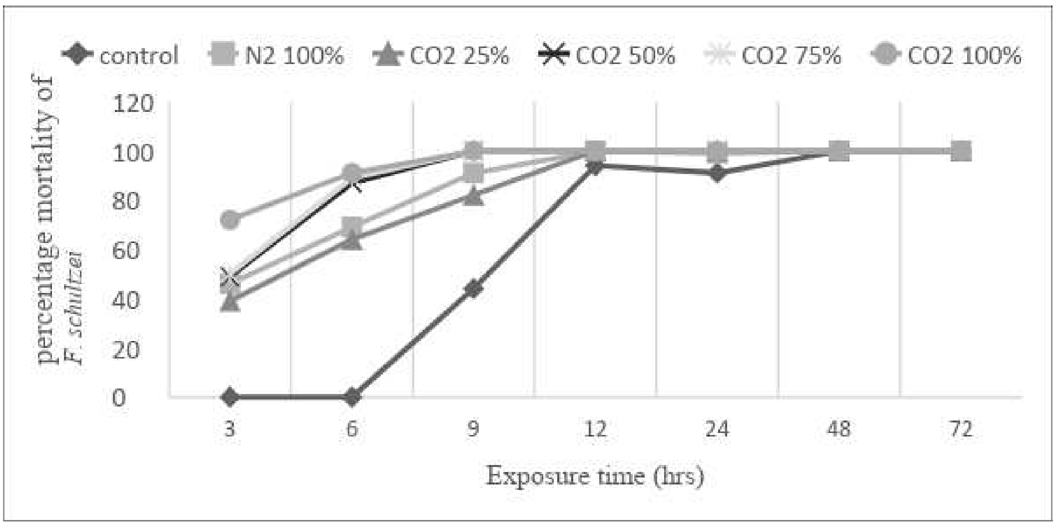

Figure 1. *Mortality of F. schultzei on lotus cut flowers under different concentration of CO_2 fumigation and exposure time*

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Table 3. *Effect of CO_2 and N_2 fumigation on mortality of F. schultzei on lotus cut flowers (N = 30)*

Treatment	Mortality percentage of thrips after (h) treatment[1]						
	3	6	9	12	24	48	72
Control	0.00d	0.00c	44.44b	94.73a	91.00a	100.00a	100.00a
100% N_2	46.25b	69.34b	91.66a	100.00a	99.00a	100.00a	100.00a
25% CO_2	39.21c	64.68b	82.77a	100.00a	100.00a	100.00a	100.00a
50% CO_2	49.30b	86.61a	100.00a	100.00a	100.00a	100.00a	100.00a
75% CO_2	50.09b	88.88a	100.00a	100.00a	100.00a	100.00a	100.00a
100% CO_2	72.22a	90.74a	100.00a	100.00a	100.00a	100.00a	100.00a

[1]Means followed by different letters indicate significant difference between concentrations

Effects of the combination of CO_2 and N_2 on lotus flower color

The color of lotus flower was measured using miniscan (EZ4500, Hunter). The color parameters L* (lightness), a* (red–green), and b* (yellow–blue) of lotus flowers under MA treatments were compared. No differences ($p > 0.05$) in the value of L* and a* were observed at 1, 2, and 3 days of exposure (*Table 4*). The a* value in the control was less than that of all levels of CO_2 and 100% N_2 on day 1 and 2 after exposure. On day 3, all treatments showed no significant difference in L*, a*, and b* values ($p > 0.05$).

Table 4. *Color of Lotus flowers after fumigation treatment (N = 30)*

Treatment	Day after treatment[1]								
	1			2			3		
	L	a	b	L	a	b	L	a	b
Control	59.90^{ns}	1.37b	18.79^{ns}	57.32^{ns}	2.91ab	18.98^{ns}	58.32^{ns}	4.77^{ns}	16.56^{ns}
100% N_2	56.89	5.80a	17.70	59.43	5.37a	23.35	61.67	5.47	15.12
25% CO_2	58.71	4.81a	17.62	59.29	4.88a	17.88	58.21	5.44	16.47
50% CO_2	57.15	4.57a	16.82	58.51	4.60a	16.75	58.50	5.76	17.14
75% CO_2	57.41	4.24a	18.35	57.61	2.17b	18.92	59.72	4.66	18.91
100% CO_2	57.86	4.98a	19.27	58.48	4.31a	17.12	58.62	3.64	18.26

[1]Mean followed by different letters indicate significant difference between concentrations

Effect of O_3 on F. schultzei

The lethal concentration of ≥ 50 ppm O_3 to *F. schultzei* larvae in a plastic chamber was 100% thrip mortality at 48 and 72 h of exposure time (*Table 5*). After 12 h of exposure to ozone fumigation, ≥ 500 ppm O_3 caused 100% thrip mortality. The results observed indicated that 1,500 ppm O_3 was the highest effective concentration as compared with the other treatments, and it caused 100% thrip mortality at 3 h of exposure.

Effects of O_3 on *F. schultzei* adults had similar patterns of response to O_3 fumigation as in the larvae (*Table 6*). At 12 h of O_3 fumigation exposure, ≥ 500 ppm O3 caused 100% mortality of *F. schultzei* adults. The results revealed that 1,500 ppm O_3 at 3 h of exposure caused 100% mortality of adults.

Effects of O_3 on F. schultzei on lotus flowers

The experiment was conducted to evaluate the different levels of O_3 fumigation (50-250 ppm) for disinfestation of *F. schultzei* on lotus flowers. Significant effect of O_3

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concentration and exposure time on thrips mortality was detected ($F_{6,1421}$ = 2762.65, p < 0.00001; $F_{6,1421}$ = 9,318.07, p < 0.00001, respectively). Moreover, there was a significant interaction between different O_3 concentration and exposure time ($F_{36,1421}$ = 34.88, p < 0.00001). Whereas, the results were displayed in *Table 7,* indicated that all these concentrations of O_3 could not eliminate the entire thrip infestation on lotus flowers. *F. schultzei* mortality increased, with increased O_3 levels and exposure time.

Table 5. *Toxicity of O_3 on mortality of F. schultzei larvae (N = 10)*

Ozone conc	Mortality percentage of thrips after (h) treatment						
(ppm)	3	6	9	12	24	48	72
Control	0.00e	0.00 f	0.00 e	19.73 c	39.17 bc	54.25 c	74.22 c
50	10.00 d	14.00 e	18.00 d	20.00 c	32.00 c	100.00a	100.00a
75	12.00 d	17.00 e	20.00 d	21.00 c	32.00 c	100.00a	100.00a
100	12.00 d	16.00 e	19.00 d	23.00 c	34.00 c	100.00a	100.00a
125	12.00 d	13.00 e	20.00 d	23.00 c	36.00 c	100.00a	100.00a
150	12.00 d	16.00 e	22.00 d	24.00 c	47.00b	100.00a	100.00a
250	22.00 c	28.00 d	30.00 c	38.00 b	46.00 b	100.00a	100.00a
500	66.00 b	79.00 c	94.00 b	100.00 a	100.00 a	100.00a	100.00a
750	67.00 b	86.00 b	95.00 b	100.00 a	100.00a	100.00a	100.00a
1500	100.00 a	100.00 a	100.00 a	100.00 a	100.00 a	100.00a	100.00a

[1]Mean followed by different letters indicate significant difference between concentrations

Table 6. *Toxicity of O_3 on mortality of F. schultzei adults (N = 10)*

Ozone conc	Mortality percentage of thrips after (h) treatment[1]						
(ppm)	3	6	9	12	24	48	72
Control	0.00f	1.00g	0.00d	12.00d	38.00c	48.00b	60.00b
50	10.00 e	12.00 f	20.00 c	21.00 c	42.00 c	96.00a	100.00a
75	10.00 e	12.00 f	22.00 c	23.00 c	47.00 bc	96.00a	100.00a
100	11.00 e	13.00 ef	23.00 c	24.00 c	48.00 bc	96.00a	100.00a
125	11.00 e	14.00 ef	23.00 c	24.00 c	49.00 bc	96.00a	100.00a
150	13.00 e	20.00 df	23.00 c	25.00 c	52.00 bc	100.00a	100.00a
250	23.00 d	24.00 d	28.00 c	30.00 b	55.00 b	100.00a	100.00a
500	48.00 c	59.00 c	65.00 b	100.00 a	100.00 a	100.00a	100.00a
750	60.00 b	72.00 b	70.00 b	100.00 a	100.00 a	100.00a	100.00a
1500	100.00 a	100.00 a	100.00 a	100.00 a	100.00 a	100.00a	100.00a

[1]Mean followed by different letters indicate significant difference between concentrations

Table 7. *Effect of O_3 fumigation on F. schultzei in lotus flowers (N = 30)*

Ozone conc	Mortality percentage of thrips after (h) treatment[1]						
(ppm)	3	6	9	12	24	48	72
Control	8.46 g	12.40 g	22.72 f	29.17 f	44.25 d	50.34d	74.22 c
50	23.81 f	27.03 f	38.88 e	58.73 e	69.47 c	75.22c	85.43 b
75	33.50 e	36.73 e	38.66 e	57.61 e	70.89 c	78.43b	94.90 a
100	36.42 d	38.57 d	44.82 d	61.65 d	85.20 b	90.32a	96.48 a
125	45.33 a	42.68 c	50.06 c	63.99 c	88.46 a	92.46a	98.23 a
150	42.76 c	47.93 b	54.06 b	68.77 a	84.84 b	93.33a	94.12 a
250	42.80 b	51.47 a	55.71 a	68.11 b	85.03 b	95.45a	96.50 a

[1]Mean followed by different letters indicate significant difference between concentrations

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Effects of O_3 on lotus flower color

The L*a*b* value of lotus petals exposed to different levels of O_3 fumigation (50-250 ppm) were different from those of the control (*Table 8*). The b* value of lotus petals in 48 and 72 exposure hours were not significantly different among treatments ($p > 0.05$). The color of lotus petals exposed to O_3 fumigation were slightly yellow (negative a*values), and the petals from control treatments were red (positive a* values). The color change of lotus flower petals was a response to ozone fumigation. It might not be enough to show much variation in flower color. Ozone fumigation made lotus flower color paler and yellowish. Slight malformation of lotus flowers was detected.

Table 8. *Effect of ozone fumigation on color of lotus flowers (N = 30)*

Ozone conc (ppm)	Day after treatment[1]								
	1			2			3		
	L	a	b	L	a	b	L	a	b
Control	60.90a	1.43a	18.79a	57.32a	2.91a	18.98^{ns}	58.32a	3.97a	16.06^{ns}
50	60.57a	1.33a	18.13a	49.30ab	-0.19b	21.74	51.06ab	-0.71b	16.91
100	51.65b	-0.21b	14.45b	47.62b	-0.81b	21.38	46.35b	-0.84b	14.92
150	51.06b	-0.01b	15.39ab	45.41b	-0.525b	19.91	46.70b	-0.74b	14.74
250	48.38b	-0.55b	13.06b	53.41a	-0.835b	21.43	51.75ab	-0.99b	15.15

[1]Mean followed by different letters indicated significant difference between concentrations

Discussion

Arthropods reduce their metabolic rate and increase memebrane permeability under high levels of carbondioxide atmosphere (Mitcham et al., 2006; Zhou et al., 2001). Spiracular opening due to low level of CO_2 can cause insect death, whereas at higher CO2 concentrations, metabolic reduction is an important factor for mortality (Janmaat et al., 2001). Held et al. (2001) observed that exposure over 12 h to >99% N_2 or CO_2 accounted for 100% mortality of aphids, mites, thrips, and whiteflies. CO_2 levels > 20% are toxic to insects and higher concentration cause insect mortality (Carpenter and Potter, 1994; Mitcham et al., 2006; Zhou et al., 2001). Consistent with the previous studies, using different combination of CO_2 and N_2 for the disinsection of *F. schultzei* on lotus flowers in polypropylene plastic packaging could eliminate all thrips on lotus flowers at 12 h of exposure time.

The results obtained in this study agree with Niakousri et al. (2010) whereby lower ozone concentration required longer exposure time to have effective fumigation. Ozone is unstable and has a short half-life and converts to oxygen during ozonation. Our results indicated that under ≥ 150 ppm O_3 fumigation caused color change and slight malformation of lotus flowers. This caused the flowers to be unacceptable for the markets. All of the ornamental plants tested were blemished due to O_3 treatments (Hollingsworth and Armstrong, 2005). The studies indicated that ≥ 500 ppm of O_3 is needed to have complete control of *F. schultzei,* but the lotus flowers are damaged from this fumigation. The previous studies showed that agricultural products with waxy surfaces could tolerate the high concentrations of ozone fumigation such as waxy leaves, waxed oranges (Hollingsworth and Armstrong, 2005; Leesch et al., 2003). There was more moisture among lotus petals in the bag, which increased the control of thrips. Cut flowers lose water through transpiration (Sankat and Mujaffar, 1994). This might be one factor responsible for thrip mortality.

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Gas concentration, fumigation time and temperature are three important key variables for the success of MA (Armstrong and Whitehead, 2005). An increase in concentration of CO_2 or O_3 in fumigation and exposure time would increase *F. schultzei* mortality. Dhouibi et al. (2015) use CO and CO_2 fumigation for *Ephestia kuehniella* and *Ectomyelois ceratoniae* in dates and indicated that insect mortality depended on gas levels and exposure time. Controlled atmosphere and temperature treatment system can control C. sasakii found in apples (Son et al., 2012). However, insect pest could become more resistance to MA treatment via a physiological adaptation (Chervin et al., 1996).

Conclusions

MA treatments are environmentally friendly methods and have no harmful effects to human health. Gas combination treatments and longer storage time along with low temperature during storage are variable factors for effective disinfestation control. MAP of any concentration of CO_2 balance N_2 at 9 h of exposure time had complete mortality of *F. schultzei*. Increased concentrations of CO_2 and longer exposure times increase thrip mortality. Ozone fumigation has the effect as CO_2, whereas the mortality of thrips increased as the concentration of O_3 increased. $O_3 \geq 500$ ppm caused 100% mortality in adult and larvae. However, at this concentration, petals had spot discoloration and slight deformation. Fumigation of lotus flowers with CO_2 balance N_2 could be used to replace methyl bromide for disinsection. Further research would be necessary to determine how the concentration of CO_2 changes during storage time and the moisture contents from plant transpiration in packaging and interaction among them effect thrip disinfestation.

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DETERMINATION OF ENVIRONMENTAL PERCEPTIONS AND AWARENESS TOWARDS REDUCING CARBON FOOTPRINT

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Abstract. In today's world where humans have exceeded limits of the planet, natural resources are becoming increasingly important. Together with the concept of sustainable living, making the agenda to conduct ecological measurements in the early 1990s, the concept of ecological footprint offers great contributions to creating ecological risk profiles. Today, it can be seen that many countries' Ecological Footprint exceeds their biocapacity. One of the most important components of the concept, carbon footprint is strongly associated with society's consumption forms, perceptions and attitudes. In this study carried out in Izmir province, in Turkey, trends and perceptions towards reducing ecological footprints left by society were analysed within the scope of ecosystem services using survey method. As a result of the study, it has been observed that ecological deficit emerging as a result of humans' perspectives on the natural environment and their consumption habits is increasing with each passing day.
Keywords: *natural resources, ecosystem services, ecological footprints, society trends, sustainability*

Introduction

Environmental and social effects caused by modern societies measuring prosperity level with consumption are one of the most important current problems of humanity. In terms of seriousness of its magnitude, a need has emerged to develop new policies at global level. Within the framework of sustainable growth and due to economic and environmental problems including recent economic crisis, climate change etc., and new concepts have emerged such as environmental sustainability, green economy, green growth, low-carbon economy, sustainable production and consumption (Öztürk, 2012; Velioğlu and Aydın, 2017). In terms of Resource Efficiency Policies, it is necessary to keep damages given to natural life at minimum, ensure economic growth in a way to sustain world resources, thus, there is a need to determine level of efficient consumption of resources in terms of exhaustible resources (Reyhan, 2014).

Within the scope of United Nations Development Programme (UNDP) and United Nations Environment Programme (UNEP), an organization was established in which economic development and environment were discussed as two inseparable aspects together with "Climate Partnership" and "Poverty and Environment Fund" initiatives launched in Nairobi-Kenya as of February 2007. While Poverty and Environment Fund aims at improving environmental management and increasing environmental investments, Climate Partnership Initiative emphasizes capacity increase in terms of technology transfer and more in developing countries' fight against climate change. Thus, boundaries of ecology which was initially considered only as a natural sciences discipline rapidly

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expand towards both economic and social fields, it is becoming increasingly possible for economy and ecology to meet on a common ground with social content.

Exceeding biophysical boundaries leads to damaging of systems that form basis of natural balance and economy. This situation, however, makes it more difficult to reach a certain quality of life in the future and maintain such a life. However, complex structure of natural systems makes it difficult to identify at which point lifestyle associated with consumption level strays from sustainability. Ecological Footprint is an indicator that measures ecological footprint in certain categories within the framework of narrow definition of natural services benefited from. Developed by Mathis Wackernagel and William Rees in early 1990s, this concept is defined in the form of production of natural resources containing agriculture, livestock, fishery and forest products and CO_2 emission, and in the form of biological productive field required to meet these demands (Ewing et al., 2010). Calculation of ecological footprint is extremely important in terms of seeing how much humans behave over the biocapacity while meeting their needs and in terms of determining how much humans use their natural resources by comparing countries' ecological footprints with biocapacity (Coşkun, 2013).

Ecological Footprint of Turkey in 2007 was 2.7 global hectare (gha), and this rate is equal to world average, and lower than the average of Mediterranean countries. Ecological Footprint of consumption in Turkey is 50% over global biocapacity per capita. This case is the sign of a non-sustainable lifestyle in Turkey as in the rest of the world. Since national biocapacity of 1.3 gha per capita is below world average (1.8 gha/person) in Turkey, its national ecological debt is much higher than its global deficit. This deficit called as "ecological limit excess" results in supplying biocapacity need partially from abroad.

Countries that calculate ecological footprint and analyse its outcomes will be able to determine ways to eliminate risks associated with ecological deficit. For our country and more importantly for the future of our world, we need to reduce our ecological footprint. Beyond our individual responsibilities with a view to reduce ecological footprint and promote more sustainable lifestyles, there are also some other designs that we should adopt globally (Keleş, 2007). One of the most important components of ecological footprint concept is carbon footprint. Carbon footprint is strongly associated with society's consumption forms (Ebadı, et al., 2016), perceptions and attitudes.

The aim of this study is to examine ecological footprint data of Turkey within the scope of ecosystem services, and to find out society's economic and social attitude on the subject. To do this, participants' carbon footprints were calculated based on their consumption data. Thus, it is considered that this study will contribute to definition of development goals that also consider environmental sustainability by means of preparing ecological footprint statement, and will contribute to determination of individual and social responsibilities to reduce adverse ecological affects emerging depending on the use of natural resources. In this sense, children and the young who will be individuals of the future and who should receive environmental education at an early age will see the concept of ecological footprint and limits of our consumerism as a society (Öztürk, 2010).

Theoretical foundations

Ecosystem services

Scientific history of the concept of ecosystem services dated back to the 1970s, use of this concept in the literature gained speed in the 1990s (De Groot et al., 2010). The

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concept of "Ecosystem Services" was defined firstly by Daily (1997) as "ecosystems required for maintaining human life, and situations and processes carried out by species". Costanza et al. (1997) describe ecosystem services as benefit from ecological functions; De Groot et al. (2002) describe them as benefits from both processes and functions; Boyd and Banzhaf (2007) describe them as only directly benefited products (Çoban and Yücel, 2018).

This concept has become more widespread with the United Nations Millennium Ecosystem Assessment (MEA) report published in 2015. In this report, while ecosystem services were described as natural capital, it was emphasized that natural capital's capacity of supporting future generations with human activities decreased. The second major international study following the MEA report is the research titled "The Economics of Ecosystem and Biodiversity (TEEB)" which emerged with the initiative of UN Environment Program. One of the most important objectives of TEEB report in which biodiversity, economics and interdisciplinary work was "to emphasize increasing costs of biodiversity loss together with degradation in ecosystem" (Demiroğlu and Karadağ, 2015). The concept of ecosystem services was discussed, interpreted and defined in different ways by associating more with ecosystem process and biodiversity subjects. Basically, ecosystem services are also defined as "situations, processes, functions, benefits and all products provided by ecosystems to maintain human life and ensure welfare among humans" (Albayrak, 2012).

Ecosystem services, in addition to the manufacture of products, also includes many life support functions such as regulation of climate, maintaining soil and flood control, clean-up, recycling and renovation of natural resources. In MEA report, ecosystem services were discussed by welding classifying them in 4 major ecological function groups including resource providing services, regulating services, cultural services and supporting services, and under 30 categories. In the TEEB report, ecosystem services were discussed by classifying in 4 major ecological function groups including resource providing services, regulatory services, culture and comfort services and habitat services, and under 22 categories (*Table 1*).

Today, the subject of ecosystem services is addressed as an important issue by government agencies, academicians, non-governmental organizations and the private sector. For example, one of the most important international conventions in which Turkey is also a party, United Nations Biodiversity Convention defines actions towards the protection of ecosystems and services they provide within the scope of Aichi Biodiversity Objectives.

The importance of ecosystem services is accepted not only with regard to processes of biodiversity but also in the field of sustainable development. In "Sustainable Development Summit" held in 2015, 17 Sustainable Development Objectives were determined by United Nations member countries to end poverty, to eliminate inequality and injustice and to fight against climate change until 2030. In this respect, protection, restoration and maintenance of ecosystems and their services were defined under various objectives. Addressing ecosystem services in such distinct conventions and legal processes is an indication of further increase in importance of this issue at global scale (URL-1, 2018).

However, social and economic development of community and thus providing a quality life have brought along many problems. Today, more than 50% of the world's population live in cities, and it is estimated that this figure will reach 70% in the year 2050 (Anna et al., 2016). Since urbanization changes forms of personal land use, transportation, industrial and agricultural production, consumption and social activities, it adversely affects natural resources. Scattered development of modern cities and expansion to large areas have adversely affected natural habitats. For example, Thailand has lost 96% of its wetlands, Australia has lost 95% of its wetlands, and USA has lost 53% of its wetlands (Çoban and Yücel, 2018). This situation has resulted in increasing importance of natural resources and growth of human-induced ecological footprints threating natural resources. In this context, the concept of Ecological Footprint has become one of most popular and widespread indicators for sustainability assessment and resource management.

Table 1. *Classification of ecosystem services: MEA (2015) and TEEB (2015) classifications*

Resource providing services (MEA)		Resource providing services (TEEB)	
1	Food	1	Food
2	Biological raw material	2	Raw materials
3	Decorative resources	3	Ornamental resources
4	Genetic resources	4	Genetic resources
5	Fresh water	5	Water
6	Biochemicals and medicinal products	6	Medical resources
Regulating services		**Regulatory services**	
7	Air quality arrangement	7	Cleaning the air
8	Climate regulation	8	Climate regulation (including C-sequestration)
9	Water flow control	9	Regulation of water flow
10	Erosion control	10	Erosion prevention
11	Water treatment and waste control	11	Waste treatment (especially water treatment)
12	Epidemic control	12	Biological control
13	Pest control	13	Corruption prevention or mitigation
14	Pollination	14	Fertilization
15	Natural risk mitigation	15	Continuity of soil productivity
Cultural services		**Culture and comfort services**	
16	Recreation and ecotourism	16	Recreation and tourism
17	Moral and ethical values	17	Spiritual experience
18	Social relationships	18	Social relationships
19	Information system	19	Information for cognitive process
20	Educational value	20	Educational value
21	Sense of place and space		
22	Aesthetic values		
23	Inspiration		
24	Cultural heritage value		
25	Cultural diversity		
Supporting services		**Habitat services**	
26	Nutrient recycling	21	Continuity of the life cycle
27	Hydrological cycle	22	Protection of gene pool
28	Photosynthesis		
29	Soil formation		
30	Primary production		

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Concept of ecological footprint was firstly developed by Mathis Wackernagel and William E. Rees in early 1990s and used to conduct ecological measurements. This criterion denominates biologically productive soil and water field in global hectares that are required for production of resources consumed and disposal of waste created in the meantime by means of existing technology and resource management (Wackernagel and Rees, 1996; Tosunoğlu, 2014). Biocapacity, on the other hand, is indicator of production capacity of renewable natural resources in a geographical region. In other words, ecological footprint refers to used resource, and biocapacity refers to available resources. Identification of global hectare is used for measurements. A global hectare represents the production capacity of 1-hectare field over world's average efficiency. Thus, total amount of resources obtained from different types of field within a certain period of time and demand for these resources are degraded to a common unit and expressed in numeric values (WWF, 2012). This allows consistent measurement of global ecological footprint and biocapacity and comparison with each other (Wilson and Anielski, 2005; Wackernagel et al., 2014).

Ecological footprint is an important criterion that reveals how much land and water are needed to reproduction of natural resources consumed and recovery of wastes emerging in the meantime. This, in a way, describes the load that mandatory consumption of natural resources by humans to survive in this world establishes over sustainability of ecosystem (Keleş, 2007). As a part of nature, humans meet their biological and cultural needs from nature. Everyone has a powerful effect on the world. As a result of production and consumption, the sum of these effects creates ecological footprints. Many behaviors we do with habits brought along by our lifestyles actually result in growth of our carbon footprints (Kaypak, 2013). Ecological footprint, in general, is obtained by calculating components such as carbon footprint, cropland footprint, forest land footprint, grazing land footprint, built-up land footprint and fishing grounds footprint (WWF, 2012; Mancini et al., 2016).

Ecological footprint components and status in the world and Turkey

Natural balance of world's ecosystem is broken every day, and natural resources are insufficient to meet humans' needs. According to data from WWF (2012) Turkey's Ecological Footprint Report, Turkey's ecological footprint has tripled since 1961. In world ranking, Turkey is in the 63rd place among 154 countries (WWF, 2012). Analysing global ecological footprint and biocapacity rates per capita in *Figure 1,* it is remarkable that global ecological footprint started to exceed world's biocapacity in the 1970s and ecological deficit began to occur. Today, this deficit still continues to increase. Continuation of such increase in global footprint will bring along many problems.

In *Figure 2,* Turkey's ecological footprint and biocapacity rate are given. Analysing *Figure 2,* it can be seen that total ecological footprint in Turkey began to exceed national biocapacity for the first time in 1977, and this deficit continued increasing in following years. While Turkey's ecological footprint per capita was equal to global footprint average in 2005 with 2.7 gha, biocapacity per capita in the country was 1.3 gha. This situation shows that national level ecological debt among people living in Turkey is higher than world average. According to data from WWF (2012) Turkey's Ecological Footprint Report, percentage distributions of Turkey's global ecological footprint as per components are given in *Table 2*. Accordingly, carbon footprint created 55% of total footprint. Following the carbon footprint, cropland footprint is the second

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biggest component threating the global ecological system with a rate of 21%. These are followed by forest land, grazing land, built-up land and fishing grounds footprints respectively. The fact that the carbon footprint ratio has such a large share requires focusing primarily on the carbon footprint. For example, the use of renewable resources instead of fossil resources will contribute significantly to reducing carbon footprint and thus reducing ecological footprint (Özsoy, 2015).

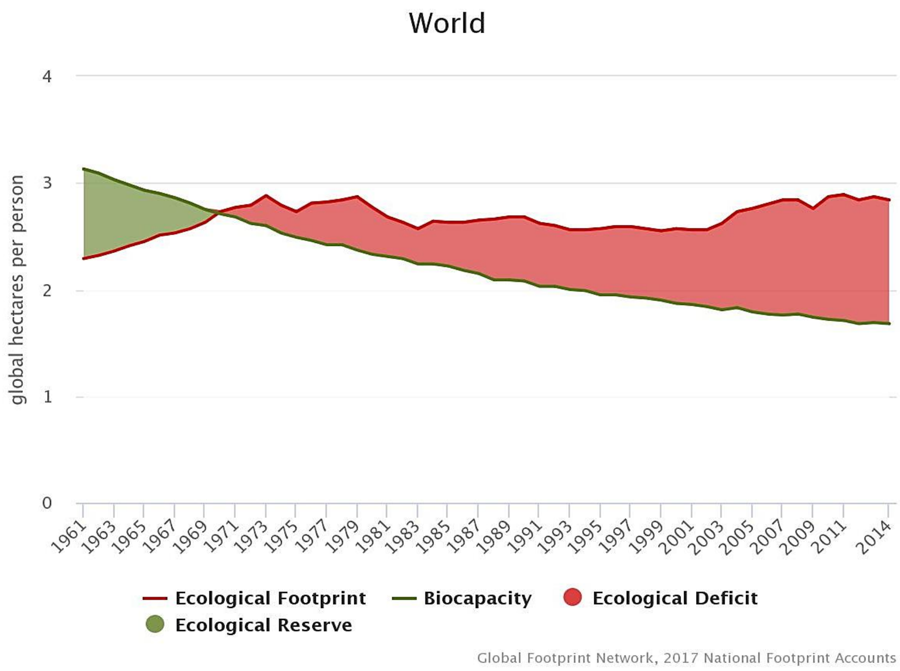

Figure 1. *The world's ecological footprint and biocapacity status (URL-2, 2018)*

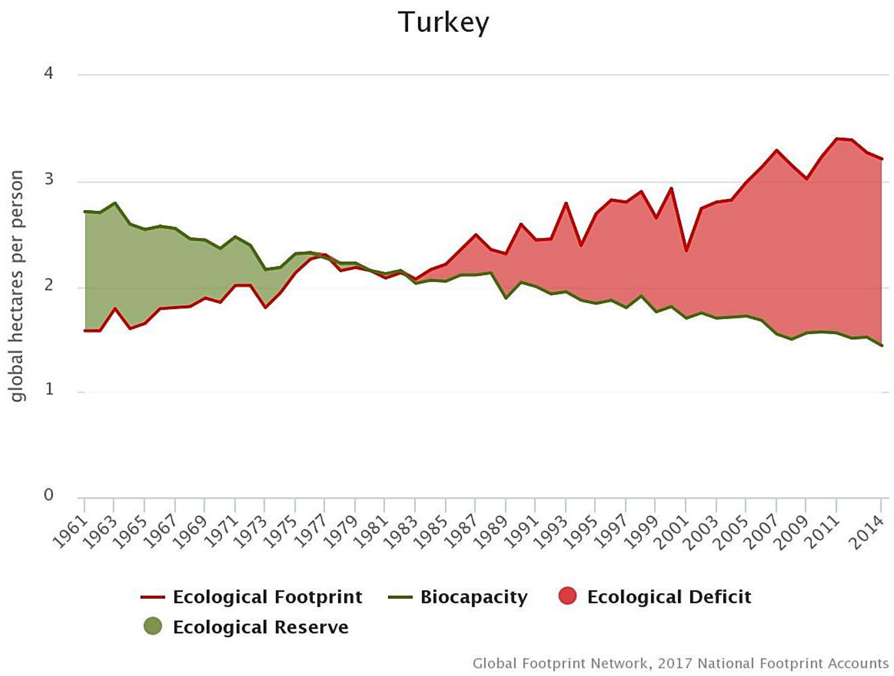

Figure 2. *Turkey's ecological footprint and biocapacity status (URL-2, 2018)*

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Table 2. *Turkey's ecological footprint components (WWF, 2012)*

Ecological footprint components	Portion
Carbon footprint	0.55
Cropland	0.21
Forest land	0.10
Grazing land	0.08
Built-up land	0.03
Fishing grounds	0.03
Total	1.00

Seeing from which consumption category our personal footprint is originated is important in terms of understanding the relationship between our daily activities and usage of natural resources. Price of footprints is paid by the whole world in different forms. Humanity must find way to live in limits of capacity of natural resources as soon as possible and implement this way rapidly for a sustainable life, change their understanding of development and differ their consumption/management of natural resources. That is because there are no other planets to live. For this reason, it is necessary to maintain global biocapacity to ensure that current resources we use for meeting our needs will also be beneficial to future generations (Dinç, 2015). These socially, economically and ecologically left traces are not only personal but also social traces. To reach sustainability, both individualistic lifestyles should be analysed and resource productivity in social expenses should be questioned (Akman et al., 2000).

Materials and methods

Damages towards nature due to insensible usage of resources are increasing day by day, consequent adverse effects are felt more seriously especially in metropolises. In addition, consumptions among people living in metropolises and congruently their calculated global footprints tend to be higher. Therefore, the 3rd largest country of Turkey, İzmir, was selected in the study. İzmir province is located in the West of Turkey, in the coast of Aegean Sea. In İzmir, Mediterranean climate is dominant with hot and dry conditions in summer and warm and rainy condition in winter, annual precipitation is between 700-1200 mm. In terms of flora, its flora is under the influence of Mediterranean climate, and all kinds of Mediterranean plants grow in the province. In terms of field distribution, forest fields take 41% share, agricultural lands take 28% share, grass-pasture lands take 4.5% share, and other type of lands take 26.5% share (URL-3, 2017).

Research data was collected with a survey study carried out with observation and interview operations. Survey study was carried out with survey forms created for this purpose in February-April 2017. In this sense, the path followed in the study was in the form of (1) collecting necessary files-documents required for the research by means of literature analysis, (2) survey-interview studies towards participants, and (3) statistical analysis and evaluation of obtained data. In survey forms, questions towards determining socio-demographic characteristics and multiple-choice questions towards

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participants' attitudes and perceptions were used. To determine participation in propositions, five-point Likert-type scale was used. In preparation of survey form, studies conducted by William (1992), Wackernagel and Rees (1996), Wilson and Anielski (2005), Keleş et al. (2008), Deniz and Ok (2016) Pamukoğlu et al. (2017) and Özmış and Tolunay (2017) were also used as reference. In accordance with objective of the study, questions prepared for calculating carbon footprint data among components of ecological footprint were based on questions used worldwide to calculate carbon footprint with a view to meet the standard and conduct comparisons. Carbon footprint was preferred in calculations since it is a component that can be increased or decreased depending on people's, thus society's, perception, attitudes and consumptions, and since it can be reduced with education, personal awareness and sense of responsibility. For this purpose, data was collected related to participants' food choices, organic food consumption amounts, dressing preferences, imported food and product consumption preferences, furniture and electronic appliances consumption preferences, recycling preferences, amusement activities, annual energy consumption amounts and annual travel conditions. CO_2 emissions occurring as a result of these activities were calculated with "Carbon Footprint Calculator" (URL-4, 2017).

Survey was carried out with the participation of 221 people. Survey forms were analysed using SPSS 20.0. In analyses, primarily all questions and answers were digitized as per order statistics, and frequencies and percentages were utilized according to characteristics of question. Kolmogorov-Smirnov and Shapiro-Wilk-W tests were used to determine whether data was parametric or not, it was determined that, at 95% confidence level, data did not have normal distribution ($P < 0.05$), in other words, not parametric (Mendes and Pala, 2003). Chi-square ($\chi 2$) independence test was used to check whether there were relationships among some variables (Eymen, 2007; Kalaycı, 2010). As a result of reliability analysis conducted for expressions provided in five item Study five-point Likert-type, Cronbach's Alpha value was calculated as 0.702. Alpha is an important concept in assessment of surveys and measurement of internal consistency, it tests how much closer a group of items is associated as a group and how much reliability a study offers (Tavakol and Dennick, 2011).

Results

Profile characteristics

Within the scope of the research, participants were asked some socio-demographical questions on their age, gender, education, work/professional status and monthly income. Analysing *Table 3,* it can be seen that a total of 221 people has participated in the study, 127 of them are female (57.5%), 94 of them are male (42.5%). In the study, five different education groups were formed. Educational status is one of most important factors for affecting, changing and guiding studies for participants' perceptions and attitudes. Obtained data shows that rate of participants with university graduate and postgraduate education is high. The participants were categorized into seven different professional groups. Especially unemployed and housewives were defined as separate groups. That is because housewives' attitudes towards food shopping, dress shopping and energy consumption have a decisive role on the footprint left by household. The participants were categorized into five income groups based on income level. The minimum wage is approximately 2000 TRY in Turkey (CSGB, 2018). This situation shows that 53.4% of participants have an income at minimum wage level.

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Table 3. *Profile characteristics of participants*

Characteristics	Group	n	%
Gender	Female	127	57.5
	Male	94	42.5
Age	18-30	80	36.2
	31-40	63	28.5
	41-50	41	18.6
	51-60	23	10.4
	60<	14	6.3
Education	Primary school graduate	41	18.6
	Secondary school graduate	15	6.8
	High school graduate	61	27.6
	University graduate	88	39.8
	Postgraduate	16	7.2
Occupation	Officer	69	31.2
	Self-employed	40	18.1
	Engineer	38	17.2
	Housewives	21	9.5
	Unemployed	21	9.5
	Retired	19	8.6
	Educator	13	5.9
Income level (TRY)	0-1000	43	19.5
	1001-2000	75	33.9
	2001-3000	58	26.2
	3001-4000	31	14.0
	4000<	14	6.3

Participants' preferences related to reduction of carbon footprints

Generally accepted questions were used in survey form with a view to calculate participants' carbon footprints, ensure consistency and make comparisons. Calculations were carried out with "Carbon Footprint Calculator" tool (URL-4, 2017). Analysing participants' answers given to these questions, participants' total carbon footprint, carbon footprint per participant and average carbon footprint amount per participant were calculated approximately. Besides, number of trees to be planted for reducing and compensating carbon footprint was determined approximately (*Table 4*).

Table 4. *Information regarding participants' ecological footprints*

Number of participants	Average CO_2 emission (ton/person)	Total CO_2 emission (ton)	Number of trees to be planted for reduction	
			Per person	Total
127 (Female)	5.22	662.94	8	976
94 (Male)	8.62	810.28	12	1162
221	6.67	1473.22	10	2210

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Analysing *Table 4,* while the amount of CO_2 emissions per capita among women is 5.22 tons, the amount of CO_2 emissions per capita among men is 8.62 tons. Assessing the situation in terms of total amount of CO_2, women's total amount of CO_2 emission was calculated as 662.94 tons approximately, and men's total amount of CO_2 emissions was calculated as 810.28 tons approximately. As long as a tree lives, it converts approximately 0.73 tons of CO_2 (URL-4, 2017). Accordingly, to compensate 810.28 tons of CO_2 produced by male participants, approximately 12 trees per capita and a total of 1162 trees should be planted. To compensate 662.94 tons of CO_2 produced by women, approximately 8 trees per capita and a total of 976 trees should be planted. Total amount of CO_2 emission produced by 221 participants in the study is approximately 1473.22 tons. The amount of average CO_2 emission per capita is 6.67 tons, number of trees to be planted per capita for compensation of 6.67 tons of CO_2 produced is approximately 10 pieces. Number of trees to be planted for compensation of 1473.22 tons of CO_2 produced is approximately 2210 pieces.

One of the most important services that forest ecosystems are received within the scope of ecosystem services is carbon storage and thus reduction of carbon footprint (Coşkun and Gençay, 2011). In this context, participants' opinions on whether they will provide financial support to afforestation studies for reducing their carbon footprints they have left are presented in *Table 5.* According to findings obtained, 71.5% of participants reported that they may support afforestation in different amounts, and 28.5% of them reported that they would not support such studies. Analysing *Table 5,* 54 of participants indicated that they would contribute an amount of TRY 10, 25 of them would contribute TRY 20, 21 of them would contribute TRY 30, 14 of them would contribute TRY 40, 8 of them would contribute TRY 50 and 36 of them would contribute TRY 100.

Table 5. *Participants' amounts of contribution to afforestation works*

Amount of support (TRY)	**10**	**20**	**30**	**40**	**50**	**100**	**Not giving support**	**Total**
Participants	54	25	21	14	8	36	63	221
Percent (%)	24.4	11.4	9.5	6.3	3.6	16.3	28.5	100
Total amount of support (TRY)	540	420	630	560	400	3600	0	6150

With reference to these data, calculations towards afforestation works to be conducted for compensation of carbon footprint left by participants and relevant costs were made in accordance with the communiqué no. 298 issued in 2014 by General Directorate of Forestry (GDF) (OGM, 2014). Red pine species (*Pinus brutia* Ten.) was selected for calculations, since this species was suitable for ecological conditions of study area and it has been used in afforestation works by GDF. Accordingly, according to bill of costs for afforestation in 2016 issued by GDF, afforestation cost for 1 hectare of pine trees (*Pinus brutia* Ten.) is around TRY 12480 for İzmir province. Number of pine trees that should be included in 1 ha varies between 150-250 units depending on yield strength. Since number of trees to be planted for 1473.22 tons of CO_2 produced by 221 participants is a total of 2210 and considering the fact that number of pine trees to be planted in a hectare is accepted as 200 pieces, there is a need for approximately 2210/200 = 11 ha of afforestation. Since afforestation of 1 ha field is TRY 12248, there

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is a need for a total of 11*12480 = TRY 137280 investment for 11 ha field. Participants indicated that they would contribute an amount of approximately TRY 6150. Deducting the obtained contribution amounts from the total amount of investment needed, an investment amount of 137280-6150 = TRY 131130 should be met by the government.

These calculations are quite overall calculations, these obtained figures are open to debate. Therefore, what is important here is services that forest ecosystems receive within the scope of ecosystem services and the fact that these services are offered to public for free. Forest ecosystems reduce carbon footprint left by the humanity thanks to their carbon storage services. However, how and until when this process will continue shape humans' perspectives on natural environment and their consumption formats. In this context, chi square test was used to analyse participants' socio-demographic characteristics and whether they would support afforestation studies to reduce carbon footprint. According to the findings of analysis, statistically significant relationship was found between participants' age, education, profession and monthly income and the subject of "*whether they would provide financial support in afforestation works towards reducing carbon emission*". Statistically significant relationships were not determined between gender and participants' responses (*Table 6*).

Table 6. *Participants' state of contribution to afforestation works*

Expression	Characteristics	$\chi 2$	sd	p
"Whether they would provide financial support in afforestation works towards reducing carbon emission"	Gender	4.536	6	0.605
	Age	38.773	24	**0.029***
	Education	59.498	24	**0.000***
	Occupation	65.801	36	**0.002***
	Income level	68.628	24	**0.000***

*<0.05

Analysing the situation in terms of participants' ages according to assessments made, while individuals between 18-30 age group preferred contributing to afforestation works, other age groups did not prefer supporting these works. Analysing the situation in terms of participants' educational levels, it was determined that, as participants' educational level increased, their rate of financial contribution also increased. Analysing the situation in terms of participants' professions, it can be seen that housewives group preferred not to support and academician/educators group preferred supporting more. Analysing the situation in terms of monthly incomes, while low income groups preferred not to support, higher income groups preferred supporting.

Participants' perceptions and attitudes towards ecosystem services and carbon footprints

According to the results of reliability analysis conducted with regard to reliability of expressions towards measuring participants' perceptions and attitudes for their ecological footprints and their answers, Cronbach's Alpha was calculated as 0.702. This situation shows that expressions towards measuring participants' perceptions and attitudes and their answers were reliable. Analyses conducted towards these statements were listed based on averages of answers given, and they are presented in *Table 7*. Accordingly, the statement "*I contribute to sustainable management of forests using*

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certificated forest products" is the highest-level expression with a rate of 4.01. The expression "*I leave places clean in recreation and picnic fields*" was the lowest rated expression with 1.48 rate. Analysing statements included in *Table 7,* it is remarkable that participants have quite complex attitudes towards reducing their ecological footprints.

Discussion and conclusion

The concept of ecological footprint was developed in the early 1990s and it is used for conducting ecological measurements. This criterion denominates biologically productive soil and water field in global hectares that are required for reproduction of resources consumed and disposal of waste created in the meantime by means of existing technology and resource management. In ecological footprint calculations, distinct components are used such as carbon footprint, cropland, forest land, grazing land, built-up land and fishing grounds. Among these footprints, carbon footprint was chosen in the study since it is dependent upon humans' consumption forms and it can be reduced by improving personal perceptions, attitudes and responsibilities.

Table 7. *Participants' expressions on their perceptions and attitudes towards footprints*

Expressions	Mean	Std. deviation	Variance
I contribute to sustainable management of forests using certificated forest products	4.01	1.254	1.573
I prefer driving LPG vehicles rather than petrol-driven vehicles	3.44	1.579	2.493
I collect domestic wastes such as cardboard, paper, metal, plastic etc. in different bags	3.38	1.424	2.029
I plant saplings to reduce carbon emissions	3.29	1.410	1.988
I do not prefer air transport which results in high CO_2 emissions	3.19	1.459	2.127
I prefer consuming white meat rather than red meat	3.01	1.468	2.154
I prefer organic foods rather than hormone-injected foods	2.74	1.088	1.183
I carry out my responsibilities to maintain ecological functions of forest ecosystems	2.69	1.216	1.478
I prefer public transportation if travel distance is distant	2.58	1.401	1.962
I travel to place in walking distance on foot or by bike	2.41	1.364	1.861
I plug out electronic appliances even when they are off since they continue to spend electricity	2.29	1.249	1.559
I use energy saving bulbs at home, workplace or in office rather than classical incandescent lamps	2.23	1.347	1.815
Forest ecosystems provide many benefits to society other than their wood products	2.16	1.163	1.352
Services offered to society by forest ecosystems are important in terms of their vital functions	1.68	0.884	0.781
Forest ecosystems are the most important component in prevention of global warming	1.65	0.885	0.783
I care not to waste water	1.64	0.855	0.731
I leave recreation and picnic fields clean	1.48	0.795	0.633

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Accordingly, the participants indicated that they can support afforestation works by a total amount of TRY 6150 to reduce their carbon footprint. Deducting this support amount from required investment amount, remaining investment of TRY 131130 should be met by the government. In other words, these services observed by forest ecosystems are provided to society for free by the Government. However, global footprint left by the humanity as of the 1970s has exceeded World's biocapacity, and emerging ecological deficit is increasing with each passing day. However, how and until when this process will continue will determine humans' perspectives on natural environment and their consumption habits. Besides, in the study, it is also seen that participants have quite complex attitudes towards reducing their ecological footprints. Participants' complex responses to the statements that were specified in terms of reduction of their ecological footprints also show that they should develop new behavior forms related to their perceptions, attitudes and responsibilities. In this case, it will be suitable that decision makers and authorities will take measures that will allow development of behavior patterns towards changing society's consumption habits and reducing their ecological footprints.

According to the results of study, humans leave their ecological footprints on the Earth event in their most basic choices (for instance, housing, nutrition, travel etc.). It is possible to reduce these footprints with some measures implemented (especially reduction of consumption, prevention of wasting resources etc.). However, forest ecosystems have a crucial role in reducing footprints. This service provided by forest ecosystems is of vital importance. Afforestation investments bear an important task in completing this service. Usually, governments do not demand a direct charge from citizens for such investments. However, this situation does not mean that society does not require environmental responsibility, on the contrary, it makes it necessary for society to become more sensitive. Besides, afforestation works contribute to not only carbon storage services but also provision of many services including regulation of water regime and precipitation, prevention of erosion, visual and aesthetic values etc. On the other hand, the carbon footprint is the total amount of carbon dioxide emissions that occur at each stage of the product life cycle (Wiedmann ve Minx, 2008). For this reason, forest ecosystems are used to store every ton of carbon released into the atmosphere. If the size and productivity of this forest ecosystem is not sufficient to store the amount of carbon released to the atmosphere, then the ecological deficit arises (Özsoy, 2015). Since 1961, Mediterranean countries have been in a situation of biocapcity deficit, with its demand for ecological services increasingly exceeding supply. In order to maintain this situation, the import of ecological assets from regions outside the Mediterranean is necessary. When Turkey is compared with Mediterranean countries in this aspect, only two countries provide approximately 50 percent of the natural endowment (biocapacity) of the Mediterranean basin: France (31%) and Turkey (16%). The ecological footprints of the Mediterranean countries show great differences among themselves. For example, three countries among Mediterranean countries alone contribute to more than 50 percent of the Mediterranean region's ecological footprint: France (20%), Italy (19%) and Spain (15%) (Moore et al., 2018). Differences among countries' ecological footprint values are most likely driven by socio-economic factors, such as disposable income, infrastructure, and cultural habits (Baabou et al., 2017). Also, differences in consumption habits and quantities of these countries cause their footprints to be different. Especially food accounts for a large part of the Mediterranean countries' overall ecological footprint. According to result of other study, in

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Mediterranean countries food and beverages represents 28% of the regional ecological footprint (Galli, 2017). In also Turkey, ecological footprint of personal consumption is predominantly made up of food (52%) (WWF, 2012). Mediterranean countries also vary considerably in terms of their food supply. Most countries in the region have a daily food supply that is 20% to 40% higher than the average FAO determined minimum daily dietary energy requirement benchmark of 2500 kcal cap^{-1} day^{-1}. Moreover, comparison of countries' food footprint intensities reveals a considerable spread, with the lowest value found in Egypt and the highest in Portugal (FAO et al., 1985; Pimentel and Pimentel, 2003). These comparisons and assessments show that our personal and social consumption behaviors need to be revised.

According to research findings, participants have quite complex attitudes towards reducing their ecological footprints. For example (*Table 7*), the statement "*i contribute to sustainable management of forests using certificated forest products*" achieved the highest average, on the other hand, the expression "*forest ecosystems are the most important component in prevention of global warming*" achieved one of the lowest score averages with a rate of 1.65. Again similarly, while the idea of recycling wastes has a higher average score as a positive expression, reducing water waste has relatively lower average. These findings show that participants have some basic information and environmental responsibilities, also show that participants do not consider some components. A similar situation occurs between expressions "*i plant saplings to reduce carbon emissions*" (mean: 3.29) and "*forest ecosystems are the most important component in prevention of global warming*" (mean: 1.65). Accordingly, participants know that tree planting is effective in reducing carbon emissions, but they do not exhibit more sensitive approach. However, it shows that participants have low levels of awareness and sensitivity in terms of functions of forest ecosystems in prevention of global warming caused by carbon accumulation in the atmosphere (CO_2). These findings are important for future studies that will be conducted in the form of training, raising awareness and providing responsibility (Aoki and Akai, 2013; Sarıkaya et al., 2016). In addition, these data provide convenience for individuals following their resource use and sustainability (Mattila et al., 2011). On the other hand, these findings contribute to determination of social perceptions for planning and managing natural resources in a sustainable way. Thus, measures can be taken that will allow participants to reduce their ecological footprints. That is because people consume their natural resources rapidly and increase their ecological footprint amounts with each passing day.

Consequently, forest ecosystems carry out many vital services apart from being sources of wood origin products, and they bear vital importance for a sustainable life. Increasing society's level of knowledge and awareness related to forest ecosystems is significant in terms of ensuring continuity of ecosystem services and reducing ecological footprints left. However, it was found out that participants shaped components constituting their ecological footprints as per their consumption forms and they did not consider the effect of these behavior patterns in reducing their footprints. Therefore, public awareness should be raised in terms of vital importance of reducing ecological footprints, factors establishing ecological footprint and their relationships with each other. By acting as a whole for a sustainable life, ecological footprints should be reduced. This disrupted balance of the world is actually humanity's own balance.

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APPENDIX

Survey Form

"Determination of Environmental Perceptions and Awareness Towards Reducing Carbon Footprint."

1. Gender: ( ) Female ( ) Male

2. Age group: ( ) 18-30 ( ) 31-40 ( ) 41-50 ( ) 51-60 ( ) 60<

3. Education level: ( ) Primary school graduate ( ) Secondary school graduate
( ) High school graduate ( ) University graduate ( ) Postgraduate

4. Work/Professional: ( ) Officer ( ) Self-employed ( ) Engineer ( ) Housewife
( ) Unemployed ( ) Retired ( ) Educator

5. Income level: ( ) 0 - 1000 TRY ( ) 1001 - 2000 TRY ( ) 2001 - 3000 TRY
( ) 3001 - 4000 TRY ( ) 4000< TRY

6. Your meal preference: ( )I'm a vegetarian. ( )I usually consume fish. ( )I usually consume white meat.
( ) I consume red and white meat. ( ) I consume red meat every day.

7. Your organic food consumption preference:
( ) I only buy organic products. ( ) Some of the products I buy are organic.
( ) I do not buy any organic products. ( ) I don't know if you buy organic products.

8. Your clothing preference: ( ) I use second-hand clothes. ( ) I buy new clothes if I need them.
( ) I follow the latest fashion.

9. Imported food and product consumption preference:
( ) I just consume domestic products.
( ) I usually consume domestic products.
( ) I rarely prefer to consume domestic products.
( ) Domestic or imported products, it does not matter to me.

10. Your furniture and electronics goods preference:
( ) I usually buy new products, but I use them for at least 5 years.
( ) I buy the latest technology or fashionable products.

11. Your recycling preference:
( ) All my garbage is recycled. ( ) Most of my garbage is recycled.
( ) Some of the my garbage is recycled. ( ) My garbage is not recycled.

12. Your entertainment activities:
( ) I do activities that do not produce carbon (e.g. walking, cycling).
( ) I usually go to movies, bars and restaurants.
( ) I make intensive carbon-producing activities (e.g. flight, motorcycle).

13. How many cars do you have?: ( ) 1 ( ) 2 ( ) Three and over ( ) I do not have a car

14. Your annual energy consumption:
( ) Electricity use (kWh)............ ( ) Natural gas (m3)………..
( ) Coal (Ton)……… ( ) LPG (Liter)……….. ( ) Other………

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15. Number of round-trip flights per year:
( ) Short-haul flights (Turkey-Europe) ....
( ) Medium-haul flights (Turkey-China) ....
( ) Long-haul flights (Turkey-America) ....

16. Traveling by car: ( ) Car model…………… ( ) The annual amount of distance (km)………

17. The annual distance from your travel by bus and train (km)?
( ) Bus……..… ( ) Train………… ( ) Light rail……...… ( ) Subway…..…..…..

18. Trees are one of the most important elements that reduce carbon dioxide emissions and store carbon. Therefore do you want to provide financial support for afforestation efforts to reduce carbon emissions?

( ) 10 TRY ( )20 TRY ( ) 30 TRY ( ) 40 TRY ( ) 50 TRY ( ) 100 TRY ( ) No

Mark the following expressions as appropriate.

Expressions	Always	Often	Sometimes	Rarely	Never
I travel to place in walking distance on foot or by bike.					
I prefer public transportation if travel distance is distant.					
I prefer driving LPG vehicles rather than petrol-driven vehicles.					
I do not prefer air transport which results in high CO2 emissions.					
I plug out electronic appliances even when they are off since they continue to spend electricity.					
I use energy saving bulbs at home, workplace or in office rather than classical incandescent lamps.					
I collect domestic wastes such as cardboard, paper, metal, plastic etc. in different bags.					
I care not to waste water.					
I leave recreation and picnic fields clean.					
I plant saplings to reduce carbon emissions.					
I prefer organic foods rather than hormone-injected foods.					
I prefer consuming white meat rather than red meat.					
I contribute to sustainable management of forests using certificated forest products.					
I carry out my responsibilities to maintain ecological functions of forest ecosystems.					
Forest ecosystems provide many benefits to society other than their wood products.					
Services offered to society by forest ecosystems are important in terms of their vital functions.					
Forest ecosystems are the most important component in prevention of global warming.					

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ARTIFICIAL INTELLIGENCE APPLICATIONS FOR PREDICTING SOME STAND ATTRIBUTES USING LANDSAT 8 OLI SATELLITE DATA: A CASE STUDY FROM TURKEY

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Abstract. Forest resources inventory is one of the essential parts of the sustainable forest management. Remote sensing applications have broad usage areas for this aim, since field measurements are costly, time consuming and laborious. Monitoring forest resources with various satellite images has found wide usage areas in forestry. In this study, the relationships between some stand attributes (mean diameter, basal area, stand volume and number of trees) and texture values obtained from Landsat 8 OLI satellite image were investigated for Crimean pine (*Pinus nigra* J.F. Arnold subsp. *pallasiana* (Lamb.) Holmboe) stands in Kastamonu region of Turkey. The multiple linear regression analysis and artificial neural networks (ANN) were utilized to fit stand parameters using texture values. To form the ANN architectures, various transfer functions in hidden and output layers and number of nodes ranged from 1 to 20 in hidden layer were used, and a total of 180 architectures were designed for each stand attribute. The results indicated that the regression models had low R^2 values (0.399 for mean diameter, 0.337 for basal area, 0.332 for stand volume, and 0.183 for number of trees), and the most of the ANN models were better than the regression models for predicting stand attributes. The model containing hyperbolic tangent transfer functions in both hidden and output layers for mean diameter (R^2 = 0.593), logistic transfer function in hidden layer and hyperbolic tangent function in output layer for basal area and stand volume (R^2 = 0.632 and 0.650, respectively), and hyperbolic tangent function in hidden layer and linear function in output layer for number of trees (R^2 = 0.610) were the best ANN models. This study concluded that the ANN models developed with Landsat 8 OLI were useful to predict stand parameters better than the regression models in Crimean pine stands located in Kastamonu, Turkey.
Keywords: *forest inventory, satellite image, artificial neural networks, multiple linear regression*

Introduction

Forest ecosystems provide so many different economic, ecological and social products and services such as wood and non-wood products, carbon sequestration, biodiversity conservation, wildlife contribution, water and soil protection, and recreation. However, these ecosystems face deforestation and desertification problems because of human activities, unusual climatic conditions due to global warming, insect attacks, erosion, wildfires, etc. In this negative situation, sustainable management of forests becomes more important. The inventory and monitoring of forest resources are the main parts of the sustainable forest management.

Traditional forest inventory based on ground measurements is very hard, time-consuming and costly in a great forest area (Lu et al., 2004). In addition to ground measurements, remote sensing data are also widely used for forest management planning (Holmgren and Thuresson, 1998). Particularly for predicting stand-level circumstances across great forest areas, remote sensing data have been utilized efficiently to offer valuable information regarding forest constructions for forest

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managers (Cohen et al., 1995). Since the early 21st century, Landsat satellite data have had broad usage in many forestry studies such as land cover or land use change (Günlü et al., 2008; Huang et al., 2009; Hu et al., 2016), stand parameters predictions (Kahriman et al., 2014; Günlü and Başkent, 2015; Günlü and Kadıoğulları, 2018) and aboveground biomass estimations (Zheng et al., 2004; Lu et al., 2012; Günlü et al., 2014a).

Forest attributes such as mean diameter, stand basal area, stand volume and numbers of trees are important for forest management planning activities. In studies on predicting stand parameters using Landsat satellite images, the models were developed by using reflectance and vegetation indices values obtained from Landsat satellite data (Hall et al., 2006; Mohammadi et al., 2010; Günlü and Kadıoğulları, 2018). Besides, there are several studies to predict the stand parameters (especially for predicting aboveground biomass) by using texture values generated from Landsat satellite data (Kelsey and Neff, 2014; Safari and Sohrabi, 2016). In general, regression analysis was used to predict stand parameters with remote sensing data (Hyde et al., 2006; Gama et al., 2010). Recently, some studies have been performed to estimate the stand parameters using artificial neural networks (ANN) techniques (Ercanli et al., 2016; Reis et al., 2018).

The aims of this research are (*i*) to generate the ANN models estimating relationships between the stand parameters (mean diameter, stand basal area, stand volume and numbers of trees) obtained from ground measurements and texture values generated from Landsat 8 OLI satellite image, (*ii*) to assess the utilization of the ANN models for attaining the estimation of stand parameters by matching the regression analysis outcomes in pure Crimean pine stands of Kastamonu Regional Directorate of Forestry.

Materials and methods

Study area

This study was carried out on pure Crimean pine stands within the boundaries of Kastamonu Regional Directorate of Forestry located in the Black Sea Region of Turkey (*Fig. 1*). This directorate is the first among 28 regional directorates of Turkey in terms of growing stock (201.4 million m^3) and annual volume increment (4.3 million m^3), and these amounts equal to about 13% of the whole country. Forests cover a total of 1.26 million ha, which is about 66% of the total area of the region (General Directorate of Forestry, 2015). Crimean pine is also the most common tree species with 0.38 million ha distribution area in the region (General Directorate of Forestry, 2006).

Mean annual temperature and precipitation of the study area are 9.8 °C and 480 mm, respectively. The slope varies from 0% to 80%, and the elevation ranges between 604-1579 m above sea level, with an average of 1149 m. All sampled areas consist of naturally regenerated pure Crimean pine stands.

Field measurements

Field measurements for this study were conducted in 184 circular sample plots during summer seasons of 2015 and 2016. Sample plot sizes were determined considering stand crown closures, which is a key parameter to decide the sample plot sizes for forest inventory activities in Turkey. According to the crown closure classes of the stands (i.e., 11-40%, 41-70% and >71%), the sizes of circular sample plots were set

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as 800, 600 or 400 m^2 with the radii of 15.96, 13.82 or 11.28 m, respectively. For some sample plots including excessive number of trees, the radii of the plots were reduced to 7.98 m (i.e., 200 m^2 in size). Sample plots were randomly selected to represent different stand characteristics, which directly affect the growth rate, such as stand densities, diameter classes and site qualities.

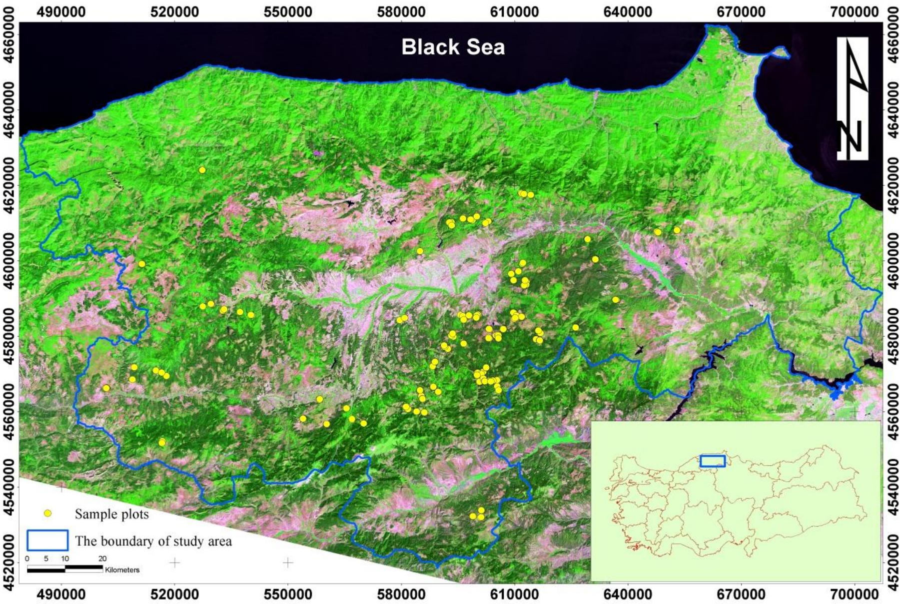

Figure 1. *Landsat 8 OLI satellite image of study area*

Measurements in the sample plots were initiated by recording UTM coordinates using a Global Positioning Systems (GPS) receiver placed at the center of every sample plot. In each sample plot, the diameter at breast height (*dbh*) over-bark was measured to the nearest 0.1 cm using calipers for each trees greater than 7.9 cm *dbh*, and the number of trees measured were counted. In total, 5757 trees were measured for *dbh*, and the number of trees measured in sample plots were ranged from 7 to 92 trees. Basal areas of each tree in sample plots were calculated. Volumes of the trees were predicted using the single-entry volume equation developed by Sakici et al. (2018) for Crimean pine stands located in the study area. After measuring and calculating the sample trees’ dendrometric values, total basal area and total volume of each sampling plot were calculated by summing basal areas and volumes of sample trees in sample plot, respectively. The total basal area and total volume of sample plots were ranged between 0.341-4.371 m^2 and 0.848-45.203 m^3, respectively. Then, stand parameters such as mean diameter (d_q), stand basal area (G), stand volume (V) and number of trees per hectare (N) were calculated using *Equations 1*, *2*, *3* and *4* for each sample plots.

$$d_q = \sqrt{\frac{\sum dbh^2}{n}} \quad \text{(Eq.1)}$$

$$G = \frac{10000}{A} g \quad \text{(Eq.2)}$$

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$$V = \frac{10000}{A} v \qquad \text{(Eq.3)}$$

$$N = \frac{10000}{A} n \qquad \text{(Eq.4)}$$

where, d_q is the quadratic mean diameter (cm), *dbh* is the tree diameter measured at 1.30 m from the ground, G is the stand basal area (m^2 ha^{-1}), g is the total basal area of sample plot (m^2), V is the stand volume (m^3 ha^{-1}), v is the total volume of sample plot (m^3), N is the number of trees per hectare, n is the number of trees in the sample plot, and A is the sample plot area (m^2).

The 184 sample plots were randomly divided into two groups to generate the model development and validation data sets. The data from 138 sample plots (75% of total data) were used to develop the models. As independent data set, the data from remaining 46 sample plots (25% of total data) were reserved for validation process of the developed models. The summary statistics for the sample plots were given in *Table 1* for modeling and validation data groups, separately.

Table 1. *Summary statistics of sample plots*

	Mean diameter (cm)	Basal area (m^2 ha^{-1})	Stand volume (m^3 ha^{-1})	Number of trees (ha^{-1})
Modeling data (n = 138)				
Minimum	10.4	5.685	14.128	88
Maximum	51.7	75.873	882.648	1800
Mean	28.3	31.160	273.305	570
Standard deviation	10.3	16.172	177.385	345.6
Validation data (n = 46)				
Minimum	12.9	7.224	19.976	88
Maximum	53.7	66.113	639.660	1850
Mean	29.7	36.288	323.589	634
Standard deviation	10.8	14.202	150.482	380.6

Remote sensing data and processing

The Landsat 8 Operational Land Imager (OLI) satellite image used in this research was free downloaded from United States Geological Survey Global Visualization Viewer (URL-1, 2014). The Landsat 8 OLI satellite image has already included atmospheric and geometric corrections, and radiometric calibration. In this study, five bands (Band 2, 3, 4, 5 and 7) of Landsat 8 OLI satellite image with a spatial resolution of 30 meters were used. Eight different texture metrics (contrast, correlation, dissimilarity, entropy, homogeneity, mean, second moment and variance) for each band were produced using four different window sizes (3 x 3, 5 x 5, 7 x 7 and 9 x 9). To produce texture values, ENVI software was used. After that, depending on the size of the sample areas, buffer zones (with radius of 7.98, 11.28, 13.82 or 15.96 m) were composed around the sample plots' centers in accordance with UTM coordinates recorded by GPS receiver. The texture images produced for each band were overlaid using GIS with the sample plots. The texture values of each sample plot were calculated

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by two different methods. If the sample plot centers were at or near the center of a pixel, the texture values of the sample plots were calculated as the value of a single pixel for 200, 400 and 600 m^2 sample plots, while calculated by taking the average of some pixel values in the buffer zone for 800 m^2 sample plots (*Fig. 2a*). However, in the second method, if the sample plot centers were far from the pixel centers, the texture values of each sample plot were computed by taking the average of pixel values in the buffer zone for all sample plot sizes (*Fig. 2b*). In this way, the texture values for five bands, four window sizes and eight different texture metrics were calculated. Thus, a total of 160 texture values were obtained for each sample plot.

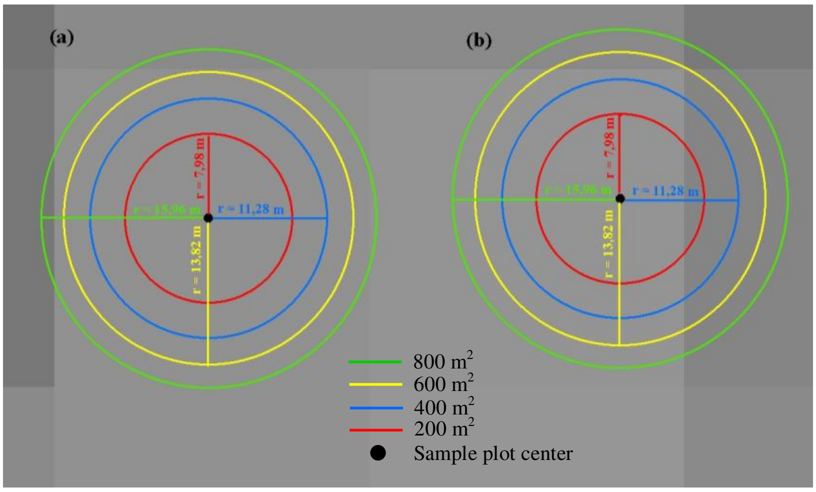

Figure 2. *Texture value calculation of sample plots according to buffer zones on satellite image*

Regression models

The multiple linear regression analysis was used to develop the equations modeling the interactions between stand parameters (mean diameter, stand basal area, stand volume and number of trees) and texture values obtained from Landsat 8 OLI satellite image. To obtain multiple linear regression models based on stepwise variable selection method, the ordinary least squares technique was used. The dependent variables in these models were quadratic mean diameter, stand basal area, stand volume and number of trees per hectare, and independent variables were 160 texture values of modeling data group containing 138 sample plots. The dependent variables were observed values from field works, while the independent ones were produced values from satellite image. The stepwise regression procedure in IBM SPSS 23 software was used to select the statistically significant ($p < 0.05$) texture values as predictor variables to estimate stand parameters. The relationships between stand parameters and texture values were assumed linear as given formula below (*Eq. 5*):

$$Stand\ Parameter = b_0 + b_1X_1 + b_2X_2 + \ldots + b_nX_n + e \quad \text{(Eq.5)}$$

where *Stand Parameter* is quadratic mean diameter, stand basal area, stand volume or number of trees per hectare, X_i (i = 1 to n) are texture values, b_i represent the model coefficients, and e is the error term.

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Artificial neural networks

Artificial intelligence techniques were also employed to model stand parameters beside multiple linear regression analysis to determine prediction success of these techniques and to compare with regression modeling. For this purpose, artificial neural network (ANN) models were developed for each stand parameter examined in this study. For defining the architecture of neural networks, there are several criteria such as number of layers, learning algorithms, transfer function forms, number of nodes in hidden layer and determination of data sizes for training, verification and test processes. The ANN models developed in this study consist of three layers: input, hidden and output layers. The feed-forward back-propagation network structure was selected because of its success popularity in forestry literature (e.g., Özçelik et al., 2014; Diamantopoulou et al., 2015). The learning algorithm used in ANN models was the Levenberg-Marquardt algorithm. Three transfer function forms (linear, logistic and hyperbolic tangent) were examined in hidden and output layers, separately (*Eqs. 6*, *7* and *8*).

$$f(s) = purelin(s) = s \qquad \text{(Linear function)} \qquad \text{(Eq.6)}$$

$$f(s) = logsig(s) = \frac{1}{1 + e^{(-s)}} \qquad \text{(Logistic function)} \qquad \text{(Eq.7)}$$

$$f(s) = tanh(s) = \frac{1 - e^{(-2s)}}{1 + e^{(-2s)}} \qquad \text{(Hyperbolic tangent function)} \qquad \text{(Eq.8)}$$

where $s = \sum w_i x_i$, w_i are weights and x_i are the input variables.

To determine the most predictive alternatives, the number of nodes in hidden layers ranged from 1 to 20 adding one by one in training process of ANN models. Thus, a total of 180 ANN model architectures were created for each stand parameter (*Fig. 3*). The ANN models were built using the neural network toolbox in R2015a version of MATLAB software. The modeling data obtained from 138 sample plots were used for ANNs' fitting. Input variables of ANN models were texture values of satellite image determined as the best predictor for each stand parameter in multiple linear regression analyses, and output (target) variable was observed mean diameter, stand basal area, stand volume or number of trees according to the stand attributes fitted.

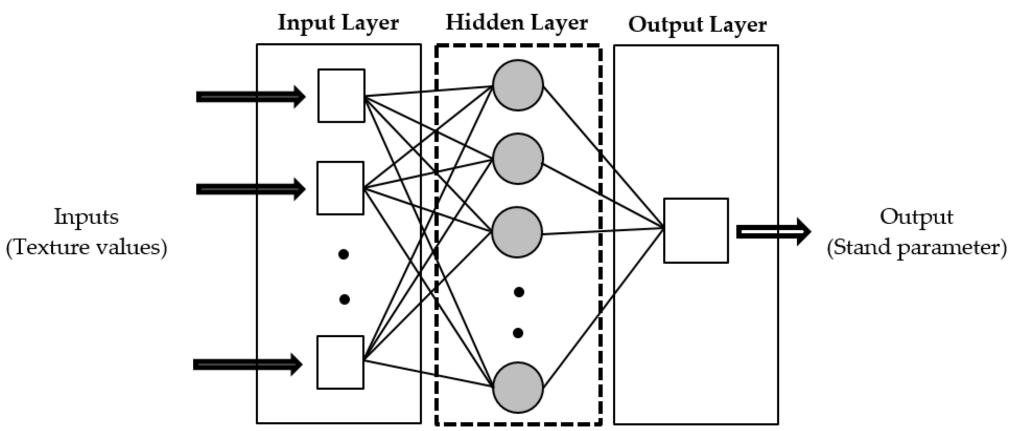

Figure 3. *ANN models architecture for stand parameters*

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Model comparisons and validation tests

The regression and ANN models were evaluated based on four goodness-of-fit statistics; the coefficient of determination (R^2), root mean square error (*RMSE*), bias and mean absolute error (*MAE*). Corresponding mathematical forms of statistical criteria utilized were defined as *Equations 9*, *10*, *11* and *12*.

$$R^2 = 1 - \frac{\sum(y_i - \hat{y}_i)^2}{\sum(y_i - \bar{y})^2} \quad \text{(Eq.9)}$$

$$RMSE = \sqrt{\frac{\sum(y_i - \hat{y}_i)^2}{n-1}} \quad \text{(Eq.10)}$$

$$Bias = \frac{\sum(y_i - \hat{y}_i)}{n} \quad \text{(Eq.11)}$$

$$MAE = \frac{\sum|y_i - \hat{y}_i|}{n} \quad \text{(Eq.12)}$$

In these equations; y_i and $\hat{y}_i$ are observed and estimated values of corresponding stand attribute, and n is sample size.

When comparing alternative models, it is desirable that the R^2 values are high while the others (*RMSE*, *Bias* and *MAE*) are low. For ranking of models, taking into account of all goodness-of-fit statistics together is better than the ranking of each criteria separately. The relative ranking method proposed by Poudel and Cao (2013) was used for model comparisons. In this method, the relative rank of model *i* for a statistical criterion is defined using *Equation 13*.

$$R_i = 1 + \frac{(m-1)(S_i - S_{min})}{(S_{max} - S_{min})} \quad \text{(Eq.13)}$$

where R_i is the relative rank of model i (i = 1, 2, …, m), S_i is the goodness-of-fit statistic of model i, S_{min} and S_{max} are the minimum and maximum values of S_i, respectively.

For each stand parameter, relative rankings of ANN models were first implemented according to number of nodes in hidden layer for transfer function pairs of hidden and output layers, separately, for each statistical criterion. So, four rankings with 20 ANN models were formed for nine transfer function pairs (i.e., linear, logistic and hyperbolic tangent functions were used in both hidden and output layers) for every stand parameter fitting. The model with the highest R^2 was ranked as 1 and the lowest R^2 was ranked as 20 for coefficient of determination, while the model with the lowest value was ranked as 1 and the highest value was ranked as 20 for *RMSE*, *Bias* and *MAE*. Then, four relative ranks of each model according to statistical criteria were summed. The second relative ranking was generated using total relative ranks of ANN models based on number of nodes ranged from 1 to 20 for each transfer function pairs. Thus, the most successful ANN models were specified for every transfer function pairs of each stand parameter fitting.

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To compare estimation success of regression and ANN models, the relative ranks of 10 models (a regression model and the best ANN models for nine transfer function pairs) were rebuilt for each stand parameter. So, the most successful models were identified for predicting stand parameters evaluated in this study. The validities of successful models were tested by comparing the validation data observed from 46 sample plots and models' results by Student's t-test (also called paired samples t-test) procedure in IBM SPSS Statistics 23 software. To exhibit the prediction abilities of the models, residual graphs based on observed vs. predicted stand parameters were also prepared.

Results

In this study, multiple regression and artificial intelligence techniques were conducted for predicting some stand parameters such as mean diameter, basal area, stand volume and number of trees using texture values obtained from Landsat 8 OLI satellite image, and regression and ANN models were developed with these techniques. To determine the best predictor texture values for predicting stand attributes and to develop linear regression models for each stand parameter, multiple linear regression technique was used. In this analyses, all stand parameters were tried to fit using 160 texture values as candidate independent variables. These variables were produced by combining five bands of Landsat 8 OLI satellite image, four window sizes and eight texture metrics. The results of regression analyses are given in *Table 2*. As seen in this table, six, seven, six and three variables included in regression models of mean diameter, basal area, stand volume and number of trees, respectively. All coefficients of these variables were statistically significant as well as constants of models except for number of trees ($p < 0.05$). It might be said that all stand parameters were fitted poorly with regression models due to the low R^2 values. The maximum coefficient of determination value was obtained for mean diameter ($R^2 = 0.399$), while the minimum was for number of trees ($R^2 = 0.183$). The R^2 values of basal area and stand volume models were similar, which were 0.337 and 0.332, respectively.

In regression models developed for stand parameters, texture value combinations containing Band 2 were not statistically significant for number of trees as well as Band 4 for mean diameter and number of trees and Band 7 for all stand parameters except mean diameter, while some combinations with Band 3 and Band 5 were significant for all. The combinations including 7 x 7 window sizes were found insignificant for number of trees, and 9 x 9 ones were for the parameters except basal area. Some combinations with 3 x 3 and 5 x 5 window sizes were significant for all stand parameters. Texture values comprising *contrast* for mean diameter, *dissimilarity* for all, *entropy* and *homogeneity* for the parameters except mean diameter, *mean* for number of trees, *second moment* for all except number of trees, and *variance* for basal area and number of trees were not statistically significant. Some combinations with *correlation* were significant for all stand parameters.

ANN models were also fitted for stand parameters using input variables found statistically significant in regression models. Totally 180 ANN architectures including various transfer functions in hidden and output layers and number of nodes from 1 to 20 in hidden layer were designed for every stand parameter. Thus, a total of 720 ANN procedures were implemented in this study. For all stand parameters evaluated, all ANN models were successfully completed the training process except architectures

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comprising logistic transfer function in output layer. The ANN models with logistic transfer function in output layer could not give any result. It means that 120 ANN models could be ranked instead of 180 models for each stand parameter.

Table 2. *Multiple linear regression results for stand parameters*

Stand parameter	Independent variable	Variable combination (Texture value)			Coefficients (b_i)	R^2	SEE[a]
		Band	Window size	Texture metric			
Mean diameter (d_q, cm)	Constant				49.531*	0.399	8.158
	X_1	2	3x3	Correlation	-0.026*		
	X_2	3	7x7	Entropy	-0.149*		
	X_3	5	5x5	Homogeneity	-0.086*		
	X_4	5	5x5	Mean	0.072*		
	X_5	5	5x5	Variance	-0.133*		
	X_6	7	3x3	Variance	0.073*		
Basal area (G, m^2 ha^{-1})	Constant				31.709***	0.337	15.348
	X_1	2	9x9	Mean	-0.774**		
	X_2	3	9x9	Mean	0.556**		
	X_3	4	7x7	Correlation	0.066**		
	X_4	5	3x3	Contrast	-0.195***		
	X_5	5	5x5	Contrast	0.583***		
	X_6	5	9x9	Contrast	-0.196*		
	X_7	5	9x9	Correlation	-0.078**		
Stand volume (V, m^3 ha^{-1})	Constant				225.797***	0.332	172.487
	X_1	2	3x3	Mean	-6.949**		
	X_2	3	3x3	Mean	4.149*		
	X_3	4	7x7	Correlation	0.640**		
	X_4	5	3x3	Contrast	-1.514**		
	X_5	5	5x5	Contrast	5.536***		
	X_6	5	5x5	Variance	-2.619**		
Number of trees (N, ha^{-1})	Constant				-21.209^{ns}	0.183	425.452
	X_1	3	5x5	Contrast	20.264***		
	X_2	5	3x3	Correlation	1.693**		
	X_3	5	3x3	Second moment	1.402**		

[a]Standard error of estimate
*p < 0.05, **p < 0.01, ***p < 0.001, ^{ns}non-significant (p > 0.05)

Four statistical criteria (R^2, *RMSE*, *Bias* and *MAE*) were used to rank the ANN models. These rankings were applied within six transfer function pairs, i.e. linear, logistic and hyperbolic tangent functions in hidden layer combined with linear and hyperbolic tangent functions in output layer, separately. Hence, a total of 24 ranking lists were produced due to the presence of four stand attributes and six transfer function pairs. Finally, the best ANN architectures within each ranking list were determined to compare with each other and regression models.

The regression models and all of the best ANN models for each stand parameter were re-ranked to decide the most successful models for predicting stand parameters.

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The final ranking lists are given in *Table 3*. In this table, it is seen that the regression models and the ANN models including linear transfer function in hidden layer were unsuccessful than the other ANN models. The ANN_4 models (i.e. the logistic function in hidden layer and hyperbolic tangent function in output layer) were the most successful for predicting basal area (with 12 nodes in hidden layer) and stand volume (with 9 nodes in hidden layer) having high R^2 values as 0.632 and 0.650, respectively. The ANN_6 model (i.e. the hyperbolic tangent functions in both hidden and output layers, and 10 nodes in hidden layer) was the best for mean diameter prediction ($R^2 = 0.593$), while ANN_5 (i.e. the hyperbolic tangent function in hidden layer, the linear function in output layer, and 9 nodes in hidden layer) was the best to predict number of trees ($R^2 = 0.610$). The other goodness-of-fit statistics had similar trends to the coefficient of determinations among the models compared. In the other words, the ANN models having higher R^2 values had lower *RMSE*, *Bias* and *MAE* values. As it can be understood from these conclusions, the powers of stand parameter predictions were considerably increased with artificial intelligence applications. These increases of prediction powers are approximately 50% for mean diameter, nearly 100% for basal area and stand volume, and more than 200% for number of trees.

If the ranking lists in *Table 3* are examined in detail, the regression models were weaker than the ANN models except the architectures containing linear transfer functions in both hidden and output layers (ANN_1). For number of trees prediction, the regression model was also stronger than the ANN model comprising the linear transfer function in hidden layer and the hyperbolic tangent transfer function in output layer (ANN_2). According to the transfer function in hidden layer, the ANN models with hyperbolic tangent function were more successful for mean diameter and number of trees predictions, while the models with logistic function for basal area and stand volume. Besides, according to the transfer function in output layer, the ANN model with linear function were more successful for number of trees prediction, while the models with hyperbolic tangent function for the other stand parameters. There was not any general trend for the ANN models with respect to the number of nodes in hidden layer. However, the node numbers were around 10 in the best models for all parameters.

The validities of the developed regression models and the best ANN models were tested with Student's t-test using independent data set obtained from 46 sample plots. For all the models tested, there were no significant differences between observed and predicted values ($p > 0.05$) and consequently it was decided that the models were statistically usable for predicting the aforementioned stand parameters (*Table 4*). Thus, because of their statistical successes, the ANN_6 model for mean diameter, the ANN_4 models for basal area and stand volume, and the ANN_5 model for number of trees estimations can be used.

The residual distributions of predicted stand parameters obtained by the best ANN models and regression models using all data from 184 sample plots are shown in *Figure 4*. When the residual distributions are examined, it is seen that the residuals were distributed randomly in all regression and ANN models. The regression and ANN models had similar residuals and the mean residuals were close to zero for each stand parameter. Mean residual values for mean diameter, basal area, stand volume and number of trees estimates were -0.4 cm, -0.37 m^2 ha^{-1}, -3.96 m^3 ha^{-1} and 18.6 ha^{-1} for regression models (*Fig. 4a-d*), and -0.1 cm, 0.64 m^2 ha^{-1}, -10.04 m^3 ha^{-1} and 5.5 ha^{-1} for the best ANN models (*Fig. 4e-h*), respectively.

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Table 3. *Relative ranks of models based on goodness-of-fit statistics*[1,2]

Stand parameter	Model	ANN architecture: Transfer function in hidden layer	ANN architecture: Transfer function in output layer	ANN architecture: Number of nodes in hidden layer	R^2 (R_i)	*RMSE* (R_i)	*Bias* (R_i)	*MAE* (R_i)	Total R_i	General rank
Mean diameter (d_q, cm)	Regression				0.399 (6.94)	7.950 (6.93)	-0.13 (3.86)	6.49 (7.00)	24.72	6.04
	ANN_1	Linear	Linear	6	0.397 (7.00)	7.967 (7.00)	-0.24 (7.00)	6.49 (7.00)	28.00	7.00
	ANN_2	Linear	Hyp. Tangent	16	0.398 (6.97)	7.956 (6.95)	-0.03 (1.00)	6.48 (6.96)	21.89	5.22
	ANN_3	Logistic	Linear	15	0.550 (2.32)	6.879 (2.41)	-0.03 (1.00)	5.37 (2.80)	8.52	1.32
	ANN_4	Logistic	Hyp. Tangent	8	0.497 (3.94)	7.273 (4.07)	0.09 (2.71)	5.86 (4.64)	15.36	3.31
	ANN_5	Hyp. Tangent	Linear	9	0.538 (2.68)	6.971 (2.79)	0.09 (2.71)	5.10 (1.79)	9.98	1.74
	ANN_6	**Hyp. Tangent**	**Hyp. Tangent**	**10**	0.593 (1.00)	6.546 (1.00)	0.15 (4.43)	4.89 (1.00)	7.43	**1.00**
Basal area (G, m^2 ha^{-1})	Regression				0.337 (6.80)	14.897 (6.83)	-0.0873 (1.00)	12.0117 (7.00)	21.63	6.92
	ANN_1	Linear	Linear	5	0.327 (7.00)	15.011 (7.00)	-0.0977 (1.07)	11.8592 (6.76)	21.83	7.00
	ANN_2	Linear	Hyp. Tangent	11	0.359 (6.37)	14.655 (6.45)	-0.1442 (1.36)	11.8403 (6.73)	20.92	6.63
	ANN_3	Logistic	Linear	16	0.544 (2.73)	12.354 (2.92)	-0.2436 (2.00)	9.3266 (2.79)	10.44	2.34
	ANN_4	**Logistic**	**Hyp. Tangent**	**12**	0.632 (1.00)	11.100 (1.00)	0.5824 (4.15)	8.1835 (1.00)	7.15	**1.00**
	ANN_5	Hyp. Tangent	Linear	8	0.465 (4.29)	13.387 (4.51)	1.0289 (7.00)	10.2516 (4.24)	20.04	6.27
	ANN_6	Hyp. Tangent	Hyp. Tangent	18	0.491 (3.77)	13.057 (4.00)	0.6217 (4.41)	9.8674 (3.64)	15.82	4.54
Stand volume (V, m^3 ha^{-1})	Regression				0.332 (6.94)	168.055 (6.95)	0.0467 (1.00)	130.3944 (6.93)	21.82	6.23
	ANN_1	Linear	Linear	11	0.329 (7.00)	168.471 (7.00)	-2.9128 (2.84)	130.8383 (7.00)	23.84	7.00
	ANN_2	Linear	Hyp. Tangent	4	0.392 (5.82)	160.306 (5.95)	-3.2678 (3.07)	125.7314 (6.16)	21.01	5.92
	ANN_3	Logistic	Linear	8	0.540 (3.06)	139.422 (3.28)	-0.1988 (1.10)	101.8162 (2.24)	9.68	1.62
	ANN_4	**Logistic**	**Hyp. Tangent**	**9**	0.650 (1.00)	121.599 (1.00)	-6.3624 (5.05)	94.2181 (1.00)	8.05	**1.00**
	ANN_5	Hyp. Tangent	Linear	8	0.585 (2.21)	132.460 (2.39)	-5.9340 (4.78)	101.5374 (2.20)	11.58	2.34
	ANN_6	Hyp. Tangent	Hyp. Tangent	5	0.513 (3.56)	143.435 (3.80)	-9.4027 (7.00)	103.9747 (2.60)	16.95	4.38
Number of trees (N, ha-1)	Regression				0.183 (6.91)	419.241 (6.92)	0.03 (1.00)	289.77 (7.00)	21.82	6.62
	ANN_1	Linear	Linear	14	0.176 (7.00)	421.113 (7.00)	-9.25 (2.00)	284.57 (6.61)	22.61	6.88
	ANN_2	Linear	Hyp. Tangent	7	0.216 (6.46)	410.800 (6.54)	-22.41 (3.44)	283.41 (6.52)	22.97	7.00
	ANN_3	Logistic	Linear	10	0.599 (1.30)	293.818 (1.37)	-12.20 (2.33)	209.70 (1.00)	5.99	1.30
	ANN_4	Logistic	Hyp. Tangent	19	0.621 (1.00)	285.546 (1.00)	-55.11 (7.00)	216.10 (1.48)	10.48	2.81
	ANN_5	**Hyp. Tangent**	**Linear**	**9**	0.610 (1.15)	289.685 (1.18)	6.92 (1.75)	209.71 (1.00)	5.08	**1.00**
	ANN_6	Hyp. Tangent	Hyp. Tangent	17	0.551 (1.94)	310.820 (2.12)	-8.17 (1.89)	217.52 (1.59)	7.54	1.82

[1]Values in parenthesis indicate the relative ranks of the models based on statistical criteria
[2]The best models are marked in bold for each stand parameters

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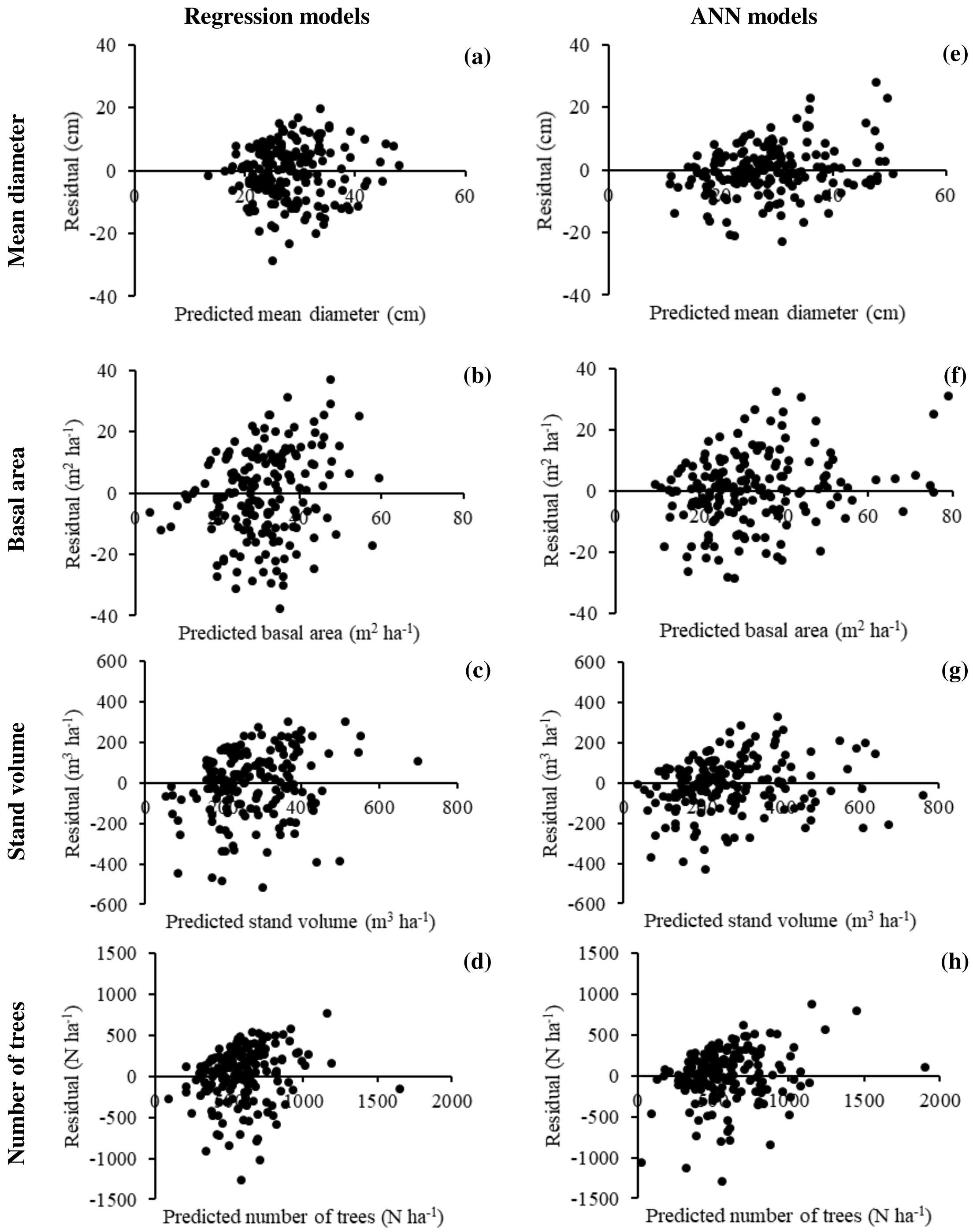

Figure 4. *Residual distributions of the regression models (a-d) and selected ANN models (e-h)*

When the observed stand parameters and predicted values of these parameters with two modeling approaches were compared considering *Table 4*, the mean diameter predictions of the regression and ANN_6 models were similar to observed values with mean differences of 1.4 and 1.5 cm, respectively. The basal area predictions of the ANN_4 model were very close to the observed values with mean difference of 0.860 m^2, while the regression model's predictions were quite different with of 5.221 m^2 per hectare. Since mean differences of the regression and ANN_4 models were 65.586 and

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44.559 m^3 per hectare, the ANN_4 model was more prosperous for the stand volume estimations. The comparison for the number of trees was resulted different from the other stand parameters, since the mean difference of the regression model was positively 45.4 trees while of the ANN_5 model was negatively 63.5 trees per hectare. However, the ANN_5 model was superior than the regression model for the number of trees prediction when the mean residuals of the models took into consideration.

Table 4. *Student's t-test results for the regression and ANN models selected*

Stand parameter	Data	Model	Mean	Paired differences			t	p
				Mean	SD[a]	SEE[b]		
Mean diameter (d_q, cm)	Observed		29.7					
	Predicted	Regression	28.3	1.4	10.5	1.55	0.894	0.376
		ANN_6	28.1	1.5	10.8	1.60	0.948	0.348
Basal area (G, m^2 ha^{-1})	Observed		36.288					
	Predicted	Regression	31.067	5.221	22.949	3.384	1.543	0.130
		ANN_4	35.427	0.860	23.279	3.432	0.251	0.803
Stand volume (V, m^3 ha^{-1})	Observed		323.589					
	Predicted	Regression	258.003	65.586	238.683	35.192	1.864	0.069
		ANN_4	279.030	44.559	312.397	46.060	0.967	0.339
Number of trees (N, ha^{-1})	Observed		633.7					
	Predicted	Regression	588.3	45.4	469.7	69.26	0.655	0.516
		ANN_5	697.2	-63.5	673.3	99.27	-0.640	0.525

[a]Standard deviation
[b]Standard error of estimate

Discussion and conclusions

In this study, the relationships between some stand attributes (mean diameter, basal area, stand volume and number of trees) and texture values obtained from Landsat 8 OLI satellite image were predicted for Crimean pine stands in Kastamonu region of Turkey. The multiple linear regression analysis and ANN technique were performed to fit stand attributes using texture values. The stand attributes estimation models developed by ANN models performed better than multiple linear regression models except ANN_1 for basal area, stand volume and number of trees and except ANN_1 and ANN_2 for mean diameter.

The band reflectance and vegetation indices were used as independent variables in some published studies performed to estimate the stand parameters using the Landsat satellite images (Mohammadi et al., 2010; Kahriman et al., 2014; Günlü and Kadıoğulları, 2018). However, in the literature no studies have been found to predict mean diameter, basal area, stand volume and number of trees using texture values obtained from Landsat satellite images. On the other hand, there are some studies on estimating stand parameters such as aboveground biomass and carbon stock using textures values obtained from Landsat satellite data images (Kelsey and Neff, 2014; Zhao et al., 2016; Safari and Sohrabi, 2016 In addition, there are many studies on estimating stand parameters such as stand volume, basal area, number of trees, aboveground biomass and carbon stock using texture values obtained from the other satellite data images (Castillo-Santiago et al., 2010; Özdemir and Karnieli, 2011;

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Eckert, 2012; Pu and Cheng, 2015; Wallner et al., 2015; Xie et al., 2017). In general, regression analysis is used in the studies to estimate stand parameters using remote sensing data images (Castillo-Santiago et al., 2010; Mohammadi et al., 2011; Günlü et al., 2014b; Kahriman et al., 2014).

The reflectance values, vegetation indices and texture values were utilized as independent variables of a multiple linear regression model in some studies on the subject. For instance, the models using vegetation indices obtained from Landsat ETM+ data had the R^2 values as 0.43 for stand volume and 0.73 for tree density (Mohammadi et al., 2010), while the texture variables of IKONOS-2 explained 35% of the variation in basal area of even-aged stands in temperate forests (Kayitakire et al., 2006). In other studies, the reflectance and vegetation indices values obtained from Landsat TM satellite data explained 61% and 70% of tree density, respectively, in mixed forest areas, and generated from pan-sharpened IKONOS satellite data explained 41% and 43% of stand volume, and 55% and 59% of basal area, respectively (Kahriman et al., 2014; Günlü et al., 2014b). In the multiple linear regression models with digital number values of Landsat TM, the R^2 values for mean diameter, basal area, stand volume and number of trees were founded as 0.25, 0.32, 0.37 and 0.44, respectively (Günlü and Kadıoğulları, 2018). In the same study, the R^2 values of the multiple linear regression analyses obtained from vegetation indices values for mean diameter, basal area, stand volume and number of trees were founded as 0.17, 0.34, 0.36 and 0.28, respectively.

The results of our study indicated that the R^2 values of the multiple linear regression analyses generated with texture values from Landsat 8 OLI satellite image for mean diameter, basal area, stand volume and number of trees were founded as 0.40, 0.34, 0.33 and 0.18, respectively. When the results obtained from our study are compared with some other studies mentioned above (Mohammadi et al., 2010: Kahriman et al., 2014; Günlü and Kadıoğulları, 2018), the regression model generated from texture values is not suitable for estimating mean diameter, basal area, stand volume and number of trees due to the low R^2 values.

In recent years, the ANN method used in some published studies on predicting leaf area index (Shoemaker and Cropper, 2010), stand volume and basal area (Shataee, 2013; Santi et al., 2015), stand carbon stock (Ercanli et al., 2016), and aboveground biomass (Ni et al., 2017; Yan et al., 2018). We also used the ANN method to predict the stand parameters in this study. The comparisons between multiple linear regression and ANN models for estimating stand parameters show that the abilities of the ANN models are higher. In other words, the R^2 values of the best ANN models increased between 48% and 239% for the stand parameters compared to the regression models. Similar results were published in some rare studies. For instance, Ercanli et al. (2016) modelled aboveground stand carbon stock using multiple linear regression analysis and ANN with vegetation indices obtained from Landsat TM satellite data and founded that the R^2 values of 0.43 and 0.65, respectively. In another study, a comparative analysis was conducted for different satellite data and modeling techniques (e.g. ANN, random forest, linear regression, *k*-nearest neighbor, support vector regression) for aboveground biomass prediction in different forest types (Gao et al., 2018). The results obtained from this study showed that the ANN models were more accurate for mixed, broadleaf and conifer forest types (*RMSE* values ranged 30.0-36.0 Mg/ha, 24.0-26.4 Mg/ha and 28.2-30.9, respectively) than linear regression model (*RMSE* values ranged 32.6-37.0 Mg/ha, 24.4-31.0 Mg/ha and 28.2-32.4, respectively). It is seen that there are few studies to predict the stand parameters using both regression analysis and ANN method. Thus, our

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study is one of the rare studies that use both the multiple linear regression and ANN models in estimating of stand parameters using remote sensing data, especially texture values. Therefore, investigating the estimation success of the ANN models using satellite data for stand attributes is essential for further studies.

The ANN models can be suggested as more suitable than regression models for predicting stand attributes such as mean diameter, basal area, stand volume and number of trees using remote sensing data, since the estimation power of the ANN models are higher than the regression models. The texture values might be used successfully as predictor variables in these models. The utilization of different model techniques such as random forest, *k*-nearest neighbor, support vector regression and mixed effect modeling and various satellite data such as optical, active and combined can improve the model achievement criteria in various forest ecosystems.

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THE STUDY OF SURFACE WATER QUALITY IN BUYUK MENDERES RIVER (TURKEY): DETERMINATION OF ANIONIC DETERGENT, PHOSPHATE, BORON AND SOME HEAVY METAL CONTENTS

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Abstract. The Buyuk Menderes basin is very wide and the people living here deal with farming, which increases the importance of the river to the region. The pollution of the river increases because the industry, urbanization and agricultural activities are very intense in the river basin. For this reason, the aim of this study is to map the extent of pollution of Buyuk Menderes River, to detect sources causing pollution in the river and also to suggest solutions for taking necessary precautions. Anionic detergent, phosphate, boron and heavy metal concentrations were determined in water samples taken from Buyuk Menderes River in this study. Mean concentrations of anionic detergent, phosphate, boron, copper, chrome, nickel and lead were found 0.2345 mg l^{-1}, 0.0181 mg l^{-1}, 0.8352 mg l^{-1}, 0.0035 mg l^{-1}, 0.0045 mg l^{-1}, 0.0247 mg l^{-1}, 0.0002 mg l^{-1}, respectively.
Keywords: *surfactant, freshwater, nickel, pollution, water quality classes*

Introduction

Rapid population growth, the spreading of industrialization and mechanization of agriculture, cause pollution of the environment and the waters. Pollution is caused by the inability of people to perceive the importance of environmental conditions for life. Due to uncontrolled domestic and industrial wastes and agricultural activities, it is known that pollution reaches important dimensions in many water resources today (Akın and Akın, 2007). Büyük Menderes River is one of Turkey’s important and large river. The River has been polluted by factors such as domestic wastewater, industrial wastewater, excessive, untimely and incorrect use of fertilizer and pesticide. These incidents require investigating pollution levels of the river. For this reason, the aim of this study is to map the extent of pollution of Buyuk Menderes River, to detect sources causing pollution in the river and also to suggest solutions for taking necessary precautions.

There are also detergents among pollutants coming from domestic and industrial wastes to aquatic environments. Detergents are used in general cleaning work. The detergents contain anionic surfactants such as alkyl sulphate or alkyl aryl sulphonate as the main cleaner and other materials that help the cleaning process. Detergents are mixtures of powder, granule, soft consistency or liquid. The detergents cause problems

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like disturbing the taste of drinking water, making foam, preventing the water cleaning process, damaging the aquatic organisms by reducing the amount of dissolved oxygen, shortening the aging process of lakes by eutrophication due to phosphate content and damaging the aquatic life (Egemen, 2011).

Approximately half of phosphorus comes from the phosphate in the composition of detergents used in domestic wastewater. 91% of the phosphorus comes from domestic and industrial wastes and 9% from agricultural areas to water resources (Egemen, 2011).

Boron compounds are commonly found in surface and ground waters. The toxic limit value of the boron element is 1.0 mg l^{-1}. This value is determined for plants, animals and humans. Also this value is an acceptable for drinking and irrigation waters and wastewater (Kabay et al., 2006).

Elements like selenium, iron, manganese and cobalt are mixed from the soil to the water. Magnesium, potassium and cobalt are passed from the sea to the air. Toxic elements such as zinc, copper, cadmium, mercury, antimony, arsenic, argon, lead, chromium, selenium mix to the air, water and soil by anthropogenic activities (Samsunlu, 1999).

Materials and methods

The cities of Aydın and Denizli are located in the Aegean region. These cities are important industrial and agricultural centers of Turkey.

The Buyuk Menderes River comes from Afyon, passes through Usak, Denizli and Aydın, reaches to the Aegean Sea. The total length of the Buyuk Menderes is about 584 km and its basin covers 25 000 km^2 (Boyacioglu et al., 2004).

In this research, six stations were determined in the discharge regions in Buyuk Menderes River (*Fig. 1*). The coordinates of the stations were showed in the *Table 1*.

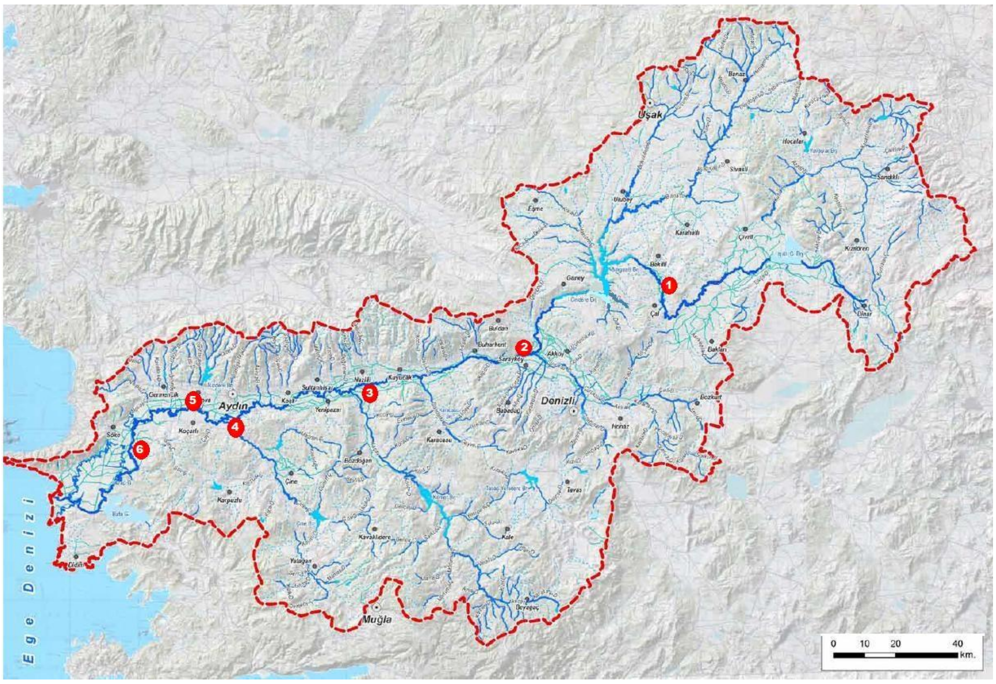

Figure 1. *Sampling points*

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***Table 1.** Coordinates of stations*

	Stations	Coordinates
1	Kumralli	38° 6' 1.62" N, 29° 25' 22.26" E
2	Saraykoy	37° 57' 5.73" N, 28° 54' 58.00" E
3	Nazilli	37°52'33.28" N, 28°19'39.26" E
4	Aydın	37°46'56.76" N, 27°50'22.99" E
5	Kocarlı	37°48'36.57" N, 27°42'47.00" E
6	Soke	37°42'26.93" N, 27°28'36.62" E

As a research material, water samples taken from six stations were selected. The water samples were taken on a monthly basis. They were collected between July 2012–June 2013. pH, temperature, dissolved oxygen, total dissolved substance and conductivity parameters were measured by TOA WQC (Water Quality Checker) – 20A.

The method in the determination of anionic detergent is based on the spectrophotometric measurement of methylene blue by being dissolved in the chloroform of the blue colored salt resulting from the anionic surfactant reaction (Anonymous, 1995). Orthophosphate phosphorus is determined by measuring the blue-colored phosphomolybdic acid in the spectrophotometer resulting from the reaction with ascorbic acid, ammonium molybdate and potassium antimony tartrate in acidic environment (Parsons et al., 1984). Determination of the amount of boron is based on the spectrophotometer measurement of the color compound formed with the carmin, a specific reagent of the tube (Hatcher and Wilcox, 1950; Anonymous, 2005). Heavy metal analysis of prepared water samples were made in ICP-MS (Inductively Coupled Plasma – Mass Spectrophotometer).

Statistical analyzes were performed using Windows, Graphpad Prism software package. "One-way Anova" variance analysis was applied to detect whether the amounts of anionic detergent, phosphorus, boron and heavy metals showed significant difference among months and stations. "Tukey test" was applied to detect in which stations and months the significant differences.

Result and discussion

In this study, anionic detergent, phosphate, boron and heavy metal concentrations were detected in the water samples from Buyuk Menderes River that flows from Denizli and Aydın cities centers. pH, temperature, dissolved oxygen, total soluble substance and conductivity parameters were measured. On average, pH 8.24, temperature 15.6 °C, dissolved oxygen 6.04 mg l^{-1}, total soluble substance 426 mg l^{-1} and conductivity 470 µS/cm were found. The river water in terms of pH and dissolved oxygen parameters 2nd quality and in terms of all other parameters 1st quality in water class were found, according to the Water Pollution Control Regulation (Official Gazette, 2004) (*Table 2*).

These mean values were compared with the Aboveground Water Quality Regulation "Quality Criteria According to Classes in Terms of General Chemical and Physicochemical Parameters of Coastal Waters" (Official Gazette, 2012). The river water was determined as the 2nd quality in terms of pH and conductivity parameters and the 3rd quality in terms of dissolved oxygen parameter (*Table 3*).

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Table 2. *Quality criteria (Official Gazette, 2004)*

Water quality parameters	**Water quality classes**				**This study**
	1st	**2nd**	**3rd**	**4th**	
Temperature (°C)	25	25	30	>30	15.6
pH	6.5-8.5	6.5-8.5	6.0-9.0	6.0-9.0 except	**8.24**
Dissolved oxygen (mg l^{-1})	8	6	3	< 3	**6.04**
Total soluble substance (mg l^{-1})	500	1500	5000	>5000	426
Surfactant (mg l^{-1})	0.05	0.2	1	>1.5	**0.2345**
Boron (mg l^{-1})	1	1	1	>1	0.8352
Total phosphorus (mg l^{-1})	0.02	0.16	0.65	>0.65	0.0181
Copper (µg l^{-1})	20	50	200	>200	3.5
Chrome (µg l^{-1})	20	50	200	>200	4.5
Nickel (µg l^{-1})	20	50	200	>200	**24.7**
Lead (µg l^{-1})	10	20	50	>50	0.2

Table 3. *Quality criteria (Official Gazette, 2012)*

Water quality parameters	**Water quality classes**				**This study**
	1st	**2nd**	**3rd**	**4th**	
pH	6–9	6–9	6–9	6–9	**8.24**
Conductivity (µs)	< 400	1000	3000	> 3000	**470**
Dissolved oxygen (mg l^{-1})	> 8	6	3	< 3	**4.94**

Mean concentrations of anionic detergent, phosphate, boron, copper, chrome, nickel and lead were found 0.2345 mg l^{-1}, 0.0181 mg l^{-1}, 0.8352 mg l^{-1}, 0.0035 mg l^{-1}, 0.0045 mg l^{-1}, 0.0247 mg l^{-1}, 0.0002 mg l^{-1}, respectively. Anionic detergent, phosphate, boron and heavy metal concentrations mean values were presented in *Table 4*. These concentrations were compared with the Water Pollution Control Regulation (Official Gazette, 2004). Buyuk Menderes River is in the class of less polluted water (2nd class) in terms of anionic surfactant and nickel (*Table 2*). Because the Buyuk Menderes River is close to the settlements, it is thought that the discharges are reaching to the river. Due to domestic waste load, the anionic detergent load increases. The average anionic detergent concentration (0.2345 mg l^{-1}) is below the surfactant concentration, which is considered to be ≤ 0.3 mg l^{-1} in European Union quality criteria. The average heavy metal values were compared with the heavy metal concentrations allowed in the irrigation waters specified in the Water Pollution Control Regulation Technical Procedures Bulletin (Official Gazette, 1991) and the metal concentrations were found to be low.

According to the One Way ANOVA test, statistically significant differences were not found for heavy metals, pH, dissolved oxygen, total soluble substance and conductivity ($p > 0.05$) and differences were found for phosphate ($p < 0.05$). Also the difference among the stations was statistically insignificant ($p > 0.05$) and the difference among the months was statistically significant ($p < 0.05$) for anionic detergent and boron amounts (*Table 5*).

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Table 4. *Monthly average concentrations of detergent, phosphate, boron, copper, chromium, nickel and lead in water samples of Buyuk Menderes River (mg l^{-1}, ± SD < 0.001)*

Months	Detergent	Phosphate	Boron	Copper	Chrome	Nickel	Lead
July	0.1812	0.0117	0.6397	0.0203	0.0010	0.0277	0.0000
August	0.1615	0.0102	0.6733	0.0032	0.0014	0.0317	0.0003
September	0.0583	0.0087	0.3215	0.0012	0.0017	0.0283	0.0004
October	0.2297	0.0228	0.8938	0.0018	0.0009	0.0320	0.0004
November	0.3345	0.0207	1.0681	0.0025	0.0010	0.0557	0.0002
December	0.3260	0.0173	0.3407	0.0037	0.0004	0.0637	0.0001
January	0.1442	0.0182	0.7017	0.0012	0.0008	0.0310	0.0000
February	0.3385	0.0237	0.6259	0.0032	0.0015	0.0051	0.0001
March	0.2227	0.0153	0.8025	0.0018	0.0094	0.0081	0.0002
April	0.2442	0.0245	1.1856	0.0008	0.0169	0.0070	0.0002
May	0.3200	0.0228	1.3985	0.0010	0.0123	0.0036	0.0002
June	0.2528	0.0208	1.3715	0.0019	0.0070	0.0034	0.0002
MEAN	**0.2345**	**0.0181**	**0.8352**	**0.0035**	**0.0045**	**0.0247**	**0.0002**

Each value is the average of 3 samples from 6 stations with three repetitions (n = 54)

Table 5. *The result of the one-way ANOVA test between months and stations*

Table analyzed	Between months			Between stations		
One-way analysis of variance	**Detergent**	**Phosphate**	**Boron**	**Detergent**	**Phosphate**	**Boron**
P value	**P<0.0001**	**0.0002**	**P<0.0001**		**0.0067**	
P value summary	***	***	***		**	
Are means signif. different? (p < 0.05)	**Yes**	**Yes**	**Yes**	**No**	**Yes**	**No**
Number of groups	12	12	12		6	
F	8.752	4.071	4.233		3.549	
R squared	0.6161	0.4274	0.437		0.2119	

The differences among the months and stations were determined by the Tukey test. Tukey test results are shown in *Tables 6, 7* and *8*. Especially in summer, the decline in detergent quantities can be attributed to the decline in population at residential areas as well as the decline in domestic waste loads in these months (*Table 6*). 50% of the amount of phosphorus in domestic wastewater is caused by the detergent-based phosphates used in houses. As a result, the amount of phosphate has decreased in parallel with the decrease in the amount of detergent (*Table 7*). Increase in detergent values in winter months, can be considered as an increase of domestic waste load transport to river with the increase of rainfall. Significant boron differences were showed in May and June (*Table 8*). Boron concentrations were high in May and June. Especially in May and June, together with agricultural irrigation using groundwater resources, abundant amounts of boron in underground waters are mixed in to Buyuk Menderes River.

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Table 6. *The result of the Tukey test among the months (anionic detergent) (only significant values)*

	Jul	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**
Jun			0.0011				
May		0.017	<0.0001				0.0047
Apr			0.0022				
Mar			0.0112				
Feb	0.0185	0.0043	<0.0001				0.0011
Jan					0.0015	0.003	
Dec	0.0428	0.0111	<0.0001				
Nov	0.0244	0.0059	<0.0001				
Oct			0.0067				

Table 7. *The result of the Tukey test between months and stations (phosphate) (only significant values)*

	Aug	**Sep**	**St 1**	
May		0.0232	**St 6**	0.0182
Apr	0.0205	0.0063	**St 5**	0.0445
Feb	0.0378	0.0123	**St 3**	0.0058
Oct		0.0232		

Table 8. *The result of the Tukey test among the months (boron) (only significant values)*

	Sep	**Dec**
Jun	0.004	0.0051
May	0.0028	0.0036
Apr	0.0384	0.0474

When we compared the values with the results of other studies which investigated anionic detergent, phosphate, boron and heavy metal levels of Buyuk Menderes, we measured similar values in present study. In the study in Buyuk Menderes River, average copper 0.011 ppm, chromium 0.012 ppm, nickel 0.01 ppm, lead 0.011 ppm, iron 0.006 ppm, mangan 0.09 ppm, zinc 0.051 ppm, cobalt 0.08 ppm were found (Akcay et al., 2003). The average boron value of the Buyuk Menderes River was determined as 0.6 mg l^{-1} in another study. According to all parameters, it was stated that the water was mostly polluted in Saraykoy and Nazilli regions (Kucuk, 2007). Boron content was found to be between 0.33 and 6.41 mg l^{-1}. The boron concentration is high especially in the regions of under ground thermal water resources (Aydın and Seferoglu, 2000). And the boron concentration was found to be 1.1 mg l^{-1} due to geothermal wastewater discharges to the river and river water used for agricultural irrigation affected the production of agricultural products (Akar, 2007). Buyuk Menderes River is

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in the third class pollution level due to domestic and industrial wastes and agricultural activities (Akın and Akın, 2007).

"Pollution of the Buyuk Menderes is to pollute the future" in the project final report, the mean values were compared with the Water Pollution Control Regulation. Buyuk Menderes River is 2nd class, namely in the class of less polluted water in terms of lead and 1st class, namely it is high quality water in terms of copper, chrome and zinc. It is below the permissible limits when compared with the maximum allowable concentrations of heavy metals and toxic elements in irrigation waters (Durdu et al., 2012).

It is stated that the most serious problems in terms of water quality in rivers in Buyuk Menderes basin are excessive organic matter, nitrogen, pH, boron and heavy metal pollution (Tubitak Mam, 2013).

Isıklı Lake is on resources that feed the Buyuk Menderes River. Isıklı Stream is a branch of the Buyuk Menderes River. Isıklı Lake and Isıklı Stream were determined to be as 2nd class (less contaminated water) in terms of anionic detergent parameter (Cakir and Minareci, 2015). Also in Isıklı Lake, it was found that organic pollution significantly increased and the amount of dissolved oxygen decreased in summer in some stations of the lake. It is stated that the important source that pollutes the lake is Buyuk Menderes River (Bulut et al., 2012). Adıguzel Dam Lake is on the Buyuk Menderes River. Adıguzel Dam Lake was categorized as less contaminated water in terms of anionic detergent parameter (Minareci and Cakir, 2018).

The measured values in the different rivers in the same region are higher than the measured values in our study. The Gediz River is highly polluted surface water in the Gediz Basin. Due to domestic and industrial wastes and agricultural activities, the river is determined as IV. class water quality in terms of nitrogen, organic matter and heavy metals (Akın and Akın, 2007). In the study carried out in Gediz River, copper 0.034 ppm, chromium 0.099 ppm, nickel 0.062 ppm, lead 0.218 ppm, iron 0.121 ppm, manganese 0.053 ppm, zinc 1.181 ppm, cobalt 0.006 ppm, cadmium 0.0036 ppm were found. The river was found dirty in terms of chrome and lead (Minareci et al., 2009a). In another study in Gediz River, boron concentrations are found between 0.19–2.25 mg l^{-1} (Demirbas and Orhun, 2008). The average boron value was 2.428 mg l^{-1} in Gediz River and Gediz River was determined as very polluted water for boron parameter (Minareci, 2014).

In Kucuk Menderes River, copper concentrations 0.01–0.02 ppm, chrome concentrations 0.01–0.02 ppm, nickel concentrations 0.03–0.08 ppm and lead concentrations 0.08–0.12 ppm were found between these amounts (Egemen et al., 2005). In the study conducted by Gundogdu and Ozkan (2006) in Kucuk Menderes River in 2006, it was determined that the iron value is 3rd class water quality. In another study, it has been reported that the Kucuk Menderes River is in the 4th class pollution level in terms of nitrogen, organic matter and heavy metals from domestic and industrial wastes and agricultural activities (Akın and Akın, 2007). It was determined that other heavy metal concentrations except Ni, Cu and Zn were low in surface water of Kucuk Menderes River (Turgut, 2003).

Anionic detergent and heavy metal levels of Berdan Stream have been found higher than the standard levels (Kumbur and Vural, 1989).

The anionic detergent values are shown comparatively in *Table 9*. The obtained phosphate values from Buyuk Menderes River were lower than the obtained values from many other rivers (*Table 10*).

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Table 9. *Anionic detergent concentrations of studies*

Rivers name	Detergent concentrations	References
Gediz River	0.023–4.48 mg l^{-1}	Tugrul, 1992
Yuvarlak Stream	0.12 mg l^{-1}	Balık et al., 2002
Bakırcay River	0.01–0.29 mg l^{-1}	Basaran, 2004
Kucuk Menderes River	0–0.93 mg l^{-1}	Egemen et al., 2005
Gediz River	0.951 mg l^{-1}	Minareci et al., 2009b
Karacay Stream	0.071–1.122 mg l^{-1}	Minareci et al., 2009b
Halifax Harbour River	0.001–mg l^{-1}	Gagnon, 1983
Tama River	0.035–0.219 mg l^{-1}	Yoshikawa et al., 1984
Hyogo River	0.004–2.5 mg l^{-1}	Kobuke, 1985
Tsurumi River	0.01–0.29 mg l^{-1}	Yoshikawa et al., 1985
Sumida River	0.005–0.01 mg l^{-1}	Kikuchi et al., 1986
Saar River	0.01–0.09 mg l^{-1}	Matthijs and De Henau, 1987
Teshiro River	0.01–0.27 mg l^{-1}	Kojima, 1989
Teganuma River	0.019–1.4 mg l^{-1}	Nonaka et al., 1989
Yodo River	0.043–0.089 mg l^{-1}	Nonaka et al., 1990
Miami River	< 0.05 mg l^{-1}	Hand et al., 1990
Oohori River	0.5–1.6 mg l^{-1}	Amano et al., 1991
Mississippi River	0.01–0.3 mg l^{-1}	McAvoy et al., 1993
Litheos River	0.1 mg l^{-1}	Dassenakis et al., 1998
Yorkshire River	0.05–0.25 mg l^{-1}	Fox et al., 2000
Itter River	0.007–0.011 mg l^{-1}	Wind et al., 2004
Buyuk Menderes River	**0.0583–0.3385 mg l^{-1} (mean 0.2345 mg l^{-1})**	**This study**

Table 10. *Phosphate concentrations of studies*

Rivers name	Phosphate concentrations	References
MericRiver	0.0001–0.02 mg l^{-1}	Kontas, 1990
Gediz River	0.016–4.054 mg l^{-1}	Tugrul, 1992
Gediz River	0.02–7.41 mg l^{-1}	Okur et al., 1997
Asi River	0.002–2.44 mg l^{-1}	Tasdemir and Goksu, 2001
Bakırcay River	0.0018–0.0229 mg l^{-1}	Basaran, 2004
Bakırcay River	0–0.7 mg l^{-1}	Gundogdu and Turhan, 2004
Karacay Stream	0.22– 6.82 mg l^{-1}	Kara and Comlekcioglu, 2004
Kucuk Menderes River	0–1.88 mg l^{-1}	Egemen et al., 2005
Gediz River	0.0044–0.2481 mg l^{-1}	Minareci et al., 2009b
Karacay Stream	0.002–0.225 mg l^{-1}	Minareci et al., 2009b
Rheraya River	0.043–1.286 mg l^{-1}	Khebiza et al., 2006
Nhue River	3.5 mg l^{-1}	Duc et al., 2006
Dun River	0.086 mg l^{-1}	Neal et al., 2006
Kenet River	0.083 mg l^{-1}	Neal et al., 2006
Fuji River	0.01–0.13 mg l^{-1}	Shrestha and Kazama, 2007
Yangtze River	0.64–1.31 mg l^{-1}	Zhang et al., 2007
Buyuk Menderes River	**0.0087–0.0245 mg l^{-1} (mean 0.0181 mg l^{-1})**	**This study**

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Conclusions and recommendations

The Buyuk Menderes River water is 2nd quality in terms of pH, conductivity, anionic surfactant and nickel parameters. Also the river water is 3rd quality in terms of dissolved oxygen parameter. Because the Buyuk Menderes River is close to the settlements, the discharges reach to the river. Due to domestic and industrial waste load, pollution load increases.

For the sustainable productivity of the Buyuk Menderes Basin lands and the protection of all aboveground and underground waters from pollution,

- The protection of the Buyuk Menderes River from all kinds of pollution (especially domestic and industrial wastes),
- Analysis of surface and groundwater should be done periodically,
- The water not suitable for irrigation should not be used,
- Fertilizer should be used as much as necessary,
- The farmers are directed to organic farming,
- Use of pesticides should be minimized,
- Industrial facilities and local governments should establish and use wastewater treatment facilities,
- The chemical fertilizers and agrochemicals must be controlled by authorized institutions.

As a result, the investigation of the underground and surface water quality of Buyuk Menderes Basin should be continued periodically, the factors that decrease the quality should be revealed and necessary precautions should be taken.

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THE EFFECTS OF DIFFERENT POSTHARVEST APPLICATIONS ON SOME PHYSICOCHEMICAL PROPERTIES IN 'RUBYGEM' AND 'SABRINA' STRAWBERRY (*FRAGARIA* X *ANANASSA* DUCH.) CULTIVARS

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Abstract. This study aimed to determine the efficacy of two standards strawberry (*Fragaria* x *ananassa* Duch.) cultivars 'Rubygem' and 'Sabrina' grown under protected cultivation system in Mersin province in Turkey. The fruit quality preservation during storage in Modified atmosphere packaging (MAP) at different storage temperatures as well as preservative compatibility under Ultraviolet-C (UV-C), hot water and combined applications were studied. Strawberry fruits kept in cold temperatures (0 °C and 5 °C) for 20 days and biochemical changes (total phenolic content, total antioxidant capacity, vitamin C content and sugar content) were measured after harvest and storage The 'cv. Rubygem' kept its quality for 16 days in all treatments and for 20 days in UV-C and UV-C + hot water applications when kept at 0 °C. Also, the fruits stored at 5 °C kept their quality for 12 days and it was observed that hot water and UV-C + hot water treatments got better results in the same period and at the same storage temperature. In the 'cv. Sabrina' results showed that fruit samples at 0 °C were successfully maintained in control as well as the other 3 treatments until the 12th day. The preservation was continued in UV-C, hot water and combined treatments on the 16th day of the storage and the best ones were the hot water and UV-C + hot water at the end of the 20th day. As a result, it was observed that total phenolic contents, total antioxidant activity and sugar were found decrease at the end of storage for both cultivars while fluctuations in vitamin C.
Keywords: *biochemical changes, hot water, UV-C, sugar, vitamin C, total phenolic, antioxidant*

Introduction

Strawberry (*Fragaria* x *ananassa* Duch.) fruit is consumed abundantly by consumers and has many positive effects (Çağlarırmak, 2006) such as strengthening defense mechanisms and protecting against cancer and infections, protection against DNA damage in the organism (Vinson et al., 2001), protection against cardiovascular disease and lipid peroxidation, because it is rich in antioxidants (Wang et al., 1996; Araque et al., 2018) including anthocyanins, flavanoids and phenolic materials, as well as its unique color, flavor (Gao et al., 2016), aroma, texture, high-order elagic acid (Aybak, 2005).

It is very important to protect the post-harvest quality of strawberries, because they are located in a group of fruit berries that break down quickly and easily after harvest.

The main soluble sugars found in the structure of strawberry berry are glucose, fructose and sucrose, and glucose and fructose are known to dominate sucrose in total sugar content (Cordenunsi et al., 2003).

Strawberry fruit contains as high as 92% water. For this reason, water loss is one of the important issues after harvest. In addition to water loss, a number of insecticides and fungicides are used both on the field and before harvesting to prevent the losses caused by diseases and pests. Applications that are environmentally friendly to prevent postharvest decay include applications such as hypobaric applications (Romanzzi et al., 2001), modified atmosphere packaging (Silva et al., 1999), Ultraviolet-C (UV-C) and hot water applications (Keskin et al., 2014, 2015).

For the last ten years UV-C (180-280 nm λ = 254 nm) in horticulture products has been considered as an alternative to fungicides to control post-harvest diseases. Moreover, many investigators have reported that UV-C protects fruits and vegetables against decay after harvesting (Marquenie et al., 2003; Allende and Artes, 2003; Allende et al., 2006; Keskin et al., 2014, 2015). It has also been used to delay the ripening of fruits and vegetables by extending their shelf life (Darvishi et al., 2012). When UV-C is applied in appropriate dose and duration, it increases the accumulation of phytoalexins (Keskin and Kunter, 2017), which is effective on the mechanism against disease and damage plants (Keskin et al., 2014, 2015). However, there is still a lot of work to be done to optimize UV-C applications.

In recent years, hot water applications have been implemented to increase the shelf life of fruits and vegetables and to reduce the use of chemical substances in the fight against diseases and pests (Lurie, 1998). Hot water applications are made by immersion (Barkai-Golan and Phillips, 1991) or by special hot water spraying (Fallik et al., 1996).

Due to short life time of post harvested strawberry fruit, the effect of modified atmosphere packaging (MAP) with different treatments have been widely considered.

In this study, effects of different postharvest treatments including UV-C and hot water were studied on biochemical changes of two commercially strawberry varieties stored at different temperatures in in MAP (modified atmosphere packaging) condition.

Materials and methods

Cultivars used in research

In the study, ‘Sabrina’ and ‘Rubygem’ strawberry cultivars, which are grown under greenhouse by a private company named Roseland and harvested in similar maturity in Tarsus district in Mersin province in Turkey, were used as research materials.

Strawberry fruit was first pre-cooled at 0 °C for 1 day. Later, the fruits of the same maturity were divided into 4 different groups. The first group of fruits was separated as controls. The second group of fruits was irradiated with 254 nm UV-C (0.25 kJ m^{-2}) with Vilber Lourmat UV-C lamp on both surfaces as 5 min at 20 cm distance. The third group of fruits was kept at 60 °C for 10 seconds in a hot water bath. In the fourth group, both UV-C and hot water applications were applied. Then fruit samples were placed in 250 gr plastic bags.

Fruits were maintained at 0 °C and 5 °C in Van Yüzüncü Yıl University Faculty of Agriculture Department of Horticulture’s cold-air storages with a relative humidity of 90-95% for 20 days.

Total phenolic substance amount and total antioxidant activity

Total phenolic contents in ‘Rubygem’ and ‘Sabrina’ strawberry cultivars were determined by spectrophotometer (Varian Bio 100, Australia) by Folin-Ciocaltaeu calorimetric method (Swain and Hillis, 1959). Absorbance values of the solutions

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were read in spectrophotometer (Thermo Fisher Scientific, G10S UV-Vis) at 725 nm wavelength and the total amount of phenolic substance expressed as gallic acid equivalent (GAE) mg kg^{-1} fresh weight (FW).

Ferric Reducing Antioxidant Power (FRAP) (Iron (III) Reduction Antioxidant Power) method was used to determine antioxidant activity (Benzie and Strain, 1996). 300 µ L freshly prepared FRAP reagent [25 mL acetate buffer (300 mmol/L, pH 3.6), 2.5 mL TPTZ (10 mmol/L) in 40 mmol/L HCl, and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution (20 mmol/L) were mixed as required to prepare working FRAP reagent] was warmed to 37 °C, and a reagent blank reading was taken at 593 nm; 10 µ L of each fraction extract solution of an appropriate concentration was then added along with 30 µ L H_2O. The 0–4 min absorbance change ($\Delta A_{593\ nm}$) was calculated for each sample and related to $\Delta A_{593\ nm}$ of a Fe^{2+} standard solution tested in parallel to obtain the FRAP value of each sample. Antioxidant activity values were reported as µmol trolox equivalent (TE) mg^{-1}.

Vitamin C (ascorbic acid)

The method specified by Cemeroğlu (2007) was applied to determine the content of vitamin C. 3 g of the strawberry fruit were weighed and homogenized in 6 ml of 6% metaphosphoric acid, then centrifuged at 12000 rpm for 15 min at 4 °C. In the HPLC (Agilent 1100 series) analyzes, C vitamins C18 (Phenomenex Luna C18, 250 x 4.60 mm, 5 µ) were performed. Readings was carried out in the DAD (DE33225146) detector at a wavelength of 254 nm. L-ascorbic acid (Sigma A5960), prepared at different concentrations, was used to identify vitamin C pick and to determine the amount of vitamin C (Karadogan and Keskin, 2017).

Sugar content

After the fruit samples of ‘cv. Rubygem’ and cv. ‘Sabrina’ were homogenized, a 5 g sample from each replicate was added in 10 ml of 6% metaphosphoric acid and then centrifuged at 15.000 rpm for 15 min at 4 °C. Sugar determinations were made on HPLC (Agilent 1100 series) after filtration through filters with a hole diameter of 0.45 mm in the supernatant sample. For this purpose, Phenomenex Rezex RCM monosaccharide column, 80% acetonitrile and refractive index detector were used as carrier phase. The definitions for sucrose, glucose, fructose and maltose were made using external standards, taking into consideration the structural changes of ‘Rubygem’ and ‘Sabrina’ (Özgökçe, 2016).

Statistical analysis

Experiments were conducted with three replication. The descriptive statistics for the traits studied are expressed as mean and standard error. In terms of these characteristics, factorial (Four Factors) Variance Analysis was performed to determine whether there was a difference between storage period, applications, temperature and cultivars. Following the variance analysis, Duncan test was used to identify the different groups. The statistical significance level was taken as $p < 0.05$ in the calculations and the SPSS statistical package program was used for the calculations.

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Results

Total phenolic content

During storage, it was observed that total phenolic contents of the ‘cv. Rubygem’ and ‘cv. Sabrina’ strawberry cultivars decreased at both storage temperatures. In the ‘cv. Rubygem’ strawberry cultivar; the highest total phenolic content at 0 °C was found to be 498.486 mg kg^{-1} with UV-C on day 16 and the lowest value was found in the control group as 247.946 mg kg^{-1} on day 20. At 5 °C, the highest total phenolic content was determined in hot water application on day 8 as 496.054 mg kg^{-1}, while the lowest value was found in the control group with 206.189 mg kg^{-1} on the 12th day.

The highest total phenolic content in ‘cv. Sabrina’ was found to be in hot water application with 515.919 mg kg^{-1} on 8th day and the lowest value was found in UV-C application on the 20th day with 117.405 mg kg^{-1}. At 5 °C, it has been identified that the highest value application was 484.297 mg kg^{-1} with UV-C + hot water application on day 0 and the lowest total phenolic compound value was with UV-C application on day 8 with 385.784 mg kg^{-1} (*Fig. 1*).

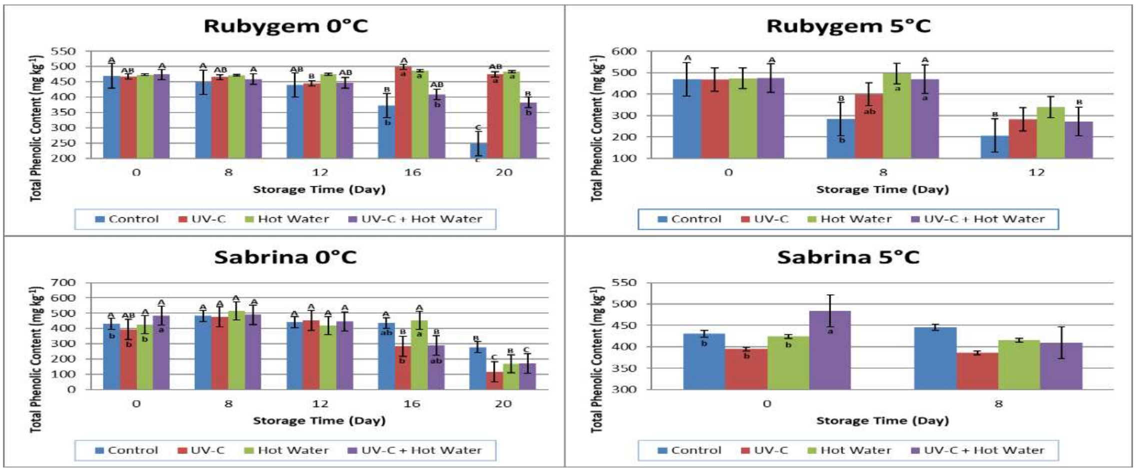

Figure 1. *Changes in total phenolic content as a result of storage of ‘cv. Rubygem’ and ‘cv. Sabrina’ strawberry cultivars at 0 and 5 °C. (a, b, c, d: The difference between the applications of the same kind, same storage period and different lower case letters at the same storage temperature is significant (p < 0.05) [Comparison of applications]. A, B, C, D: The difference between storage times of the same kind, same application and different capital letters at the same storage temperature is significant (p < 0.05) [Comparison of storage periods])*

Total antioxidant activity

Total antioxidant activity was found to decrease at the end of storage at both types and at the storage temperature. Therefore, in the ‘cv. Rubygem’, the highest value in total antioxidant activity at 0 °C was found to be 73.650 mg 100 g^{-1} in the hot water application and the lowest value in the control group with 9.600 mg 100 g^{-1} in the 20th day. The highest total antioxidant capacity at 5 °C was found to be 66.300 mg 100 g^{-1} in hot water at 0th day, and the lowest total antioxidant value in 12th day at 3.250 mg 100 g^{-1} in UV-C + hot water application. The ‘cv. Sabrina’ has the highest antioxidant value at 0 °C, Hot water application with 47.250 mg 100 g^{-1} on the 8th day and hot water application on the 20th day with the lowest value of 6.600 mg 100 g^{-1}. At 5 °C, the

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highest antioxidant amount was 40.100 mg 100 g^{-1} UV-C + hot water application on day 0, while the lowest was 23.050 mg 100 g^{-1} UV-C on day 8 (*Fig. 2*).

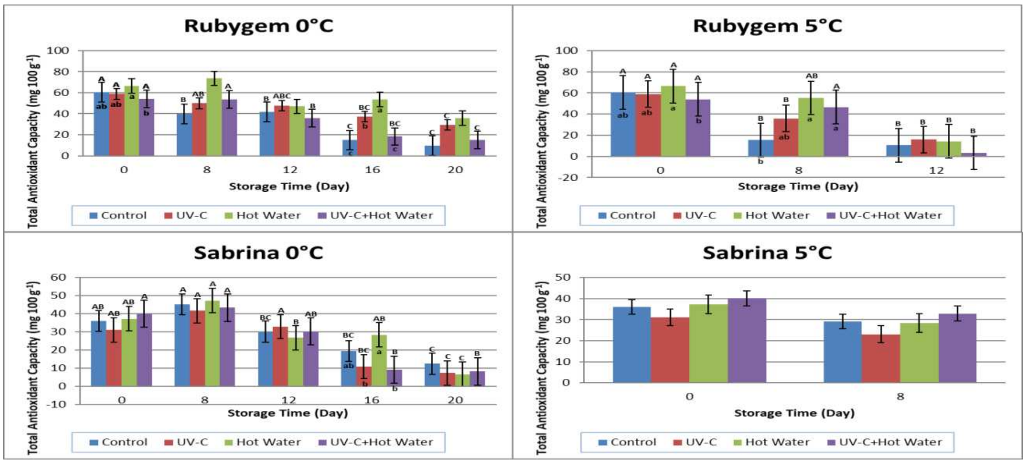

Figure 2. *Changes total antioxidant capacity resulting from storage of 'Rubygem' and 'Sabrina' strawberry cultivars at 0 and 5 °C*

Vitamin C (ascorbic acid)

During storage, fluctuations in vitamin C value are observed. In the 'cv. Rubygem', the highest vitamin C concentration at 0 °C was on 0th day with 343.723 mg 100 g^{-1} and the lowest vitamin C concentration was 16th day with 78.041 mg 100 g^{-1} in UV-C + hot water application. According to analysis results, at 5 °C, the highest value of vitamin C was detected in the application of UV-C + hot water with 343.723 mg 100 g^{-1} on day 0, while the lowest value of vitamin C was in the control group with 32.492 mg 100 g^{-1} on day 8. When the 'cv. Sabrina' was examined, it was determined that the highest value at 0 °C was in hot water application with 382.854 mg 100 g^{-1} on day 0 (*Fig. 3*).

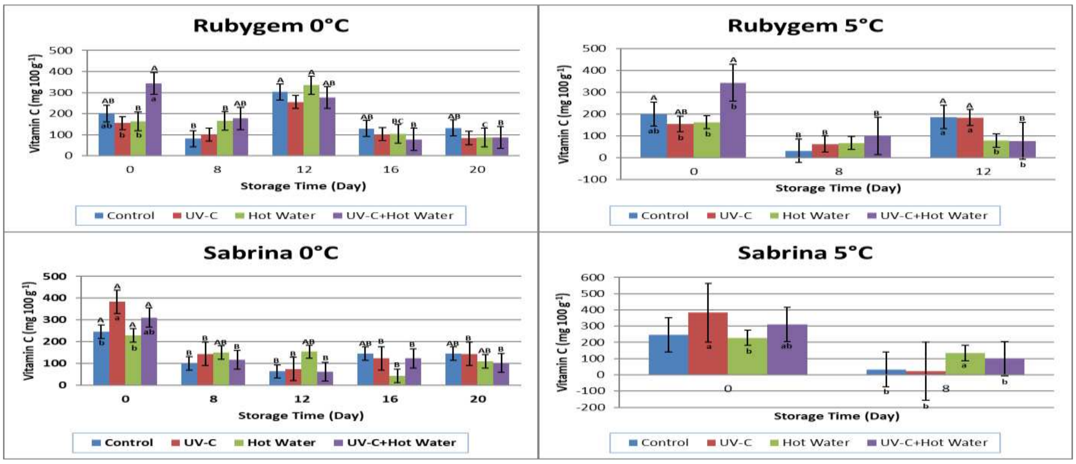

Figure 3. *Changes in the value of vitamin C as a result of storage of 'Rubygem' and 'Sabrina' strawberry cultivars at 0 and 5 °C*

The lowest values were found to be 43.273 mg 100 g^{-1} and 16 days in hot water application. The highest value of C vitamins at 5 °C was found in UV-C application at

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382.854 mg 100 g^{-1} on day 0. The lowest value was determined in UV-C application on day 8 with 22.906 mg 100 g^{-1}.

Sugar content

At the end of storage of the 'cv. Rubygem' at 0 °C, the application with the highest fructose value was found in the control group on day 0 with 30.404 mg g^{-1}, while the lowest value was found in the control group on day 20 with 4.180 mg g^{-1}. At 5 °C, the application with the highest fructose content was 30.404 mg g^{-1}, while the lowest value application was with hot water at 3.876 mg g^{-1} on the 12th day. When the variations in the 'cv. Sabrina' at 0 °C were examined, it was found that the highest fructose value was 22.793 mg g^{-1} on 8th day in UV-C application and the lowest value was 1.539 mg g^{-1} on 20th day in UV-C application were determined according to the analysis results. At 5 °C, the highest fructose value was found to be 22.556 mg g^{-1} with UV-C application on day 0 and the lowest value was found with UV-C application on day 8 with 11.372 mg g^{-1} (*Fig. 4*).

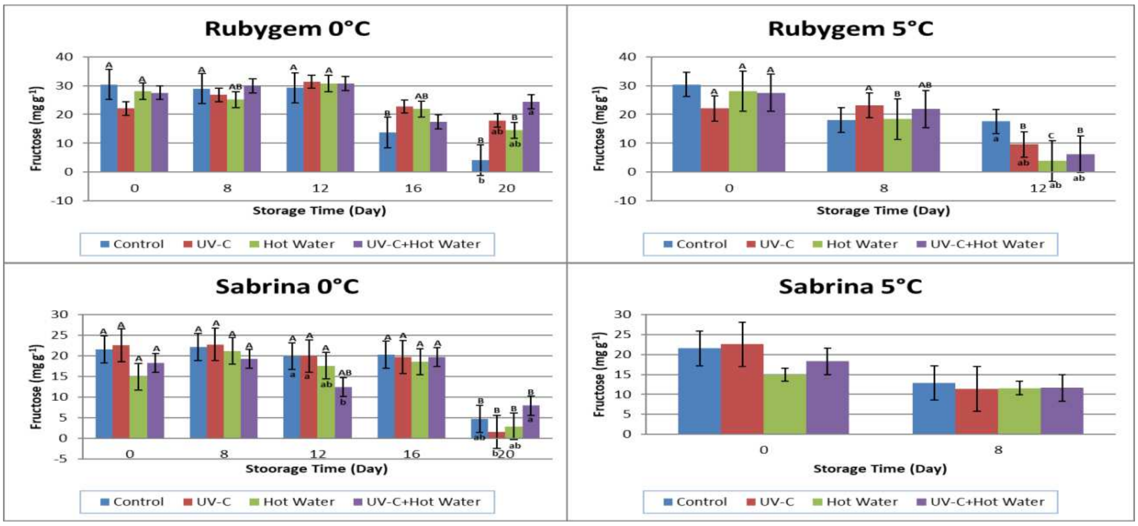

Figure 4. *Changes in fructose values resulting from storage of 'Rubygem' and 'Sabrina' strawberry cultivars at 0 and 5 °C*

Glucose has decreased regularly at both cultivars and storage temperatures. In this context, it has been determined that the lowest values in both cultivars and storage temperatures are in hot water application. As a result of storage of the 'cv. Rubygem' at 0 °C, the highest glucose value was found to be in the control group with 26.539 mg g^{-1} on day 0, while the lowest value was found to be in hot water application with 0.577 mg g^{-1} on the 20th day. The highest glucose value for 5 °C was the control group with 26.539 mg g^{-1} on day 0, while the lowest glucose value was found to be 1.086 mg g^{-1} on the 12th day with hot water application. In the 'cv. Sabrina', it was determined that the maximum value at 0 °C was 16.945 mg g^{-1} in UV-C application on 8th day, while the lowest value was 0.762 mg g^{-1} in hot water on 20th day. The highest glucose value at 5 °C was found to be 16.284 mg g^{-1} in UV-C application on day 0, while the lowest value was found in hot water application on day 8 with 5.676 mg g^{-1} (*Fig. 5*).

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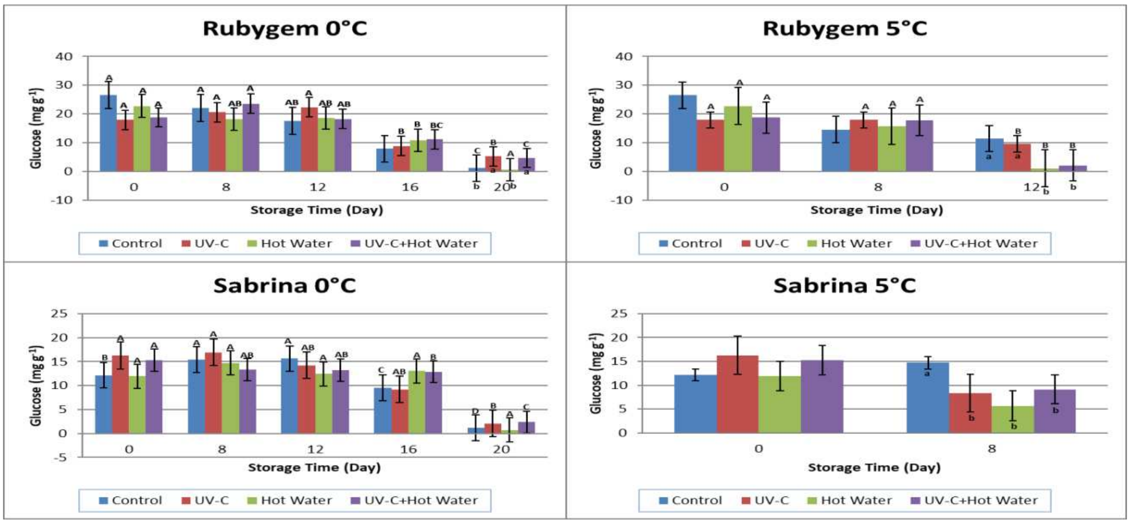

Figure 5. *Changes in glucose values resulting from storage of 'Rubygem' and 'Sabrina' strawberry cultivars at 0 and 5 °C*

When the changes in the sucrose value were examined, it was determined that there were fluctuations in both strawberry cultivars and storage temperatures. In the 'cv. Rubygem', it was determined that the highest value at 0 °C was 0.304 mg g^{-1}, the UV-C + hot water application on 16th day, and the lowest value was UV-C application on day 0 with 0.027 mg g^{-1}. At 5 °C, the highest sucrose level was detected in fruit samples treated with 0.300 mg g^{-1} of UV-C + hot water on day 8, while the lowest value was found to be in UV-C application on day 0 with 0.027 mg g^{-1}. According to the results of the analysis, in the 'cv. Sabrina', the highest sucrose value at 0 °C was found in the control group at 0.466 mg g^{-1} in the hot water application on 0th day and the lowest value application was in the control group on the 16th day with 0.033 mg g^{-1}. The highest sucrose value at 5 °C was determined to be 0.466 mg g^{-1} in hot water application on day 0, while the lowest value was found to be in the control group with 0.094 mg g^{-1} on the 8th day (*Fig. 6*).

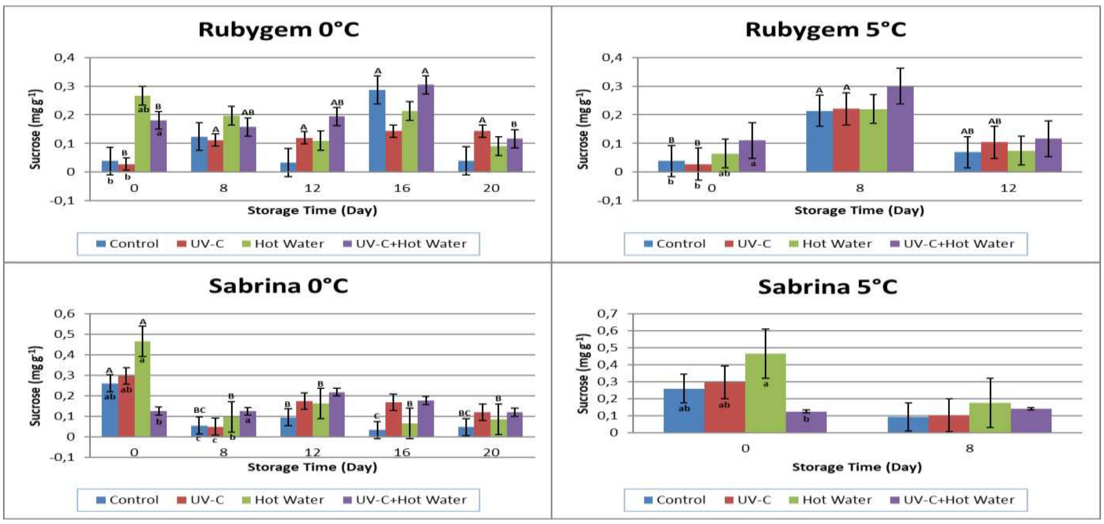

Figure 6. *Changes in sucrose value resulting from storage of 'Rubygem' and 'Sabrina' strawberry cultivars at 0 and 5 °C*

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Looking at the changes in maltose value, it was determined that the variations for the ‘cv. Rubygem’ were parallel to each other, while the ‘cv. Sabrina’ was found to be fluctuated. In this case, it was determined that the lowest value of the ‘cv. Rubygem’ was at 0 °C, the highest maltose value was in the control group with 29.497 mg g^{-1} on day 0. The lowest value was found in the control group with 4.20 mg g^{-1} on the 16th day. The highest maltose concentration at 5 °C was determined in the control group with 29.497 mg g^{-1} on 0th day, whereas the lowest value application was found to be the control group with 1.178 mg g^{-1} on 12th day. In the ‘cv. Sabrina’, the highest maltose value at 0 °C was found to be 20.094 mg g^{-1} in UV-C application on day 0, while the lowest value was found to be 2.592 mg g^{-1} in UV-C + hot water application on day 8. At 5 °C, the application of the highest maltose value was found to be 20.095 mg g^{-1} on day 0 with UV-C application, while the lowest value was found to be in the control group with 3.285 mg g^{-1} on day 8 (*Fig. 7*).

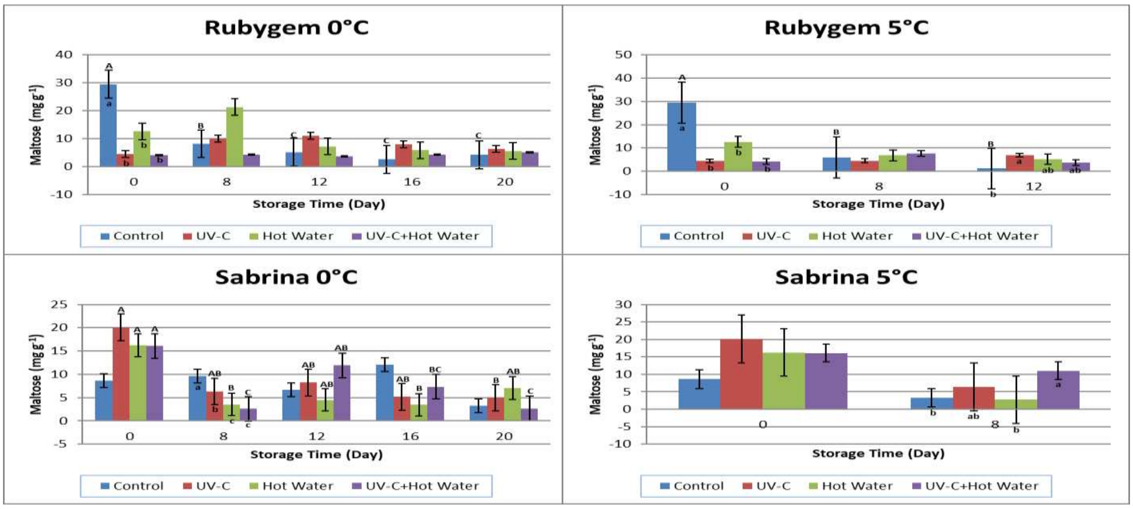

Figure 7. *Changes in the maltose value resulting from storage of ‘Rubygem’ and ‘Sabrina’ strawberry cultivars at 0 and 5 °C*

Discussion

Statistical analysis among different treatments on total phenolic contents in ‘cv. Rubygem’ and ‘cv. Sabrina’ cultivars showed that there was no significant difference in ‘cv. Rubygem’ stored at 0 °C in 0, 8 and 12 days. On the other hand, the difference between UV-C and hot water applications on the 16th day and also difference between control and UV-C + hot water applications on the 20th day was found statistically significant. The difference between the applications of hot water, UV-C + hot water, and control application on the 8th day was found to be significant, whereas the applications on the 0th and 12th day were not statistically significant when considering the differences between applications at the other storage temperature of 5 °C (*Fig. 1*). In the ‘cv. Sabrina’; the differences between the applications on days 8, 12 and 20 were not significant. The difference between UV-C + hot water application on day 0 and UV-C application and hot water application on day 16 were significant. Differences between applications at the ‘cv. Sabrina’ at 5 °C, another storage temperature; UV-C + hot water application was statistically significant at day 0, whereas it was not significant at day 8 applications (*Fig. 1*). In both types and temperatures there was a decrease in the total

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amount of phenol in almost all applications. These findings are similar to those of Pan et al. (2004). Results of this study showed that the highest amounts of phenolic substances in 'cv. Rubygem' could be observed at 0 °C storage temperature by using hot water and at 5 °C by using UV-C and hot water treatments. In this study that we have conducted, it has been determined that the application that protects the phenolic materials, which are so important in terms of human health, is hot water application at 0 °C, and UV-C and hot water applications at 5 °C in the 'cv. Rubygem'. In the 'cv. Sabrina', the result is that the hot water application is the best at both storage temperatures. Previous studies have shown that the total amount of phenolics in strawberries depends on the storage temperature and the composition of the atmosphere (Wang and Zheng, 2001; Ayala-Zavala et al., 2004; Cordenunsi et al., 2005). This high phenolic material in strawberries has increased the shelf life of fruit.

Differences in application with respect to total antioxidant capacity; the difference between the 'cv. Rubygem' and the 'cv. Sabrina' stored at 0 °C was statistically significant. The difference between the applications in the 'cv. Rubygem' type stored at 5 °C is significant, while the 'cv. Sabrina' is not significant (*Fig. 2*). Antioxidant substances naturally occur in plants. These plant antioxidants include anthocyanins and phenolic compounds. It was determined that the amount of antioxidant decreased regularly in both cultivars and 4 different applications. When the applications are evaluated on the basis of the amount of antioxidant in the 'cv. Rubygem', UV-C and hot water application is the application that best protects the amount of antioxidant compound at both storage temperatures. In the 'cv. Sabrina', it is determined that UV-C and hot water applications are the best application for strawberry fruit stored at 0 °C and control and UV-C + hot water applications for the fruits stored at 5 °C. The successful results have been obtained in terms of protection of antioxidants, especially in applications that we have made in the 'cv. Rubygem'. The study we conducted is similar to that of Vicente et al. (2004) with a study of grape fruits, indicating that the amount of antioxidants decreased both at the end of storage and that hot water and UV-C treatments were effective.

The comparison of different treatments on amounts of vitamin C showed that in 'cv. Rubygem' at 0 °C, the difference between UV-C + hot water application with UV-C and hot water applications on day 0 was statistically significant. Also, there wer no significant differences among storage days (8, 12, 16 and 20 days). At 5 °C, the difference between applications on day 0 and 12th day of storage were statistically significant, whereas on 8th day was not meaningful (*Fig. 3*). Difference between applications in the 'cv. Sabrina': at 0 °C, the difference between control and hot water applications of UV-C application was statistically significant at day 0, whereas the difference between applications at 8, 12, 16 and 20 days was not significant. At 5 °C, the difference between UV-C and hot water was significant at day 0 and hot water at day 8 and other applications (*Fig. 3*). Vitamin C values during storage: In the 'cv. Sabrina', except for UV-C applications, there was a decrease in vitamin C value at both temperatures and applications. Both types and at both storage temperatures (except for the 'cv. Sabrina' stored at 5 °C) have been the control group that best protects Vitamin C. With this study, both UV-C applications and hot water applications are thought to reduce the amount of vitamin C.

When the changes in sugar content were examined, the difference between the applications of fructose in both types of storage (except the 'cv. Sabrina' at 5 °C) was found to be significant (*Fig. 4*). The difference between the applications at the end of

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the storage of the ‘cv. Rubygem’ strawberry in terms of glucose was found statistically significant. In the ‘cv. Sabrina’, the difference between applications was not statistically significant at 0 °C. At 5 °C, the difference between treatments was found to be insignificant on day 0, whereas on day 8, control was statistically significant (*Fig. 5*). When the difference between the applications for sucrose value was examined, the difference between UV-C + hot water application and control and UV-C applications on day 0 in the strawberry cultivars stored at 0 °C was statistically significant, while the difference between the practices on the day was not statistically significant on days 8, 12, 16 and 20. When the difference between the applications is examined in the ‘cv. Rubygem’, the difference between UV-C + hot water application and control and UV-C applications was statistically significant on day 0 at 5 °C, whereas the difference between the applications on days 8 and 12 found to be insignificant (*Fig. 6*). The difference between the application of UV-C and UV-C + hot water at 0 °C on the 0^{th} day was found to be statistically significant when looking at the difference between applications for the changes in the value of sucrose in the ‘cv. Sabrina’. On the 8^{th} day, the difference between control and UV-C applications was not statistically significant, while UV-C + hot water application was statistically significant with hot water, control and UV-C applications. The difference between the applications of storage on the 12^{th}, 16^{th} and 20^{th} day was not statistically significant. When the difference between the applications was observed at 5 °C storage temperature of the ‘cv. Sabrina’ fruits, the difference between the applications of hot water and UV-C + hot water on the 0^{th} day was significant, but the difference between the applications on the 12^{th} day was not statistically significant (*Fig. 6*). At 0 °C, maltose content of the ‘cv. Rubygem’ was statistically significant at day 0, whereas applications on 8, 12, 16 and 20 days were not statistically significant. At 5 °C, the control application on 0^{th} day and the difference between control and UV-C applications on 12^{th} day was found to be statistically significant in the ‘cv. Rubygem’ (*Fig. 7*). The difference between the applications in the ‘cv. Sabrina’: While the difference between hot water and UV-C + hot water applications at 0 °C on the 8^{th} day was not statistically significant, the difference between control and UV-C applications of these applications was found statistically significant. The difference between the applications on 0, 12, 16 and 20 days was not statistically significant. At 5 °C, the difference between control and hot water applications with UV-C + hot water application on the 8^{th} day of the ‘cv. Sabrina’ was found to be statistically significant (*Fig. 7*). When fructose, glucose, sucrose and maltose sugar compositions were examined in strawberry fruit, these values decreased in almost all applications at the end of storage. This is why the fruits need energy to perform various reactions inside the cell so that they can survive even when they are picked up. This energy is obtained by using sugars, organic acids and oils present in the fruit. In other words, sugar is used during respiration.

Conclusion

In this study, the ‘cv. Rubygem’ and the ‘cv. Sabrina’ strawberry cultivars were placed in the plastic bags with control, UV-C, hot water and UV-C + hot water applications and stored at 0 and 5 °C, the fruits of the ‘cv. Rubygem’ were successfully preserved in 4 groups of applications until the 16^{th} day and at the end of 20^{th} day UV-C, hot water and UV-C + hot water applied fruit samples were successfully preserved. Changes in the same fruit samples at 5 °C were observed for 12 days and during this

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period it was determined that hot water and UV-C + hot water treatments could last 12 days at this storage temperature. When the changes occurred at the end of preservation of the 'cv. Sabrina' are examined, it is thought that fruit samples at 0 °C can be successfully preserved in all applications up to 12th day. It has been determined that UV-C application in this cultivar is preserved for 16 days, hot water and UV-C + hot water applications are preserved up to 20 days. It was determined that the 'cv. Sabrina' fruit samples kept at 5 °C lasted for only 8 days. As a result, it was observed that total phenolic contents, total antioxidant activity and sugar were found decrease at the end of storage for both cultivars while fluctuations in vitamin C.

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