SPATIAL AND TEMPORAL DIVERSITY OF MACROFUNGI IN THE WESTERN GHAT FORESTS OF INDIA

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Abstract. This study presents results of macrofungal inventory in the Western Ghat forest of Karnataka (reserve forest, shola forest, sacred grove and coffee agroforest) during wet season (June–November). A total of 157 species belonging to 87 genera was recovered. A maximum of 53 species was found in the coffee agroforest with highest diversity, exclusive (confined to a specific forest: 42 sp.) and core-group (frequency of occurrence, ≥10%: 17 sp.) species. Irrespective of forest, the species richness attained peaks during June and September. Rarefaction indices of species against sporocarps also showed the highest expected number of species in coffee agroforest. Of a total of 9256 sporocarps, the coffee agroforest consists of highest sporocarps than other forests (3715 *vs*. 1676–2999). Two-way ANOVA revealed significant spatial difference in richness of species ($P < 0.01$) as well as sporocarps ($P < 0.05$) without significant difference temporally. Low species similarity among forests surveyed (2.4–8.5%) depicts uniqueness of macrofungi in these forests. This survey yielded 45 new records to the Western Ghats and 47 economically valuable core-group fungi (edible, medicinal and ectomycorrhizal). Maintenance of suitable edaphic conditions along with enrichment organic matter (woody and leaf litter) in coffee agroforests seems to maximize economically viable macrofungi.
Keywords: *diversity, forests, macrofungi, mushrooms, Western Ghats*

Introduction

Fungi constitute one of the widely distributed biological entities involved mainly in decomposition of organic matter, biogeochemical cycles, mutualistic associations and pathogenicity (Kendrick, 2000). A conservative global estimate of fungi ranges between 1.5 and 3 million species (Hawksworth, 2001, 2012) and macrofungi has worldwide distribution ranging from 53,000 to 110,000 species based on the ratio of plant/macrofungi (Mueller et al., 2007). Forest ecosystems in Himalayan and the Western Ghat ranges of India are the major hotspots of fungal diversity consisting of about 850 species of macrofungi (Manoharachary et al., 2006).

The Western Ghats of India is a stretch of 1,600 km mountain range distributed in 160,000 km^2 in six political States (Gujarat, Maharashtra, Goa, Karnataka, Tamil Nadu and Kerala). The Western Ghats in Karnataka State endowed with a wide range of vegetation (grasslands, shola forests, deciduous forests, moist-dry deciduous forests, evergreen forests, semi-evergreen forests and scrub jungles) at different altitudinal ranges (~500 to 1,200 m asl). Although a variety of macrofungi have been reported from the Western Ghats, investigations in Karnataka are sporadic and quantitative studies are meager (e.g. Natarajan et al., 2005a; Brown et al., 2006; Swapna et al., 2008; Karun and Sridhar, 2013, 2014; Karun et al., 2014). Moreover, there is a gap in temporal assessment of macrofungi in the forests of Western Ghat. Therefore, the

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present inventory compares the occurrence of macrofungi in three natural forests (reserve forest, shola forest and sacred grove) and one agroforest (coffee agroforest) in the Western Ghats to draw insight on their diversity and temporal distribution pattern.

Materials and methods

Study area and survey

The forests surveyed for macrofungi include Makutta reserve forest in Perambadi (12°8'N, 75°47'E; 897 m asl), Monnangeri shola forest in Sampaje (12°28'N, 75°37'E; 608 m asl), Kadnur sacred grove in Virajpet (12°13'N, 75°46'E; 891 m asl) and coffee agroforest in B'Shettigeri (12°7'N, 75°52'E; 846 m asl) located in Kodagu District of Karnataka State (*Figure 1*). The survey was carried out throughout the wet season on monthly basis during monsoon (June-September 2012) and post-monsoon (October–November 2012) seasons. The rainfall data in each forest was recorded from the nearest meteorological observatory and it was highest in reserve forest (533.4 cm) followed by shola forest (482.6 cm), coffee agroforest (419.1 cm) and sacred grove (393.7 cm). Among the forests studied, coffee agroforest is fragmented owing to plantation practices.

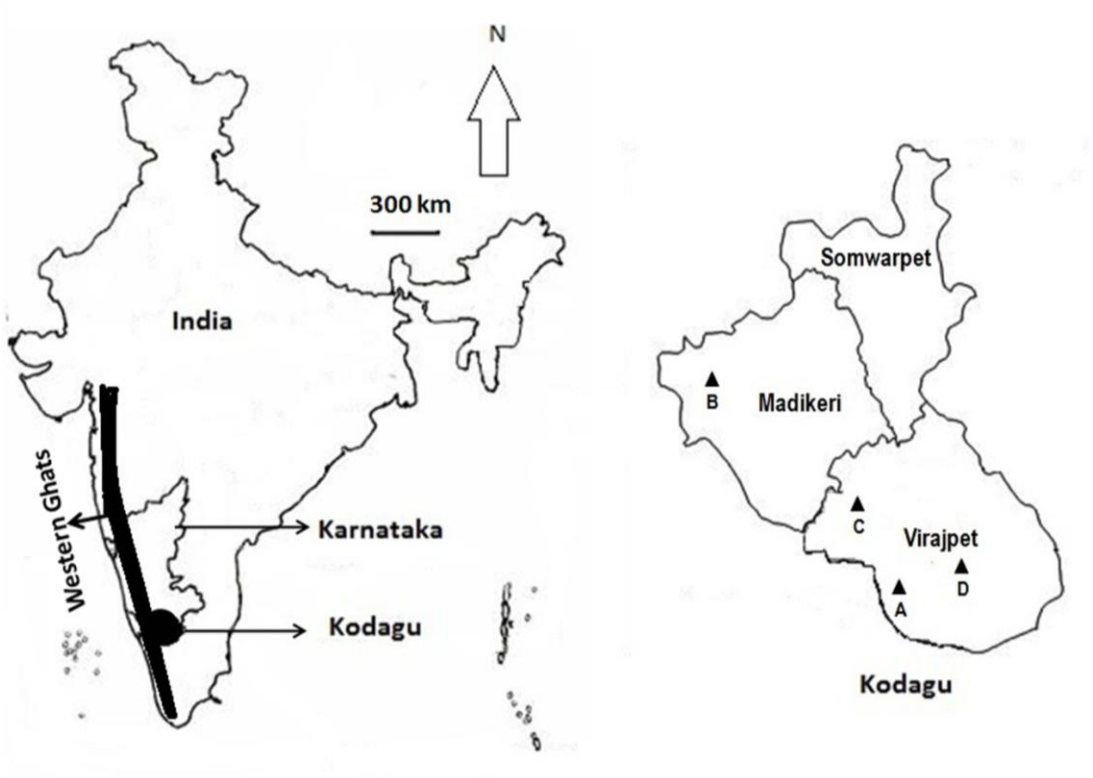

Figure 1. *Map of the study area in Kodagu District of the Western Ghats: A, Makutta reserve forest near Perambadi; B, Monnangeri shola forest near Sampaje; C, Kadnur sacred grove near Virajpet; D, B'Shettigeri coffee agroforest near Ponnampet.*

In each forest, macrofungal assessment was carried out on monthly basis during wet season in one quadrat (25 × 25 m) without overlapping. Qualitative and quantitative assessment of sporocarps of macrofungi occurring on soil, leaf litter, woody litter,

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standing dead or live trees (bark or branches) and other substrates (e.g. dung and dead insects) was carried out. Macromorphological characteristics of representative species of macrofungi sampled were assessed on the sampling site, blotted and preserved in ziploc bags in cool packs for further assessment. Microscopic observation was carried out in the laboratory (Olympus # CX41RF; magnification, 1000X) using diagnostic keys to confirm the identity (Jordan, 2004; Phillips, 2006; Sathe and Daniel, 1980; Sathe and Deshpande, 1980; Tibuhwa et al., 2010; Mohanan, 2011; Buczacki, 2012; Tibuhwa, 2012). Blotted representative specimen were preserved by transferring into a preservative (water-ethanol-formaldehyde: 14:5:1) and additional blotted mushrooms were oven-dried (55–60°C) and preserved in ziploc bags.

Data analysis

The mean number of sporocarps of each macrofungus/quadrat in each forest (MSF) (n = 6; in six months; irrespective of months) as well as mean number of sporocarps of each macrofungus/quadrat in each month (MSM) (n = 4; in four forests; irrespective of forests) were plotted along with overall mean number of sporocarps/quadrat (MS) (n = 24).

$$MSF = (TSL \div TQ) \quad \text{(Eq.1)}$$

$$MSM = (TSM \div TQ) \quad \text{(Eq.2)}$$

$$MS = (TS \div TQ) \quad \text{(Eq.3)}$$

(where, MSF, mean sporocarps of a species/forest/quadrat; TSL, total sporocarps of a species in a forest; TQ, total number of quadrats surveyed; MSM, mean sporocarps of a species/month/quadrat; TSM, total sporocarps of a species/month; MS, mean sporocarps of a species/quadrat; TS, total sporocarps of a species). Those macrofungi having ≥10% frequency of occurrence was considered as core-group fungi.

The Simpson (D') and Shannon (H') index (Magurran 1988) and Pielou's evenness (J') (Pielou 1975) of macrofungi in different forests and months were calculated:

$$D' = 1 \div \sum (pi)^2 \quad \text{(Eq.4)}$$

$$H' = -\sum (p_i \ ln \ p_i) \quad \text{(Eq.5)}$$

$$J' = (H' \div H'_{max}) \quad \text{(Eq.6)}$$

(where, p_i is the proportions of sporocarps that species i contributes to the total number of sporocarps of all species; H'_{max} is the maximum value of diversity for the number of species present)

Sorensen's similarity coefficient (%) of macrofungi between different forests and months was determined based on Chao et al. (2005):

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$$C_S = [(2c) \div (a + b)] \times 100 \quad \text{(Eq.7)}$$

(where, a is total number of species in forest or month 1; b is total number of species in forest or month 2; c is number of species common to forest or month 1 and 2).

To compare the richness of species based on the number of sporocarps in each forest or month, the expected number of species [E_s] was calculated using rarefaction index by Ludwig and Reynolds (1988). The E_s in a random sample of n sporocarps from a grand total of N sporocarps was estimated:

$$E_s = \sum_{i=1}^{s} \left\{ 1 - \left[\binom{N - n_i}{n} \Big/ \binom{N}{n} \right] \right\} \quad \text{(Eq.8)}$$

(where, n_i is the number of sporocarps of the ith species).

Two-way ANOVA was employed to ascertain spatial (forest) and temporal (month) impact on the richness of species and sporocarps of macrofungi by multiple comparisons using Holm-Sidak method (SigmaPlot, version 11, Systat Inc., USA).

Results

Forest and season

The present inventory revealed occurrence of 157 species of macrofungi in 87 genera encompassing 45 new records of macrofungi to the Western Ghats consisting of 16 and 29 species identified up to species and genus level, respectively (*Appendix 1)*. Some of the representative species of macrofungi are projected in *Figure 2* and *Figure 3*. Among the four forests surveyed, irrespective of seasons, the highest number of macrofungi (53 *vs*. 37-42 species) (*Figure 4A*), exclusive species (those confined to a specific forest: 42 *vs*. 33-35 species) and core-group species (17 *vs*. 6-11 species) were highest in coffee agroforest (*Figure 4B*). Based on the rarefaction indices out of 625 random number of sporocarps, the expected number of species was also higher in the coffee agroforest than in other forests (45 *vs*. 31-37 species) (*Table 1*). The Simpson and Shannon indexes were peaked in the coffee agroforest. The Sorensen similarity coefficient showed low overlapping in different forests ranging from 2.4% (reserve forest *vs*. shola forest) to 8.5% (sacred grove *vs*. coffee agroforest) depicting uniqueness of macrofungi in these forests (*Table 2*). The trend was also same in different forests as reflected in the rarefaction curves (*Figure 5A*). Similar to species richness, the sporocarp richness was highest in coffee agroforest (3715) followed by shola forest (2999) (*Figure 6A*).

Irrespective of forests, seasonal survey for six months revealed two peaks in species richness (June and September) (*Figure 4C*). Overall, the monsoon season (June-September) consists of higher species richness compared to post-monsoon season (October-November). The exclusive species were steeply declined, while the core-group species were highest during September (*Figure 4D*). The rarefaction index out of 625 random number of sporocarps revealed the highest expected number of species during June (42 species) followed by September (41) (*Table 1*). The Simpson index was also

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highest in June/July, while the Shannon diversity was highest during September. The Sorensen's similarity coefficient in different months ranged between 2.5% (June *vs*. October) and 55.6% (October *vs*. November) (*Table 2*). The Sorensen's similarity coefficient between consecutive months was steadily increased from June to November (*Figure 5B*). Similar to species richness, the sporocarp richness was also highest during September followed by August (*Figure 6B*).

Figure 2. *Sporocarps of selected core-group fungi: A, Amylosporus campbellii (edible); B, Auricularia auricula-judae (edible); C, Boletinellus merulioides (edible and ectomycorrhizal); D, Cookeina tricholoma; E, Coprinus disseminates (edible); F, Cyathus striatus; G, Dacryopinax spathularia; H, Entoloma theekshnagandhum (ectomycorrhizal); I and J, Immature and mature Filoboletus manipularis (edible).*

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Figure 3. *Sporocarps of selected core-group fungi: A, Inocybe viridiumbonata (ectomycorrhizal); B, Lentinus squarrosulus (edible); C, Marasmius guyanensis; D, Phellinus gilvus (medicinal); E, Pleurotus cornucopiae (edible); F, Pleurotus djamor (edible); G, Pycnoporus cinnabarinus (medicinal); H, Scutellinia setosa; I, Termitomyces microcarpus (edible) and J, Trametes versicolor (medicinal).*

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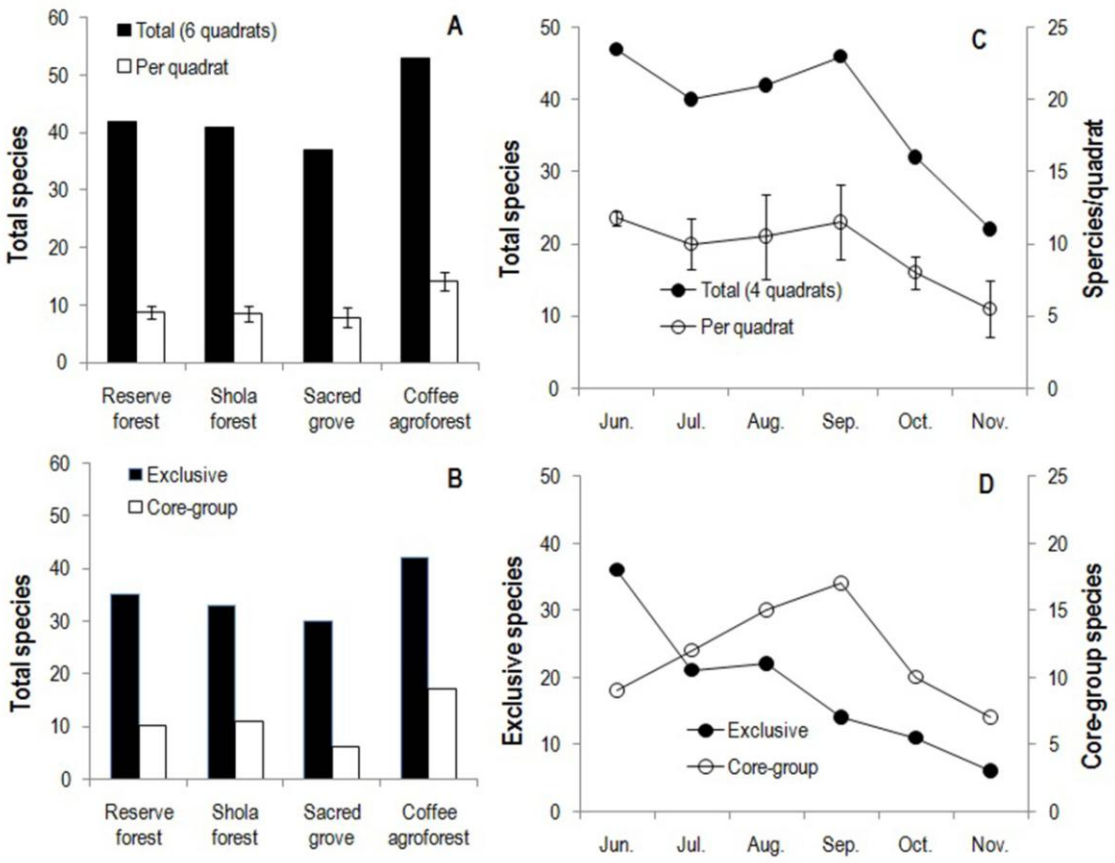

Figure 4. *Total species, species/quadrat, exclusive species and core-group species in different forests (A, B) and months (C, D) (species/quadrat in forest, A: n=6, mean±SE; species/quadrat in months, C: n=4, mean±SE).*

Table 1. *Species richness, diversity and evenness of macrofungi in different forests and months of the Western Ghats (*Expected number of species, Es, out of 625 random number of sporocarps)*

	Species richness		Diversity index		Pielou's
	Total species	$E_{(s625)}$*	Simpson	Shannon	evenness
Forest					
Reserve forest	42	37	0.928	4.349	0.806
Shola forest	42	36	0.868	3.685	0.683
Sacred grove	36	31	0.804	3.056	0.591
Coffee agroforest	53	45	0.939	4.576	0.799
Month					
June	47	42	0.934	4.044	0.728
July	40	37	0.934	4.355	0.818
August	42	35	0.881	3.843	0.713
September	46	41	0.933	4.504	0.815
October	32	31	0.907	4.065	0.813
November	22	22	0.929	4.078	0.914

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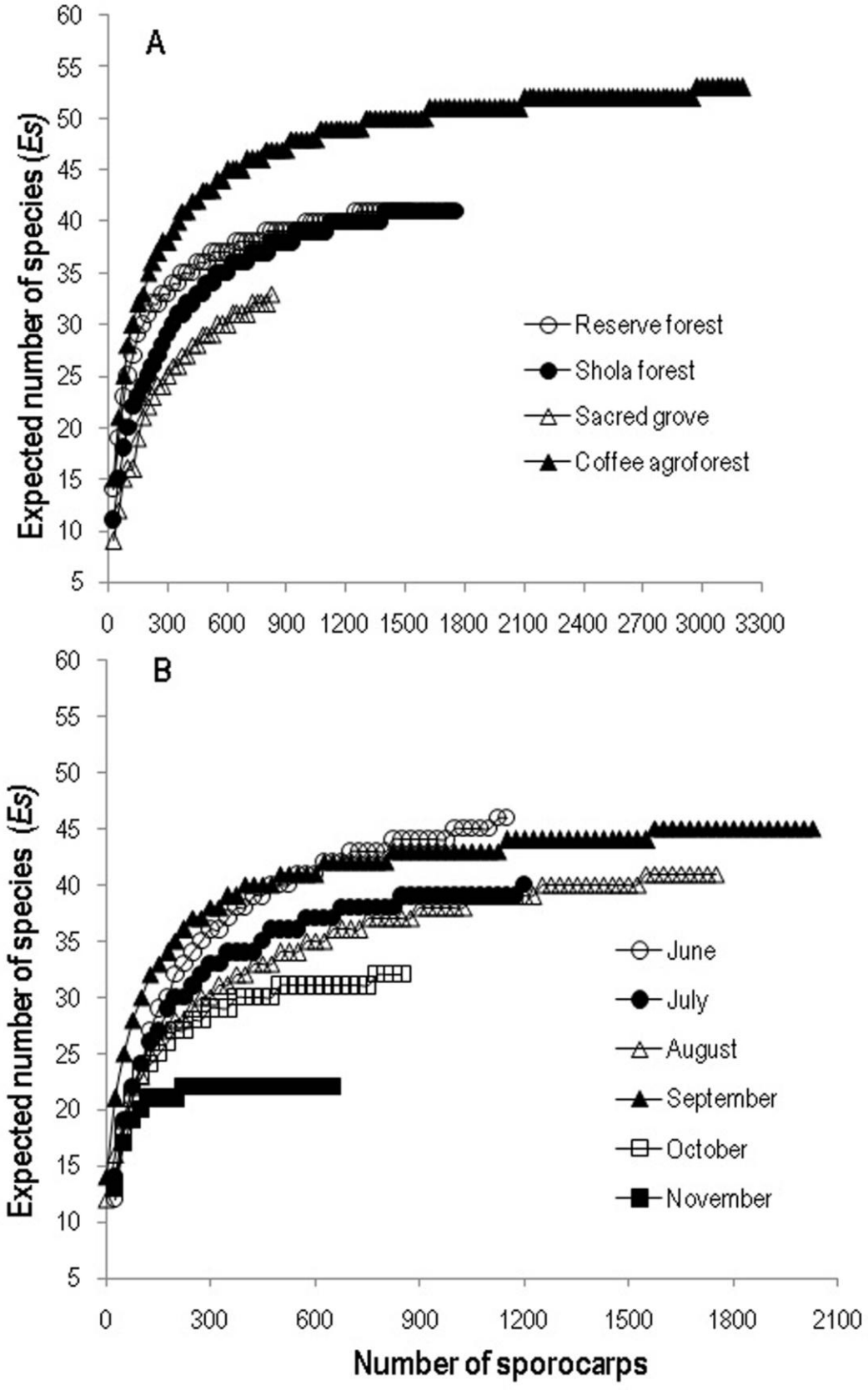

Figure 5. *Rarefaction curves of macrofungal expected number of species [Es] against the number of sporocarps in different forest (A) and months (B).*

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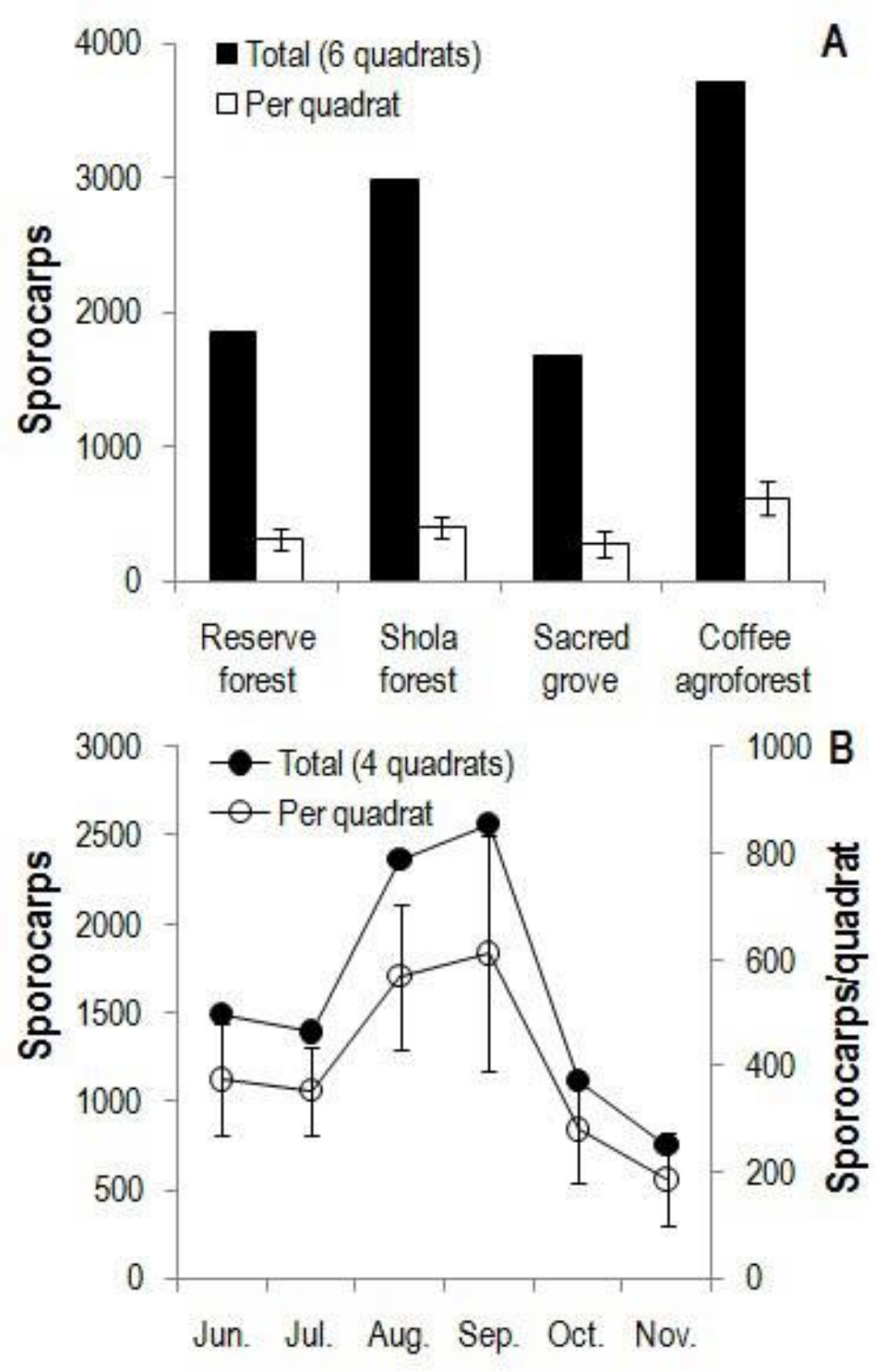

Figure 6. *Total sporocarps, and sporocarps/quadrat in different forests (A) and months (B) (sporocarps/quadrat in forest, A: n=6, mean±SE; sporocarps/quadrat in months, B: n=4, mean±SE).*

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Table 2. *Sorensen's similarity coefficient (%) of macrofungi in four forests (RF, Reserve forest; SF, Shola forest; SG, Sacred grove; CAF, Coffee agroforest) during six months (Monsoon: June-September; post-monsoon: October-November) in the Western Ghats*

Forest				Month					
	SF	SG	CAF		Jul.	Aug.	Sep.	Oct.	Nov.
RF	2.4	5.1	8.4	Jun.	9.2	9.0	10.8	2.5	2.9
	SF	7.7	8.5		Jul.	26.8	27.9	11.1	12.9
		SG	6.7			Aug.	29.6	13.5	12.5
							Sept.	38.5	29.4
								Oct.	55.6

Two-way ANOVA revealed significant difference in overall species richness ($P<0.01$) and overall sporocarp richness ($P < 0.05$) among different forests but not among different months. Holm-Sidak multiple comparisons resulted in significant difference in the species richness between coffee agroforest *vs*. other forests (shola forest, $P < 0.001$; reserve forest, $P < 0.01$; sacred grove, $P < 0.01$).

Core-group fungi and substrate preference

The core-group fungi have a major role to play in the ecosystem services compared to those occur in low frequency. Among 157 species of macrofungi, 47 belonged to core-group (*Appendix 1*) and 43% of species possess economic importance (edible, 25 species; medicinal, 25 species; ectomycorrhizal, 17 species). Besides saprophytes, up to 50% of core-group fungi are economically valuable (edible, 11 species; medicinal, 11 species; ectomycorrhizal, 3 species). Nine core-group fungi are new records for the Western Ghats (*Ganoderma oregonense, Marasmiellus ramealis, Marasmius guyanensis, M. pellucidus, Ophiocordyceps nutans, Phellinus pini, Pleurotus cornucopiae, Pluteus* sp. 2 and *Xylaria filiformis*) and some are likely to be new species or varieties. Among the core-group fungi, edible species were higher in shola forest followed by coffee agroforest than other forests (5 spp. *vs*. 1 sp.). The core-group fungi were recorded on different substrates like bark, dead insects, leaf litter, soil, twigs and wood (*Appendix 1*). Majority of them preferred woody litter (bark, twig and wood) (83%), the rest confined to soil/elephant dung (13%) and leaf litter/dead insects (4%). Although six species of macrofungi were confined to the elephant dung, none belongs to core-group category.

Discussion

Even though some studies are available on the diversity, taxonomy and distribution of macrofungi in the Western Ghats (e.g. Farook et al., 2013; Sathe and Daniel, 1980; Sathe and Deshpande, 1980; Mohanan, 2011), quantitative studies are scanty (e.g. Natarajan et al., 2005a, 2005b; Brown et al., 2006; Swapna et al., 2008). Bhagwat et al. (2005) and Brown et al. (2006) surveyed reserve forests, sacred groves and coffee plantations distributed in different parts of the Western Ghats.

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They sampled macrofungi in three occasions during monsoon (May-September) and recorded 163 species. Similarly, Swapna et al. (2008) surveyed several sampling stations of semi-evergreen and moist deciduous forests of the Western Ghats up to two years and reported 315 species. Surprisingly, even though less area as well as a few forest types were surveyed, 157 species were recorded in our study. As the similarity coefficient between consecutive months was steadily increased from June through November, overlapping species was lower during monsoon than in post-monsoon as supported by the rarefaction index. Overall, two peaks in species richness (June and September) coincided with peaks of exclusive species (June), core-group species (September) and sporocarp richness (September). A sudden change due to the onset of south-west monsoon in May and stability of edaphic factors during September in the Western Ghats might be plausible reasons for peaks in species as well as sporocarps in forests surveyed.

According to Brown et al. (2006), among the three forests (reserve forests, sacred groves and coffee plantations), the sacred groves possess the highest species as well as sprorcarps. In coffee plantations in their study, although species and sporocarp richness were low, Shannon index was higher than reserve forests and sacred groves. On the contrary, the coffee agroforest in our study revealed higher richness of species as well as sporocarps compared to other forests although samples and periodicity of survey was uniform. This has also been supported by the two-way ANOVA with significant difference in species richness between coffee agroforest against other forests. As the species similarity was low among the forests surveyed, it can be predicted that macrofungi existing in these forests are fairly unique. Likewise, the evergreen forests of the Western Ghats showed uniqueness in macrofungi as reported by Brown et al. (2006) and Swapna et al. (2008).

Brown et al. (2006) also opined that the degradation of habitat is a major threat for diversity of macrofungi than the habitat fragmentation. However, in spite of human interference and fragmentation, the coffee agroforest showed highest species richness as well as diversity possibly due to prevalence of suitable edaphic conditions (e.g. moisture and temperature) and deposition of substrates (e.g. woody and leaf litter) on the floor. The standing dead wood, leaf and woody litter accumulated on the forest floor are valuable lignocellulosic materials in maintaining suitable conditions for the growth and perpetuation of macrofungi (Hammel, 1997).

Bhagwat et al. (2005) recorded negligible occurrence of entomophagus and coprophilous macrofungi in reserve forests, sacred groves and coffee plantations (1.8–3.8%) corroborating our study (4.5%). Edible macrofungi were relatively more in sacred groves than in reserve forests and coffee plantations (Bhagwat et al., 2005). Out of a total of 163 species reported by Bhagwat et al. (2005), only one species belonged to *Termitomyces*. Similarly, out of 315 species reported in semi-evergreen and moist deciduous forests, none of them belonged to *Termitomyces* (Swapna et al., 2008). Surprisingly, our study revealed occurrence of five species of *Termitomyces* (*T. clypeatus, T. eurrhizus, T. heimii T. microcarpus* and *T. tylerianus*) and *T. microcarpus* was core-group species in shola forest as well as coffee agroforest.

Macrofungal species composition vary at altitudinal ranges and between the habitats of the Western Ghats (e.g. evergreen, semi-evergreen, moist deciduous and deciduous forests), which is depending on the tree species, substrate availability (quality, quantity and season), surface area and penetration of sunlight (Bhagwat et al., 2005; Brown et al., 2006). Brown et al. (2006) surveyed representative locations in the reserve forests,

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sacred groves and coffee plantations of entire Kodagu District. Swapna et al. (2008) confined their inventory to the semi-evergreen and moist deciduous forests of Shimoga District. Natarajan et al. (2005a) surveyed the Nilgiri Biosphere Reserve forest spread over in three States of the Western Ghats (Tamil Nadu, Kerala and Karnataka). Another study by Natarajan et al. (2005b) mainly focused on the occurrence of ectomycorrhizal fungi in forest dominated by dipterocarp tree species (*Dipterocarpus indicus, Hopea parviflora* and *Vateria indica*) in the Uppangala forest region of Kodagu. The above studies corroborates with the present survey with low overlap of species between the forests of different geographical regions predicting uniqueness of macrofungi.

Conclusions

Inventory of macrofungi in four forest types in the Western Ghats of India up to six months during wet season yielded 157 species in 87 genera. Low species similarity among forests (2.4–8.5%) depicts uniqueness of macrofungi possibly due to prevelance of forest-specific edaphic conditions. Coffee agroforest showed the highest species richness, sporocarp richness and diversity of macrofungi. Irrespective of the forest type, June and September showed peaks in species as well as sporocarp richness. Out of 47 core-group fungi, up to 50% species was economically viable (edible, medicinal and ectomycorrhizal) preferred woody litter. It is predicted that maintenance of coffee agroforest surveyed in the Western Ghats with diverse tree species, edaphic conditions (e.g. temperature, moisture and humidity) and ample substrates on the floor favoured perpetuation of economically viable macrofungi. Blending traditional knowledge of tribals on macrofungi in different forests of the Western Ghats and enforcing modern agricultural practices in agroforestry in favour of macrofungi will facilitate sustainable production for future benefits.

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APPENDIX

Appendix 1. *Occurrence of macrofungi (abundance of sporocarps/625 m^2) in four locations of the Western Ghats (RF, Reserve forest; SF, Shola forest; SG, Sacred grove; CAF, Coffee agroforest) during monsoon (June–September) and post-monsoon (October–November) season; new records for the Western Ghats are in bold-face); importance (Ed, edible; Me, medicinal; Em,ectomycorrhizal) and substrate preference (B, Bark; I, Insect; L, leaf litter; S, Soil; T, Twigs; W, Wood) are given for core-group fungi (MSF, mean sporocarps of a species/forest in a quadrat; MSM, mean sporocarps of a species/month in a quadrat; MS, mean sporocarps of a species per quadrat)*

Macrofungus	Sporocarps/quadrat										Sporocarps/ quadrat (mean; n=24) (MS)
	Forest (mean; n = 6) (MSF)				Month (mean; n = 4) (MSM)						
	RF	SF	SG	CAF	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	
Microporus vernicipes (Berk.) Kuntze (T, W)	30.0	100.0	–	–	11.3	–	–	93.8	65.0	25.0	32.50
Pleurotus cornucopiae (Paulet) Rolland (B, W) (Ed)	–	–	90.8	–	–	–	136.0	–	–	–	22.70
Trametes versicolor (L.) Lloyd (W) (Me)	4.7	–	–	85.7	–	25.5	30.0	37.0	29.0	14.5	22.60
***Pluteus* sp. 2** (B)	–	83.3	–	–	–	–	125.0	–	–	–	20.80
Coprinus disseminatus (Pers.) Gray (B, W) (Ed)	–	20.8	–	50.0	–	–	–	106.0	–	–	17.70
Schizophyllum commune Fr. (B, W) (Me)	–	–	–	60.0	–	5.0	17.5	30.0	20.0	17.5	15.00
Psathyrella lucipeta (Berk. & Broome) Pegler	–	–	58.3	–	87.5	–	–	–	–	–	14.60
Dacryopinax spathularia (Schwein.) G.W. Martin (B, W)	–	–	–	50.0	–	–	50.0	25.0	–	–	12.50
Termitomyces microcarpus (Berk. & Broome) R. Heim (S) (Ed)	–	33.3	–	11.0	–	–	–	50.0	–	16.5	11.10
Xylaria multiplex (Kunze) Fr.	44.0	–	–	–	–	–	–	50.0	11.0	5.0	11.10
Gymnopilus bryophilus Murrill (B, W)	42.0	–	–	–	–	50.0	–	12.5	–	–	10.40
Pycnoporus cinnabarinus (Jacq.) P. Karst. (W) (Me)	–	–	–	41.7	–	–	32.4	29.0	1.2	–	10.40
Cyathus striatus (Huds.) Willd. (S, W)	–	1.7	–	33.3	52.5	–	–	–	–	–	8.75
Xylaria obovata (Berk.) Berk. (W) (Me)	–	–	32.5	–	–	37.5	11.3	–	–	–	8.13

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Xylaria longipes Nitschke (W) (Me)	4.7	–	–	26.0	–	11.3	14.0	7.5	12.0	1.3	7.68
Lentinus squarrosulus Mont. (W) (Ed)	–	–	–	27.7	–	–	22.1	–	14.0	5.9	6.93
Marasmius pellucidus Berk. & Broome (T, W)	–	–	27.0	–	40.5	–	–	–	–	–	6.75
Xylaria filiformis (Alb. & Schwein.) Fr. (L) (Me)	–	25.0	–	–	37.5	–	–	–	–	–	6.25
Ophiocordyceps nutans (Pat.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora (I) (Me)	–	–	–	22.2	–	17.0	16.3	–	–	–	5.55
Marasmius guyanensis Mont. (L)	–	–	–	22.0	–	14.3	7.4	11.3	–	–	5.50
Phellinus pini (Brot.) Bondartsev & Singer (B, W) (Me)	–	–	–	21.7	–	–	–	–	13.0	20.0	5.43
Cookeina tricholoma (Mont.) Kuntze (B, W)	–	20.9	–	–	–	20.0	11.4	–	–	–	5.23
Hypholoma subviride (Berk. & M.A. Curtis) Dennis (B, W)	3.3	–	4.2	12.5	–	6.3	20.0	3.8	–	–	5.00
Phellinus gilvus (Schwein.) Pat. (B, W) (Me)	20.0	–	–	–	–	25.0	–	2.3	1.0	1.9	5.00
Boletinellus merulioides (Schwein.) Murrill (S) (Ed, Em)	–	–	–	19.7	–	14.5	–	15.0	–	–	4.93
Clavulinopsis laeticolor (Berk. & M.A. Curtis) R.H. Petersen (S)	–	18.1	–	–	–	26.3	–	0.8	–	–	4.53
Royoporus spathulatus (Jungh.) A.B. De (B, W) (Ed)	–	13.3	–	2.0	–	–	–	2.5	21.0	–	3.83
Cyclomyces setiporus (Berk.) Pat. (W)	15.0	–	–	–	–	–	–	18.8	3.7	–	3.75
Auricularia auricula-judae (Bull.) Quél. (B, W) (Ed)	–	–	–	14.2	–	13.8	6.3	1.3	–	–	3.57
***Hypholoma* sp.** (B, W)	14.0	–	–	–	–	21.3	–	–	–	–	3.55
Irpex lacteus (Fr.) Fr. (B, W)	–	–	14.2	–	–	–	–	–	16.0	5.0	3.55
Stereum hirsutum (Willd.) Pers. (W)	14.0	–	–	–	–	–	–	–	15.0	6.3	3.55
Entoloma theekshnagandhum Manim., A.V. Joseph & Leelav. (S) (Em)	–	13.3	0.8	–	21.2	–	–	–	–	–	3.53
Amylosporus campbellii (Berk.) Ryvarden (W) (Ed)	13.0	–	–	–	–	–	20.0	–	–	–	3.33
***Phellinus* sp.** (W)	13.0	–	–	–	–	–	–	20.0	–	–	3.30
Pleurotus djamor (Rumph. ex Fr.) Boedijn (B, W) (Ed)	–	13.0	–	–	–	–	13.3	6.2	–	–	3.25
Ganoderma oregonense Murrill (W) (Me)	13.0	–	–	–	–	–	–	6.3	7.5	5.0	3.13
Lenzites elegans (Spreng.) Pat. (W)	–	–	–	12.5	–	–	–	–	–	18.8	3.13
Pleurotus flabellatus Sacc. (B, W) (Ed)	–	11.2	–	–	–	–	11.8	5.0	–	–	2.80

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Scutellinia setosa (Nees) Kuntze (B, L, W)	–	–	–	10.8	–	–	–	13.8	2.5	–	2.70
Inocybe viridiumbonata Pegler (S) (Em)	–	–	10.0	–	–	–	–	15.0	–	–	2.50
***Inonotus* sp.** (W)	9.2	–	–	–	–	–	–	13.8	–	–	2.30
Coprinopsis fibrillosa (Berk. & Broome) Redhead, Vilgalys & Moncalvo (B)	–	–	–	8.3	–	–	–	–	–	12.5	2.08
Xylaria polymorpha (Pers.) Grev.	–	8.3	–	–	–	–	–	6.8	2.9	0.7	2.08
Ganoderma lucidum (Curtis) P. Karst.	–	–	7.5	0.7	–	–	–	3.5	3.8	5.0	2.05
Ganoderma applanatum (Pers.) Pat.	–	–	7.7	–	–	–	–	2.6	9.0	–	1.93
Marasmiellus ramealis (Bull.) Singer (B, T)	–	–	–	7.5	11.3	–	–	–	–	–	1.88
Favolaschia tonkinensis (Pat.) Kuntze	–	–	–	7.2	–	–	–	10.8	–	–	1.80
Filoboletus manipularis (Berk.) Singer (B, T, W) (Ed)	–	–	–	7.2	10.8	–	–	–	–	–	1.80
Crepidotus sp.	6.7	–	–	–	10.1	–	–	–	–	–	1.68
Marasmius kuthubutheeni Y.S. Tan, Desjardin, Vikinesw. & N. Abdullah	–	–	–	6.7	–	5.0	–	5.1	–	–	1.68
Chondrostereum purpureum (Pers.) Pouzar	5.8	–	–	–	–	–	–	–	8.8	–	1.45
Coriolopsis telfari (Klotzsch) Ryvarden	–	–	–	5.8	–	–	–	–	–	8.7	1.45
Oxyporus cervinogilvus (Jungh.) Ryvarden	5.8	–	–	–	–	–	–	–	–	8.7	1.45
Podosordaria elephanti J.D. Rogers & Y.M. Ju	5.8	–	–	–	–	8.7	–	–	–	–	1.45
***Pluteus* sp. 1**	–	–	–	5.7	1.4	7.2	–	–	–	–	1.43
Ascocoryne cylichnium (Tul.) Korf	5.5	–	–	–	8.3	–	–	–	–	–	1.38
Termitomyces heimii Natarajan	5.0	–	–	–	–	–	7.5	–	–	–	1.25
Conocybe pubescens (Gillet) Kühner	0.7	–	–	–	–	1.1	–	–	–	–	1.18
***Lactarius* sp.**	–	4.7	–	–	7.1	–	–	–	–	–	1.18
***Agaricus* sp.**	0.2	–	–	4.2	6.3	–	0.3	–	–	–	1.10
Coprinus micaceus (Bull.) Fr.	–	–	–	4.2	–	–	6.3	–	–	–	1.05
Marasmius rotula (Scop.) Fr.	–	4.2	–	–	6.3	–	–	–	–	–	1.05
Pholiota squarrosa (Vahl) P. Kumm.	4.2	–	–	–	–	–	–	6.3	–	–	1.05

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Termitomyces eurrhizus (Berk.) R. Heim	2.2	–	1.8	–	3.3	2.3	0.5	–	–	–	1.00
Entoloma haematinum Manim., Leelav. & Noordel.	3.8	–	–	–	5.7	–	–	–	–	–	0.95
Phellinus chrysoloma (Fr.) Donk	–	–	–	3.7	–	–	–	–	2.8	2.8	0.93
***Pleurotus* sp. 1**	3.7	–	–	–	–	2.7	–	2.9	–	–	0.93
***Ascocoryne* sp. 2**	3.3	–	–	–	5.0	–	–	–	–	–	0.83
Auricularia mesenterica (Dicks.) Pers.	–	–	–	3.3	–	5.0	–	–	–	–	0.83
Termitomyces clypeatus R. Heim	–	–	–	3.3	–	3.8	1.2	–	–	–	0.83
***Inocybe* sp.**	–	–	3.0	–	4.5	–	–	–	–	–	0.75
Crepidotus mollis (Schaeff.) Staude	–	–	2.9	–	–	–	–	4.4	–	–	0.73
Hygrocybe conica (Schaeff.) P. Kumm.	2.8	–	–	–	4.2	–	–	–	–	–	0.70
Hexagonia tenuis Speg.	–	–	–	2.7	–	–	–	–	1.6	2.5	0.68
Lenzites betulina (L.) Fr.	–	–	–	2.7	–	–	–	4.1	–	–	0.68
Marasmius trichotus Corner	–	–	2.7	–	4.1	–	–	–	–	–	0.68
Clitocybe pallida Velen.	–	–	–	2.5	3.8	–	–	–	–	–	0.63
Gloiocephala resinopunctata Manim. & K.A. Thomas	–	2.5	–	–	–	–	3.8	–	–	–	0.63
Gymnopilus dilepis (Berk. & Broome) Singer	–	–	–	2.5	–	–	–	3.8	–	–	0.63
***Nigroporus* sp.**	2.5	–	–	–	–	–	–	–	–	3.8	0.63
***Oxyporus* sp.**	–	2.5	–	–	–	–	–	–	3.8	–	0.63
Pleurotus eous (Berk.) Sacc.	–	2.5	–	–	–	–	3.8	–	–	–	0.63
Ramariopsis kunzei (Fr.) Corner	–	1.7	0.8	–	1.3	2.5	–	–	–	–	0.63
Coprinus leiocephalus P.D. Orton	–	–	–	2.2	–	–	3.3	–	–	–	0.55
Nigroporus vinosus (Berk.) Murrill	–	2.2	–	–	–	–	–	3.3	–	–	0.55
Daldinia concentrica (Bolton) Ces. & De Not.	–	–	–	2.0	–	3.0	–	–	–	–	0.50
***Fomitopsis* sp.**	–	–	–	–	–	–	–	3.0	–	–	0.50
Xylaria escharoidea (Berk.) Sacc.	–	2.0	–	–	–	–	–	3.0	–	–	0.50
Laetiporus sulphureus (Bull.) Murrill	–	–	–	1.8	–	–	–	–	2.7	–	0.45
***Pleurotus* sp. 2**	–	–	1.7	–	–	–	2.6	–	–	–	0.43

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***Scleroderma* sp. 3**	1.7	–	–	–	–	2.6	–	–	–	–	0.43
***Amauroderma* sp.**	1.7	–	–	–	2.6	–	–	–	–	–	0.43
***Irpex* sp.**	–	1.5	–	–	–	–	–	–	2.3	–	0.38
Lepiota metabola (Berk. & Broome) Sacc.	1.5	–	–	–	2.3	–	–	–	–	–	0.38
Scleroderma areolatum Ehrenb.	–	–	1.5	–	2.3	–	–	–	–	–	0.38
***Scleroderma* sp. 1**	–	–	1.5	–	0.5	–	1.5	0.3	–	–	0.38
***Ascocoryne* sp. 1**	1.3	–	–	–	2.0	–	–	–	–	–	0.33
Geastrum saccatum Fr.	–	–	–	1.3	–	–	2.0	–	–	–	0.33
Lepiota phlyctaenodes (Berk. & Broome) Sacc.	–	1.3	–	–	2.0	–	–	–	–	–	0.33
Lepiota thrombophora (Berk. & Broome) Sacc.	–	–	–	1.3	2.0	–	–	–	–	–	0.33
Merulius tremellosus Schrad.	–	1.3	–	–	1.2	0.8	–	–	–	–	0.33
***Scleroderma* sp. 2**	–	–	1.3	–	–	2.0	–	–	–	–	0.33
Aleuria rubra L.R. Batra	–	–	–	1.2	–	–	1.8	–	–	–	0.30
Entoloma vernum S. Lundell	–	1.2	–	–	–	1.8	–	–	–	–	0.30
Gymnopilus subbellulus Hesler	–	1.2	–	–	–	–	1.8	–	–	–	0.30
Hygrocybe alwisii (Berk. & Broome) Pegler	–	–	1.2	–	–	–	1.8	–	–	–	0.30
Lentinus dicholamellatus Manim.	1.2	–	–	–	1.8	–	–	–	–	–	0.30
Phaeolus schweinitzii (Fr.) Pat.	–	–	1.2	–	–	–	–	1.0	0.8	–	0.30
Phlebopus portentosus (Berk. & Broome) Boedij	–	–	–	1.2	0.5	–	0.5	0.8	–	–	0.30
Trogia infundibuliformis Berk. & Broome	–	–	–	1.2	1.8	–	–	–	–	–	0.30
***Amanita* sp. 2**	–	–	–	1.0	1.5	–	–	–	–	–	0.25
Cookeina indica Pfister & R. Kaushal	–	1.0	–	–	–	1.5	–	–	–	–	0.25
Lepiota erythrogramma (Berk. & Broome) Sacc.	–	–	1.0	–	1.5	–	–	–	–	–	0.25
Ramaria gracilis (Pers.) Quél.	–	–	1.0	–	–	–	1.5	–	–	–	0.25
***Panus* sp.**	–	1.0	–	–	–	1.5	–	–	–	–	0.25
Polyporus arcularius Lázaro Ibiza	–	–	–	1.0	–	–	1.5	–	–	–	0.25
***Gymnopus* sp. 1**	–	–	0.9	–	1.0	–	–	0.4	–	–	0.23

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Panus conchatus (Bull.) Fr.	–	0.8	–	–	1.2	–	–	–	–	–	0.20
Piloporia indica Ganesh & Ryvarden	0.8	–	–	–	–	–	–	–	1.2	–	0.20
Postia stiptica (Pers.) Jülich	–	–	–	0.8	–	–	1.2	–	–	–	0.20
Psilocybe fimetaria (P.D. Orton) Watling	–	0.8	–	–	–	–	–	–	1.2	–	0.20
Clavulinopsis aurantiocinnabarina (Schwein.) Corner	–	0.5	0.2	–	–	1.1	–	–	–	–	0.18
Coprinus cinereus (Schaeff.) Gray	0.7	–	–	–	–	1.1	–	–	–	–	0.18
Coprinus patouillardii Quél.	–	0.7	–	–	–	–	–	–	1.1	–	0.18
Lysurus brahmagirii C. Mohanan	–	–	–	0.7	–	–	1.0	0.1	–	–	0.18
***Marasmiellus* sp.**	–	–	–	0.7	1.1	–	–	–	–	–	0.18
Panaeolus fimicola (Pers.) Gillet	–	0.7	–	–	–	–	–	–	1.1	–	0.18
Psilocybe coprophila (Bull.) P. Kumm.	–	0.7	–	–	–	1.1	–	–	–	–	0.18
Trametes maxima (Mont.) A. David & Rajchenb.	–	0.7	–	–	–	–	–	–	1.1	–	0.18
Xylaria symploci A. Pande, Waingankar, Punekar & Randive	0.7	–	–	–	–	1.1	–	–	–	–	0.18
Agaricus caribaeus Pegler	–	–	0.3	0.2	0.5	–	–	0.3	–	–	0.13
Clavulinopsis luteoalba (Rea) Corner	–	–	0.5	–	–	–	–	–	–	0.8	0.13
***Entoloma* sp.**	–	–	0.5	–	–	–	–	0.8	–	–	0.13
Oudemansiella canarii (Jungh.) Höhn.	–	–	0.5	–	–	–	–	–	0.8	–	0.13
Ramaria flava (Schaeff.) Quél.	–	0.5	–	–	–	–	0.8	–	–	–	0.13
Ramariopsis pulchella (Boud.) Corner	–	0.5	–	–	–	0.8	–	–	–	–	0.13
***Tricholoma* sp.**	–	–	–	0.5	–	0.8	–	–	–	–	0.13
***Amanita* sp. 1**	–	–	–	0.3	0.5	–	–	–	–	–	0.08
Boletus huronensis A.H. Sm. & Thiers	–	–	0.3	–	–	0.5	–	–	–	–	0.08
Geastrum fimbriatum Fr.	–	–	0.3	–	–	0.5	–	–	–	–	0.08
Geastrum sp.	0.3	–	–	–	–	–	0.5	–	–	–	0.08
Lepiota plumbicolor (Berk. & Broome) Sacc.	–	–	–	0.3	–	–	0.5	–	–	–	0.08
Lycoperdon nigrescens Pers.	–	0.3	–	–	0.5	–	–	–	–	–	0.08
***Pholiota* sp.**	–	0.3	–	–	0.5	–	–	–	–	–	0.08

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Xerula furfuracea (Peck) Redhead, Ginns & Shoemaker	–	–	0.3	–	–	–	0.5	–	–	–	0.08
Amauroderma rugosum (Blume & T. Nees) Torrend	–	0.2	–	–	0.3	–	–	–	–	–	0.05
Clitocybe minuta C. Mohanan	–	–	0.2	–	–	–	–	0.3	–	–	0.05
Cystoagaricus trisulphuratus (Berk.) Singer	–	–	0.2	–	0.3	–	–	–	–	–	0.05
Dictyophora cinnabarina W.S. Lee	–	0.2	–	–	0.3	–	–	–	–	–	0.05
Geastrum pseudostriatum Hollós	–	–	0.2	–	–	–	0.3	–	–	–	0.05
Hericium erinaceum Hesler	0.2	–	–	–	–	0.3	–	–	–	–	0.05
Lycoperdon utriforme Bull.	–	–	–	0.2	0.3	–	–	–	–	–	0.05
***Lepista* sp.**	–	–	0.2	–	–	–	0.3	–	–	–	0.05
Phellinus rimosus (Berk.) Pilát	–	–	0.2	–	–	–	–	–	0.3	–	0.05
Russula aciculocystis Kauffman ex Bills & O.K. Mill.	0.2	–	–	–	0.3	–	–	–	–	–	0.05
Termitomyces tylerianus Otieno	0.2	–	–	–	–	0.3	–	–	–	–	0.05

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THE ROLE OF SELECTED BIOPHYSICAL FACTORS IN LONG-TERM LAND-USE CHANGE OF CULTURAL LANDSCAPE

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Abstract. The paper addresses the importance of biophysical factors in land-use change over a period of time of more than 150 years beginning in the middle of the nineteenth century. Biophysical factors define the environmental capacity of a territory and can be viewed as a predisposition for changes in land use. The article investigates the impact of selected biophysical factors on land-use changes in the Trkmanka Watershed in the southeast part of the Czech Republic. The results of a canonical correspondence analysis demonstrated a relationship between selected variables and land-use changes. The most significant variables were slope grade, elevation and soil fertility, while aspect was of limited importance. Extensification processes are conditioned on less favourable environmental circumstances, especially steeper slope grade, higher elevation, less fertile soil and a north-facing slope. Intensive land use is associated with a lower slope grade and elevation, more fertile soils and a more favourable southern exposure. Although the results of the statistical analyses confirmed the importance of environmental factors, they explain only some of the overall land-use changes. It is clear that socioeconomic factors are the main forces influencing the character of land use.
Keywords: *land-use change, biophysical factors, canonical correspondence analysis, Trkmanka Watershed, Czech Republic*

Introduction

Land-use changes are tied to changes in the ecosystem services of the agricultural landscape, which have a direct impact on man's well-being (Duraiappah and Naeem, 2005; Vitousek et al., 1997). In addition to food production, these services also include soil erosion prevention measures (Feng et al., 2010; Zorn and Komac, 2009), protection of biodiversity (Pereira et al., 2010), climate change mitigation (Stone, 2009) and the cultural and aesthetic quality of the landscape (Xu et al., 2005). The study of changes in the development of the agricultural landscape and an understanding of the drivers of these changes serve as a basic guideline for anticipating the trajectory of future development (Veldkamp and Lambin, 2001) and assessing the influence of land related policies (Vliet et al., 2015). Better understanding of the interactions between land cover, land use and function may enhance our ability to properly model land changes (Verburg et al., 2009). This knowledge is important for establishing sustainable land management and for protecting basic land-use functions (Turner II, 1997) and related ecosystem services. Avoiding simplifications regarding the causes of land-cover land-use changes is a substantial prerequisite for sound and sustainable environment-development policies (Lambin et al., 2001).

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Land-use change and its drivers are usually investigated in local studies that often capture the period of the economic and/or social transition of society in recent decades. In this context, Vliet et al. (2015) identified three main contemporary processes impacting land-use change: the globalization of agricultural markets, the transition from a rural to an urban society and the shift to post-socialism in central and eastern Europe. Land-use change in the period of the adoption of a market-oriented economy after the era of socialism and the implementation of European Union policies during and after EU accession in post-socialist countries is a typical example of a relatively short-term transition with a more distinct dynamic attracting the interest of researchers. Articles on this regional research have recently been published from Slovakia (Pazúr et al., 2014; Lieskovský et al., 2014a), Albania (Müller et al., 2013), Ukraine (Baumann et al., 2011), Hungary (Szillasi et al., 2010) and the European part of Russia (Prishchepov et al., 2013). A common feature of these studies is the investigation of driving forces against the background of the extensification of the agricultural landscape.

The drivers of land changes can be divided into those of a biophysical and a socioeconomic nature (Mitsuda and Ito, 2011). Underlying drivers conditioned by people can be further divided into economic and technological factors, demographic factors, institutional factors and cultural factors (Geist and McConell, 2010). Verburg et al. (2004) presents a slightly different classification of land change determinants, which can be biophysical, economic, social, interactive, neighbourhood, and/or political nature. Finally, according to the Food and Agriculture Organization of the United Nations (FAO), the various reasons for agricultural land abandonment can be grouped in the following categories: natural constraints, land degradation, socio-economic factors, demographic structure and the institutional framework. The mentioned sources list various categories of human-related factors, all of which agree on the separate role of biophysical/natural constraints. It is not unusual for the study of land-use change to be focused on human-related factors (Łowicki, 2008; Song et al., 2013; Hietel et al., 2007; Krausmann et al., 2003) or, in contrast, environmental biophysical factors (Fu et al., 2006; Opršal et al., 2013). Recent studies that include selected socioeconomic and biophysical factors (Serra et al., 2008; Müller et al., 2009; Prishchepov et al., 2013; Pazúr et al., 2014) have two common traits. First of all, they are almost always extensive regional studies (involving an entire country or several regions) in which socioeconomic factors (e.g. the demographic structure of the population, migration, distance from regional economic centres) can be meaningfully modelled. Secondly, the studies are short-term in nature and are usually focused on changes occurring over the past several decades.

The research of drivers is common in geographically explicit historical studies of larger territories covering a time period of one-hundred years and more (Bičík et al., 2001; Cousins, 2001; Petit and Lambin, 2002). This situation can be explained in several ways, the most important being the apparent lack or absence of sufficiently accurate maps from historical time periods and the difficulty of converting them to digital form. For this reason, studies of historical land-use change often focus on smaller territories, typically municipalities (Kanianska et al., 2014; Druga and Falťan, 2014). Another selection factor is the aforementioned preference for periods of major change, especially the period following the Second World War with a higher land-use change dynamic (Antrop, 2005). The third bias in case study selection has a geographical rather than a temporal nature – cases are more than can be expected by chance in areas which are considered hotspots for change (Vliet et al., 2015). These hotspots are usually located in marginal areas (Verburg and Overmars, 2009) typically in mountains (Schneeberger et al., 2007; Kuemmerle et al., 2009;

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Li et al., 2009; Alvarez Martinez et al., 2011) and/or dry-land areas (Lasanta and Vicente-Serrano, 2012; Zhang et al., 2004; Li et al., 2007). Although it is understandable that scientists are primarily interested in change and not necessarily in regions that maintain the status quo (Vliet et al., 2015), such a selection might not be representative of all agriculture land in a respective region or country. Finally, Gerard et al. (2006) pointed to the fact that the degree of thematic detail and level of spatial detail of the land cover measured may determine the type, amount and rate of change detected and therefore to a certain extent determine the reliability of the results.

The aim of this article is to identify selected physical conditions and constraints that influence long-term land-use changes. The study of socioeconomic factors is tied to time-limited studies that often capture a period of the economic and/or social transition of society. However, socioeconomic factors in particular are highly variable and time-specific, and therefore their explanatory potential loses reliability in longitudinal studies (with the exception of certain demographic factors, especially out-migration). In contrast, natural conditions are relatively constant and hence their relevance is also retained in studies of long-term land-use development exceeding a timeframe of 100 years. The study is based on the reconstruction of the landscape in the middle of the nineteenth century using data from the Second Military Mapping Survey from 1836-1842. This time layer is compared to current land use, and the study thus captures a period of more than 150 years.

Materials and Methods

Study area

The Trkmanka Watershed is located in the southeast part of the Czech Republic (*Figure 1*). The Trkmanka Watershed represents a diversified area from the perspective of natural conditions and topography (*Figure 2*), and can be regarded as a representative type of agricultural landscape in the southeast part of the Czech Republic. The land slopes from the northeast to the southwest, and its total area is 380 km^2. The watershed is an old settlement region characterised by a warm climate and fertile loessic soils. Long-term annual average precipitation in the entire territory is around 500 mm (Demek and Novák, 1992). The lowest southwest part of the watershed is characterised by flat alluvial relief, whereas the middle and northwest part is hilly (Demek and Mackovčin, 2006). The highest point in the region is at an elevation of 437.4 m above sea level, while the lowest point is the confluence of the Trkmanka and Dyje rivers at 158 m above sea level.

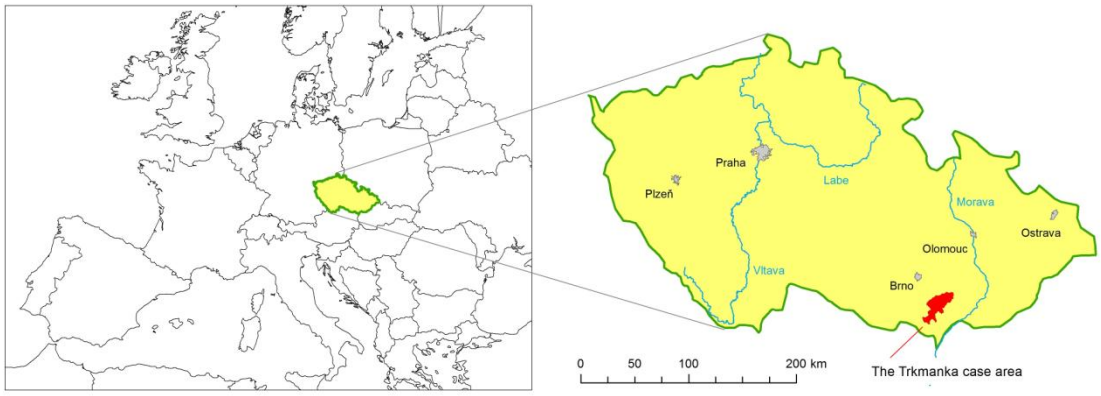

Figure 1. *Location of the Trkmanka Watershed in Europe and the Czech Republic*

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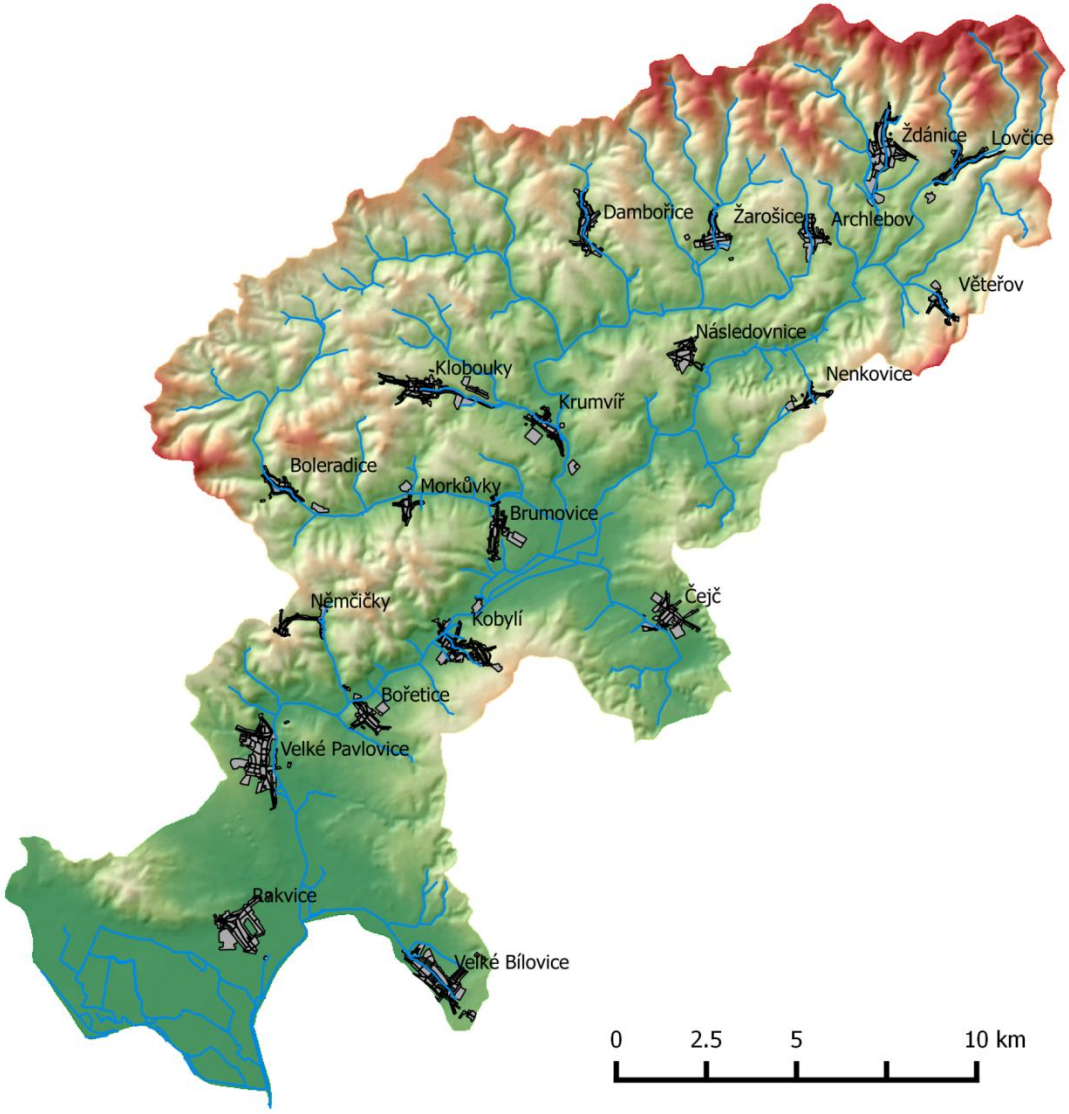

Figure 2. *The study area of the Trkmanka Watershed, marking the current watercourses and settlements*

Agricultural land was and continues to be a significant landscape element in a large part of the watershed (Kiliánová et al., 2009). The greatest land-use changes occurred after the middle of the nineteenth century in connection with innovation in the forms of agricultural production. The loss of grazing land in favour of arable land was connected with imports of cheap Australian wool, growth in stable cattle operations and later also the mechanisation of agricultural production. The intensification of agricultural production was also responsible for the demise of many natural and artificial bodies of water, of which only a fraction remain today. Among the unique wetlands that no longer exist were the Čejč and Kobylí salt lakes, which were drained during the nineteenth century (Kiliánová et al., 2009).

The forced collectivisation and intensification of agricultural production in the second half of the twentieth century represents another significant intervention in the

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structure of the landscape. Collectivisation was accompanied by the consolidation of land parcels and led to both a reduction in landscape diversity but also to the disruption of the traditional ties between owners and farm land (Bičík et al., 2001). The current form of the agricultural landscape is the result of long-term development reflecting changes in society, the economy, institutions and available technologies (Geist and McConell, 2010). In our article we work under the assumption that in addition to these time-variable socioeconomic factors, the current appearance of the land was also influenced by environmental and biophysical conditions that can be regarded as constant over the chosen time span of 150 years.

Identification of historical and contemporary land use

As the goal of the study is to assess the importance of biophysical factors in long-term land-use change, the first half of the nineteenth century was chosen as the baseline. This historical land use is compared to the current state of the land in 2008 (*Figure 3*). Historical land use was reconstructed using maps from the Second Military Mapping Survey (Franziszeische Landesaufnahme) conducted in Moravia (at the time part of the Czech lands within the Austro-Hungarian Empire) in 1836-1840. The survey has its foundation in the mapping for the Stable Cadastre established by the Land Tax Act (Grundsteuerpatent) from 1817 (Lisec and Navratil, 2013). The graphic topographic basis was the reduced content of the cadastral maps in a scale of 1:2 880. Topographic data of military importance (areas) were shown in eleven colours; terrain configuration was represented using Lehmann hachure. Elevation was given only at trigonometric points (Boguszak and Šlitr, 1962), the basic unit was the 'Vienna fathom' (Wiener Klafter) (Timár and Molnár, 2013). Six map sections (georeferenced and digitised) from the Second Military Mapping Survey were used for the analysis of the watershed. The digitisation and interpretation of orthophotos from 2004-2006 were used to determine current land use. Basic information on the area was refined and updated by supplementary field survey in 2008 (Kiliánová et al., 2009). All created vector data are in the ESRI shapefile format in the S-JTSK coordinate system.

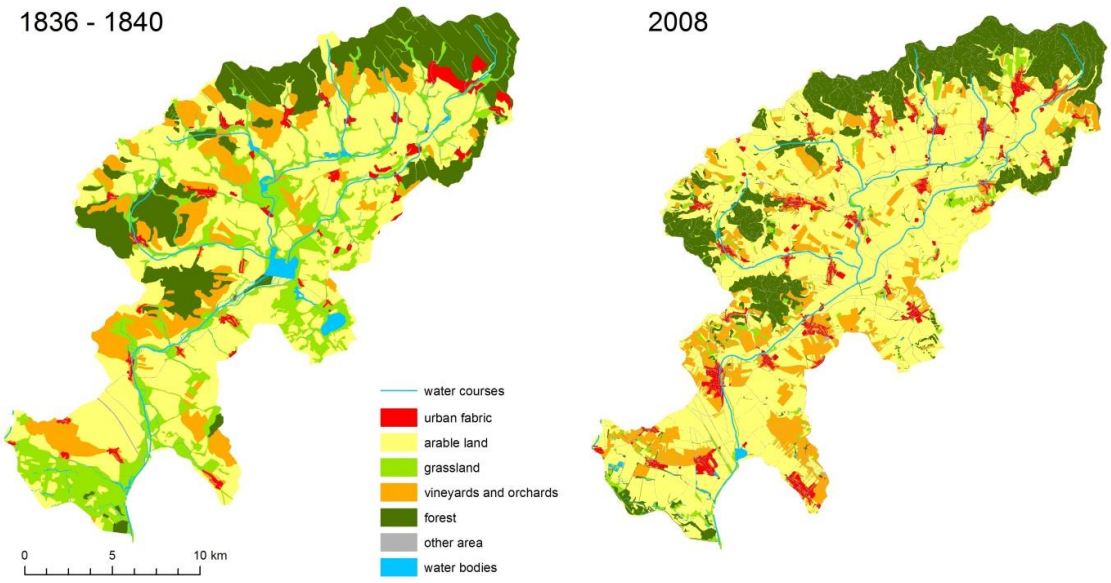

Figure 3. *Land-use of the studied area in the years 1836-1840 and 2008*

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The 'land cover' category used in the paper is based partially on the CORINE Land Cover classification (Bossard et al., 2000); for the needs of the study it was aggregated into five basic categories and labelled with unique codes: water bodies (1); forest (2); grassland (meadows and pastures) (3); arable land (4), orchards and vineyards (5). These five types, which in combinations create thirteen types of land cover trajectory, were included in the analysis. These trajectories capture land-use change (and include both extensification and intensification processes) and the stability of land use (i.e. the occurrence of categories in both studied periods).

Stable land use:

- Stable forest (22); stable grassland (33); stable arable land (44); stable vineyards and orchards (55)

Changes in land use:

- Forest to grassland (23); forest to arable land (24)
- Grassland to forest (32); grassland to arable land (34)
- Arable land to forest (42); arable land to grassland (43);
- Vineyards and orchards to forest (52); vineyards and orchards to grassland (53); vineyards and orchards to arable land (54)

Biophysical determinants of land use change

The study analyses selected biophysical variables representing specific limiting or facilitative factors determining land-use changes. Naturally, the link between land-use changes and local natural conditions is not entirely positive. While works such as Chen et al. (2001), Fu et al. (2006) and Pazúr et al. (2014) demonstrated close ties between natural factors and land-use changes, other studies (Schneider and Gil Pontius, 2001; Hietel et al., 2005) suggested only a weak connection between land-use changes and natural conditions. Opršal et al. (2013) confirmed the assumption that environmental factors are more influential in areas with greater topographic heterogeneity. Bajocco et al. (2016) pointed out the important fact that different land-use land-cover change trajectories were associated to different combinations of biophysical factors.

The actual selection of natural factors (*Figure 4*) was made based on two criteria: i) the possibility of their operationalisation with the use of GIS, and ii) their relevance with respect to representation and variability in the studied area. Firstly, this concerns biophysical factors connected with the topography of the area: slope grade, elevation and aspect (indicating sun exposure). We added also one variable tied to the soil condition – fertility. On the other hand, climatic variables were not included in the analysis. Climatic variables (e.g. average temperature, average amount of rainfall) are part of several studies involving vast territories where variability can be anticipated (e.g. Pazúr et al., 2014; Ya et al., 2014, Bajocco et al., 2016). However, as it has been documented (Tolasz et al., 2007) that average annual precipitation and temperature do not show variability in the studied area, the inclusion of these variables was not justified.

Elevation, slope grade and aspect were derived from a digital elevation model (DEM) with a resolution of 1 m. The model was generated from the contour lines which were obtained from the geographic base data of the Czech Republic - ZABAGED®. The values of average slope grade, average elevation and aspect were acquired from a digital terrain model in a scale of 1:10,000 using spatial operations in the ArcGIS 10.2 program. Aspect data correspond to azimuth values 0°-360°; however, they could not be used in this form in statistical analyses. Orientation data was therefore rescaled into four categories: a slope with

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a predominantly northern exposure (N), a slope with a predominantly eastern exposure (E), a slope with a predominantly southern exposure (S) and slope with a predominantly western exposure (W). After reclassification, aspect data acquired the character of nominal data, and as part of statistical analyses their occurrence or non-occurrence (0/1) was assessed for the given area. In our study we worked on the assumption that higher elevation and higher slope grade variables are associated with extensification processes in land-use change, whereas low elevation and more favourable slope grades have an impact on persistence or intensification of agriculture. As is the case with aspect, with respect to sun exposure extensification processes can also be anticipated in areas with a northern orientation (e.g. the transition of the arable land category to grasslands or grasslands to forest). On the other hand, southern tracts are suitable for agricultural production, and the greater probability of the persistence of the vineyard and orchard category can be expected in the studied area.

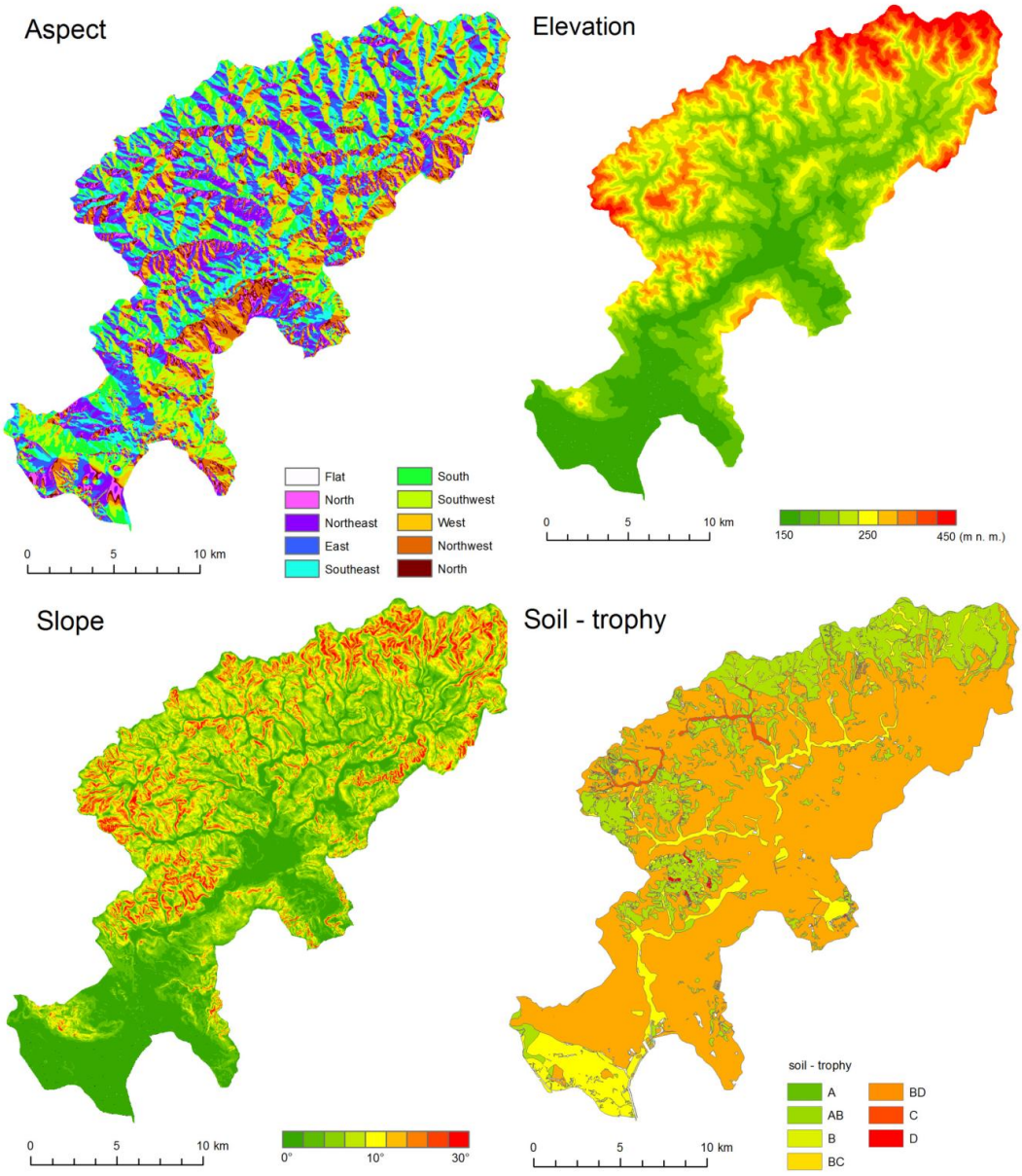

Figure 4. *Spatial variation of biophysical factors in the Trkmanka Watershed*

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In addition to terrain morphology, soil characteristics represent a significant biophysical constraint to land-use change; nevertheless, a certain lack of uniformity exists in the selection and classification of soil parameters. Used most often are various taxonomic soil categories (see, for instance, Fu et al., 2006; Baumann et al., 2011), the sand/clay ratio in the soil or soil pH (Prishchepov et al., 2013), and these variables are then associated with soil fertility. Studies by Slovak authors were based on the unique map database of the Bonited Pedo-Ecological Units (Lieskovský et al., 2014; Pazúr et al., 2014), which has also been processed for the Czech Republic. Still, the basic limitation of this map database is the fact that it was created only for agricultural land and not forest land. This means that these resources can only be used for land-use change occurring on agricultural land. However, as our study also includes forest land and other non-agricultural land, an alternative approach was chosen in which the selected parameters of agricultural and forest land were converted to fertility categories using a conversion key (Buček and Lacina, 1999). The transformation of the agricultural land was based on the Main Soil Units, which are expressed in the second and the third position in the five-digit code of the Bonited Pedo-Ecological Units (Novotný et al., 2013). The classification system comprises 78 Main Soil Units (agronomical groupings of similar soil types, subtypes, soil-forming substrates and other characteristic) which were converted into the soil trophy categories (*Table 1*) derived from the geobiocenological classification system (Buček and Lacina, 1999). Transformation of forest land was based on the map of Forest Types Groups which are a key part of the soil typology used in forestry in the Czech Republic. Forest Types Groups are expressed as three-digit symbols where second symbol indicates edaphic category of the soil (Plíva, 1971). There are 24 edaphic categories of the forestry classification system which can be converted into the above mentioned soil trophy classification based on geobiocenological classification system. The conversion key (Buček and Lacina, 1999) was employed to assign Main Soil Units of agriculture land and edaphic categories of forest land to corresponding soil trophy categories. The result of the process is a common soil classification according to fertility (*Table 1*). We assumed that extensification processes are associated to a greater degree with less fertile oligotrophic soils.

Table 1. *Fertility categories and sub-categories*

code	trophy/fertility	code	trophy/fertility
A	oligotrophic	AB	oligotrophic-mesophilic
B	mesophilic	BC	mesophilic-nitrophilic
C	eutrophic-nitrophilic	BD	mesophilic-alkaline
D	eutrophic-alkaline	CD	nitrophilic-alkaline

In order to establish overall land changes and to determine the relationship between the main transformation processes in the landscape and natural conditions, spatial analyses were conducted by superimposing the digital layers of chosen time horizons. First, the layers representing the transformation of the land cover were created by connecting the digital layers of land use (for the years 1836-1840 and 2008). These newly created layers were then joined with the soil nutrient layer. Spatial operations were used to calculate the values of average slope grade, average elevation and aspect for all of the polygons representing individual types of land development. The results in

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the form of attribute tables served as material for individual statistical analysis of landscape changes and for a canonical correspondence analysis.

The territory as a whole did not enter into the final analyses because only selected types of land-use trajectories (both stable land use and changes in land use) were included. In the process of GIS analysis, the area of 335 km^2 was divided into more than 15,000 patches (larger than 1,000 m^2). Using a simple method of weighted averages (see formula);

$$\bar{x} = \frac{\sum_{i=1}^{k} x_i . n_i}{\sum_{i=1}^{k} n_i} \qquad \text{(Eq.1)}$$

where: x_i is the value of the given characteristic (e.g. elevation, aspect) of the individual land patch, and n_i is the statistical weight of the given land patch, the average characteristics of the studied territory were first established.

The relationship between land-use change (response variables) and selected biophysical characteristics (explanatory variables) was tested using a multidimensional statistical method – canonical correspondence analysis (Lepš and Šmilauer, 2003). All of the more than 15,000 land patches with their characteristics were entered in the CCA (soil fertility, slope grade, elevation, orientation), and each unit was also coded with the type of transformation (or stability). The CCA also included an evaluation of the success of the calculated model, a calculation of the correlation between variables and extracted axes and graphic interpretations. The CCA calculation was conducted using Canoco software for Windows.

Results

The general characteristics of the analysed variables in the studied territory confirm favourable conditions for agricultural production. The average elevation of the territory is 241 m above sea level, and the majority of land (42%) faces south; 29% of the territory faces east, 26% faces west and only 3% has a northern orientation. From the perspective of soil fertility, mesotrophic alkaline dominates with a nearly two-thirds share (64.4%), followed by mesotrophic (22.8%) and mesophilic-nitrophilic (12%). The representation of soils from other fertility categories is minor. Due to the favourable values of biophysical factors, it is therefore not surprising that arable soil is dominant with a nearly 70% share (68.9%), followed by forest land (21.7 %), orchards, gardens and vineyards (5.3 %); meadows and vineyards currently occupy only 4% of the territory, despite the fact that at the beginning of the studied period they covered an area nearly five times as large.

An analysis of the relationship between land-use change and selected biophysical conditions using CCA confirmed several assumptions formulated in the introduction. The ordination graph (*Figure 5*) visualises the relationship of studied variables and land-use transformation.

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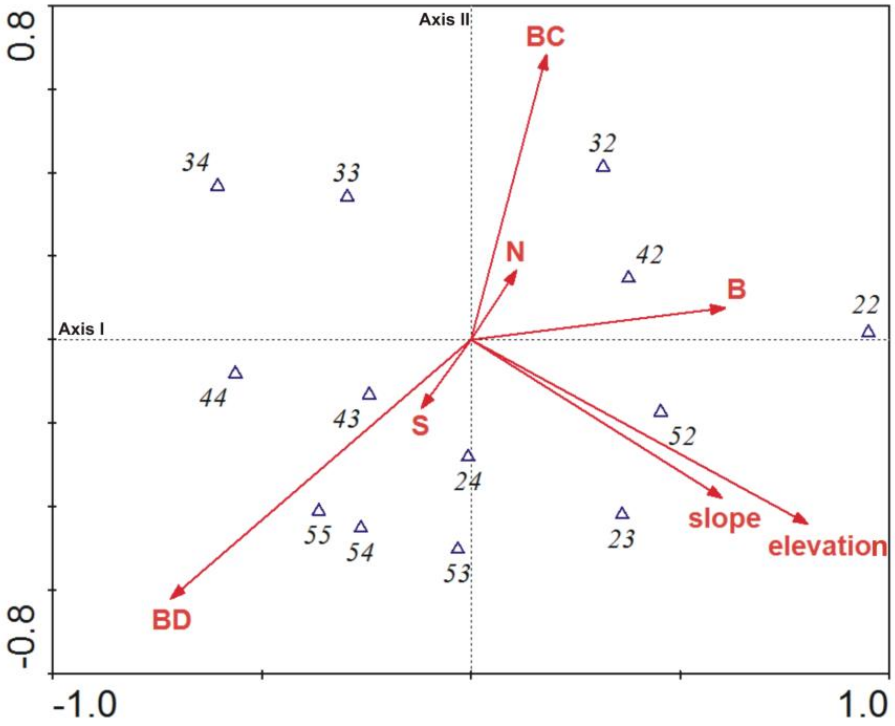

Figure 5. *Changes in the use of the landscape and biophysical variables with respect to the first two extracted axes. The length of the arrows determine the correlation between the given variable and the extracted ordination axis; the points represent individual transformation processes. Their location suggests the relationship to individual variables.*

Figure 5 shows that the transformations of all types of land-use to forest (32, 42, 52) are exclusively concentrated on the right side of the graph and positively correlate with the rising slope of the land and increasing elevation. These two variables are positively correlated with the first extracted axis (see *Table 2*). Also related to these two variables is the stability of forest areas (represented by code 22), which demonstrates the existence of forest stands in both time periods. The mentioned extensification processes are also positively correlated with relatively less fertile (mesotrophic) soils (B). The analysis likewise suggests the role of an unfavourable northern aspect (N), despite the fact that this variable is not statistically conclusive.

In contrast, all intensification processes with ties to a lower slope grade and elevation are found on the left side of the graph. This specifically concerns the t of forest into arable land (24), meadows and pastures into arable land (34) and vineyards and orchards into arable land (54). In addition to intensifaction processes, the mentioned variables are determined by the persistence of intensive forms of land use (i.e. the existence of arable land – code 44, and the existence of vineyards and orchards – code 55, in both periods). Having a significant role in intensification processes from among the variables connected with soil fertility are relatively more fertile mesotrophic alkaline soils (BD). Although aspect is not statistically significant, the accuracy of the analysis is also documented by the direction of the arrows of the south orientation variable (S) toward arable land and vineyards.

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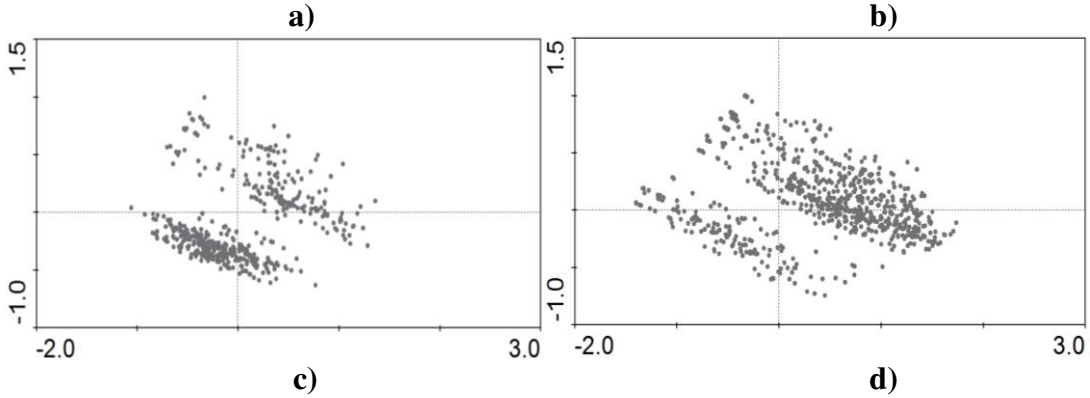
Table 2. *Variable entering the analysis and their correlation with extracted axes*

Variable	Axis 1	Axis 2
A	0.0143	-0.0008
AB	0.0119	0.0220
B	**0.4457**	0.0241
BC	0.1324	**0.2166**
BD	**-0.5272**	-0.1968
C	-0.0473	0.0431
D	0.1142	0.0079
Elevation	**0.5909**	-0.1401
Slope	**0.4406**	-0.1203
N	0.0794	0.0525
S	-0.0871	-0.0522
E	0.0477	0.0079
W	-0.0018	0.0163

The advantage of CCA is the possibility of establishing the statistical success of the entire model (Hietel et al., 2005; Opršal et al., 2013), which other geostatistical methods do not enable (Bakker et al., 2011; Druga and Falťan, 2014; Pazúr et al., 2014). At first glance, the statistical success of the model is not high (only 7% of the original information is explained by the model), a situation caused by several factors. The first is the number of units used in the CCA, the second the intensity of land-use changes in the Trkmanka Watershed. Nearly a third of the overall territory underwent transformation during the studied period. These factors impacted the analysis, and the authors encountered a similar problem at other heavily transformed sites (Opršal et al., 2013).

Despite the limited reliability of the model, the relationship between land-use changes and biophysical variables was confirmed to a certain degree using multidimensional statistics. Evidence of this is provided in *Figure 6*, which captures the land units that always underwent the relevant transformation; ovals designate the part of the graph in which their dominant share occurs. Their position with respect to the extracted axes again indicates a link to the environmental variables whose vectors are depicted in *Figure 5*.



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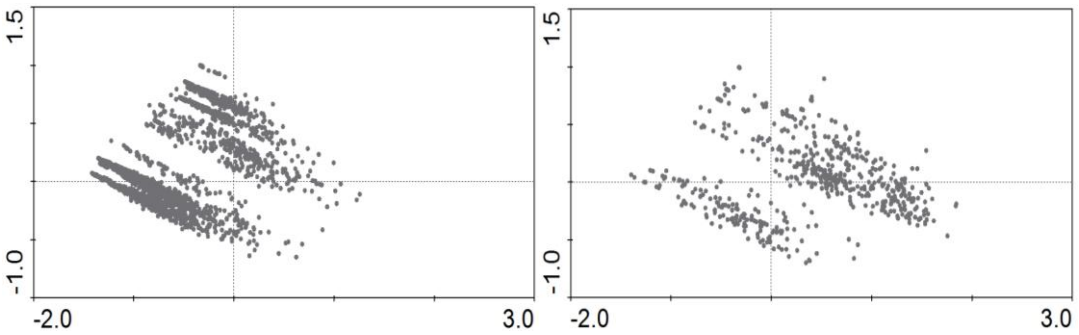

Figure 6. *Individual CCA results – the location of units of the selected land-use transformation in relation to the first two extracted axes (Axis I – horizontal; Axis II – vertical). Key: transformation type: a) forest to arable land (24); b) grassland to forest (32); c) grassland to arable land (34); d) arable land to forest (42).*

Discussion

In our analysis we were able to confirm the significance of all included variables in the land-use transformation process. We found slope grade and elevation to be extremely significant in extensification processes and land abandonment, which is consistent with the conclusions of studies from other regions (Fu et al., 2006; Kuemmerle et al., 2008; Müller et al., 2013; Liu et al., 2010; Druga and Falťan, 2014). These topographical factors seem to be dominant, and their influence is less significant in only a few cases (Pazúr et al., 2014). Moreover, slope grade and elevation can also influence other biophysical factors such as soil quality and socioeconomic factors, particularly isolation, land accessibility and the ability to use modern mechanisation to cultivate parcels. Therefore, we can designate less favourable topographic conditions as key predictive factors for extensification and land abandonment not only in agriculturally marginal (mountainous) areas but also on those with a tradition of intensive agricultural production. In contrast, favourable slope grade and elevation characteristics are associated with the intensification of agricultural production (the transition of grassland to arable land in the case of the Trkmanka Watershed).

Another significant biophysical characteristic is soil fertility, which has an influence on both extensification processes (on land with relatively low fertility) and intensification processes (on relatively fertile land). The significance of soil fertility must therefore be tied to a specific type of land-use change; for example, a great significance was confirmed in the transition of vineyards and orchards to forests, while a surprisingly low significance was found in the transition of arable land to grassland. This is possible to explain in the studied territory of the Trkmana Watershed by the fact that a large part of meadows and pastures were located on flat alluvial land that was converted to arable land after it was drained. Relatively high significance of soil fertility also appears in other studies (Szillasi et al., 2010; Baumann et al., 2011; Prishchepov et al., 2013a; Pazúr et al., 2014), while others state only small or average significance (Hietel et al., 2004; Alvarez Martinez et al., 2011). However, an evaluation and especially the direct comparison of the influence of soil factors is complicated by the lack of uniformity in the selection and classification of soil parameters (cf. Fu et al., 2006; Prishchepov et al., 2013; Baumann et al., 2011).

The final investigated variable was aspect. This factor is often omitted from studies (e.g. Müller and Munroe, 2008; Liu et al., 2010; Lieskovský et al., 2014), which is

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clearly related to methodological difficulties in geostatistical analyses. Although the orientation of the slope toward one of the four cardinal directions proved to be statistically insignificant in our study, the ordination diagram (*Figure 5*) suggests an association between slopes with a southern exposure and intensive land-use forms. On the other hand, northern exposures suggest the greater probability of transition from arable land or grassland to forest. It is logical in this context that vineyards and orchards show the highest degree of stability on slopes with a southern exposure.

Our study demonstrated the significance of selected biophysical factors on land-use change over the long-term. Nevertheless, we are aware that our chosen approach has several methodological limits, e.g. in the selection of maps, the precision of the cartographic materials from the Second Military Mapping Survey and their conversion to digital form. We believe that the relatively large territory contributed to the sufficiently robust results supporting the aforementioned analysis results. It should also be emphasised that land-use changes are a dynamic process that cannot be reduced to a single set of factors (Munroe et al., 2013; Vliet et al., 2015; Geist and McConell, 2010; Bajocco et al., 2016). The conclusions of many studies point to the importance of socioeconomic factors, some of which such as relative accessibility and isolation of areas (Olah et al., 2006; Baumann et al., 2011; Lieskovský et al., 2014) or depopulation (Renwick et al., 2013; Pazúr et al., 2014) can be modelled in broader land-use studies. It is clear that additional influences related to the economic and political context of the evaluated period and territory also play a role. For instance, Havlíček et al. (2014) identified unprofitable fish breeding, the development of sugar beet industry and higher demand for food and technical crops as main driving forces responsible for the disappearance of water bodies in the Trkmanka watershed. It can be expected that other factors such as land ownership, systems of agricultural support and the individual decisions of farmers based on personal values and motivations may affect the land-use changes. The mentioned factors cannot be included in simple deterministic models and require different approaches based on questionnaires at sites with the greatest intensity of changes (Hersperger et al., 2010). Moreover, despite the significance of socio-economic drivers land use of an area, its changes and distribution remains affected by the local natural conditions (Olah et al., 2006). Finally, the decision not to incorporate socioeconomic factors is also related to the chosen timeframe; for a period of more than 150 years it is possible to consider biophysical factors as essentially stable, whereas the explanatory potential of socioeconomic factors can be very limited. Nevertheless, as our research has shown, even an analysis of actual biophysical factors can reveal hotspots of potential changes and become a useful tool for predicting landscape development (Pechanec and Machar, 2014) and sustainable landscape management (Machar, 2013).

Conclusion

This article evaluates the impact of selected biophysical factors on land-use changes using the example of the Trkmanka Watershed in the southeast part of the Czech Republic. It is one of a small number of studies focused on the territory, which has a predominance of intensive agricultural production. Utilising unique historical cartographic documents, the study assesses the influence of biophysical factors over a time period of more than 150 years. The results of a canonical correspondence analysis (CCA) demonstrated the correlation between selected variables and land-use changes.

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The most significant variables were slope grade, elevation and soil fertility; aspect was of limited importance.

Our study showed that while extensification processes are minor on heavily farmed land compared to marginal mountainous or arid areas (Pazúr et al., 2014; Lasanta and Vicente-Serrano, 2012), they are still conditioned on less favourable environmental circumstances. The extensification of farming is especially related to a higher slope grade and elevation, while the analyses also suggest the influence of an unfavourable northern aspect and less fertile soil. Likewise, less favourable natural factors condition the persistence of extensive forms of land use (especially the likelihood of the continued existence of forest stands). In contrast, the stability of intensive forms of agricultural production or further intensification are associated with a lower slope grade and elevation, more fertile soils, and a more favourable southern exposure also plays a role.

Although the results of the statistical analyses confirmed the importance of natural factors, they explain only some of the overall land-use changes. While it is clear that socioeconomic factors are the main forces influencing the character of land use (Vliet et al., 2015), their inclusion in the CCA is significantly limited due to their nature and variability. Environmental and socioeconomic factors do not act separately but produce a mutual interaction (Geist and McConell, 2010). Nevertheless, independent analyses of natural factors are justified, since they help anticipate land-use changes in the long-term and identify hotspots of change. The results can be used to predict land-use changes and for strategic land-use planning and sustainable land management.

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ELECTRONIC APPENDIX

Electronic appendix 1: Input matrix of the RDA analysis

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WATER USE EFFICIENCY AND NET PRODUCTION OF TWO SEMI ARID GRASSLANDS IN DIFFERENT SUCCESSIONAL STAGES

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Abstract. The productivity of Mediterranean grasslands, which depends on successional stage, climatic factors and human interventions, cannot fulfil forage demands of ruminants during the dry summer period. The current paper investigates whether the successional stage of vegetation could alter the water use efficiency during the semiarid period of summer and whether the productivity of low elevation Greek grasslands may be approached by the traditional concept of succession. Seasonal trends in physiological parameters, such as net photosynthetic rate and transpiration rate as well as net productivity was recorded in the most dominant species of two grasslands at an early and late successional stage (ESS and LSS respectively). The results reveal significant differences in net photosynthetic rate, transpiration rate, and water use efficiency of the most dominant species between the two grasslands throughout the season. Net production was significantly higher in the LSS grassland especially during the drier parts of the season (middle summer). The higher water use efficiency and higher net production in late successional stages of the lowland Mediterranean grasslands that we have examined in the current study suggests a rather substantial divergence from the traditional view of the succession theory regarding productivity.
Keywords: *net photosynthetic rate, transpiration rate, leaf area index, leaf water potential, production*

Introduction

Grasslands are important terrestrial resource for Greece and other Mediterranean countries (Cosentino et al., 2014). However, today their productivity does not fulfill the forage demand of ruminants, especially during the dry period of summer, widely oscillating in space and time according to plant species composition, herbivore pressure, human activities, successional stages as well as climatic conditions (Gatti et al., 2005; Migo, 2006; Ali-Shtayeh and Salahat, 2010). The abiotic profile of an area (i.e soil, climate, landscape) as well as external factors, such as fire and grazing may affect the productivity of an ecosystem (Gurevitch et al., 2006; Fernandez et al., 2009; Salis, 2010; Long et al., 2015). In addition, the productivity of grasslands is strongly associated with the successional stage of vegetation (Würtz and Annila, 2010). There are several theories that have been put forward to explain those changes induced in composition and production of plant species at different successional stages. For example, Odum (1983) proposes that total biomass of an ecosystem increases with succession from the initial to advanced stages, while the net production is reduced. Plants at the stage of climax are the best indicator of the potential of productivity of an area (Foin, 1986; Meiners et al., 2015). However, Odum (1985) demonstrated that in stressed ecosystems the expected trends include changes in energetic, nutrient cycling, and community structure and function. In arid and semiarid regions, annual changes in productivity due to changes in precipitation

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are often much larger than those due to small changes in the composition of vegetation as a result of the improvement or_deterioration of the ecosystem (Laycock, 1989; Nippert et al., 2006; Han et al., 2015).

The stage of climax, in terms of productivity, is often not desirable because both net production and the nutritional value are low. In such cases, it would be desirable to maintain the grassland in a successional stage where the optimum composition of vegetation for grazing animals, coexists with high productivity while ensuring the balance of the ecosystem (Sharma, 2009; Han et al., 2015). In the Mediterranean basin, the productivity of grasslands depends directly on water availability and species richness, productivity and plant cover are expected to decrease with increasing aridity (Bartha et al., 2011; Meiners et al., 2015). The water is considered the more important factor for the establishment, survival and growth of plants in semiarid Mediterranean regions. Under drought conditions, natural selection favors plants with physiological adaptations that ensure the survival and growth based on the efficient use of available water (Heschel and Riginos, 2005). The low annual precipitation in the Mediterranean region combined with natural hazards of environmental degradation make an essential need to detect plants or successional stages which discourage the degradation and ensure environmental stability and high productivity (Bolle, 1995; Alcamo et al., 2007; Fernandez et al., 2009). According to Souza and coworkers (2005) there is a flexibility in plant capacity to respond to environmental changes taking into account positive or negative relationships between physiological parameters and environmental conditions.

Water use efficiency (WUE) provides a useful index for understanding the metabolism of terrestrial ecosystems as well as for evaluating the degradation of grasslands (Han et al, 2013). WUE relating two physiological parameters the photosynthetic rate (P_N) and the transpiration rate (E) is a very important index to define how efficiently individual plants use water to produce biomass (Wang et al., 2007; Bacon, 2009). Earlier models suggest that the total production of grassland increases but the net production decreases while moving from initial to advanced stages of succession (Odum, 1983; Odum, 1985). However, later experimental studies have proven that, under the influence of intense grazing, the progress of succession is correlated with an increase in net production (Casado et al., 1985; Noitsakis et al., 1992) and, therefore, alternative models should be developed to explain these deviations from Odum's theory (McNaughton and Wolt, 1973; Smith, 1988).

In Mediterranean ecosystems, the natural vegetation has been modified by the interactions of climate and human intervention and has developed coping mechanisms to further changes inhibiting a possible new environmental degradation (Fernandez et al., 2009; Păcurar et al., 2014; Zimmermann et al., 2014). Ecologists have developed many theories and models to describe those changes that succession induce (Clements, 1916; Odum, 1969; Connell and Slayer, 1977; Pickett et al., 1987; Meiners et al., 2015). However, studies in several Mediterranean ecosystems have demonstrated that out of the three models proposed (facilitation, inhibition, tolerance) by Connell and Slatyer (1977), the model of "facilitation" cannot explain the function of these ecosystems under the influence of drought and respective future climate change (Valladares and Gianoli, 2007). Apparently, traditional range successional models cannot account for the productivity of the Mediterranean grasslands and there is need for the development of new alternative models

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(Bartolome, 1989; George et al., 1992). Relationships between productivity and diversity differ not only among scales and between artificial and natural gradients, but also within scales and gradient types. A better understanding of the relationships between productivity and diversity requires a thorough study of the mechanisms the establish the relationship, and whether differing mechanisms explain the variation in patterns (Rajaniemi, 2003).

These contradictory views on the relation of vegetation dynamics, successional stage and production of an ecosystem are challenging to research. The aim of this paper was to investigate whether the successional stage of vegetation could alter the water use efficiency during the semiarid period of summer and whether the productivity of low elevation grasslands may be approached by the traditional concept of succession.

Materials and methods

Study area

The study was conducted at a low elevation grasslands in Northern Greece, which was located close to Melissohori (lat. 40^{o} 58N, log. 28^{o} 01E), 25 km north-east of Thessaloniki, at an altitude of 170m a.s.l., constitute mainly of forage species and few patches of shrubs such as *Pyrus amygdaliformis* Vilm *and Jasminum fruticans* L. The climate of the area is classified as Mediterranean semiarid with average monthly temperatures ranging from 4.9 to 25.6 ^{o}C. The mean annual air temperature ranged from 4.4 ^{o}C (January) to 24.7 ^{o}C (August) and the average annual precipitation to approximately 409 mm. During the experimental period the annual average precipitation was 476.5 mm, while the vapour pressure deficit (VPD) ranged from 1.4 kPa (April) to 4.17 kPa (end of June).

Materials

Data were collected from two fenced (10 by 20 m) neighboring areas with the same orientation (north-east) and slope 10-12%. The two experimental areas had been excluded from grazing four years before the beginning of the study. Before fencing both experimental areas were grazed by sheep and goats without any control. At the onset of the experiment the first area had just been abandoned from cereal cultivation (early stage of succession, ESS) and the dominant species were annual grass, legumes and annual forbs (old field successsion). The second one was grassland at late stage of succession (LSS) that had been grazed for at least 20 years before fencing. The dominant species, annual and perennial grasses, with stable frequency of appearance and higher percentage of cover in the ESS grassland were: *Medicago minima* (L.) Bartal (10.43%), *Onobrychis aequidentata* (Sibth and Sm) D'Urv (10.6%), *Avena fatua* L. (10.68) and *Cynodon dactylon* L. (10.37%), while in the LSS grassland: *Lotus aegaeus* L. (1.65), *Dactylis glomerata* L. (9.24%), *Dichanthium ischaemum* (L.) Roberty (9.19%), *Chrysopogon gryllus* (L.) Trin (9.13%) and *Dasypyrum villosum* (L.) P. Candargy (23.25%). Detailed description of the two grasslands are given in Karatassiou and Koukoura (2009).

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Physiological parameters

Net photosynthetic rate (P_N), and transpiration rate (E) were measured in the dominant species of each grassland with the portable infrared gas analyzer system LI-6200 (LI-COR Lincoln, NE). Seasonal measurements were obtained on clear sunny days at around solar noon (12.00h -14.00h) and approximately 15 days intervals. All measurements were conducted on mature and intact fully expanded upper leaves. The water use efficiency (WUE) was calculated as the ratio between carbon gain (P_N) to water loss (E) (Guo et al., 2006; Bacon, 2009). For each parameter, the values given are averages of the four replications of the five dominant species of LSS grassland and of four dominant species of the ESS grassland.

The above ground, dry biomass (net production) was estimated by randomly taken samples of cut vegetation at ground level every twenty days during the growing season. Each sample, for each grassland consisted of ten sampling units of 50 cm x50 cm quadrats. Following removal of the previous year, production was separated into leaves and stems. Leaf area was measured using the leaf area measurement system (Area measurement system, Delta-T-Devices). To determine the dry weight, leaves and stems of sampled species were placed in an oven for 48 hours at 70 °C. The Leaf Area Index (LAI) was also estimated following Gurevich and coworkers (2006).

Statistical analysis

To determine differences in the ecophysiological response of the species of the two grasslands during the growing season we performed a two-way analysis of variance (ANOVA) on all parameters studied (Steel and Torrie, 1980). Following a significant interaction between grassland and time one way ANOVA on effects of the five sampling times in each grasland was performed. Means of the two grasslands at each sampling date were compared using the t-test for independent samples ($\alpha = 0.05$). All statistical analyses were performed using the SPSS statistical package v. 21.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

The two-way analysis of variance reveals significant differences ($p \leq 0.01$) between the two grasslands for most of the parameters recorded (i.e. P_N, E, WUE, production). Also, time (season) significantly affected ($p \leq 0.0001$) all these parameters (P_N, E, Ψ, WUE, production). The interaction between time (during the growing season) and grassland was significant for parameters ($p \leq 0.0001$) but water potential, indicating differential physiological response of the species of the two grasslands throughout the season.

Leaf water potential (Ψ) showed a declining trend during the growing season in species of both grasslands (*Figure 1,* $F_{4, 75} = 6.2$ and $F_{4, 95} = 14.1$, $p \leq 0.001$ for ESS and LSS respetively) but no significant differences ($p > 0.05$) was found between the two areas at each sampling time ($t < 0.99$, df=34, $p > 0.05$). The species of each grassland presented significant higher values of water potential at the beginning of the growing season (middle April) and the significantly lower values in the last days of June (LSD-test among different dates within each grassland, $p < 0.05$). It is well appreciated that water deficit (intensity, and/or duration) causes a number of significant modifying

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functions in photosynthesis and other physiological processes, which may be species specific (Mojayad and Planchon, 1994; Wang et al., 2003; Zlatev et al., 2012).

Our results demonstrate that overall (including all dominant species) during the growing season the two grasslands expressed differential sensitivity to water deficit conditions (*Figures 1, 2*). The net photosynthetic rate in species of the LSS grassland was significantly higher than that of species in the ESS grassland ($t>3.48$, $df= 34$, $p\leq0.001$) for most dates during the growing season. No significant differences between the species of two grasslands were recorded only in the middle of May ($t=0.548$, $df=34$, $p =0.587$) at Ψ values ranging between -1.6 MPa and -1.86 MPa (*Figure 2a*). Apparently, the effects of micro- and macro- climatic conditions were more favorable for the perennial species compared to annual ones. In species of the ESS grassland, P_N significantly increased following a significant decrease (LSD-test among different dates, $p \leq0.001$) in Ψ up to -1.8 MPa, following a declining trend in lower values of leaf water potential. In contrast, as the growing season progressed and Ψ reduced (*Figure 1*) in species of the LSS grassland P_N peaked at higher Ψ values, decreased and a stabilized between -1.7 MPa and -2.4 MPa and finaly dropped in lower Ψ values (*Figure 2a*). The mean net photosynthetic rate of species in the LSS grassland (8.57 ± 0.46 μmol.m^{-2}.s^{-1}) was significant higher ($F_{1,170} =109.9$ $p\leq 0.0001$) than that of species in the ESS grassland (4.30 ± 0.289 μmol.m^{-2}.s^{-1}). It seems that the perennial species, which mainly consisted the vegetation of the LSS grassland, were able to maintain a high rate of photosynthesis for longer periods of time than the annual species, prevailing in ESS, under lack of water in the leaf tissue. This is in agreement with data given in McNaughton (1991), Saint Pierre and cowerkers (2004, 2004a), Lambers et al. (2008) and Souza and coworkers (2009). However, there are some annual species, such as *Dasypyrun villosum* that, departing from the expected response, in advanced stages of succession can express similar photosynthetic capacity with perennial species, in order to survive and thrive (Karatassiou and Noitsakis, 2010).

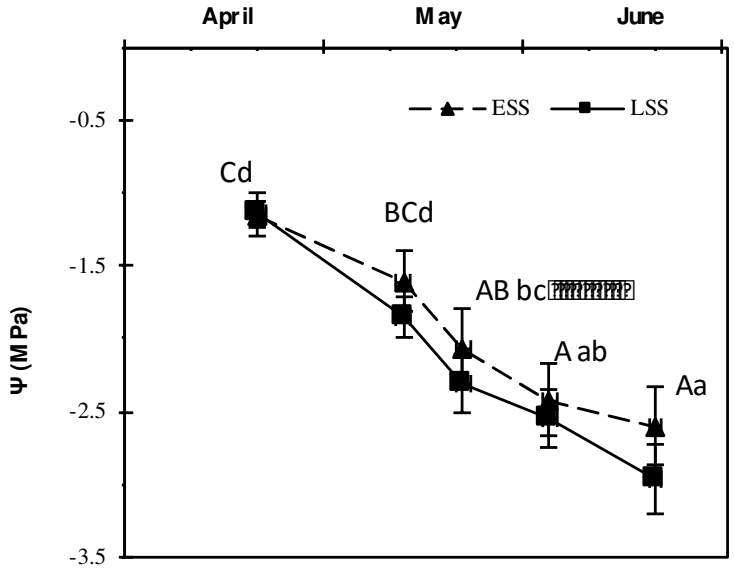

Figure 1. *Seasonal pattern of the leaf water potential (Ψ) for two grasslands early (ESS) and late (LSS) successional stages. Values present means ± SE. Small and capital letters indicate significant differences (LSD-test, $p<0.05$) within LSS and ESS grassland respectively. *Indicates significant differences (t-test,. $p<0.05$).*

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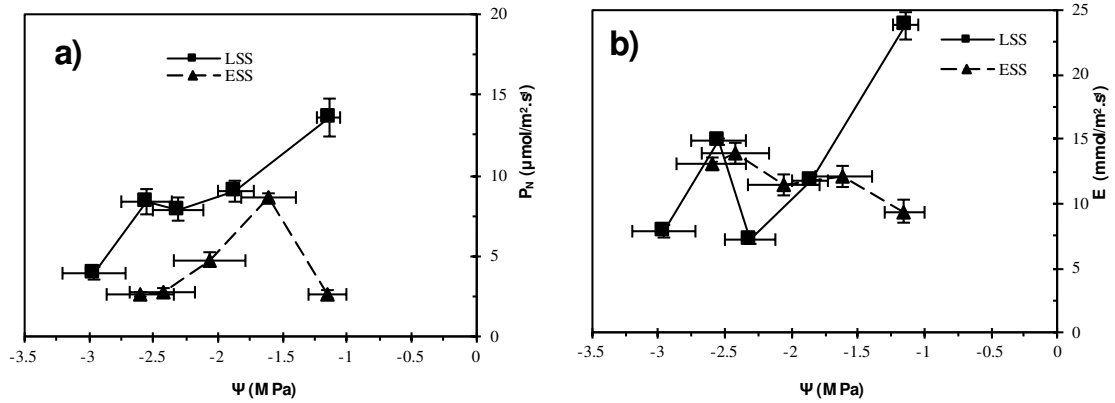

Figure 2. *The relationship between leaf water potential (Ψ) and (a) net photosynthetic rate (PN), and (b) transpiration rate (E) for two grasslands in early (ESS) and (LSS). Values present means ± SE.*

Patterns of E in relation to Ψ were different for the species of the two grasslands. Significant differences were found among sampling dates in E values within each grassland (*Figure 2b,* $F_{4,75}$= 5.1 and $F_{4,95}$= 117.9, p≤ 0.001 for ESS and LSS respetively). In species of the ESS grassland E values increased until middle of June (Ψ= -1.61MPa) and then stabilized, while those in the species of the LSS grassland following a dramatic initial decrease fluctuated in lower Ψ values. As far as transpiration rate is concerned, the species of the two grasslands expressed different response in higher values of Ψ around -1.15 MPa and and relatively similar in lower values of water potential (*Figure 2b*). As the growing season progressed to the drier summer the water potential decreased (*Figure 1*, Ψ>2.6 MPa), and those species at advanced stages of succession (LSS) showed lower rates of transpiration compared with species at earlier stages of succession (ESS), because the sensitivity of the stomatal apparatus to drought is increased in late successional species in response to the decrease in leaf water potential and increase in VPD (Karatassiou and Noitsakis, 2010). Houssard and coworkers (1992), Bazzaz (1996) and Chapin II and coworkers (2011) have demonstrated that plants at early stages of succession expressed higher transpiration rates compared to plants that are in advanced stages of succession.

The above differences in net photosynthetic and transpiration rates between plants in ESS and LSS (*Figure 2a,b*) suggest that water use efficiency would vary between the species of the two grasslands. Indeed, WUE was significantly higher (t-test, df=34, p≤0.05) throughout the growing season (except early May) in LSS plants compared to ESS ones (*Figure 3*). Only in the first days of May the species of the two grasslands expressed similar water use efficiency (t=0.842, df=34, p>0.05). Moreover, significant differences during the growing season presented in each grassland (p≤0.001).

However, the changes of WUE become more interesting when are plotted against water potential (*Figure 4*). The rate of WUE of the species of the both grasslands during the growing period showed no substantial differences up to a value of -1.8 MPa water potential. In lower leaf water potential values, species at LSS and ESS departed a great deal in WUE values (*Figure 4*). For the same relatively low value of Ψ (approximately -2.45 MPa) the WUE of plants in LSS grassland was almost three times higher than in the ESS.

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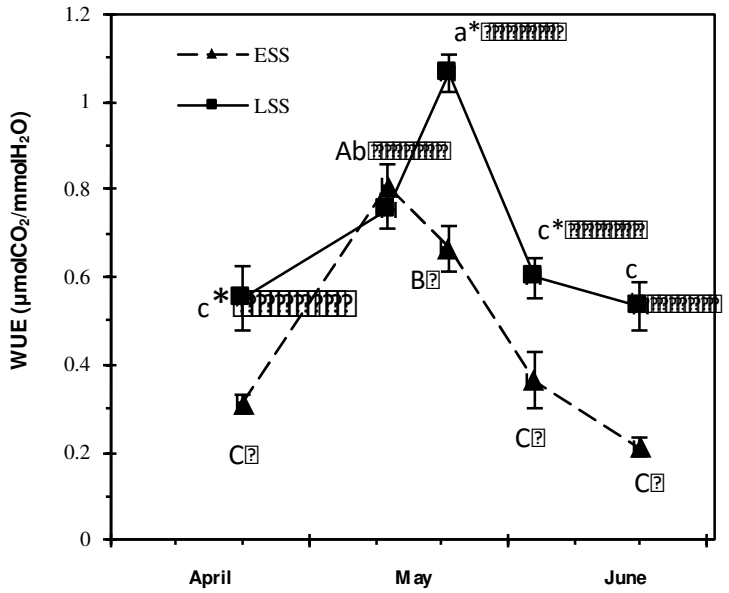

Figure 3. *Seasonal patterns of water use efficiency of two grasslands in early (ESS) and late (LSS) successional stages. Values present means ± SE. Small and capital letters indicate significant differences (LSD-test, p<0.05) within LSS and ESS grassland respectively. *Indicates significant differences (t-test, p<0.05).*

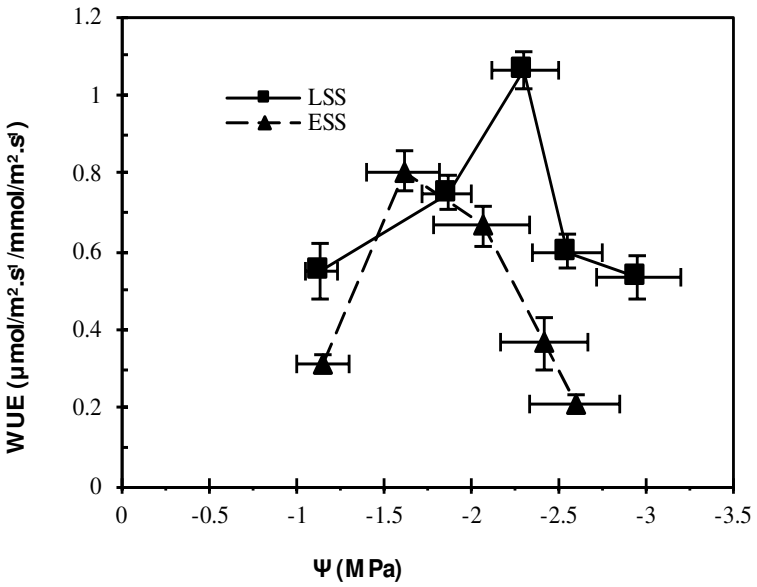

Figure 4. *Relationship between leaf water potential (Ψ) and water use efficiency (WUE) for two grasslands in early (ESS) and late (LSS) successional stages. Values present means ± SE.*

The early and late successional species differ in photosynthetic characteristics and these differences expected to increase when the plant grow in similar environmental conditions (Souza et al., 2005). The more efficient use of water under conditions of water deficit in species of grasslands at an advanced stage of succession (LSS) suggests

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that the forage species at this successional stage follow the k-selected strategy (Gurevich et al., 2006; Meiners et al., 2015). Most species at LSS are perennial species that invest in higher biomass production, because they have higher efficiency per unit of water used and survive under drought conditions (Heschel and Riginos, 2005) .

The differential behavior of the two grasslands (WUE_{ESS}<WUE_{LSS}) means that the species of LSS grassland transpire smaller quantities of water per unit of CO_2 (weight) diffuse in mesophyll. Therefore, species of the LSS grassland need to consume smaller amounts of water per unit of photosynthetic products relative to species of the ESS grassland. Consequently, species of the LSS grassland present more efficient ecophysiological adaptations under drought conditions, and achieve a favorable water balance (Han et al., 2015). Higher water use efficiency at late successional stages is directly connected with higher net production.

From the above results, we expect that the changes in net production during the growing period of both grasslands (*Figure 5*) followed a similar trend with the change of water use efficiency (*Figure 4*). However, the net production of each grassland presented significant differences throughout the growing season ($F_{3,36}$ =117.3 and $F_{3,36}$ = 58.1, p≤ 0.001 for ESS and LSS respetively).

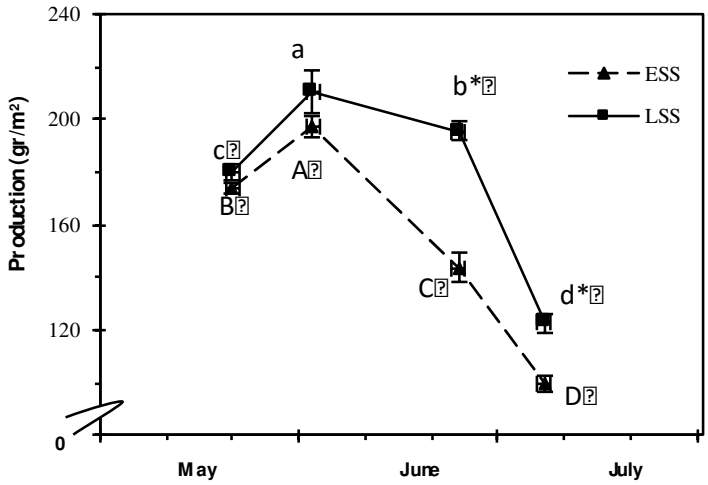

Figure 5. *Seasonal changes of net production of the two grasslands (ESS and LSS) during the semi-arid growing period. Values present means ± SE (n =10). Small and capital letters indicate significant differences (LSD-test, p<0.05) within LSS and ESS grassland respectively. *Indicates significant differences (t-test, p<0.05).*

Net production of the LSS grassland during the dry season, especially after mid-May, was significantly higher (t>4.9, df=18, p≤ 0.0001) than that of the ESS grassland that was protected from grazing for five years before these measures were taken. The production of the ESS and LSS grasslands ranged from 99 g/m^2 to 198 g/m^2 and 122 g/m^2 to 216 g/m^2 respectively. Both grasslands showed significantly higher production than that reported for low elevation grassland in Greece in other studies (Platis et al., 2004). In addition, the lower production of the ESS grassland was primarily due to the large participation of broadleaf grasses and annual legumes (Karatassiou and Koukoura, 2009) in the composition of the vegetation, which significantly limits their production under low rainfall (Rajaniemi, 2003; Nippert et al., 2006; McCain 2010).

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The largest production of the LSS grassland over the ESS grassland could be attributed to the different value of leaf area index (LAI) (*Figure 6*). The LAI controls light interception of plant and influences gas exchange (water, carbon) between vegetation and the atmosphere, and is a key variable to model the carbon and water exchange between vegetation and the atmosphere (Leuschner et al., 2006). Two way ANOVA reveals that both grassland ($F_{1,72}$= 6.2, p≤ 0.05), time of the season ($F_{3,72}$= 159.6, p≤0.0001) and their interaction ($F_{3,72}$ = 66.4, p≤0.0001) are significant predictors of the LAI (*Figure 6*). Seasonal patterns of LAI differ a great deal between the two grasslands.

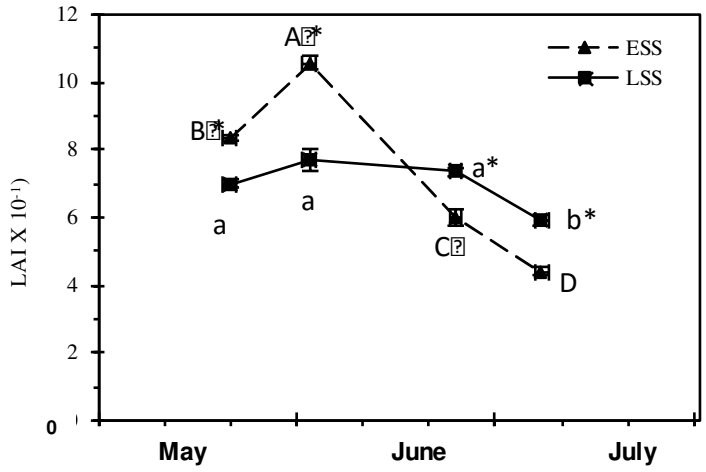

Figure 6. *Seasonal changes of leaf area index (LAI) of the two grasslands (ESS and LSS) during the semi-arid growing period. Values present means ± SE (n =10). Small and capital letters indicate significant differences (LSD-test, p <0.05) within LSS and ESS grassland respectively. * Indicates significant differences (t-test, p<0.05).*

The water deficit appears to have minimal effects on the production of the LSS grassland that exhibited low and approximately constant leaf area index (LAI) throughout the growing season (*Figure 6*), which decreased only in the end of the growing season (LSD-tests, p≤0.0001). According to Liu et al. (2015) and Meiners (2015) the high leaf area index indicates that the photosynthesis of species that compose the LSS grassland should not be limited by the intake of light or the accumulation of assimilates. In contrast, species of ESS grassland showed a decrease in leaf area index for reducing water loss, which enabled them to survive under water deficit conditions. Similar results were obtained by Bai and coworkers (2004) for grasslands at different succession stages in China under dry and hot climatic conditions. Interestingly in this study grassland at advanced successional stages expressed both higher species richness and productivity (Karatassiou, 1999; Karatassiou and Koukoura, 2009).

Although generalizations regarding response of all similar grasslands in the Mediterranean should be avoided it becomes apparent that in the low elevation Mediterranean grasslands the model proposed by Odum (1983, 1985) fails to explain net production. The species that compose the vegetation in these successional stages are mostly perennial, with mechanisms that enable them to use efficiently the water during the hot and dry season, with a favorable impact on net production of grasslands (Saint Pierre et al., 2002; Gallego and Distel, 2004; Fernandez et al., 2009)

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Conclusions

The higher water use efficiency and higher net production in late successional stages of the lowland Mediterranean grasslands that we have examined in the current study suggests a rather substantial divergence from the traditional view of the succession theory regarding productivity. Apparently, the vegetation (species) in the Mediterranean region has developed specific mechanisms of adaptation and survival to thrive and persist under the dry and hot summer conditions prevailing in the area.

Therefore, both the structure and productivity of grasslands in this region could be explained by a model in which, besides others important parameters, the water use efficiency should be taken into account as an index of productivity as well of drought adaptation in low elevation areas.

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IMPACT OF THE OCCURRENCE OF *TARAXACUM OFFICINALE* F.H.WIGG. ON FLORISTIC DIVERSITY AND UTILISATION VALUES OF MEADOW-PASTURE COMMUNITIES

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Abstract. The floristic composition of anthropogenic meadow communities undergoes transformations dependent on utilisation and habitat conditions. One of their consequences includes encroachment into and maintenance in the sward of some expansive plant species e.g. *Taraxacum officinale*. The objective of the study was to analyse the impact of the occurrence of *Taraxacum officinale* on the floristic diversity of meadow-pasture communities at varying levels of utilisation and habitat conditions. The effect of *Taraxacum officinale* presence on changes in the floristic composition of meadow communities was assessed on the basis of results of geobotanical studies carried out by Braun-Blanquet's method. The studies assumed evaluation of plant species proportions in the sward of meadow communities based on the share of *Taraxacum officinale*, floristic diversity and the natural value index of the sward. The highest share of analysed plant species was determined in extensively utilised meadows and pastures found on periodically dry habitats. Simultaneously, the high numbers of examined plant species exert a negative influence on the floristic diversity index and the natural value of plant communities.
Keywords: *common dandelion, expansive plant species, habitat conditions, manner of use, meadow communities*

Introduction

The floristic composition of anthropogenic communities, including grass communities, undergoes slow, but continuous changes, which scope is dependent e.g. on habitat conditions as well as the type and intensity of utilisation (Barabasz, 1994, 1997). Transformations of the floristic composition connected with quantitative changes in individual species of meadow and pasture swards result, among other things, from the encroachment of new species exhibiting high adaptability (Baker, 1965, 1974). Their spread is most frequently connected with the increase in the population of the species, leading to penetration of areas adjacent to the boundaries of the original range or even colonisation of new, previously unoccupied habitats within the natural range limits of the species (Pysek et al., 1995; Jackowiak, 1999; Genovesi, 2004). These species, thanks to their adaptability, become common, thus reducing the natural value of communities (Kryszak et al., 2009). An example of the species which tend to colonise new habitats is *Taraxacum officinale* F.H.Wigg. This species exhibits high competitiveness, as a result of which it is found at a high proportion and in considerable clusters in swards of permanent grassland (Vavrek et al., 1996; Molina-Montenegro et al., 2011, 2013; Martinkova and Honek, 2014).

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Thus the aim of this study was to determine causes for the increasing share of *Taraxacum officinale* in meadow and pasture communities and its effect on the utilisation value of the sward and floristic diversity.

Materials and methods

The occurrence of *Taraxacum officinale* (common dandelion) was determined based on a comparative analysis of 1000 relevés, prepared following Braun-Blanquet's method (1964), and which were entered in the Turboveg data base (Hennekens, Schamiane, 2001). Using the Twinspan programme (Hill, 1979) a preliminary hierarchic classification analysis was conducted, which showed similarities and differences between the relevés. Identified groups of relevés were classified to the phytosociological system based on the study by Matuszkiewicz (2012).

Relevés selected for further analyses represented 5 meadow-pasture communities from the class *Molinio-Arrhenatheretea* differing in the type and intensity of use (*Table 1*).

Table 1. *Analysed meadow-pasture communities*

Community	Manner of use
Ass.: *Alopecuretum pratensis* (Regel 1925) Steffen 1931	cut 3×
Ass.: *Arrhenatheretum elatioris* Br.-Bl.ex Scherr.1925	cut 2×
Com. *Deschampsia caespitosa*	cut 1×
Ass.: *Lolio-Cynosuretum* R.Tx.1937	pasture; 2-3 LSU/ha
Com. *Poa pratensis-Festuca rubra* Fijałk. 1962	pasture; 1 LSU/ha

Notes. LSU – livestock units.

Relevés in the communities were grouped depending on the occurrence of common dandelion in the phytocoenosis:
A – none,
B – share with quantity r and +,
C – share with quantity 1 and 2.

Phytocoenoses were characterised in terms of the mean sward cover in the relevé and floristic abundance based on the total number of plant species in the community and mean number in the relevé, as well as the Shannon-Wiener floristic diversity index (Magurran, 1991; Szoszkiewicz and Szoszkiewicz 1998). Natural value (NVI) was assessed using valuation numbers according to Oświt (2000). This method ascribes numerical values from 1 to 10 to each taxon depending on the natural value of the plant species. The most valuable, protected and endangered species receive the highest values, while common species are ascribed the value of 1. Natural value of phytocoenoses was determined as the arithmetic mean of the numbers ascribed to species.

The fodder value was assessed using the method, which ascribes to each plant species values from -3 to 10 (Filipek, 1973). Negative numbers refer to poisonous species, numbers 0 and 1 are given to taxa with the lowest fodder value, while 10 – to species with the highest fodder value. The fodder value of sward (FVS) in the evaluated phytocoenoses was obtained as the weighted mean taking into consideration the proportion of the plant species and the ascribed number representing its fodder value.

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Habitat conditions of the phytocoenoses were determined by the phytoindicator method and laboratory methods. Using the indicator number according to Ellenberg and Leuschner (2010) the following values were calculated for each relevé: insolation (L), moisture content (F), pH-reaction (R) and soil nitrogen content (N). In turn, laboratory methods were used to determine the following:
- soil moisture content – by the oven-dry method,
- soil reaction, i.e. soil pH in 1 mol KCl dm^{-3} – by potentiometry
- organic substance content in soil – by the gravimetric method consisting in roasting of samples at 600˚C and calculation of weight losses,
- potassium content (by flame photometry) and phosphorus content (by colorimetry):
  - in mineral soils - the Egner-Riehm method,
  - in organic soils in 0.5 mol HCl dm^{-3},
- content of available magnesium
  - in mineral soils - the Schachtschabel method,
  - in organic soils in 0.5 mol HCl dm^{-3}.

Results were analysed using PCA and RDA with the use of the Canoco for Windows 5 programme (Braak and Šmilauer, 2012), which makes it possible to arrange the collection of relevés in relation to habitat factors and determine the dependence of natural value and fodder value in communities with different shares of *Taraxacum officinale* on habitat conditions.

Results and discussion

Occurrence of Taraxacum officinale in meadow communities

Results of analyses conducted in the five communities indicate that the share of *Taraxacum officinale* is connected with the manner of utilisation and its intensity. Occurrence of the species is particularly promoted by pasture use. An increase in its share in the sward was also found at the lower number of cuttings and cattle stocking (*Table 2*). Such a dependence indicates a potential for the control of this species in swards of grassland through increased intensity of use (Jankowska, 2012). Klimeš et al. (2003) showed that in the unharvested vegetation covers rate of *Taraxacum officinale* can increase. Moreover, those authors confirmed combined way of manner (once mowing and once grazing) actually contibute to proliferate and increase common dandelion share.

Table 2. *Natural and useful characteristics of studied plant communities*

Community + utilisation		Number of plant species generally	average in relevés	Cover [%]	H'*	NVI **	FVS ***
Alopecuretum pratensis	A	76	21	88.1	2.2	2.26	7.23
2-3 × cutting	B	64	23	80.0	2.3	2.41	7.17
	C	60	20	81.9	2.2	1.98	7.64
Arrhenatheretum elatioris	A	67	24	82.5	2.4	1.93	6.99
2 × cutting	B	77	26	76.9	2.4	1.77	7.42
	C	65	24	74.4	2.3	2.00	7.59
Deschampsia caespitosa	A	79	24	81.2	2.3	2.13	3.74
1 × cutting or no cutting	B	60	22	80.6	2.5	2.37	3.80
	C	62	19	76.2	2.1	2.50	4.35

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Lolio-Cynosuretum	A	66	22	88.1	2.3	1.94	8.05
grazing: 2-3 LSU/ha	B	77	31	86.2	2.7	1.95	7.44
	C	64	22	84.4	2.3	1.75	7.92
Poa pratensis-Festuca rubra	A	68	21	85.0	2.4	2.00	6.48
grazing: 1 LSU/ha	B	50	24	82.5	2.4	2.00	8.14
	C	60	22	76.8	2.4	2.01	7.41

Notes. * H' – floristic diversity index – according to Shannon-Wiener; **NVI – nature value index – according to Oświt (2000); *** FVS – fodder value score – according to Filipek (1973); LSU – livestock units.

In low and less dense swards used as pastures in the phytocoenosis *Lolio-Cynosuretum* and the community *Poa pratensis – Festuca rubra* a higher share of this species was recorded, particularly at a low total vegetation cover in the phytocoenosis (*Fig. 1*). As it was reported by Jankowska et al. (2009), this process may be explained by allelopathic properties of common dandelion or its adaptation to changing habitat conditions. Changes in habitat conditions cause elimination of plant species of high natural value, as a result of which free spaces appear for species with a wide ecological scale. These conditions are used e.g. by common dandelion, which occupies the free spaces and as a consequence dominate the phytocoenosis.

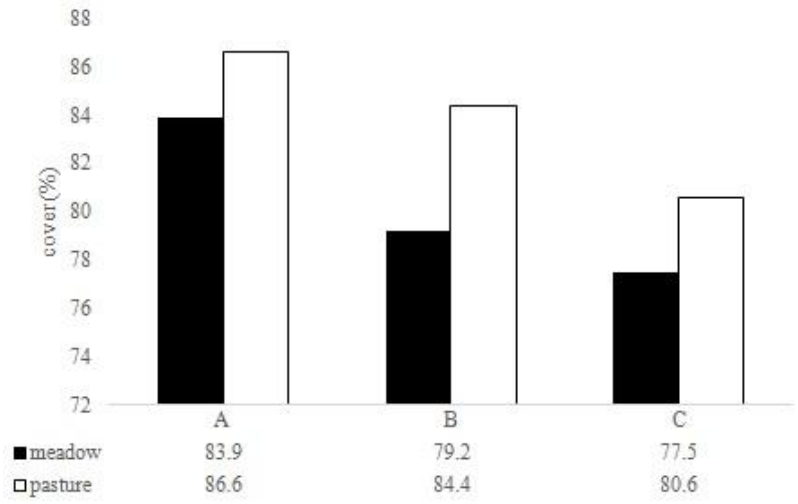

Figure 1. *The dependence of the share of Taraxacum officinale on sward cover of the relevé area. Notes. A – group of relevés without Taraxacum officinale; B – group of relevés with 'r' and '+' share of Taraxacum officinale; C – group of relevés with '1' and '2' share of Taraxacum officinale.*

The dependence of the occurrence of *Taraxacum officinale* on habitat conditions is presented in *Figs. 2, 3* and *4*. They show that insolation and soil reaction are the most significant habitat factors having a positive effect on the share of dandelion. In turn, a negative effect on the presence of this species is found for habitat moisture content and contents of available magnesium forms in soil. The other habitat factors, i.e. the level of available nitrate nitrogen, potassium and phosphorus has no effect on its share in the sward. This species was recorded in phytocoenoses developed in habitats with the lowest moisture contents and as a rule with soils containing low levels of the analysed macronutrients (*Fig. 2*).

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Moreover, it was found that periodically dry and strongly insolated areas are characterised by a lesser turf cover and promote an increased share of *Taraxacum officinale*.

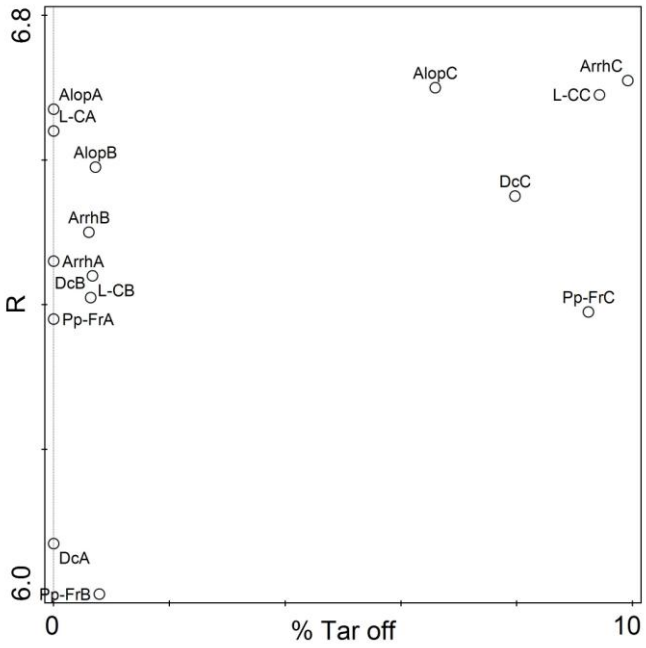

Figure 2. *The share of Taraxacum officinale in swards of phytocoenoses depending on pH-reaction. Notes. Alop – Alopecuretum pratensis; Arrh – Arrhenatheretum elatioris; Dc – community Deschampsia caespitosa; L-C – Lolio-Cynosuretum; Pp-Fr – community Poa pratensis-Festuca rubra; A – group of relevés without Taraxacum officinale; B – group of relevés with 'r' and '+' share of Taraxacum officinale; C – group of relevés with '1' and '2' share of Taraxacum officinale.*

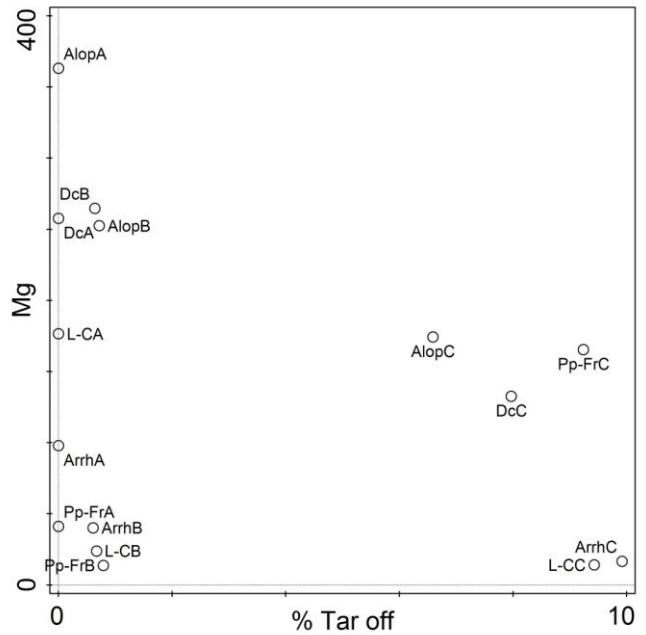

Figure 3. *The share of Taraxacum officinale in swards of phytocoenoses depending on soil magnesium content. Explanations under Figure 2.*

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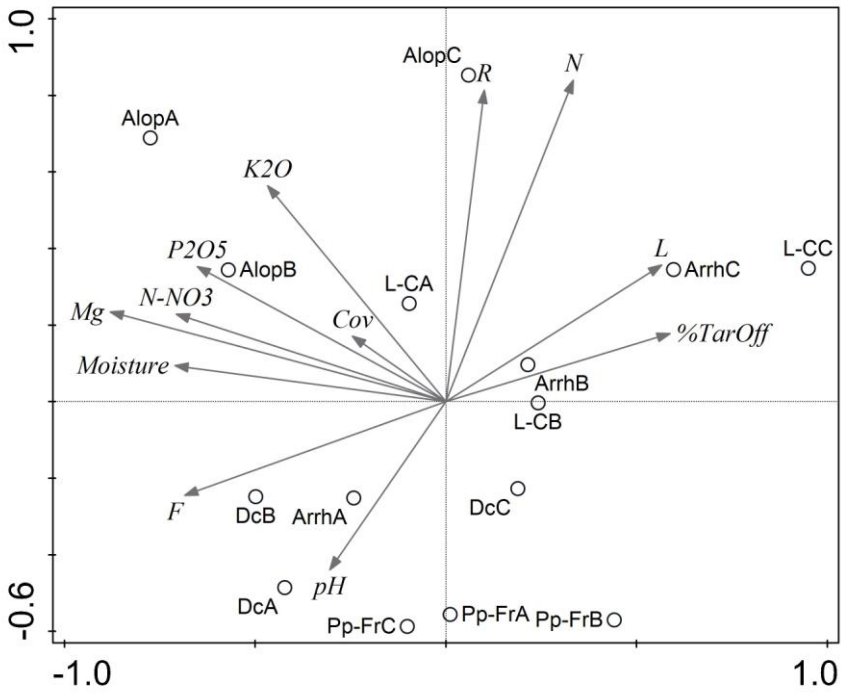

Figure 4. *Habitat conditions assessed by laboratory methods and the share of Taraxacum officinale in swards of the phytocenoses. Notes. Moisture – soil moisture content; pH – soil reaction; Cov – cover herb layer; %TarOff – share of Taraxacum officinale; Mg – content of available magnesium; K2O – potassium content; P2O5 – phosphorus content; N-NO3 – nitrogen content; L – Ellenberg's light index; F – Ellenberg's moisture content index; R – Ellenberg's pH-reaction index; N – Ellenberg's nitrogen content index. Other explanations under Figure 2.*

Brock et al. (2005) indicated a considerable phenotypic elasticity of *Taraxacum officinale* in relation to habitat conditions and its potential adaptability to light conditions. At high insolation leaves of this species considerably decrease in size, while at reduced lighting, as observed in denser swards, they elongate significantly. This promotes the occurrence of dandelion in periodically dry areas at strong insolation (Neuteboom and Lantinga, 1991; Brock, 2003).

The presence of Taraxacum officinale in swards of communities and their natural and fodder value

The presence of *Taraxacum officinale* in the sward influences both the natural value and utility value of the phytocoenoses. Greater numbers of plant species were recorded in relevés of mowed areas and their number decreased with the increase in the share of common dandelion. Swards used as pastures showed no such dependence. It needs to be stressed that the highest share of species in the sward, irrespective of the manner of use, was recorded at a slight, approx. 1% share of *T.o.* (*Fig. 5*). Similar changes were observed in the values of the calculated Shannon-Wiener index (*Table 1*).

Generally the presence of *Taraxacum officinale* to a limited extent positively influences natural value of phytocoenoses – most typically it is low. Only sporadically utilised communities of habitats with a higher moisture content such as communities

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Deschampsia caespitosa and *Alopecuretum pratensis* at a slight share of *Taraxacum officinale* present moderate natural value. In turn, this species has a positive effect on the fodder value of mowed sward. Such a dependence was not observed at pasture use (*Table 1*). It may be assumed that the positive effect on fodder value of sward is a result of the chemical composition of this species, which is reflected in its high palatability (Tsuyuzaki and Takahashi, 2007; Jankowska, 2012; Lukač et al., 2012).

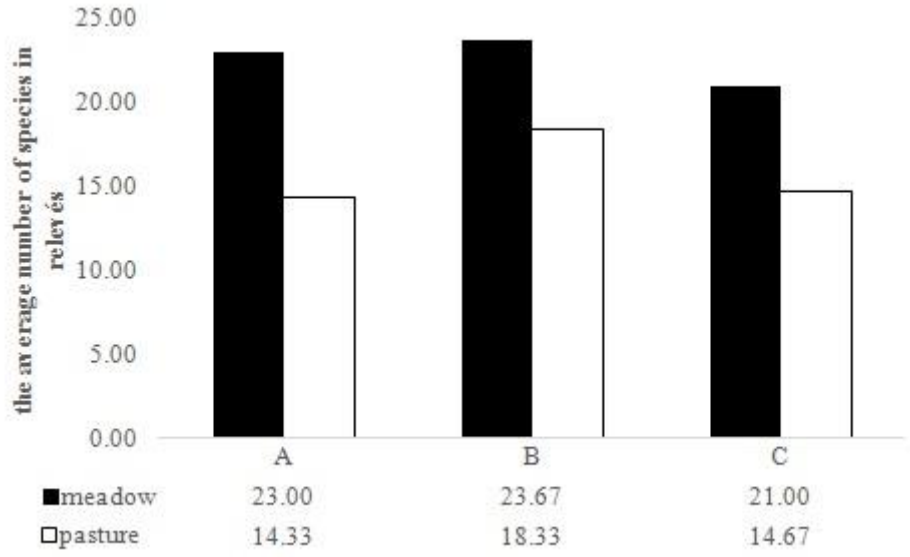

Figure 5. *Species richness in phytocenoses with different shares of Taraxacum officinale in sward and different manner of use. Explanations under Figure 1.*

Natural and utility value of the plant communities reflect the phytosociological structure of phytocoenoses containing this species. The Principal Component Analysis (*Fig. 6*) confirmed that an increased share of dandelion in relevés with *Deschampsia caespitosa* and *Alopecuretum pratensis* is correlated first of all with a greater share of species from the class *Phragmitetea*, which is manifested in the higher natural value of these phytocenoses.

Dependencies between all the assessed parameters connected with the share of *Taraxacum officinale* in swards of analysed communities, i.e. factors determining habitat conditions and its effect on natural and utility value of phytocoenoses is presented synthetically in *Fig. 7*, which confirms that an increasing share of dandelion in the sward is positively correlated with values of R and N. In turn, an increase in the share of *Taraxacum officinale* is accompanied by a greater share of segetal and ruderal species, which is connected with a greater insolation of the area. These factors contribute to the poorer floristic structure of phytocoenoses, as manifested by a lower number of recorded species (Kiss et al. 2011; Beck et al. 2014). It needs to be stressed that the absence of dandelion in the sward is frequently connected with a greater share of species representing the class of *Phragmitetea,* and thus greater natural values. These areas were also characterised by greater soil resources of available magnesium and phosphorus forms.

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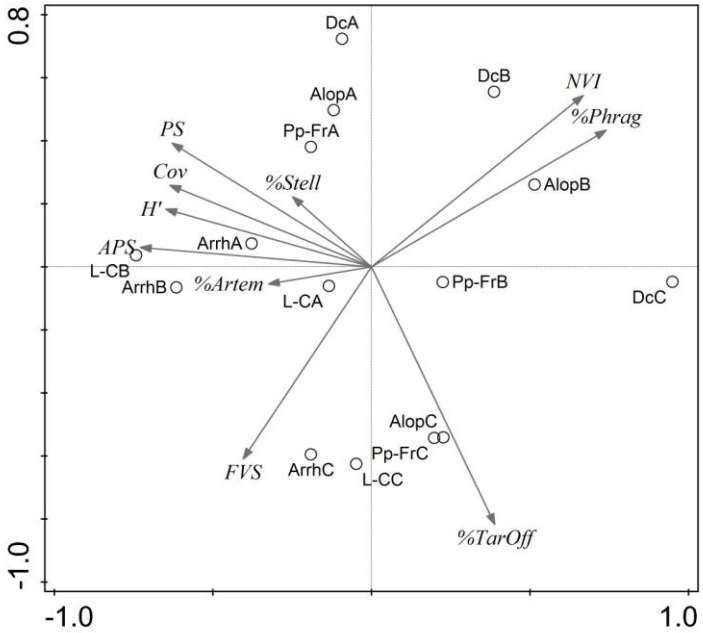

Figure 6. *Natural value and utility value of communities with different shares of Taraxacum officinale. Notes. PS – number of plant species generally; APS – average number of plant species in relevés; H' – Shannon-Wiener index; FVS – fodder value of sward; NVI – natural value index; %Stell – share of plant species of Stellarietea mediae class; %Arterm – share of plant species of Artemisietea vulgaris class; %Phrag – share of plant species of Phragmitetea class. Other explanations under Figure 2 and 4.*

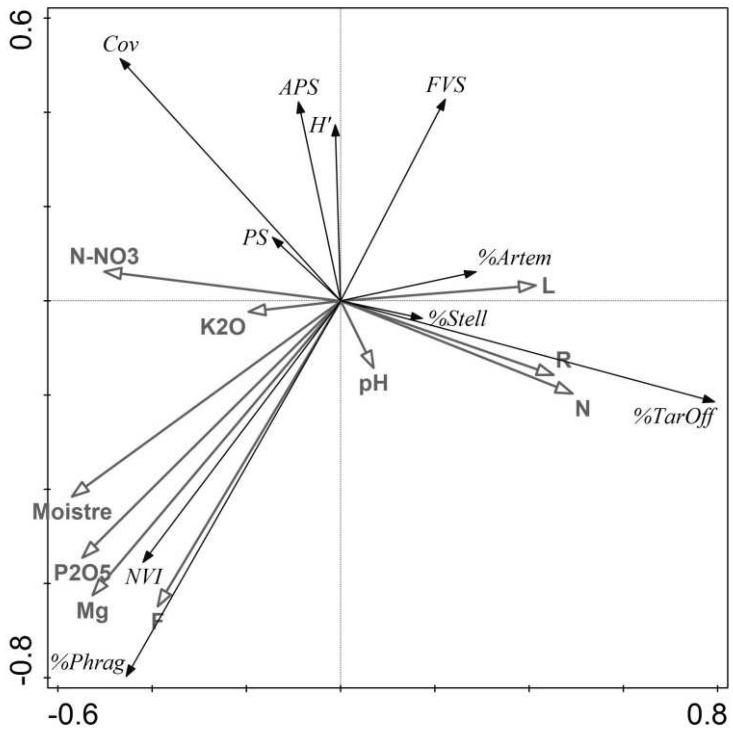

Figure 7. *The dependence of the share of Taraxacum officinale on habitat conditions and its effect on natural value and fodder value of sward. Explanations under Figure 4 and 6.*

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Conclusion

Occurrence of common dandelion (*Taraxacum officinale* F.H.Wigg.) in swards of meadow-pasture communities is promoted by: habitat conditions, particularly soil periodically dry and good insolation, as well as a slightly acidic soil reaction and low contents of available magnesium in soil, pasture use and its lower intensity.

An increased share of common dandelion in phytocenoses of class *Molinio-Arrhenatheretea* contributes to a decreased natural value, as manifested in the reduction of floristic diversity, while it has a positive effect on the fodder value of mowed swards.

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NOVEL INSIGHT INTO EVOLUTIONARY PROCESS FROM AVERAGE GENOME SIZE IN MARINE BACTERIOPLANKTONIC BIOTA

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Abstract. Genome evolution is an essential force that shapes biodiversity. Genome reduction has been demonstrated in numerous microorganisms and is interpreted as a means of decreasing material cost during DNA synthesis. However, most of the previous studies have been conducted under pure-culture or endosymbiotic conditions, which greatly restrict horizontal gene transfer between microbial species. In nature, microbes frequently interact with each other and gene exchanges are quite common within the microbiota. The evolutionary trend underlying genome size is still poorly understood. The ocean provides an ideal setting for examining the evolutionary process of microbial genomes under natural conditions. We retrieved bacterial compositional information for marine bacterioplanktonic biota from the Visualization and Analysis of Microbial Population Structures database and compared their average genome size along an ocean depth gradient. The results showed that marine bacterioplanktonic biotas tend to minimize their average genome size in the course of their evolutionary processes and that niche differentiation is probably the major force driving genome size reduction. These findings provide novel insight into the understanding of the evolutionary process underlying microbial genome size and mechanisms for maintaining microbial biodiversity in nature.

Keywords: *microbial genome evolution*; *bacterioplanktonic biota*; *microbial diversity*; *evolutionary processes*

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Introduction

Bacteria are the most abundant and diverse organisms on Earth (Kuo et al., 2009). They inhabit almost every environment in the biosphere and play crucial roles in biogeochemical cycling of essential elements (Huse et al., 2008; Zhou et al., 2015; Metcalf et al., 2016). Their diversity and maintenance mechanisms have been extensively studied in various habitats as the potential association between their diversity and function (Vellend and Geber, 2005; Chaffron et al., 2010; Monard et al., 2011; Nemergut et al., 2011; Sul et al., 2013). Currently, many ecological microbial patterns have been identified, such as the cosmopolitan distribution of microbial subgroups (Chaffron et al., 2010; de Wit and Bouvier, 2006), the taxa-area relationship (Bell et al., 2005), and the distance-decay relationship (Green and Bohannan, 2006; Bell, 2010; Ni et al., 2014). These observations have expanded our knowledge regarding microbial diversity and maintenance mechanisms. However, most of these studies have focused on diversity at the species level or were based on the species concept.

Although the evolutionary processes of microbial genomes have attracted attention (Andersson and Kurland, 1998; Dufresne et al., 2005; Giovannoni et al, 2005; Nilsson et al., 2005; McCutcheon and Moran, 2010; Wolf and Koonin, 2013), relatively few studies have addressed their role in preserving microbial diversity. Microbial genome size is primarily determined by two opposing processes: acquisition of new genomic fragments by gene duplication or by horizontal gene transfer and deletion of non-essential genomic fragments (Mira et al., 2001; Giovannoni et al., 2005; Nilsson et al., 2005). Several studies have demonstrated that pure-culture and endosymbiotic bacteria tend to reduce their genome size during the evolutionary process (Nilsson et al., 2005; Pérez-Brocal et al., 2006; Marais et al., 2008; Wolf and Koonin, 2013). For instance, Nilsson et al. (2005) report the initial rate of DNA loss is estimated to be 0.05 bp per chromosome per generation in the bacterium *Salmonella enterica* in culture. Phylogenetic analyses also reveal that bacteria with smaller genomes are derived from those with larger ones (Andersson and Kurland, 1998; Wolf and Koonin, 2013). However, this proposed trend has come into question, since interspecies horizontal gene transfer, the primary route by which bacterial species obtain new genes, is restricted under pure culture or endosymbiotic conditions (Ochman et al., 2000). In nature, microbes interact with each other and commonly form a microbial community (i.e. microbiota) within which gene exchanges are quite common (McDaniel et al., 2010; Tuller et al., 2011). For instance, McDaniel et al. (2010) report environmental gene transfer frequencies range from 6.7×10^{-3} to 4.7×10^{-1}, which are 1900 to 459 million times the frequency for transformation and 650000 to 31 million times the frequency of transduction. These exchanges could counteract the influence of genomic fragment deletion. Although Mira et al. (2001) have proposed "a pervasive bias towards higher numbers of deletions than insertions" and that "deletional bias is a major force that shapes bacterial genomes," this has yet to be proved under natural conditions. The "deletional bias" hypothesis suggests that the average genome size (AGS) of microorganisms would decrease during their evolutionary process in nature.

The evolutionary process on Earth has been maintained in the vertical plane stretching from the deep sea to the ocean surface since ancient times (Carney, 2005; McClain and Hardy, 2010). Therefore, this oceanic vertical plane contains bacterioplanktonic

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evolutionary environments, which demonstrate microbial evolutionary processes similar to those exhibited by marine multicellular organisms (Carney, 2005; Brandt et al., 2007; McClain and Hardy, 2010; Plata et al., 2015 and Unpublished data). Thus, the ocean provides an ideal setting for examining the evolutionary process of microbial genomes under natural conditions. Based on the "deletional bias" hypothesis (Mira et al., 2001), the AGS of microorganisms in microbiota would decrease during their evolutionary process in nature. Therefore, we hypothesized that the AGS of the marine bacterioplanktonic biota would decrease from deep sea to surface layers, consistent with the evolutionary processes. To test this, we retrieved bacterial composition information of marine bacterioplanktonic biota from the Visualization and Analysis of Microbial Population Structures (VAMPS) database and compared their AGS along an ocean depth gradient. To the best of our knowledge, this is the first report of AGS analysis at the microbiota level. The findings of this study provide novel insight into the evolutionary processes underlying microbial genome size and microbial biodiversity maintenance mechanisms in nature.

Materials and Methods

Data retrieval

The bacterial composition information of 63 marine bacterioplanktonic biotas at different water depths range from 0 to 4000 m was retrieved from the VAMPS database (http://vamps.mbl.edu/portals/icomm/icomm.php/microbis/data_exports/download_bacteria_gz.php) with Project codes KCK_HOT_Bv6. The genomic data of 4891 sequenced and annotated bacterial genomes, containing 3257 species were retrieved from the Integrated Microbial Genomes (IMG) system as reference genomic data (Markowitz et al., 2012).

Data analysis

Since the full genomes of most bacterial taxa are not available in public databases, their genome sizes and 16S rRNA gene copy number (GCN) cannot usually be deduced directly from environmental sequence data (Kembel et al., 2012; Tamames et al., 2012; Ni et al., 2013). Hence, the average GCN and genomic size of taxa belonging to the same higher taxonomic category were calculated based on the reference genomic data according to Ni *et al.* (2013) and used as representative of the taxon as the method excludes the bias from the possible weighting effect due to variability in the number of fully sequenced genomes per taxa.

Because of the GCN variation in different bacterial genomes, the organismal proportion of each taxon in the bacterioplanktonic biota was adjusted using the following equation (Kembel et al., 2012; Ni et al., 2013; Angly et al., 2014):

$$p_i = \frac{N_i / (AC_{16S})_i}{\sum_{i=1}^{n} (N_i / (AC_{16S})_i)} \qquad \text{(Eq. 1)}$$

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where p_i is the organismal proportion of the i^{th} taxon, N_i is the number of 16S rRNA gene reads for the i^{th} taxon, AC_{16S} is the average GCN value of the i^{th} taxon, and n is the total number of taxa in the bacterioplanktonic biota.

The AGS of each bacterioplanktonic biota was calculated as follows:

$$AGM_j = \sum_{i=1}^{n} (p_i \cdot AG_i) \quad \text{(Eq. 2)}$$

where AGS_j and AG_i are the AGS of the j^{th} bacterioplanktonic biota and the i^{th} taxon in the j^{th} bacterioplanktonic biota, respectively.

The Shannon-Wiener, Simpson, and inverse Simpson indices of the bacterioplanktonic biotas, as well as the Bray-Curtis distances between biota at the same depth were calculated using the vegan 2.0-10 package (Dixon, 2003) of the R platform (R Development Core Team, 2006). All calculations and statistical tests were conducted with the R platform.

Results

Since the complete genome information of many bacteria is not available in public databases, approximated genome sizes and GCN were calculated according to previous reports (Ni et al., 2013); these calculated values were generally consistent with our results. Next, we calculated the AGS of each marine bacterioplanktonic biota. The AGS was significantly, positively correlated with ocean depth ($R^2 = 0.694$, $F = 138.1$, $p < 2.2\times10^{-16}$; *Fig. 1*), with a regression equation of y = 0.000316x + 2.171, where y is the AGS (Mb) and x is ocean depth (m). The correlation between the average gene count per genome of each marine bacterioplanktonic biota and ocean depth exhibited the same trend ($R^2 = 0.662$, $F = 119.6$, $p = 5.17 \times 10^{-16}$). These results indicate that the AGS of marine bacterioplanktonic biota decreases from deep sea to surface, consistent with our hypothesis. Similarly, *Prochlorococcus* lineages exhibited a similar evolutionary progression in their genomes. Based on genome data from the IMG system (Markowitz et al., 2012), ecotype eMIT9313, the earliest lineage of *Prochlorococcus* branching from the last common ancestor of *Prochlorococcus* and *Synechococcus*, contains the largest genome (2.41 Mb), followed by ecotype eNATL2A (1.84 Mb), strains restricted to the deep euphotic zone. However, ecotypes eMIT9312 and eMED4, the most recently derived lineages, only contain genomes of 1.71 Mb and 1.66 Mb, respectively, and have spread to the upper euphotic zone (Dufresne et al., 2005; Morris et al., 2011).

In order to understand the mechanism underlying changes in AGS, we compared the composition of dominant species (organismal abundance >1%) in the bacterioplanktonic biota. We observed that the relative abundance of bacteria with smaller genome sizes was higher at the surface than in the deep sea (*Fig. 2*), indicating that the bacterial composition with different genome size of marine bacterioplanktonic biota changes at various ocean depths, forming a marine bacterioplanktonic biota AGS gradient along the ocean depth gradient.

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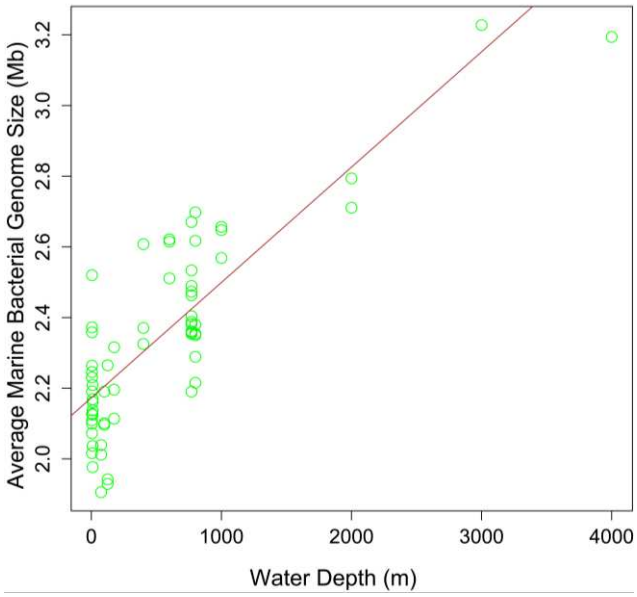

Figure 1. *The correlation between the average genome size of marine bacterioplanktonic biota and ocean water depth.*

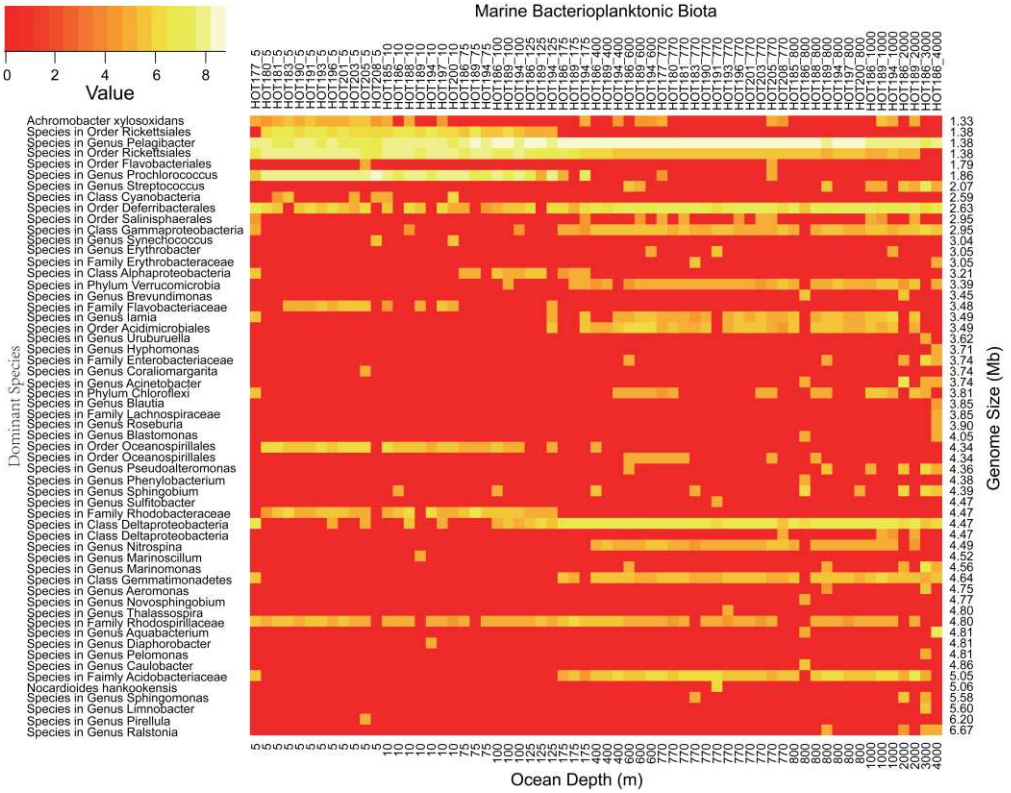

Figure 2. *Heatmap showing abundance of dominant species in marine bacterioplanktonic biota. The heatmap was generated based on the logarithmic transformation of the relative abundance of the dominant species.*

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The physical and chemical properties of marine water columns show considerable variability at the upper layer of the ocean, but remain relatively constant at greater depth (Robison, 2004; Giovannoni et al., 2014). These observations indicate that oceanic niches differentiated from deep sea layers to the surface. Oceanic niche differentiation has been demonstrated to cause genome divergence in two *Prochlorococcus* ecotypes (Rocap et al., 2003). In addition, genome reduction has been widely observed in symbiotic bacteria that have adapted to a specific symbiotic niche (Gil et al., 2002; Pál et al., 2006; Pérez-Brocal et al., 2006; McCutcheon et al., 2010). Hence, it is plausible that bacterioplanktonic biotas continuously differentiate to better suit specific marine niches formed during evolutionary progression. For example, a bacterial group might reduce its genomic material to better suit a specific marine niche, especially an oligotrophic marine zone (Morris et al., 2002) since genomic reduction could conserve materials and energy for biosynthesis during the process of bacterial reproduction. If this hypothesis is correct, the marine bacterioplanktonic biota alpha-diversity indices, such as the Shannon-Wiener index, the Simpson index, and the inverse Simpson index, would increase with water depth, while the beta-diversity indices (e.g., Bray-Curtis distances) between biotas at the same depth (isobaths) should decrease along the water depth gradient. Our results support this hypothesis with the exception of the Bray-Curtis distance showing a weak non-significant decreasing trend (*Fig. 3*).

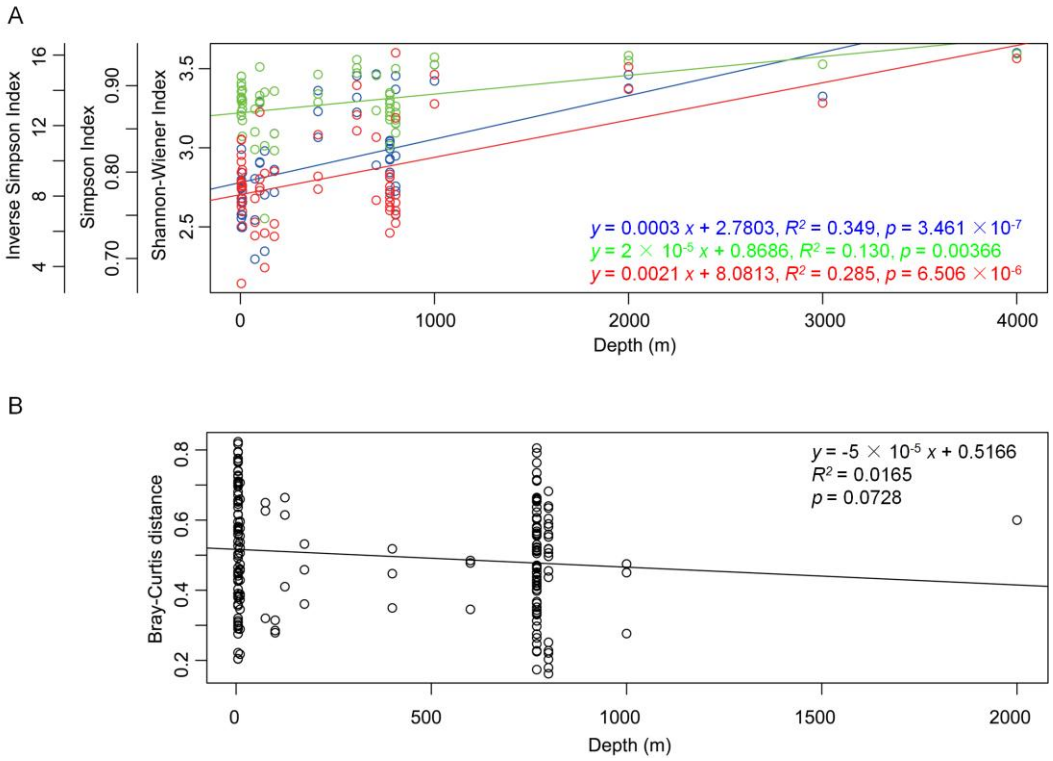

Figure 3. *Alpha-diversity indices (A) and Bray-Curtis distances (B) of marine bacterioplanktonic biota along the ocean water depth gradient. Blue, green, and red in panel (A) indicate the Shannon-Wiener index, Simpson index, and inverse Simpson index, respectively. Since only one sample was retrieved at 3,000 m and 4,000 m, the Bray-Curtis distance between the biota at the same depth was not calculated.*

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Discussion

Genomic reduction could conserve materials and energy for biosynthesis during the process of bacterial reproduction. Since bacterial generation time is relatively short, this reduction is probably quite significant in terms of bacterial competition, especially in oligotrophic ocean environments. The dominance of SAR11, which has the smallest genome known for a free-living heterotrophic cell, in the ocean surface bacterioplanktonic biota, exemplifies the advantage of genome reduction (Morris et al., 2002). Based on the linear relationship between bacterial genome size and gene counts (Mira, 2001; Giovannoni et al., 2005), our study demonstrates that marine bacterioplanktonic biota show an evolutionary trend towards efficiently utilizing their genetic material to adapt to different marine niches. These results suggest that bacteria in current ecosystems may be subjected to powerful selection to minimize the material and energetic cost of cellular replication, concurring with the streamlining hypothesis (Giovannoni et al., 2005). Morris et al. (2012) proposed a Black Queen hypothesis to explain how selection leads to the dependence of free-living organisms with gene loss on co-occurring microbes. However, it is unclear why bacteria possessed large genomes at the start of their evolutionary processes.

The benefits afforded by genome reduction are not without a cost. Since the majority of bacterial genomic sequences are functional protein-coding regions (Mira et al., 2001), AGS reduction in marine bacterioplanktonic biota could result in substantial gene loss. At the species level, gene loss might cause physiological deficiencies. For instance, the lack of catalase and additional protective mechanisms in *Prochlorococcus* genomes leads to high sensitivity to oxidative damage due to hydrogen peroxide (Morris et al., 2011). However, in a bacterioplanktonic community, *Prochlorococcus* could eliminate oxidative damage by coexisting with heterotrophs at the mixed surface layer of the oligotrophic ocean (Morris et al., 2011). Tripp and colleagues (Tripp et al., 2008) provided another example of this cost-benefit; SAR11 possesses the smallest genome among free-living heterotrophic bacteria and is the dominant and ubiquitous lineage in the euphotic zone. However, SAR11 is deficient in assimilatory sulphate reduction genes. Hence, SAR11 requires exogenously reduced sulphurs that originate from other bacterioplanktons for growth (Tripp et al., 2008). Another considerable issue is trade-off of benefits between the acquisition of a function after loss and keeping it. It is reasonable to keep the function if the cost of acquiring the function far outweighs the cost of keeping it and the function is essential for bacterial surviving. However, it is hard to distinguish which function is re-acquired after loss and which one is always retained. Therefore, it cannot compare the cost between re-acquiring a function after loss and keeping it. The trade-off of benefits between the acquisition of a function after loss and keeping it need to be further studied.

A possible explanation for the lower correlation coefficient ($R^2 = 0.694$) between AGS and water depth is the disruption of the vertical bacterioplanktonic distribution pattern by turbulence, a driver of vertical plankton distribution at the subsurface upper ocean layer (Macías et al., 2013). However, although turbulence temporally changes nutrient and bacterioplanktonic community compositions (Lewis et al., 1986; Macías et al., 2013), it does not seem to alter the distribution pattern of some bacterioplanktonic taxa, such as *Prochlorococcus*, at the ocean surface (Morris et al., 2011). The upper euphotic zone is dominated by eMED4 and eMIT9312, which are high-light adapted, while the lower euphotic

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zone is dominated by low-light adapted ecotypes including eNATL2A, eMIT9313, and eSS120 (Morris et al., 2011). In deep ocean layers, water features such as temperature, pressure, and salinity are relatively constant (Robison, 2004). Thus, although vertical turbulence would temporally disturb the bacterioplanktonic community composition, it could not change the distribution pattern of bacterioplanktonic biota in a vertical direction.

Many factors (e.g. water temperature and bacterial density) affect the AGS of marine bacterioplanktonic biota. Water temperature could constitute a crucial factor influencing the AGS. For instance, the maximal values of SAR11 relative abundance in marine bacterioplanktonic biota occurred during the summer (Morris et al., 2002). This increase in the relative abundance of this small genome free-living heterotrophic cell could reduce the AGS of the bacterioplanktonic biota. Therefore, water temperature could change the abundance of other bacterial species thus indirectly affecting the AGS. Density is another possible factor influencing AGS. Bacterial density decreases with water depth, especially at 0-1000 m (Morris et al., 2002). The decrease in bacterial density probably reduces mutuality among different bacterial species, which is crucial for the survival of bacteria with small genomes (Morris et al., 2012). Under these conditions, bacteria must maintain a genomic size sufficient to ensure they retain essential genes. The effect of these factors on AGS remains to be confirmed.

The Bray-Curtis distance values demonstrate a weak non-significant decreasing trend (*Fig. 3*). Such non-significance may be due to small sampling areas; the bacterioplanktonic biota habitat at the sampling depth probably did not completely differentiate into different niches. The decreasing trend would be more obvious if the sampling sites were at a greater horizontal distance from each other. Moreover, the evolutionary mechanisms of marine bacterioplanktonic biota could be further elucidated at the level of gene composition via metagenomic sequencing.

Based on the environmental, genome, and evolutionary data, we postulate that niche differentiation is probably the major driving force for AGS reduction in marine bacterioplanktonic biota. These finding, together with future studies, could provide considerable information for protecting the bio- and genetic diversity of microbiota.

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EFFECTS OF SUBSTRATES AND PLANT SPECIES ON WATER QUALITY OF EXTENSIVE GREEN ROOFS

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Abstract. Green roofs have multiple environmental benefits, such as saving energy, improving air quality, mitigating noise, providing habitat, beautifying the landscape, and improving urban hydrology. Among the environmental benefits of green roofs, improving runoff quality is a controversial issue. Substrate and plant litter may be transported with green roof runoff, which would increase the risk of urban nonpoint source pollution. However, the soil substrates on green roofs can filter out pollutants that can then be taken up by plants, which would improve water quality. In the present study, experimental containers with 2 substrates and 8 plant species were constructed, and data were collected from 7 real rainfall events to analyze the effluent water quality. This outdoor site was unfortunately terminated by a strong typhoon, but the idea and some results of the study might give some lights for the future study. The total solid (TS), chemical oxygen demand (COD), and total nitrogen (TN) concentrations were greater during the earlier stage and gradually decreased. However, the total phosphorous (TP) concentrations did not change with time. The limited data did not show the expected effects of the combinations of substrates and plants, but the effect of substrates was significant on the COD and TP concentrations.
Keywords: *exetensive green roofs; substrate; plant species; water quality*

Introduction

Green roofs provide various environmental benefits regarding water, energy, air, ecology, and aesthetic value (Dunnett and Kingsbury, 2004; Oberndorfer et al., 2007; Getter et al., 2009). Therefore, green roofs are commonly used component of the design of sustainable cities. Unlike in other measures taken to increase green space in urban areas, no additional land is required to implement green roofs because they use underutilized rooftops. This unique feature promotes the development of green roofs (Peck, 2003; Villarreal and Bengtsson, 2005; Mentens et al., 2006; Dvorak and Volder, 2010). Some countries and cities provide incentives to encourage or enforce the use of green roofs, including Germany, Belgium (Dunnett and Kingsbury, 2004), Singapore (Wong et al., 2003), Japan (Osmundson, 1999), Hong Kong (Zhang et al., 2012), and some cities in Canada and the United States (Carter and Fowler, 2008). In Taiwan, green roofs are a component of several central and local governmental policies and regulations regarding development (Chen, 2013).

Green roofs can improve the urban environment; however, the factors affecting green roof performance remain unclear. For example, the selection of plant species, use of substrates, integration of rainwater harvesting systems, carbon sequestration, and human

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health benefits of green roofs have not yielded consistent research results (Lundholm et al., 2010; Roehr and Kong, 2010; Rowe, 2011). In particular, the overall benefit or harm of green roofs regarding runoff quality remains uncertain. A roof with soils and plants on it might flush out solids or pollutants with rainfall runoff and impair water quaity; howerver, in the other hands, the soils and plants might be able to filter pollutants and clean up water. The water quality of green roofs remains controversy and has been discussed. Several impact factors for water quality have been defined in previous research, including plant height, root density, and substrate properties. Plants reduce pollution levels by extending water retention time and taking up nutrients from the substrate. When grown in an appropriate substrate, plants are healthy and nutrient losses decrease. Thus, the combination of substrate and plant species is a crucial design factor that determines the level of water quality improvement. The objective of this study was to determine the effects of substrates, plant species, and their combinations on green roof water quality.

Review of literature

Water quality of green roofs is mainly influenced by the properties of substrates and plants and rainfall events. Moran et al. (2005) reported that pollutants can accumulate in the bottom layers of green roof substrates. These pollutants may be flushed out during intensive rainfall events, which would result in lower water quality. Teemusk and Mander (2007) claimed that the runoff flow rate determines whether water quality will be improved or worsened by green roofs; during moderate- and low-intensity rainfall events, water quality improves as rainwater traps nutrients and sediments on green roofs. The USEPA (2009) reported that high nutrient concentrations and low flow rates from green roofs may result in reduced pollutant mass loads. Bliss et al. (2009) and Wang et al. (2013) indicated that the first flush (a typical nonpoint source pollution characteristic) did not occur with green roofs. Most previous studies have indicated that green roof runoff has poor water quality. However, these studies have also indicated that the water quality is influenced by the age of the green roofs, the substrate used, the rainfall volume, and the fertilization practices used (Czerniel Berndtsson et al., 2006; Hathaway et al., 2008; Carpenter and Kaluvakolanu, 2011; Vijayaraghavan et al., 2013). In a review, Rowe (2011) reported that mature and well-maintained green roofs improve water quality, whereas new green roofs do not. New green roofs without full-grown plants typically have high substrate nutrient concentrations and are therefore regarded as pollution sources.

In addition to the effects of rainfall, substrate characteristics and plant composition affect the water quality of green roof runoff. Emilsson et al. (2007) emphasized that the use of conventional fertilizers would reduce water quality, whereas plants are capable of reducing pollutant leaching. The health and nutrient use of plants largely affect the performance of green roofs (Rowe, 2011). Tall stems and dense roots help retain rainwater and soil and effectively reduce water runoff (Nagase and Dunnett, 2012). Diverse plant species create various ecological habitats, and the use of mixed plant species results in improved performance (Dunnett et al., 2008; Lundholm et al., 2010). Monterusso et al. (2004) tested the water quality from sedum and herbaceous plant species and found that nitrate concentrations were greater on sedum green roofs than on herbaceous green roofs. However, no changes in phosphorus concentrations were observed. Furthermore, the depth and material of the substrate affect water quality

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(Moran et al., 2005; Teemusk and Mander, 2007; Getter and Rowe, 2009; Alsup et al., 2010; Wang et al., 2013). Nutrients in the substrate are required for plant growth but should be limited to prevent the detachment and transport of nutrients from the substrate.

In summary, the water quality of green roofs can be imporved in low rainfall intensity event. The new bulit green roofs produce bad water quality because of abundant nutrients blended in substrates. But the studies on the details of substrates and plants effects on water quality are still rare. Theoretically, the deep depth of substrates and the dense roots of plants strengthen filtration level and benefit water quailty. The clarification of effects of substrates and plant species would help to develop a satisfactory green roof.

Materials and Methods

Experimental containers

To collect rainwater from different substrates and plants, individual containers were used for each experimental unit. We designed a transparent experimental container constructed of acrylic materials (*Fig. 1*). The dimensions of the container were 30×30×30 cm (length×width×height). Each container was 5 mm thick. The standard green roof structure was used, including plants, substrate, filter, and a drainage layer. The drainage layer was composed of acrylic material with 81 drainage holes (2 cm diameter). A PE material filter net was placed above the drainage layer. Next, the substrate was spread on the filter net to a depth of 10 cm. The container included a removal drawer at the bottom of the container to receive outflow water from the drainage holes.

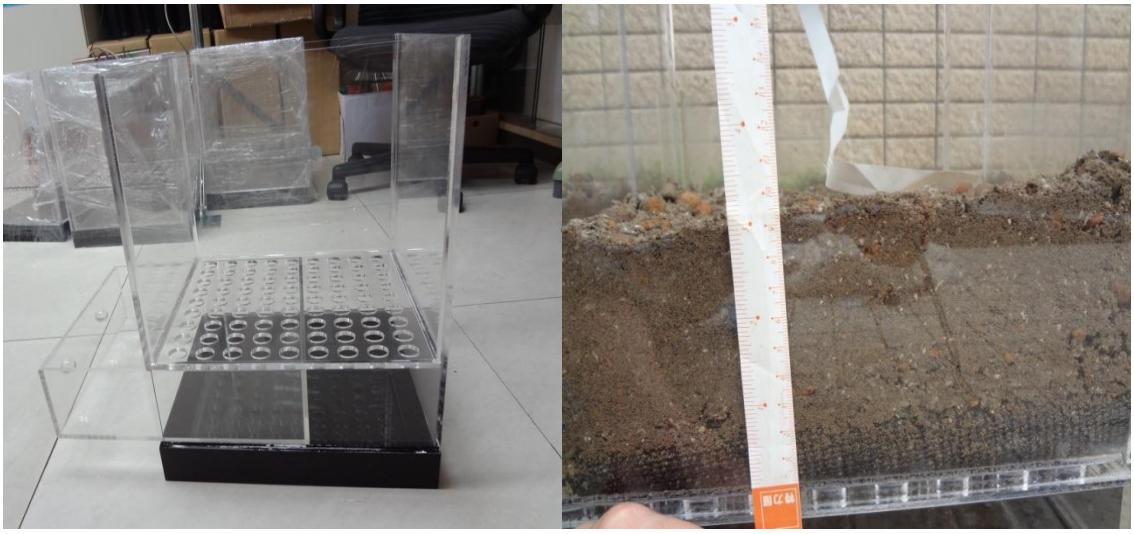

Figure 1. *The left is the experimental container. At the bottom is a removal drawer to collect outflow water. The right shows the filter net and the substrate.*

Substrates

Two substrates were tested, including a traditional cultivated soil from Taiwan and a soil that was specifically designed for green roofs by a local green roof company in Taiwan. The latter substrate was designed to mitigate roof loads by achieving improved

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drainage capacity and lighter weight. Recycled fiber and pottery stone were mixed for the light green roof (GR) soil. The chemical and physical properties of the two substrates are listed in *Table 1*. The GR soil is lighter than the traditional cultivated soil. The results of a particle analysis indicated that the two soils had very different particle distributions. Although 90% of the cultivated soil passed through a 4 mm sieve, only 60% of the light GR passed through a 4 mm sieve. In both soils, 45% of the particles were smaller than 1 mm. The cultivated soil was uniform, with particle sizes of 1 to 4 mm. However, the light GR soil included large particles, such as pottery stones, that were larger than 4 mm. The uneven particle distribution in the GR soil was used to increase the soil porosity and enhance water and air movement in the substrate layer. The result of particle size analysis is in *Fig. 2*. Becasue of the loose texture the GR soil has higher water holding capacity than the traditional cultivated soil. The organic matters, mass percentages of total organic carbon (TOC), total Kjeldahl nitrogen (TKN), and total phosphorous (TP) in the two substrates were very different. The nutrient concentrations in the light GR soil were approximately 3 times greater than those in the cultivated soil.

Table 1. *Properties of the studied substrates*

Substrates Properties	Cultivated soil	Light GR soil
Bulk density (g/cm^3)	1.13	0.84
Water holding capacity (%)	39.1	53.7
Diameter size>1mm (%)	42	52
D_{10} (mm)	0.12	0.07
D_{50} (mm)	0.7	1.2
Organic matters (g/L)	35	63
TOC (%)	1.82	4.39
TKN(mg/kg)	628	1,150
TP (mg/kg)	6.53	20.1

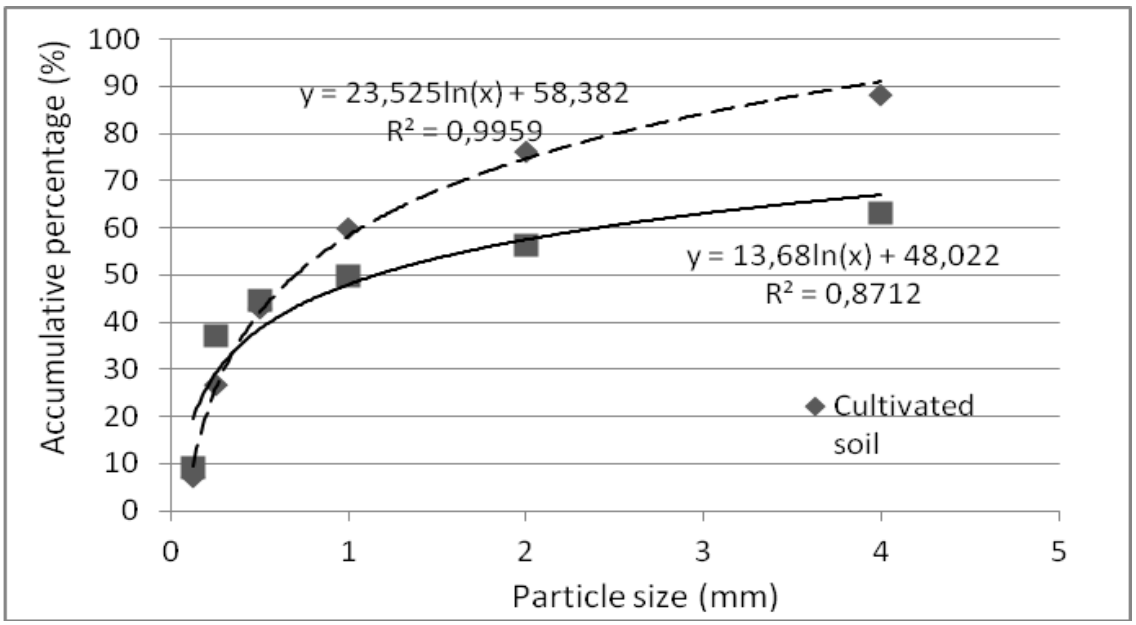

Figure 2. *Particle size analyses for the two substrates*

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This study started in 2013 and at that time, no design guideline was provided in Taiwan to follow. However, a latest official technic design guideline of green roofs was established in 2015 to suggest local design factors used in Taiwan (Architecture and Building Research Institute, 2015). This guildeline referred to the German Landscape Research, Development and Construction Society (FLL) green roof technology standards and made adjustments for Taiwanese cases. The suggestions for substrates are shown in *Table 2.* The physical properties and the organic matters percentage of the two studied substrates are fit in the range of the criteria.

Table 2. *Suggested properties of the substrates of green roofs in Taiwan(Architecture and Building Research Institute, 2015)*

Propeties	Suggested values
Diameter size>1mm	≧30%
Bulk density	0.7-1.0 g/cm^3
Permeable rate	>3.6 cm/hr
Water holding capacity	≧50% volume
Organic matter	≦65 g/L
pH	6-8.5
Electrical Conductivity	<2.5 mS/cm
Cation exchange capacity	>10 meg/100g

Plants

Sedum and herbs are generally planted on green roofs. Here, we studied plants that are commonly used on extensive green roofs in Taiwan. To examine the effects of plant species, we used plants with substantially different appearances and growth characteristics. After reviewing commonly used plants on local green roofs, we chose four types of plants and two species of each plant type. The selected plant species are also listed in the 2015 local technic guideline (Architecture and Building Research Institute, 2015). The first type of plant was the creeper forb. Creeper forbs are tiny, have dense root systems, and can grow extensively to cover the surface quickly. The creeper forb species *Ficus pumila* and *Portulaca 'Hana Misteria'* were chosen for this study. The second type of plant was sedum. *Sedum lineare* and *Sedum mexicanum* are the two most widely used plants for green roofs in Taiwan and were thus chosen for this study. The third type of plant was non-sedum succulents. *Sansevieria trifasciata* and *Aloe* were selected because they have large blade areas and a low irrigation requirement. The fourth type of plant was evergreen shrubs. Shrubs are planted one at a time and cannot fully cover the substrate surface. However, shrubs have longer and thicker roots relative to the other three plant types. We selected the shrub species *Adenium obesum* and *Euphorbia milii Desm*. *Adenium obesum* has a succulent stem, and *Euphorbia milii Desm.* is drought tolerant.

Eight plant species and 2 substrates were studied. Therefore, 16 experimental units and 2 control groups without plants were set up. *Fig. 3* provides photos of the experimental substrates and plants. The experimental units were placed outside to receive natural rainfall. Every rainfall event contributes one sample for each substrate and plant combination unit. While receiving multiple rainfalls, multiple samples are for each combination as replications. In this study period, 7 rainfall events were collected,

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meaning that 7 samples are for each combination unit. Because of the fixed collection volume, it is not able to analyze three replications for every water quality items for individual rainfall events. Therefore, we took multiple rainfall events as multiple samples of each experiment unit to run statistic.

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Figure 3. *Photos of the experimental units. From left to right and top to bottom: cultivated soil, light GR soil, Ficus pumila, Portulaca 'Hana Misteria', Sedum lineare, Sedum mexicanum, Sansevieria trifasciata, Aloe, Adenium obesum, and Euphorbia milii Desm. (A total of 18 units were tested, including 9 units with each test substrate: 1 without plants and 8 with each of the different plant species).*

Water quality analysis

The water quality items in this study characterize sediment erosion and nutrient concentrations. NO_3-N, NH_4-N, TN, TP, COD, and TS were analyzed. Heavy metal concentrations were not assessed in this study. Heavy metals generally result from fertilizer and substrate materials. In this study, we did not add fertilizers and do not discuss the inherent heavy metal concentrations in the substrates. Therefore, only the nutrient concentrations and organic substances were examined. All analysis procedures and methods complied with the national standard methods.

Results

Collected rainfall events

The experiment began in January 2013. The first three months were used for plant growth, and the first rainfall data were collected on March 28, 2013. The experimental period was expected to last for at least one year. Unfortunately, a violent typhoon,

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Typhoon Soulik, occurred on July 12, 2013, and destroyed the outdoor study sites. All experimental containers were blown away and broken, which forced the termination of the experiment. Before this unexpected event, 7 realistic rainfall events were examined between March and July 2013. *Fig. 4* shows the rainfall hyetograph during the experimental period. The rainfall volume, duration, and intensity of the sampling events were summarized in *Table 2.* We collected the waters from the bottom removal drawer of the experimental containers, which represented the mixed effluent of the sampling rainfall events. The total sample number should be 126 (7*18=126). However, some of the small rainfall events did not produce enough water for analysis, which resulted in a final total sample number of 118. Due to the only 7 sampling rainfall events, the effects of rainfall volume or intensity on water quality are not discussed.

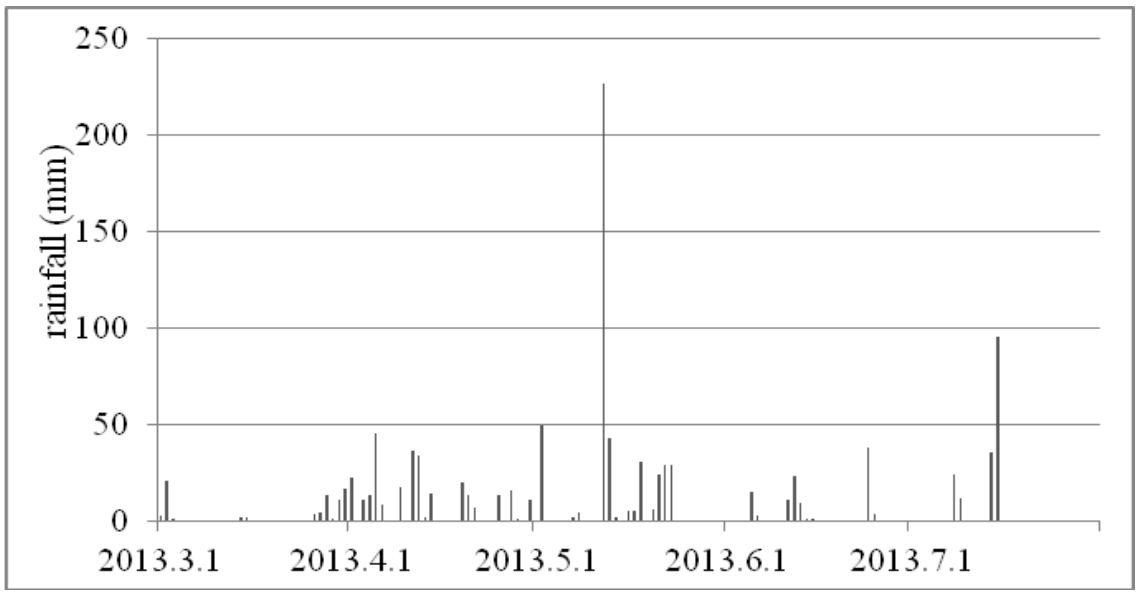

Figure 4. *Rainfall hyetograph during the experimental period.*

Table 2. *Water sampling events*

data	Rainfall volume (mm)	Rainfall duration (hr)	Rainfall intensity(mm/hr)
2013/3/28	23.0	7.0	3.29
2013/4/1	51.5	7.0	7.36
2013/4/14	87.5	13.0	6.73
2013/5/2	61.0	16.0	3.81
2013/5/13	269.5	48.0	5.61
2013/6/12	35.0	10.0	3.50
2013/7/9	36.5	8.0	4.56

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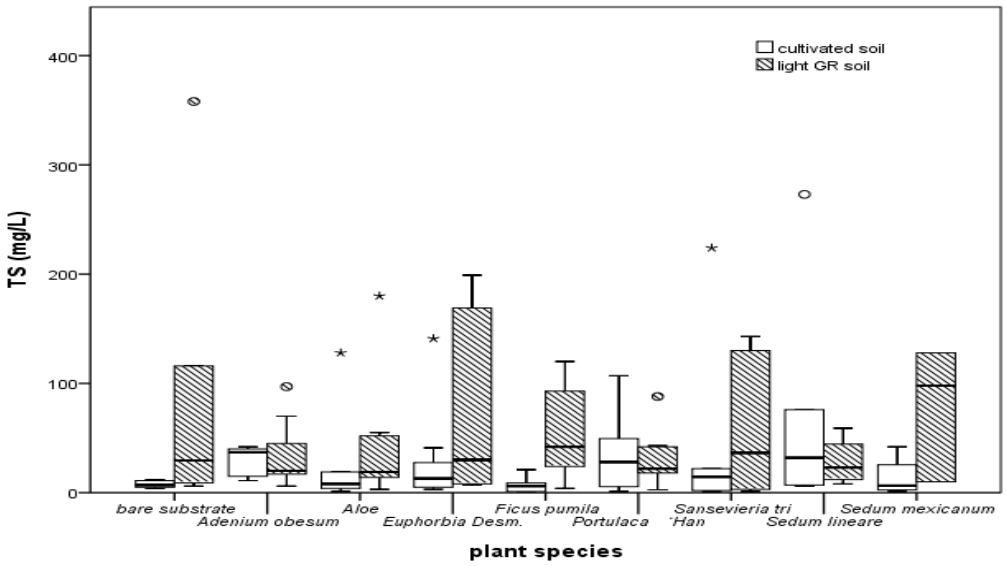
Water quality results

The water quality results that derived from the 7 rainfall events are depicted in a box-plot in *Fig. 5.* The average TS concentrations in the cultivated and light GR soils were 7.80 and 260.71 mg/L, respectively. Out of more than 100 water samples, four samples had TS concentrations of greater than 500 mg/L. Of these four samples, two were collected during the first sampling event. During this event, the substrate was not completely compacted and the plant roots had not effectively stabilized the soil. The other two turbid water events occurred on June 11. Before June 11, outflow had not occurred for one month and the gravitative substrate had accumulated at the receiving end of the container, which resulted in the high TS concentrations in the water.

The COD concentrations from the light GR soil were approximately twice as high as the COD concentrations from the cultivated soil in all plants units. The variability of the COD concentrations in the light GR soil was high, whereas the variability of the COD concentrations in the cultivated soil units was low. The COD concentrations from the sedum plants were slightly higher than those from the other plants. The average COD concentrations for *Sedum lineare* and *Sedum mexicanum* were 55.20 and 75.60 mg/L in the cultivated soil and 165 mg/L and 144 mg/L in the light GR soil, respectively.

The TN concentrations in the substrate and plant species units were not obviously different. The TN concentration variability in the light GR soil units was high. However, the average TN concentrations among the different plant species were similar for both substrates. The average TN concentrations from the bare cultivated and light GR soils were 1.22 and 10.37 mg/L, respectively. This finding potentially resulted from the 2-fold greater original TN concentration in the light GR soil relative to the cultivated soil.

The TP concentrations were 3 times higher in the light GR soil series relative to the cultivated soil series. The average TP concentration in the bare cultivated soil unit was 0.25 mg/L, whereas the average TP concentration in the bare light GR soil was 2.88 mg/L. The cultivated soil units with plants resulted in average TP concentrations that were less than 1 mg/L, except for *Sedum mexicanum*. The average TP concentrations in the light GR soil units with plants were greater than 3 mg/L, except for *Ficus pumila*, which had an average TP concentration of 1.96 mg/L. In both substrate units, *Ficus pumila* resulted in the lowest TP concentrations.



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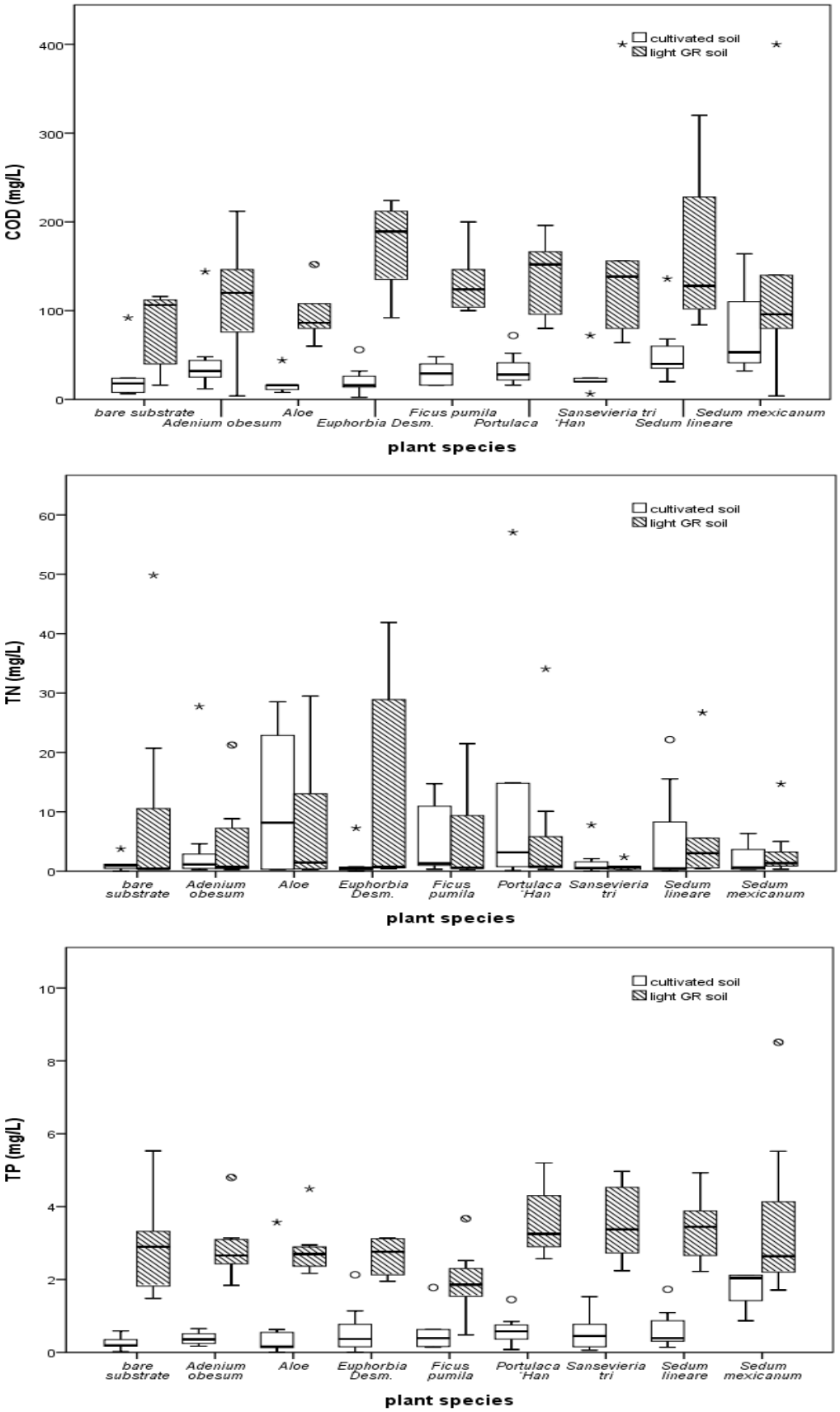

Figure 5. *Water quality results from the different substrates and experimental plant units.*

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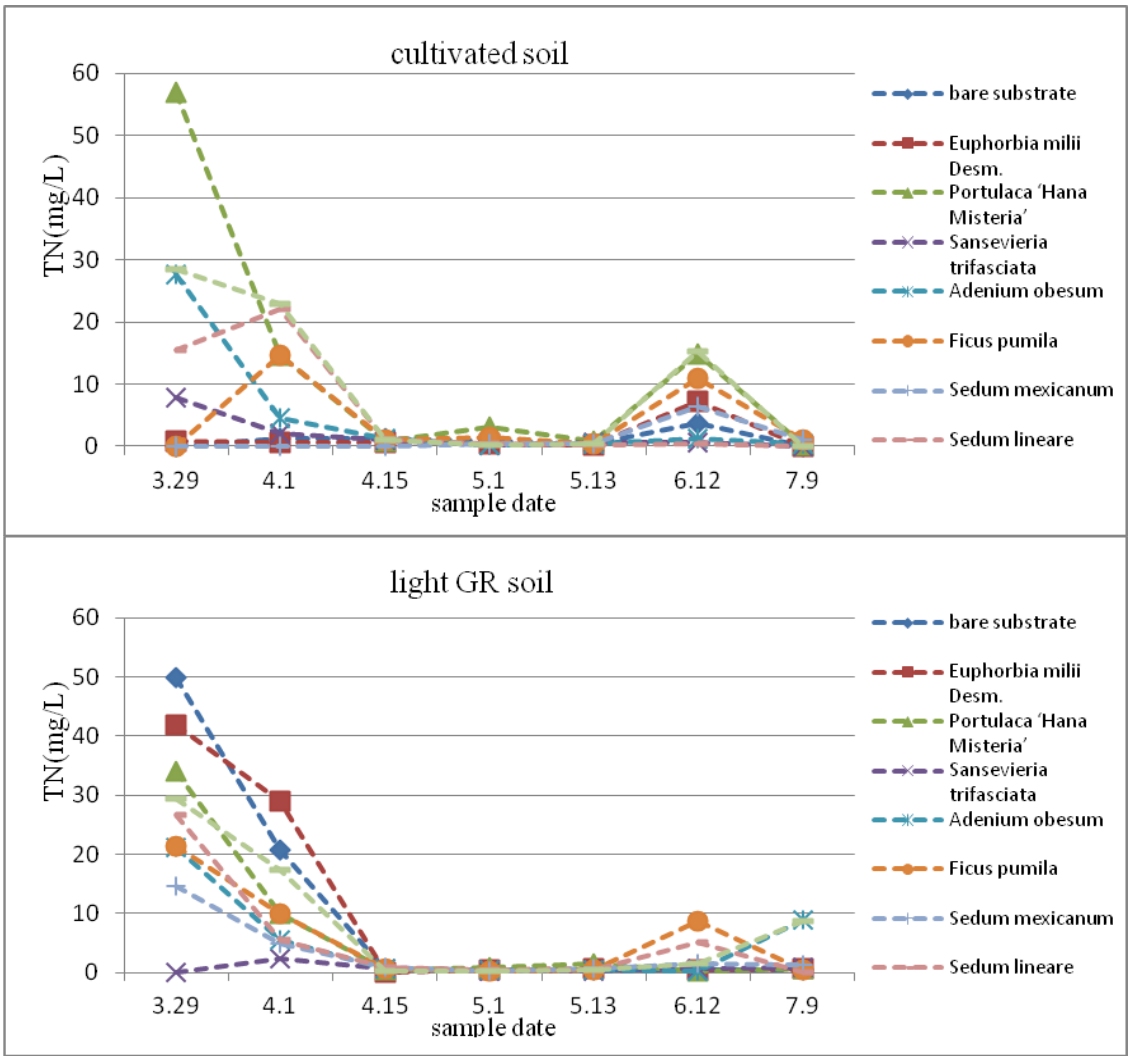
Temporal variability of water quality

Analyzing the temporal variability allowed us determine the effects of green roof age on water quality. The results showed that TS, COD, and TN concentrations decreased with time. However, TP concentrations did not substantially change. *Fig. 6* shows the temporal trends of the TN and TP concentrations. In both substrate series, the TN concentrations were higher before May 2013, which corresponded to five months after the green roof units were positioned. In contrast, the TP concentrations did not dramatically change. As the results above, the TN concentrations can be affected by both the substrate and plant types. However, the TP concentrations were only affected by the substrates. As plants grow, high TN concentrations may be reduced. The percentage of nitrate in the TN pool was 96% (on average) during the earliest stages and was reduced to less than 50% during the latest stages. This result implies that the nitrogen was effectively used by the plants. The TP concentrations in the runoff did not depend on plant growth and did not change with green roof age.



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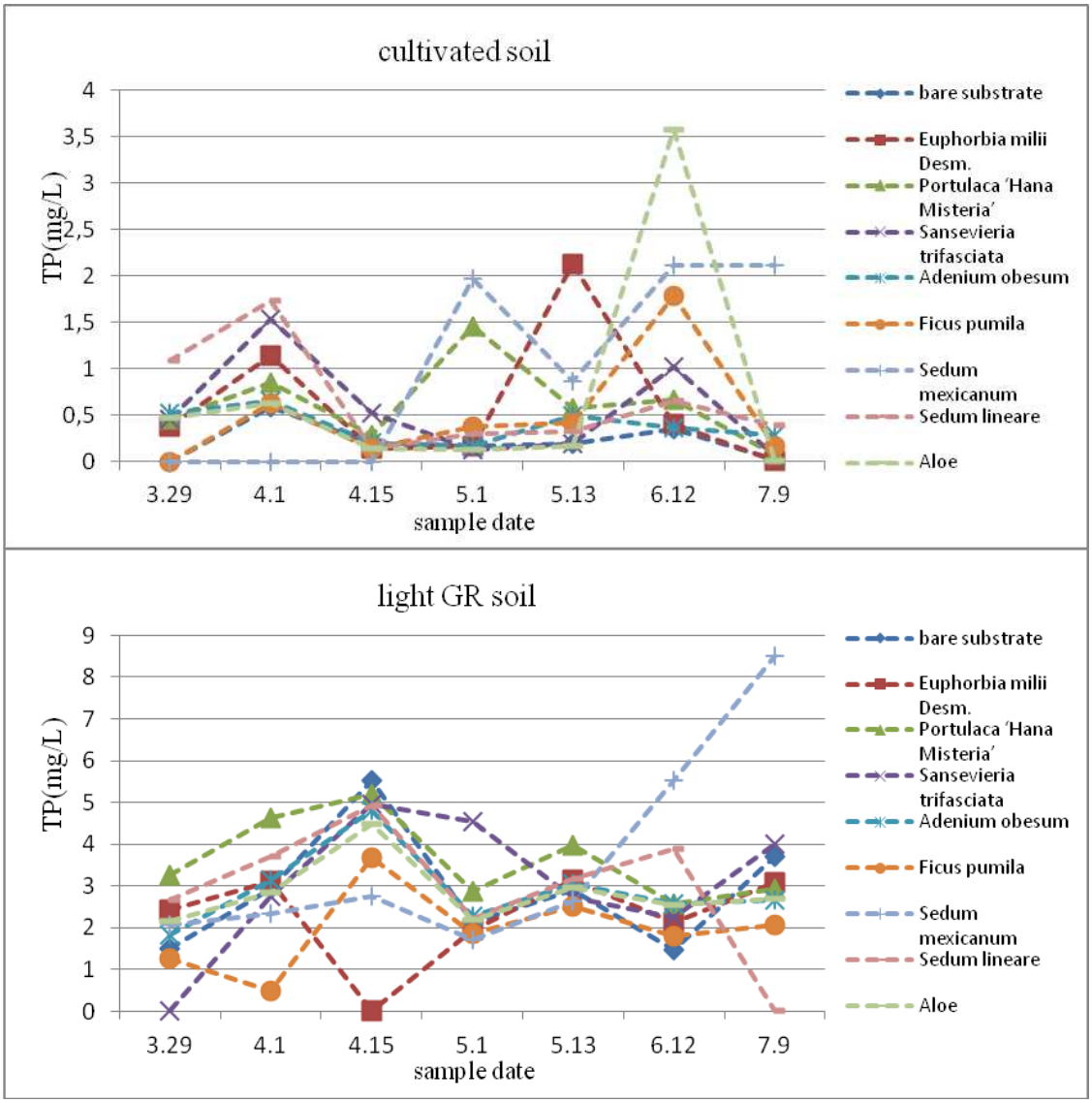

Figure 6. *Temporal trends of average TN and TP concentrations in the experimental series.*

Discussion

We used 2 substrates and 8 plants as independent variables, and 4 water quality items (TS, COD, TN, and TP) as dependent variables, to test the significant level of the interactions of substrates and plants. However, the results of the ANOVA analysis did not reach the expected significant level. There are only 7 samples for each combination and the few samples are not sufficient to reveal the combination effects. Therefore, we tested the single effects of substrates and of plants. When testing the effects of substrates, difference of plants was ignored and each substrate had a maximum total of 56 samples (8 plants and 7 rainfall events). Taking 2 substrates as independent variables and 4 water quality items as dependent variables, the results showed that the influence of substrate on the COD and TP concentrations was significant ($F(1, 99) = 63.063$, $p < 0.001$ for COD, and $F(1,116) = 155.460$, $p < 0.001$ for TP). Using the same method to test the effects of plants, each plant had a maximum total of 14 samples (2 substrates

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and 7 rainfall events). However, no significant differences in TS, COD, TP, and TN concentrations among plant species were observed.

The effect of substrate on TP concentrations was significant but no consistent effects were observed among the different plant species. Plants use phosphorus in the form of phosphate. Although we did not measure phosphate concentrations, when higher TP concentrations corresponded with higher COD and TS concentrations, we hypothesized that organic phosphate was the dominant form of phosphorous, which is mainly transported with solids (Logan, 2000). Therefore, the TP concentrations highly depend on substrate properties, such as the original phosphorous concentration and erodibility. The effects of plant species on TP concentrations were relatively small. Thus, proper filter maintenance and reducing substrate loss have a greater effect than plan growth on TP concentrations.

Unlike TP results, neither substrate nor plant species did not result in significant influence on TN concentrations. The TN concentrations include various compounds of organic nitrogen, ammonia-nitrogen, and nitrate. The distribution of these compounds should allow us to determine whether the TN results from soil nutrients or nitrification. The insufficient number of collection events and the highly variable water quality precluded the use of our data to determine the sources of TN. But, the results showed that the units with plants produce higher TN concentrations in the cultivated soil series. This implies that nitrate would be generated by nitrification and leached out. The light GR soil series demonstrated opposite results because organic nitrogen was transported with fine soil solids, which increased the TN concentration. In both substrates, *Sansevieria trifasciata, Sedum lineare*, and *Sedum mexicanum* resulted in the lowest TN concentrations. *Sansevieria trifasciata* was the only C4 plant that was used in this study, and the two sedum plants are CAM plants. These three plants exhibited different carbon transformation reactions relative to the other studied plants. However, it is unclear whether these reactions affected plant nitrogen use or resulted in different TN concentrations in the water.

In the results of TS, four samples had TS concentrations of greater than 500 mg/L. The four high TS samples came from the light GR soil units, which indicates that the larger pore space for water retention and root growth allowed the fine particles to pass through the filter layer and be carried out with the runoff. Thus, to avoid contaminating the runoff with the substrate itself, the substrate particle size distribution should be seriously considered.

Conclusions

The design of substrates and plants is an important factor to advance the green roof performance and the effect of such combinations is expected to influence the effluent water quality. In this preliminary study, the limited data did not reveal the expected effects, but the results presented that the substrates significantly affect water quality. Based on the nature of substrates that were used in this study, the highly porous soil was designed for better root growth, air movement, and water retention. However, high porosity may increase soil erosion and contaminate runoff during the early stages of green roof establishment. Although the experiment period is not long enough, it is obvious that poor water quality is unavoidable for newly built green roofs. Water quality, such as TS, COD, and TN improves with time. It might be caused by the substrates are more compact and roots develop after 5 or more months, and organic

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matters in substrates are utilized and are decreased. In order to mitigate the water quality concentration, a well-maintained filter layer is important to prevent the loss of substrate and the associated pollutants. The effects of plant species on these substances are not clear in this study; although, the factors impacting TN concentrations are related to substrate and plant types. Details regarding the effects of plants on water quality did not be analyzed, but based on the current results, C4 and CAM plants (which have special carbon transformation processes) resulted in the lowest TN concentrations. Future studies could continue to study the effects of plants on nitrogen runoff and water quality.

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ELECTRONIC APPENDIX

This article has an electronic appendix with basic data.

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EVALUATION OF FIRE ACTIVITY IN SOME REGIONS OF AEGEAN COASTS OF TURKEY VIA CANADIAN FOREST FIRE WEATHER INDEX SYSTEM (CFFWIS)

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Abstract. Çanakkale, Izmir and Muğla regions, which have much higher number of fires and larger burnt area values than Turkey's mean values, are the regions at 1st rank in terms of forest fire risk. The negative changes in climate conditions in recent years have significantly increased the forest fire risk. One of the most common methods used worldwide in order to rank the weather-related forest fire risk is the Canadian Forest Fire Weather Index System (CFFWIS). In this research, it has been determined for all of 3 regions that the SSR index calculated by using DSR index was statistically significantly successful in explaining the burnt area in forest fires. The success levels of SSR were 37.8% in Çanakkale Region, 49.8% in Izmir region, and 59.6% in Muğla region.
Keywords: *forest fire, fire index, CFFWIS, burnt area*

Introduction

Especially the Aegean and Mediterranean coastal regions of Turkey are at high fire risk due to the plant cover and climate conditions (Altan, 2011; Altan et al., 2011). Meteorological factors such as long summer drought and high temperatures are some of the factors increasing the risk. Maquis and *Pinus brutia,* among the plant cover types that are prone to be burnt, are dominant in these regions. Three of the highest fire activity regions of Turkey are located in Aegean coasts of Turkey; these are the regions investigated in this research. Of 21,137 forest fires that have occurred in Turkey between the years 2001 and 2011, the portion of these three regions is 27.92% in terms of the area (22.30% in terms of the number of fire). In other words, while the annual mean number of fire per regional directorate in Turkey is 73.90, the annual mean area burnt is 308.27 ha in 2001-2011. The same values of these three regions were 482 forest fires per year and 2115.11 ha of mean burnt area, respectively.

Various fire-weather indices have been developed throughout the world in order to rank the fire risk. These systems used under the name of National Fire Danger Rating System (NFDRS) in USA, McArthur Forest Fire Danger Index (FFDI) in Australia and Canadian Forest Fire Weather Index System (CFFWIS) in Canada constitute the Foundation of the Fire Weather Risk Indices that is used commonly in world. During the comparisons of these indices, it has been determined that there are similarities and differences between them. In study of Dowdy et al. (2010), where they compared Canadian and Australian forest fire risk indices, it has been determined that both of the indices are sensitive to wind at most, and then the relative humidity, and temperature,

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respectively. When these indices are compared, it has been determined that, in proportion to FWI (Fire weather index), FFDI is more sensitive to temperature and relative humidity and less sensitive to wind speed and precipitation (Dowdy et al., 2010).

Nowadays, the CFFWIS is the most commonly used index in the world (Natural Resources Canada, 2015). Some of those countries are New Zealand, Fiji, Northeastern China, Alaska, Minnesota, Michigan and Florida from USA and Mexico, Indonesia, Malaysia, and Argentina. Furthermore, since the system is suitable for entire continent, it is used by the research center affiliated to European Commission in order to determine the fire risk in Europe (Taylor and Alexander, 2003; Taylor, 2001; San-Miguel-Ayanz et al., 2012; Willis, 2001).

All these indices have been perfected via the data that have been collected from the numerous natural forest fires and the test fires for many years in the country, where they have been developed. In many countries, in order to rank the forest fire risk, the above-mentioned models are used either by modifying or as is. Various weather elements are utilized in ranking the forest fire risk through both of existing systems and new indices that are derived from them.

CFFWIS, which is the result of the studies carried out for many years in Canada, has been introduced firstly by Van Wagner in year 1968. Then, Van Wagner has published the new improvements for Forest Fire Weather Index in 1970, his studies about the structure of index in 1974, and recent developments in CFFWIS and index's structure in 1987 (Van Wagner, 1970; Van Wagner, 1974; Van Wagner, 1987). In the course of time, CFFWIS has been continuously improved for perfection. This system has been used in Canada officially since 1971 (Turner and Lawson, 1978). CFFWIS has been utilized in many researches to date. In year 2004, FWI and head fire intensities have been calculated in Canada for burnt areas (larger than 2 km^2) in boreal forest lands and taiga ecozones. As a result of calculations made, it has been determined that the effects of climate change are earlier and severer in regions in northern longitudes (Amiro et al., 2004).

In order to rank the forest fire risk in European countries, CFFWIS index is frequently used. Thus, it has been examined by Dimitrakopoulos et al. (2011) in Crete Island under 2 different fire season conditions; one normal precipitation and one extremely drought season. According to the research, DMC (Duff Moisture Code), DC (Drought Code), BUI (Build Up Index) and FWI were highly correlated with the occurrence of the fire. On the other hand, the correlations of these indices with burnt area were at medium level. Moreover, high level of correlation has been found between FFMC (Fine Fuel Moisture Code) and determined L (measured litter layer) and moisture values. Moreover, DC has been found to be lowly correlated with the ground surface moisture (Dimitrakopoulos et al., 2011).

The Fire Protection Department of Croatia, another country having coast to Mediterranean Sea, has been using CFFWIS in coastal regions since 1981. For this purpose, FWI is calculated daily from June to September. For the calculations, the data obtaining from 20 synoptic meteorology stations are used (Vucetic and Vucetic, 2008).

In Europe, it has been adopted that all of the EFFIS-member (The European Forest Fire Information System) countries will use CFFWIS in determining the fire danger rating. In previous studies, it has been found that FWI is useful in fire risk rating in countries having Mediterranean climate such as Greece and Italy (Viegas et al., 1999). In another study carried out in Toscany-Italy and Thessaloniki, Athens and Heraklion (Greece), this method has been utilized in determining the aggravated and protracted fire seasons. In that study, in addition to previous calculations, a non-linear functional

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relationship between the number of fires per day and FWI has been found to be roughly consistent between multiple stations (Good et al., 2008)

In a study of Sturm et al. (2012) carried out in Slovenia, the fire activity between 1995 and 2009 has been examined via the same method in SW Slovenia Sub-Mediterranean Karst forestland. In that study, the usability of CFFWIS under Middle European conditions has been tested (Sturm et al., 2012).

By the forest department in New Zealand, another system named NZFDRS (The New Zealand Fire Danger Rating System) based on CFFDRS (Canadian Forest Fire Danger Rating System) has been developed in 1980 (Fogarty et al., 1998; Anderson, 2005). The main foundation of the NZFDRS is the FWI system, and this index provides numeric proportions about the relative combustion potential and fire behavior by utilizing weather data (Van Wagner, 1987; Anderson, 2005; Pearce and Clifford, 2008).

The above-mentioned method has been utilized by Tian et al. (2011) in Daxing'anling region in northern China in order to examine the fire seasons between 1987 and 2006. Accordingly, according to FWI classification, 81.1% of the total fires have occurred at high, very high, and extremely high fire risk levels. Hence, at the end of the research, it has been determined that the FWI elements are useful indicators for forest fire risk in Daxing region of China (Tian et al., 2011).

The Canadian Forest fire weather index system (CFFWIS-FWI), with The Canadian Forest Fire Behavior Prediction (FBP) System, constitutes the Canadian Forest Fire Danger Rating System (CFFDRS). CFFWIS, one of the elements constituting the fire risk rating system, is based only on meteorological observations (Natural Resources Canada, 2015). Nowadays, as well as the CFFDRS, CFFWIS is still used by fire management agencies in Canada in predicting forest fires, and enlargement and severity of fire (Wotton, 2009). It is widely used for many years as decision-making mechanism in fire occurrence, fire behavior estimations, and other fire management activities (Martell and Sun, 2008).

Materials and Methods

Study Area

In this study, Çanakkale, Izmir and Muğla regions of Turkey (*Figure 1*) have been determined as study areas. Çanakkale region is located in $39^0$33'39"-$42^0$04'08" northern latitudes and $26^0$05'32"–$27^0$31'39" eastern longitudes, Izmir region in $39^0$25'28"-$37^0$52'30" northern latitudes and $26^0$11'42"–$28^0$52'28" eastern longitudes, and Muğla region in $36^0$16'26''-$38^0$06'32'' northern latitudes and $27^0$13'49''-$29^0$46'40'' eastern longitudes (*Figure 1*). Together with the number of fires between 2001 and 2011, also the daily meteorological data of these 3 regions have been used in this study (*Table 1*).

Table 1. *Distribution by month of forest fire in study area.*

	May		June		July		August		September	
	No	**Burnt Area**	**No**	**Burnt Area**	**No**	**Burnt Area**	**No**	**Burnt Area**	**No**	**Burnt Area**
Çanakkale	14	17.36	42	358.81	114	1860.42	88	587.63	69	57.16
İzmir	157	201.95	371	1475.07	479	3454.44	404	3667.92	245	312.27
Muğla	184	596.91	405	304.01	566	2224.69	528	6730.90	424	663.66

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During the study period (2001-2010), of the burnt areas stated above, 1621 ha in Çanakkale and 4111 ha in İzmir and 6343 ha in Muğla have been damaged in large forest fires .

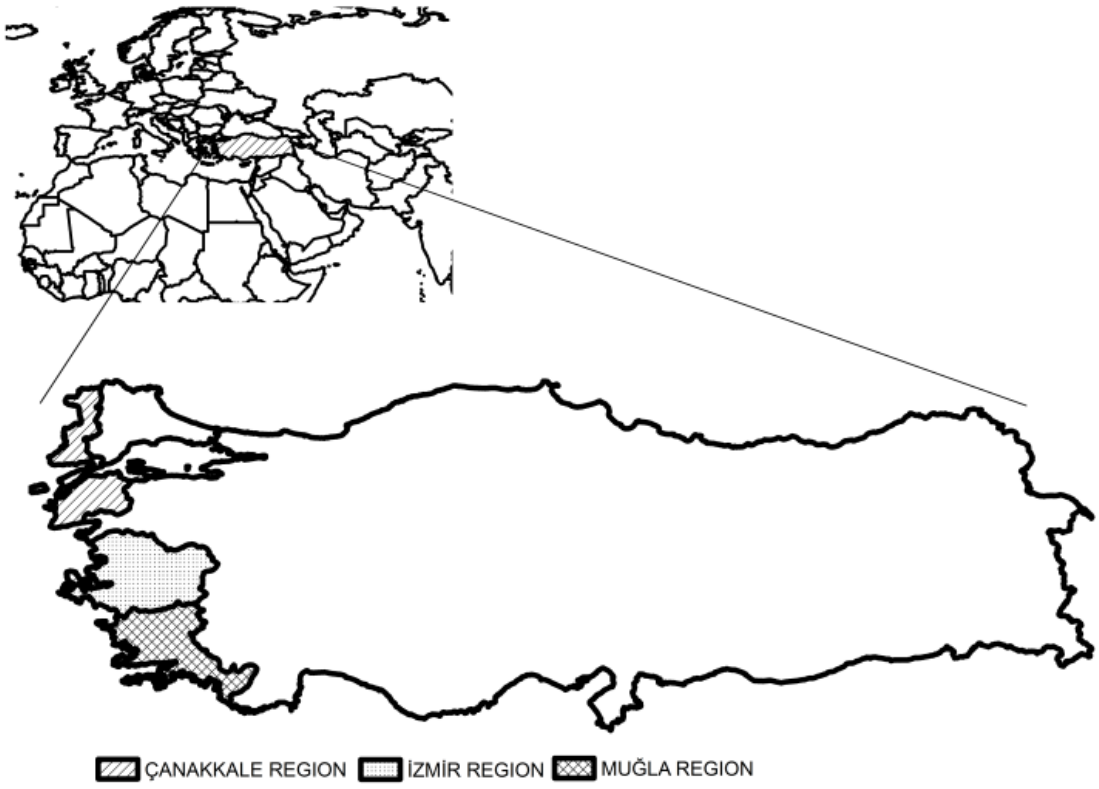

Figure 1. *Locations of Çanakkale, Izmir and Muğla regions constituting the study area*

Method

CFFWIS consists of the components such as FWI, FFMC, ISI (Initial Spread Index), BUI, DMC, DC and DSR (Daily Severity Rating) (*Figure 2*). FFMC, DMC and DC used in calculating the moisture of flammable material. They numerically express the moisture status of litter layer, other fine fuels, decomposed organic matters in medium depth, and deep compact organic matter. High level of the elements indicates the more flammable dry matters (Anderson et al., 2007). The system uses daily meteorological data in order to determine the moisture at 3 different forest layers (Wotton and Beverly, 2005). Among these variables constituting the model, FFMC is related with the moisture of litter and other fine fuels in forest surface. DMC, another variable, represents the moisture content of the decomposed organic matter (5 kg/m^2 dry weight). DC, the final variable about the moisture, represents the deep compact organic material up to 25 kg/m^2 dry weight (Lawson and Armitage, 2008).

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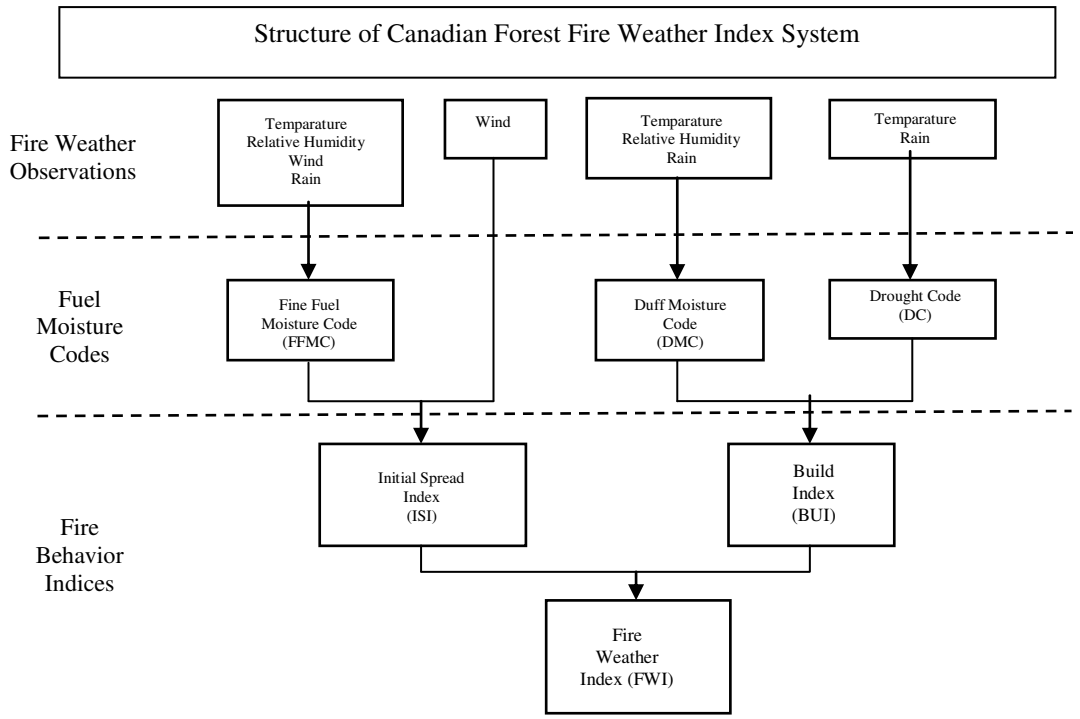

Figure 2. *Structure of the Canadian Forest fire weather index system (De Groot, 1987)*

Different from these 3 variables, the other elements constituting the system are the ISI, BUI and FWI fire behavior indices, and they represent the fire's spreading speed, flammable material ready-to-combust, and pre-severity of the fire. As is in other elements, the higher values of these elements indicate the increasing fire risk. DSR and its mean values are the extensions of FWI system. The DSR is the transformed form of daily FWI value. Seasonal sum of DSR value is the SSR (Seasonal Severity Rating) (Anderson et al., 2007). ISI is also the combination of the spreading speed represented by FFMC and the wind without considering the flammable material effect that is also a variable. BUI is the combination of DMC and DC, the flammable materials representing the spreading of the fire. They are correlated with fuel consumption. FWI is a combination of ISI and BUI representing intensity of the spreading fire that is expressed as energy rate per unit length of fire front, and lonely represents the fire weather in general (Lawson and Armitage, 2008).

DSR that can be considered as controllability difficulty of a fire is the power function of FWI (Amiro et al., 2004). In cases where the mean of many stations or a certain time period, the use of FWI is not appropriate at all. In such cases, the use of DSR will be more appropriate. The mean value of the DSR during entire fire season is named SSR. This value is used in evaluating the fire weather conditions for regions or seasons (Lawson and Armitage, 2008).

Using the meteorological data of the regions selected as study area, the average values of 5 months (May, June, July, August, and September) selected as fire season have been calculated for 10 years. By using Mann-Whitney U Test, it has been determined if there is any difference between CFFWIS elements determined for 3 regions. Then, Spearman's Rank Order Correlation has been utilized in order to

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calculate the degree of relationship between the number of fires and the burnt area. In order to mathematically explain the relationship between the elements of CFFWIS, of which relationship with number of fires and burnt area has been determined as a result of Spearman's Rank Order Correlation Test, the basic linear regression analysis has been performed. The statistical analyses used in this study have been performed through R 3.0.2 software (R Software, 2014).

Results

The CFFWIS indices have been calculated for these 3 regions in Aegean region of Turkey. The relationships of the calculated indices with the number of fires in study area and the burnt area have been investigated. The index values calculated for Çanakkale, Izmir and Muğla regions and the relationships of these values with the number of fires and the burnt area are presented in *Figure 3*. Since the data of burnt area doesn't exhibit normal distribution, the natural algorithm of these data has been used in order to better-express the values.

As a result of MANN-Whitney U test performed between 6 index values (FFMC, FWI, DC, BUI, ISI, SSR), it has been determined that there was no statistical difference in Çanakkale region between BUI and FFMC (p=0.140), in Izmir region between SSR and ISI values (p=0.974), and in Muğla region between SSR and ISI (p=0.947) and BUI and FFMC (p=0.948). p value of 0.0000 in all of the variations other than these indicate that the indices are different from each other. Since the index values calculated in 3 regions showed significant variation, Spearman analysis has been performed for all the index values. The results of Spearman analysis are presented in *Table 2*.

Table 2. *Spearman RHO coefficients by indices for Çanakkale, Izmir, and Muğla regions*

REGION		Spearman rho coef. for FFMC	Spearman rho coef. for FWI	Spearman rho coef. for DC	Spearman rho coef. for BUI	Spearman rho coef. for ISI	Spearman rho coef. for SSR
ÇANAKKALE	NOF	0.418	0.181	0.463	0.454	0.218	0.209
	Log BA	0.481	0.809*	0.491	0.754*	0.791*	0.736*
İZMİR	NOF	0.572	0.554	-0.009	0.254	0.618	0.254
	Log BA	0.491	0.509	0.263	0.581	0.527	0.709*
MUĞLA	NOF	0.481	0.572	0.054	0.409	0.61	0.572
	Log BA	0.463	0.645	0.363	0.618	0.60	0.745*

NOF: Number of fires, Log BA: Natural log of burnt area, *Statistically significant relationship at confidence level of 95%

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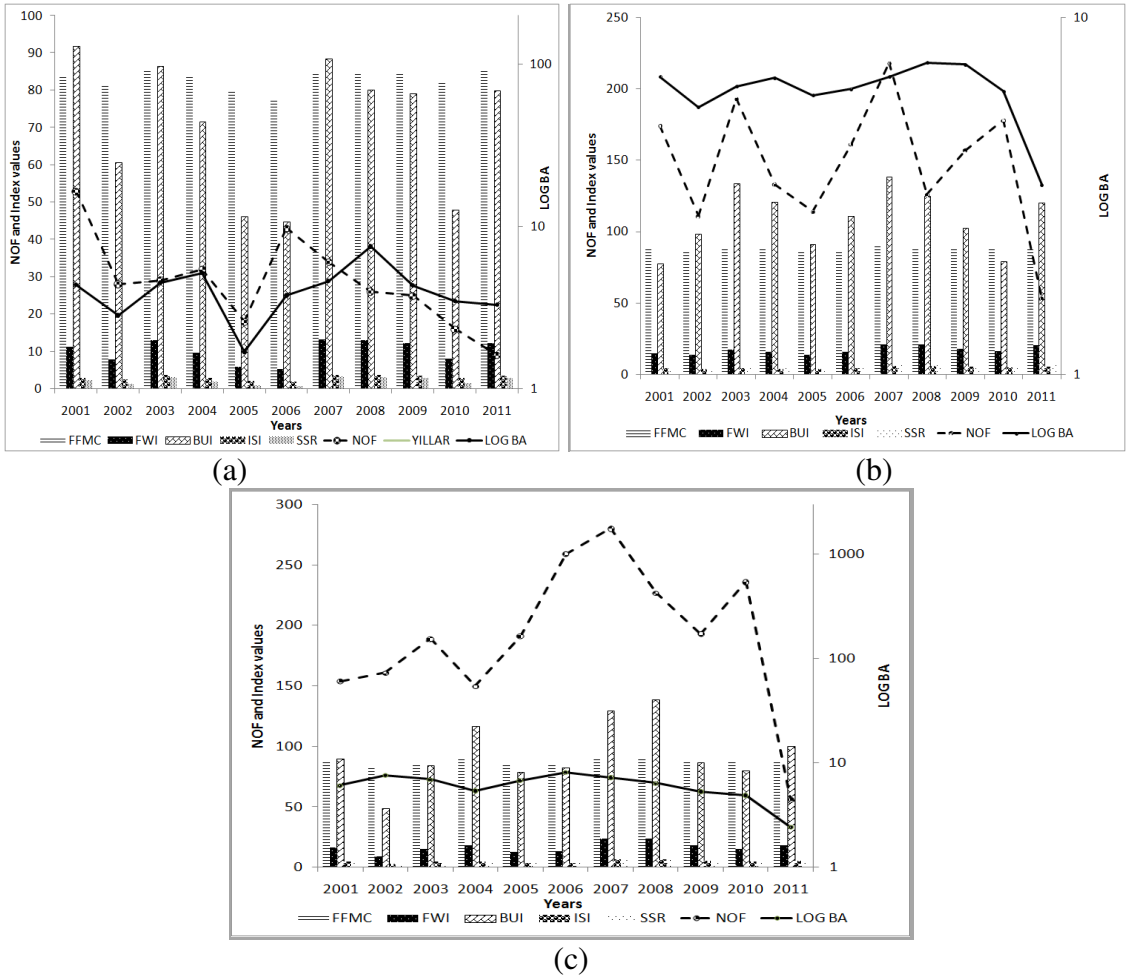

Figure 3. *The relationship of the number of fire and the burnt area in Çanakkale (a), Izmir (b) and Muğla (c) regions with CFFWIS elements (NOF: Number of fires, Log BA: Natural log of burnt area)*

In *Figure 3*, it can be seen that there was no similar trend between the index components and the number of fires in all of three regions. But, it has also been determined that the higher index values indicated higher risk of fire. Moreover, it has been determined that higher number of fires has occurred in those days, when the high index values were observed (*Figure 4*). It has been found for all three regions that there was a relation between different index components and the area burnt (*Figure 3*).

For all these 3 regions, no statistically significant relationship could be detected between the number of fires and the indices. But, a strong relationship has been detected between natural algorithms of the data about the burnt area in Çanakkale region and FWI, ISI, BUI and SSR values. The p values obtained for mentioned indices are 0.0039, 0.00549, 0.00984, and 0.0127, respectively. When the relationship between the burnt area and indices are examined for Izmir and Muğla regions, it has been seen that there was a strong relationship only between natural algorithm of the data of burnt area and SSR, and the p values of the relations determined for Izmir and Muğla were 0.018 and 0.011, respectively. The results of regression analysis performed in order to determine the mathematical relationship

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between the indices and the data of burnt area are presented in *Table 3*. Regression analysis has been performed because the significant relationships have been identified as a result of Spearman analysis.

Table 3. *Regression analysis results for Çanakkale region*

	R square	**F statistics**	**t**	**Sig.**
ISI-LOGBA	0.350	4.870	2.207	0.055
FWI-LOGBA	0.382	5.555	2.357	0.043*
BUI-LOGBA	0.301	3.897	1.967	0.081
SSR-LOGBA	0.378	5.459	2.336	0.044*

Log BA: Natural log of burnt area

Given the values in *Table 3*, it is seen that the relationship of ISI and BUI indices with the area bunt was not statistically significant. FWI and SSR values explain the 38.2% and 37.8% of the burnt area, respectively. According to the parameter estimations obtained as a result of regression analyses, it has been determined that 1 unit increase in SSR index leads to 0.959 fold increase in burnt area and 1 unit increase in FWI index leads to 0.312 fold increase in burnt area. Since the t values of these coefficients were found to be significant, the coefficients of SSR and FWI variables were found to be statistically significant.

While the linear equation for FWI is

$$y=0.968+0.312x, \quad \text{(Eq.1)}$$

The linear equation for SSR is

$$y=2.063+0.959x \quad \text{(Eq.2)}$$

For Izmir and Muğla regions, since only the relationship between SSR values and the data of burnt area has been found to be significant in Spearman analysis, the regression analysis has been performed only between these data groups. As a result of these analyses, the F statistics of the variance analysis has been found to be significant (p=0.023). This value indicates that our model, where we try to explain the data of burnt area via SSR index, is a significant model. While SSR value is capable of explaining 49.8% of the data of burnt area, the 1 unit increase in SSR value leads to 0.031 fold increase in the data of burnt area.

The linear equation obtained for SSR is

$$y=6.269+0.031x \quad \text{(Eq.3)}$$

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As a result of variance analysis in Muğla region, the F statistics has been found to be significant (p=0.042). This value indicates that our model, where we try to explain the data of burnt area via SSR index, is a significant model. While SSR value is capable of explaining 59.6% of the data of burnt area, the 1 unit increase in SSR value leads to 0.762 fold increase in the data of burnt area.

The linear equation obtained for SSR is

$$y=3.640+0.762x \quad \text{(Eq.4)}$$

Discussion and conclusion

62.85% of the forest fires occurring in Turkey are caused from human activities (GDF, 2009; GDF, 2013). Despite that, it cannot be disclaimed that the climate factors have an important effect on forest fires worldwide (Pinol et al., 1998; Crimmins, 2006; Liu et al., 2010; McKenzie et al., 2004; Westerling et al., 2003; Flannigan and Wotton, 2001).

Thus, in recent years, it has been reported in scientific studies that the summer temperature and droughts of especially the Aegean and Mediterranean regions of Turkey have shown increase (Turkes and Altan, 2013; Demir et al., 2008; Erlat and Turkes, 2013).

In this study, the fire activity in 3 regions of Aegean region of Turkey that are risky in terms of forest fires have been interpreted via CFFWIS elements. For instance; as a result of the study of Carvalho et al.(2008), it has been determined that almost 81% of the variation of the burnt area in Portugal has been explained with relative humidity and DC and monthly max. FWI, the elements of CFFWIS. The variation determined about the number of fire is lower, and is 63% (Carvalho et al., 2008). In the study of San-Miguel-Ayanz et al. (2013) large forest fires that have occurred in 3 South African countries have been examined. As a result of that research, it has been determined that all the mega-fires have occurred in high FWI and DMC periods (San-Miguel-Ayanz, 2013). Differently from South Europe, in a study of Arpaci et al. (2013) for determining the forest fire in Austria that is a middle European country, it has been determined that BUI, one of the CFFWIS elements, is one of the most useful indices for ranking the actual forest fire risk in summer season (Arpaci et al., 2013).

As a result of the study, it has been determined for the relationship of annual number of fire and burnt area with calculated indices that different indices were successful for different regions, and that SSR was successful in determining the fire activity in all 3 regions. Besides that, no relationship like the one expected could be determined between the indices and the number of fire; the days, in which the multiple fires occurred in the region, overlap with the high index values. Moreover, strong relationships have been determined between indices and the areas burnt in all 3 regions. Among these regions, it has been determined that the burnt area has strong relation with FWI, ISI, BUI and SSR in Çanakkale region, and with SSR in Izmir and Muğla region. It has also been observed that the above-mentioned indices were significantly successful in indicating the large fires (>300 ha) that occurred in various dates (*Figure 4*).

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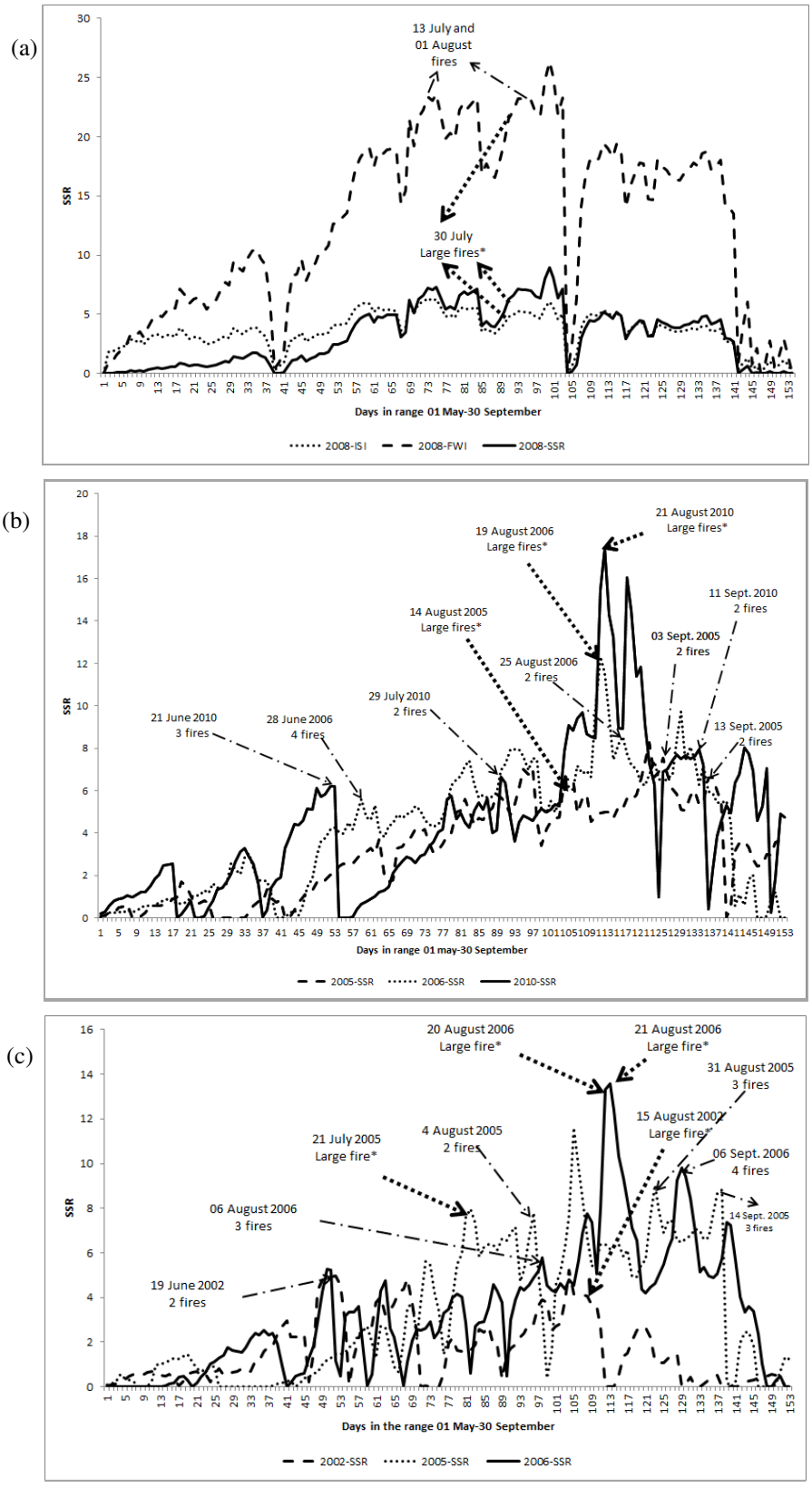

Figure 4*. The fires in 3 regions and SSR values for 5-month period (May-Sept) (a-Çanakkale region, b-Izmir region, c-Muğla region)*

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NUTRIENT REMOVAL CAPACITIES OF FOUR SUBMERGED MACROPHYTES IN THE POYANG LAKE BASIN

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Abstract. Submerged macrophytes play an important role in eutrophic water purification. Using four species of commonly submerged macrophytes, *Ceratophyllum demersum*, *Elodea canadensis*, *Hydrilla verticillata* and *Vallisneria natans* (Lour.) Hara, as research objects, and using the Junshan Lake water quality in the Poyang Lake basin as the standard, purification efficiency levels were analyzed for submerged macrophytes at low temperatures using static simulation experiments. The results showed that these four of submerged macrophyte species could partially purify eutrophic water at low temperatures. The purification efficiencies of the four submerged macrophyte species were ranked in descending order as follows: *Elodea canadensis*, *Hydrilla verticillata*, *Ceratophyllum demersum*, and *Vallisneria natans* (Lour.) Hara. For *Ceratophyllum demersum*, *Elodea canadensis*, *Hydrilla verticillata* and *Vallisneria natans* (Lour.) Hara.The maximum total nitrogen (TN) removal rates were 67.84%, 68.70%, 64.38% and 69.13% of *Ceratophyllum demersum*, *Elodea canadensis*, *Hydrilla verticillata*, and *Vallisneria natans* (Lour.) Hara, respectively; the maximum total phosphorus (TP) removal rates were 36.59%, 58.60%, 47.94% and 43.70%, respectively; the maximum NH_4^+-N removal rates were 23.86%, 24.69%, 18.47% and 22.93%, respectively; the maximum NO_3-N removal rates (percentage) were 46.69%, 41.08%, 34.19% and 36.32%, respectively; the maximum PO_4-P removal rates were 24.26%, 27.92%, 22.75% and 12.71%, respectively; and the maximum COD_{Mn} removal rates of 35.11%, 45.06%, 46.30% and 42.88%, respectively.

Keywords: *submerged macrophytes, eutrophication, purification efficiency, Junshan Lake*

Introduction

Currently, an important problem in aquatic environments is eutrophication that is primarily caused by high concentrations of nitrogen and phosphorus in the water column (Howarth and Marino, 2006; Carpenter, 2008; Hautier et al., 2009). Most experts believe that hydrophytes can be used to treat polluted water (Huang et al., 2005; Hu et al., 2008).

Submerged macrophytes fundamentally shape ecological productivity in lake systems. The plant species purifies a water body by absorbing, transferring and converting nitrogen, phosphate and other persistent organic pollutants into biomass in water columns and sediment. Submerged macrophytes can also inhibit biotic and abiotic suspensions, enhance illumination effects and dissolve oxygen in water by increasing

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the spatial niche. These processes provide the necessary conditions for establishing a complex food web development in aquatic ecosystems (Song and Chen, 1997). By regulating cycling speeds, enhancing biodiversity, and controlling algae growth, submerged macrophytes also improve water quality levels in water columns and ecological sediment environments. Hence, eco-restoration from submerged macrophyte is central to eutrophication control (Zhao et al., 2010; Ren et al., 2011).

Poyang Lake located in the north of Jiangxi Province and lies on the southern bank of Yangtze River in China. It is the biggest freshwater lake in China as well as one of the most important international wetlands. Although it is the lowest eutrophication of five great lakes in China, increased human activity and rapid development of the economy have led to great changes in the environment of Poyang Lake, of which eutrophication is the most serious. Poyang Lake (*Fig.1*), aquatic vegetation in Junshan Lake has declined gradually due to the damming of the dam and the presence of crab cultivation activities. Frequent forage flooding has compromised the eco-balance of the lake, degrading eco-system water quality and spurring eutrophication, posing a threat to water environment security of Poyang Lake. Temperature is an important factor affecting the effects of sewage treatment, because of the withering of plants and the reducing of microbial activity. At low temperature, the effect reduced (Zhang et al., 2006). Low removal rate produced by the long time low-temperature in winter of Poyang Lake becomes the key to sewage purification. Some winter growth of submerged plants can reduce the influence of the low temperature (Huang et al., 2005).

According to the survey results in 1984, the macrophytes including *Vallisneria natans* (Lour.) Hara and *Hydrilla verticillata* were dominant species, and the biomass accounted for 22.24% and 16.6% of the total biomass of the macrophytes in Poyang Lake (Guan et al., 1987). The *Ceratophyllum demersum* was common species in Poyang Lake, and the biomass accounted for 2.85% of the total biomass of the macrophytes in Poyang Lake (Guan et al., 1987). At the same time, *Elodea canadensis* was considered as pioneer submerged macrophyte for eutropihication water purification (Tian et al., 2000), this study examined the purification efficiencies of the four common submerged macrophyte species in autumn. Using the Jun-Shan Lake water quality in the Poyang Lake basin as the standards, this study also analyzed the concentrations of TN, TP, NH_4^+-N, NO_3-N, PO_4-P and COD_{Mn} affected by submerged macrophytes, as well as their purification capacities. Though this research, we developed a theory for the selection of submerged macrophytes for eutrophication treatment and ecological recovery in Poyang Lake.

Review of literature

Submerged macrophyte is one of the primary producers of the lake ecological system (Ren et al., 2011). The ecological restoration by submerged macrophytes is an important link to control eutrophication, and such research is carried out a lot in at home and abroad (Bole and Allan, 1978; Phillips et al., 1978; Ewell, 1987; Zhao et al., 2010; Blindow et al., 2002). *Ceratophyllum demersum* (Wang et al., 2008; Tian et al., 2009; Ren et al., 2011), *Elodea canadensis* (Wang et al., 2008; Zhu et al., 2008; Tian et al., 2009), *Hydrilla verticillata* (Ren et al., 2011; Wang et al., 2008; Wang et al., 2008) and *Vallisneria natans* (Lour.) Hara (Sun et al., 2012) are used as the common submerged macrophytes for eutrophication water purification. For the purification, removal rate and removal capacity are taken as the indicators for measuring the purification ability of

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submerged macrophytes (Ren et al., 2011; Sun et al.,2012), and the pollution indicators are monitored such as total nitrogen (TN), total phosphorus (TP), ammonia nitrogen(NH_4^+-N), nitrate nitrogen(NO^3-N) and COD etc. (Tian et al., 2009; Wang et al., 2008; Sun et al.,2012).

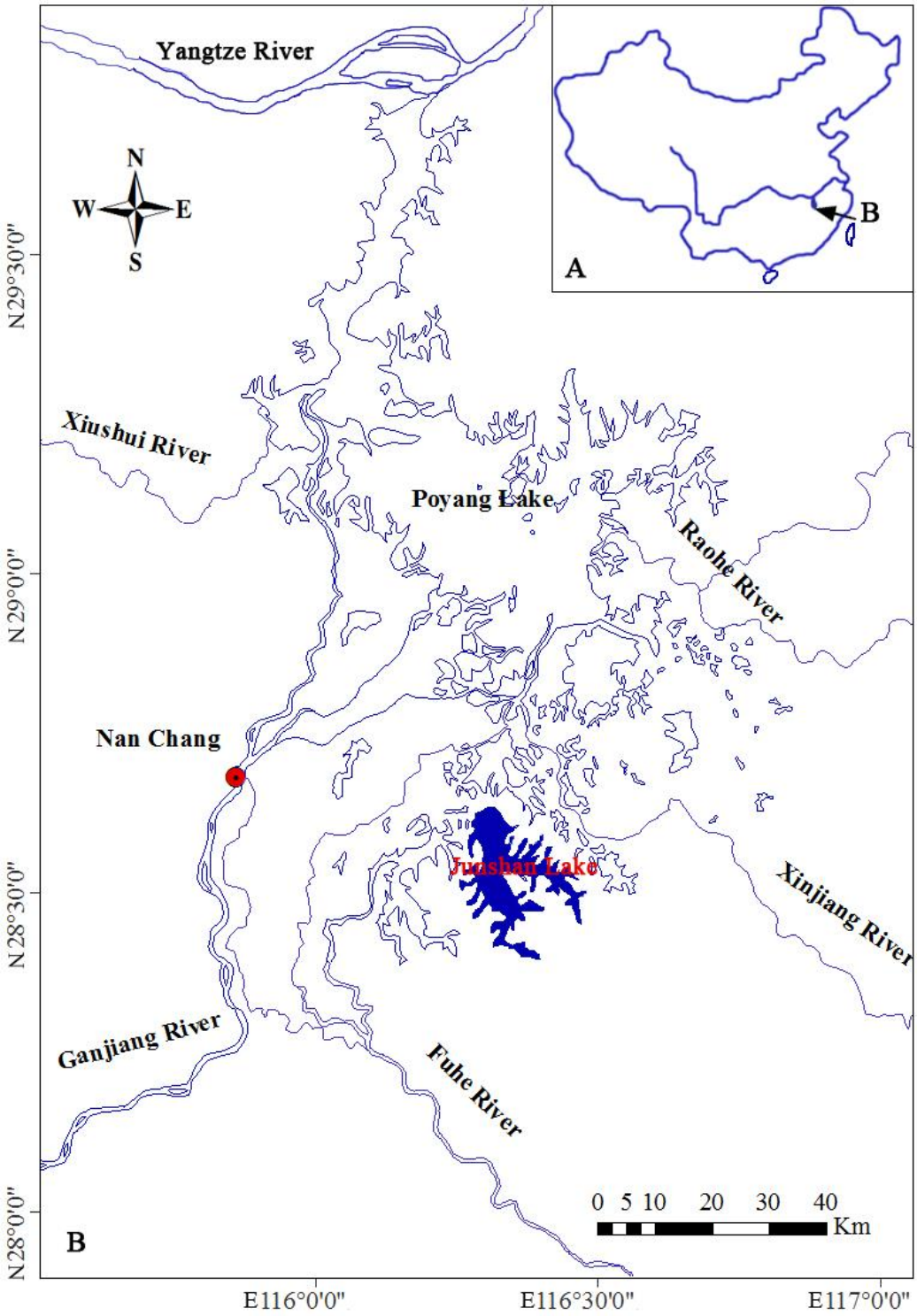

Figure 1. *Location of Junshan Lake*

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Materials and methods

Materials

Samples of the four submerged macrophyte species, *Ceratophyllum demersum Elodea canadensis*, *Hydrilla verticillata* and *Vallisneria natans* (Lour.) Hara, were collected from Chi Lake (a section of Poyang Lake basin) located in Ruichang City, Jiangxi Province, on October 16, 2012. All macrophyte samples were healthy, mature and were undergoing similar growth stages. After removing sludge and other impurities from the samples, we placed the samples into a container of a 100-dilution Hoagland nutrient solution for a 7-day acclimatization period. Then, we selected mature and healthy samples of similar growth stages.

Experimental conditions and method

All experiments were conducted from Ocbober 23, 2012 until November 26, 2012 using four submerged macrophyte species under laboratory conditions with the same water depth, and didn't use any heat preservation measures in order to keep the experimental temperature as same as the outdoor. After equipping experiment water contrasting Junshan Lake water quality and filling 30 L 2-cm-thick plastic containers with 0.5-1.0-cm diameter abluent quartz sand.Then we conducted experiments with and without submerged macrophytes. Three blanks and experiments were repeated with for each type of macrophyte.

To sample 500 ml lake water 10 cm below the water surface via siphoning, we determined initial concentrations using an aquatic quality indicator and drew evaporation samples using fresh water. The samples were collected every 7 days. Then, we determined the aquatic chemical components, e.g., TN, TP, NH_4^+-N, NO_3-N, PO_4-P and COD_{Mn}, and these were analyzed following the method described in Rules of eutrophication invesgation in lake (Jin and Tu, 1990).

Calculation method

Removal rate (%) = (initial pollutant concentration – terminal pollutant concentration) / initial pollutant concentration × 100%;

Removal capacity ($mg.d^{-1}.g^{-1}$) = (initial pollutant concentration – terminal pollutant concentration) × volume / days / average biomass.

Results and analysis

Initial concentration and submerged macrophyte growth analysis

Considering the current water quality levels for Poyang Lake basin, we monitored the water quality of Junshan Lake in August 2012, and the result showed that the TN, TP and COD were 0.75 mg/L, 0.075 mg/L and 5.5 mg/L respectively, which were close to eutrophication level. The water quality in Junshan Lake was taken as reference standard, and the initial concentrations of the submerged macrophytes are shown in *Table 1*.

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Table 1. *Individual initial concentration of four types of submerged macrophytes indicators.*

Water quality indicator		*Ceratophyllum demersum*	*Elodea canadensis*	*Hydrilla verticillata*	*Vallisneria natans* (Lour)	Blank
TN (mg/L)	Concentration	0.757	0.745	0.755	0.749	0.756
	Standard deviation	0.0085	0.0092	0.0128	0.0117	0.0098
TP (mg/L)	Concentration	0.752	0.746	0.753	0.749	0.751
	Standard deviation	0.0005	0.0006	0.0007	0.0004	0.0006
NH_4^+-N (mg/L)	Concentration	0.242	0.236	0.243	0.237	0.24
	Standard deviation	0.0069	0.0056	0.0097	0.0097	0.0085
NO_3-N(mg/L)	Concentration	0.50701	0.50072	0.50831	0.50593	0.50147
	Standard deviation	0.00292	0.00231	0.00456	0.00621	0.00351
PO_4-P (mg/L)	Concentration	0.00269	0.00250	0.00229	0.00242	0.00261
	Standard deviation	0.00059	0.00011	0.00027	0.00076	0.00018
COD (mg/L)	Concentration	5.5	5.541	5.503	5.472	5.455
	Standard deviation	0.0324	0.0529	0.0097	0.0808	0.0352

Table 2 shows limited variations in the total fresh weight levels among the *Ceratophyllum demersum, Elodea canadensis,* and *Hydrilla verticillata* samples; howerer, *Vallisneria natans* (Lour.) Hara had a heavier weight and was composed of 70% water. The other three samples contained 80% water.

This study was conducted from late October 2012 to November 2012. The average temperatures in October and November were recorded to be approximately 20℃ and 13℃, respectively. The temperature change affected the four submerged macrophyte species differently. During the experiments, *Ceratophyllum demersum* grew well, developing adventive roots that reached the bottom of the experimental apparatus. Using these adventive roots, the *Ceratophyllum demersum* sample absorbed and transferred nutrients efficiently. In a later stage of the experiments, parts of the *Elodea canadensis* sample died, and a similar but more pronounced phenomenon occurred in the *Hydrilla verticillata* and *Vallisneria natans* (Lour.) samples. Nearly the entire *Vallisneria natans* (Lour.) sample died by the end of the experiments, causing further pollution of the water body.

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Table 2. *Initial wet weight, dry weight, water content and total fresh weight of four types submerged macrophytes*

Submerged macrophytes	Wet weight/unit (g)	Dry weight/unit (g)	Water content (%)	Unit (individual plant)	Length （cm）	Total fresh weight (g)
Ceratophyllum demersum	0.3069	0.1194	0.7991	5	25	1.5343
Elodea canadensis	0.2021	0.0756	0.8133	5	21	1.0106
Hydrilla verticillata	0.3200	0.0900	0.7188	3	17	0.9600
Vallisneria natans (Lour)	1.8025	0.3500	0.8058	4	15	7.2100

TN-removal efficiency of submerged macrophytes

Because the submerged macrophytes efficiently removed TN from water bodies, we constructed a tendency chart of TN concentration change records from the four species of macrophytes submerged in water (*Fig.2*). Briefly, water bodies were purified to different extents under different experimental conditions. At different stages of the experiments, the plants clearly removed more TN compared with the control experiments. In the first 14 days, the TN concentrations decreased due to the presence of submerged macrophytes. In later stages of the *Ceratophyllum demersum* L and *Hydrilla verticillata* experiments, the TN concentrations in the water body decreased at a slower rate. Meanwhile, for the *Elodea canadensis* and *Vallisneria natans* (Lour.) experiments, the TN concentrations fluctuated along flexible curves. In the experiments without plants, the concentrations initially decreased but then followed a flexible wave pattern due to the absence of macrophytes.

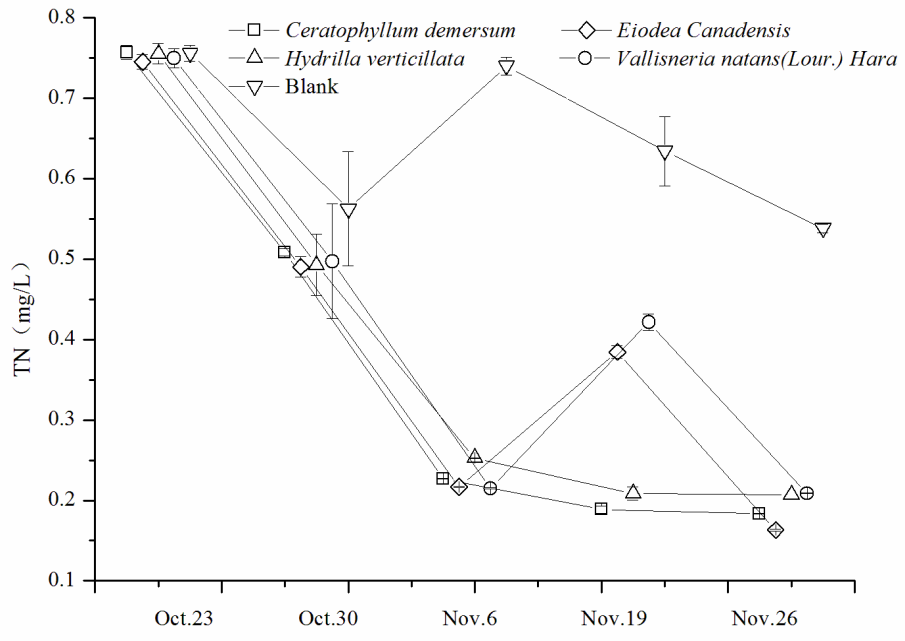

Figure 2. *Variation of TN concentration during the experiment*

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The removal efficiencies of the various experimental conditions are shown in *Fig.3.* The removal rates for the four submerged macrophyte species were calculated as the difference between the data for the experiments with plant and control experiments. The results indicate that the TN removal rates accelerated as a result of submerged macrophyte purification, reaching a peak value (i.e., a minimum TN value) on the 14th day. At this point, the removal rates of the four submerged macrophyte species approached 70%. The TN removal percentages for *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 67.84%, 68.70%, 64.38%, and 69.13%, respectively. Hence, the TN removal efficiencies for the four submerged macrophyte species are ranked in descending order as follows: *Vallisneria natans* (Lour.)*, Elodea canadensis, Ceratophyllum demersum,* and *Hydrilla verticillata*. After a sharp decline, the purification activity in the *Ceratophyllum demersum* and *Hydrilla verticillata* experiments became more gradual, and the curves of the *Elodea canadensis* and *Vallisneria natans* (Lour.) experiments became flexible.

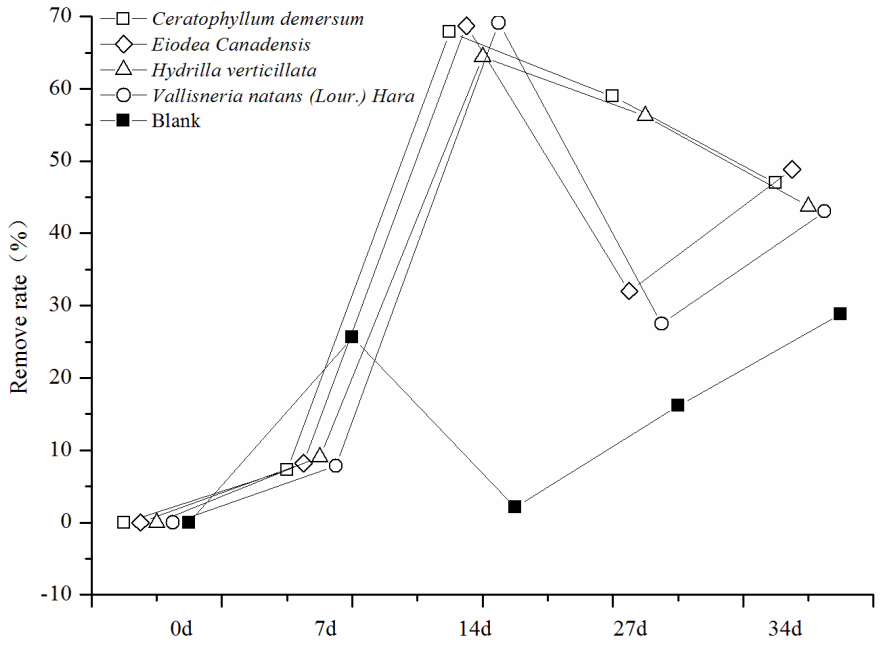

Figure 3. *Removal efficiency of TN during the experiment*

TP-removal efficiency of submerged macrophytes

Submerged macrophytes played a critical role in TP removal through anabolism processes and by adhering to PAO (phosphorus accumulation organism) surfaces (Tian et al., 2009). *Fig.4* shows decreasing curves for the four submerged macrophyte species experiments in the first 27 days. The macrophyte experiments initially showed decreasing tendencies, but the TP concentration increased thereafter because of the dead macrophytes and restrict temperatures. The terminal data for the control experiments were higher for every period.

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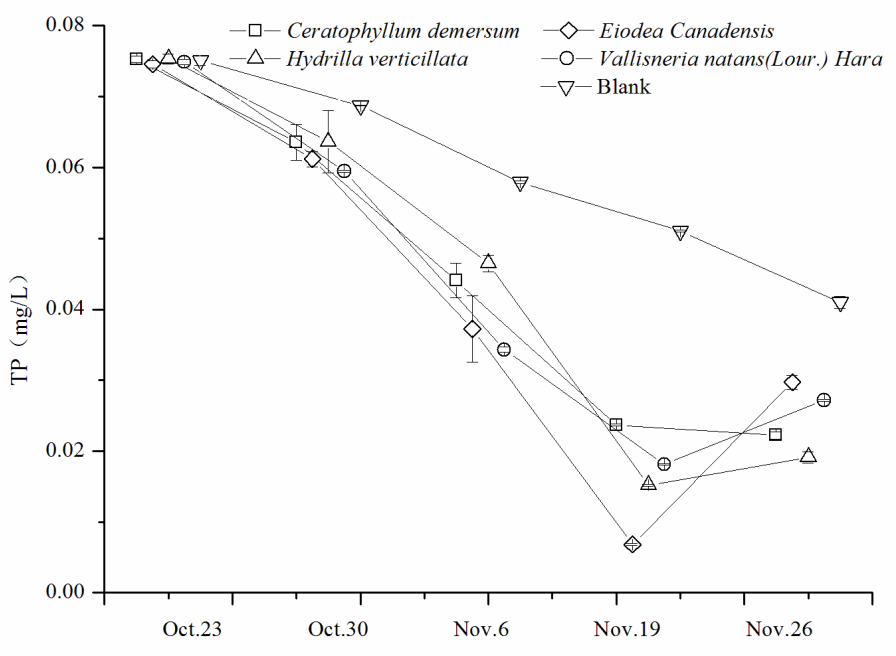

Figure 4. *Variation of TP concentration during the experiment*

Fig.5 shows that the TP removal rates in the control condition increased until reaching 45.38% at the end of the experiment. The submerged macrophyte removal rates were calculated as TN levels, which increased until reaching a maximum value on the 27th day and then declined. The TP removal rates for *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 36.59%, 58.60%, 47.94% and 43.70%, respectively. Thus, the efficiency levels for the four submerged macrophyte species are ranked in descending order as follows: *Elodea canadensis, Hydrilla verticillata*, *Vallisneria natans* (Lour.) and *Ceratophyllum demersum.*

NH_4^+-N-removal efficiency of submerged macrophytes

Fig.6 shows that the NH_4^+-N concentrations decreased linearly, and the values generated under the submerged macrophyte conditions were much lower than those of the control condition. Chang-Hua Tong's (2008) experiments conducted under winter conditions showed the NH_4^+-N removal efficiency values of only 14%-70%, which indicated that NH_4^+-N could be removed via evaporation and by directly adhering to sediment (Reddy and Kevusk, 1987). However, NH_4^+-N is typically removed via nitrification and denitrification (Li and Hu, 1995). Given the low temperature conditions used in this study, the removal efficiencies can not clearly attributable to submerged macrophytes because all values were below 25%. By the 14th day of the experiment (*Fig.7*), the removal rates of the submerged macrophyte attained the peak levels, with *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) reaching 23.86%, 24.69%, 18.47%, and 22.93%, respectively. Thus, the efficiency levels for the four submerged macrophyte species are ranked in descending order as follows: *Elodea canadensis, Ceratophyllum demersum, Vallisneria natans* (Lour.) and *Hydrilla verticillata.* Only slight differences were found among them.

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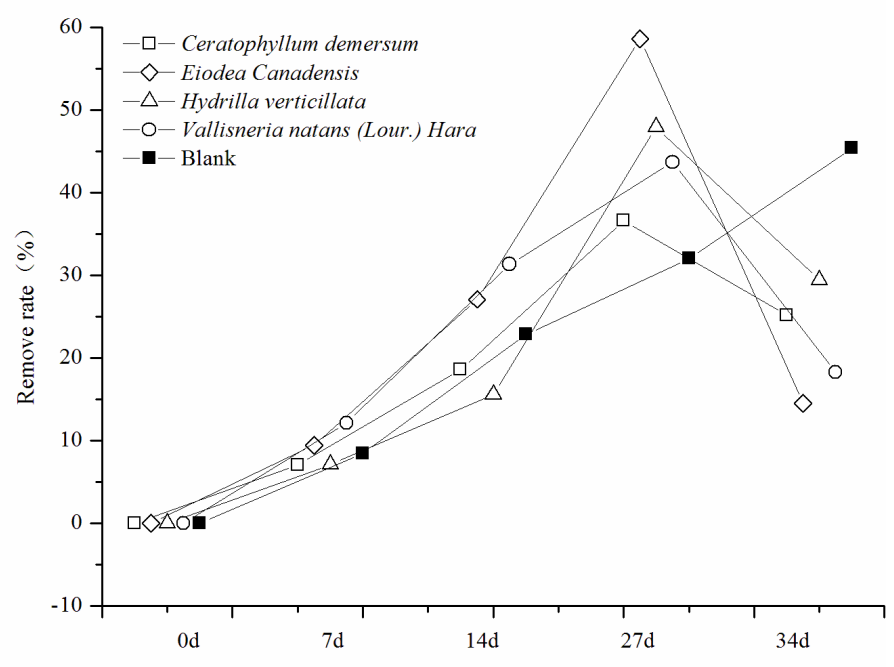

Figure 5. *Removal efficiency of TP during the experiment*

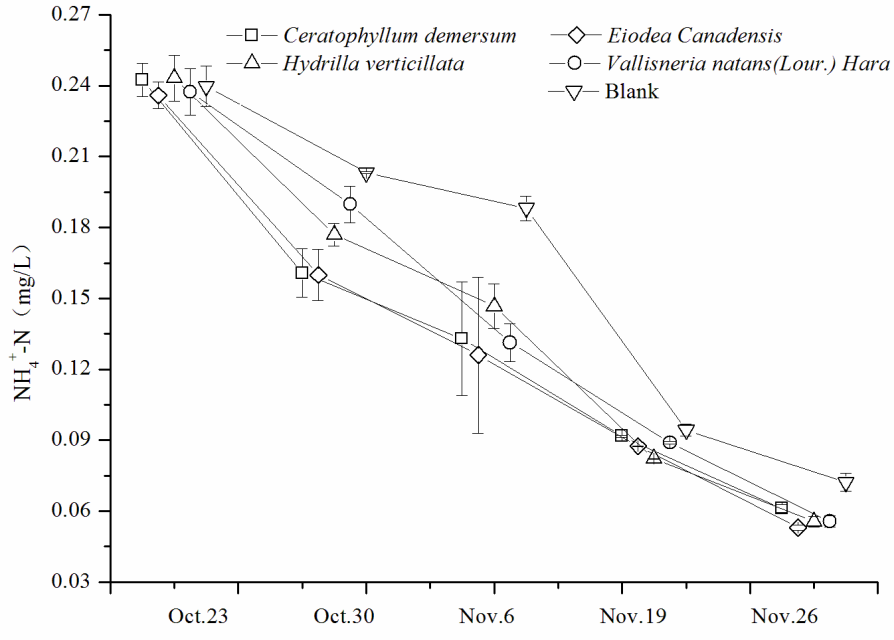

Figure 6. *Variation of NH4+-N concentration during the experiment*

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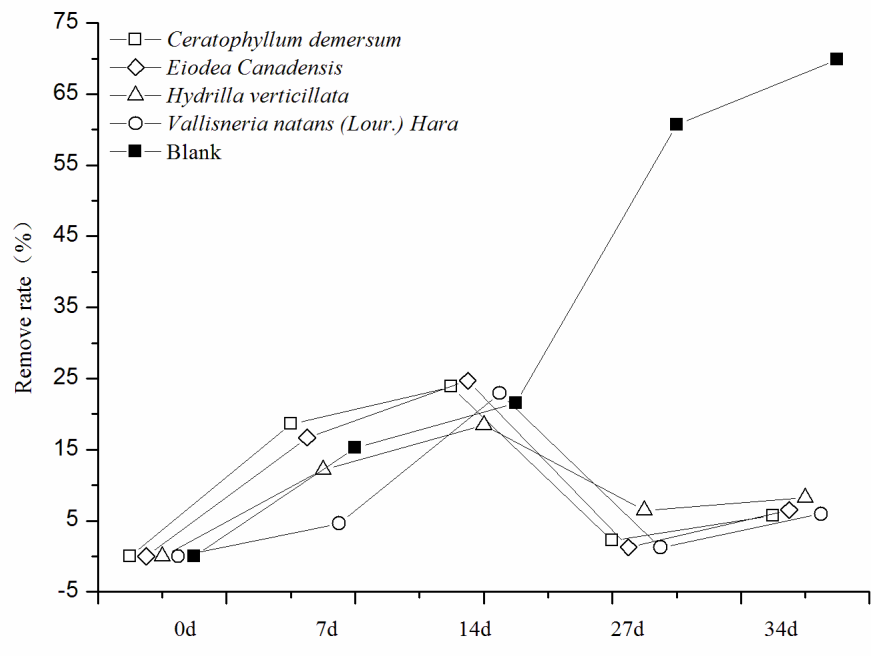

Figure 7. *Removal efficiency of NH4+-N during the experiment*

NO_3-N removal efficiency of submerged macrophytes

During the experiments (*Fig.8*), NO_3-N concentrations found under submerged macrophyte conditions were lower than those of the control condition. The submerged macrophyte experimental showed a decreasing tendency. However, the *Vallisneria natans* (Lour.) experimental conditions showed an increasing trend by the end of the experiment. NO_3-N concentrations increased gradually under the control conditions (*Fig.9*), reaching a final value of 26.89%. The removal efficiency values were calculated as the difference between the experiment with and without submerged macrophyte, which initially increased and then declined. The NO_3-N removal efficiencies of *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 46.69%, 41.08%, 34.19%, and 36.32%, respectively. Thus, the removal efficiency levels of the four submerged macrophyte species in descending order are as follows: *Ceratophyllum demersum, Elodea canadensis, Vallisneria natans* (Lour.) and *Hydrilla verticillata*.

PO_4-P removal efficiency of submerged macrophytes

The submerged macrophytes shows strong removal capabilities with PO_4-P removal, and shows decreasing tendencies in the first 27 days, increases thereafter because of the dead submerged macrophytes and restrict temperatures. In the control experiments, the concentrations of PO_4-P decreasd during the experiments with the terminal data found to be higher for every period. (*Fig.10*)

The PO_4-P removal efficiency levels initially increased but then followed a decreasing tendency. The PO_4-P removal rates were velatively low, and the removal efficiency attained a maximum value on the 7^{th} day. The removal efficiencies of *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 16.41%, 13.52%, 2.18%, and 8.71%. Thus, the removal efficiency

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levels for the four submerged macrophyte species in descending order are as follows: *Ceratophyllum demersum, Elodea canadensis, Vallisneria natans* (Lour.) and *Hydrilla verticillata.* During the experiment, the removal rate of PO_4-P was even negative (*Fig.11*). This may be caused by the disturbance of the sampling or by the adsorption and rerelease from the sediment.

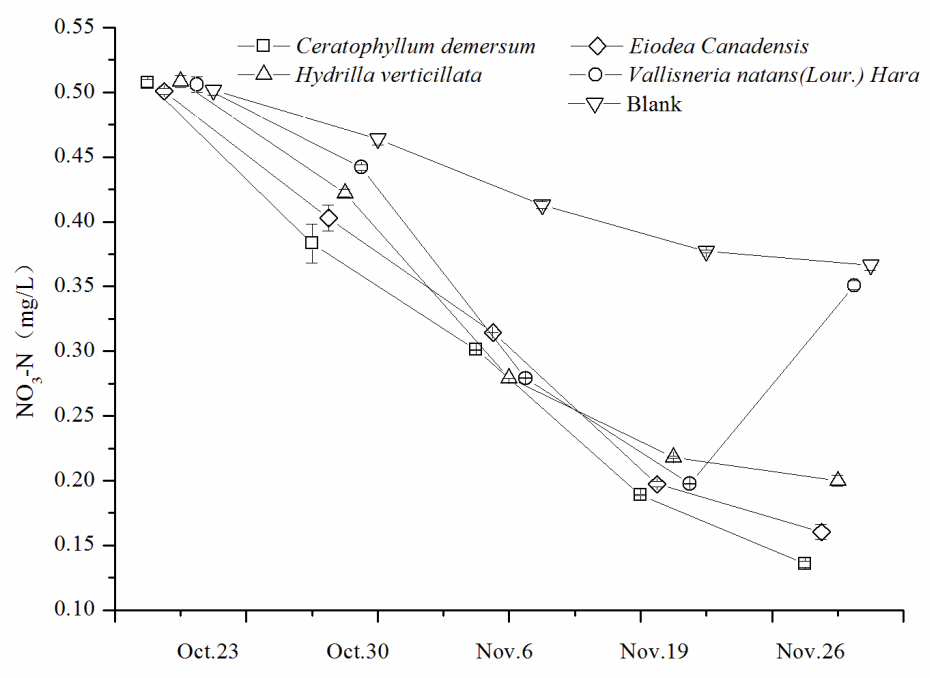

Figure 8. *Variation of NO_3-N concentration during the experiment*

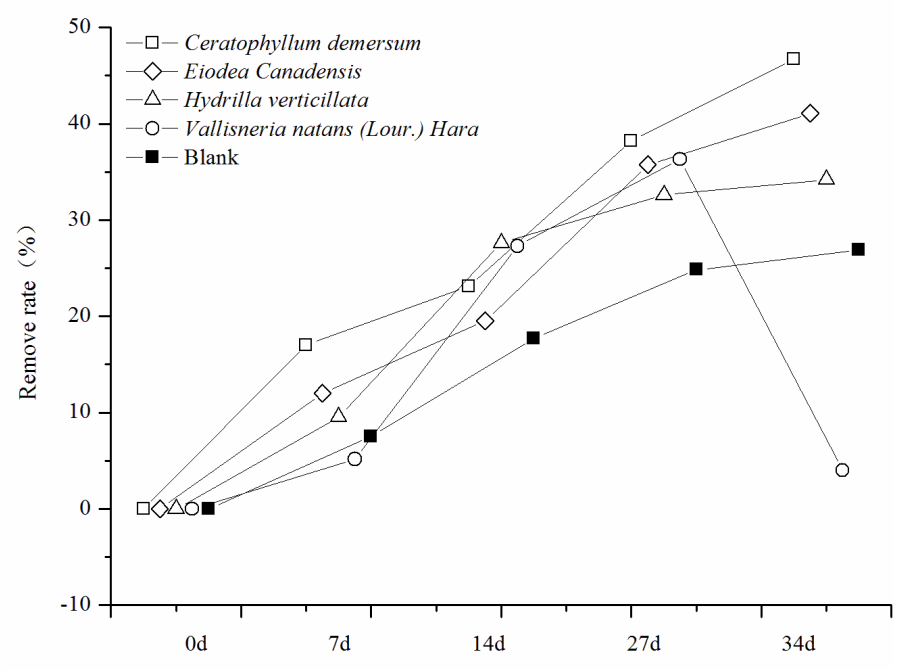

Figure 9. *Removal efficiency of NO_3-N during the experiment*

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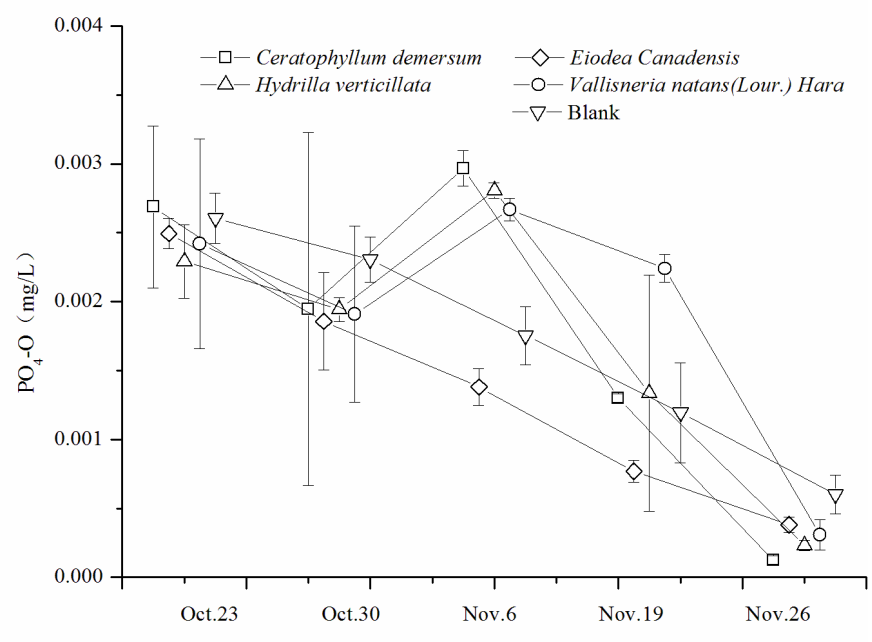

Figure 10. *Variation of PO_4-P concentration during the experiment*

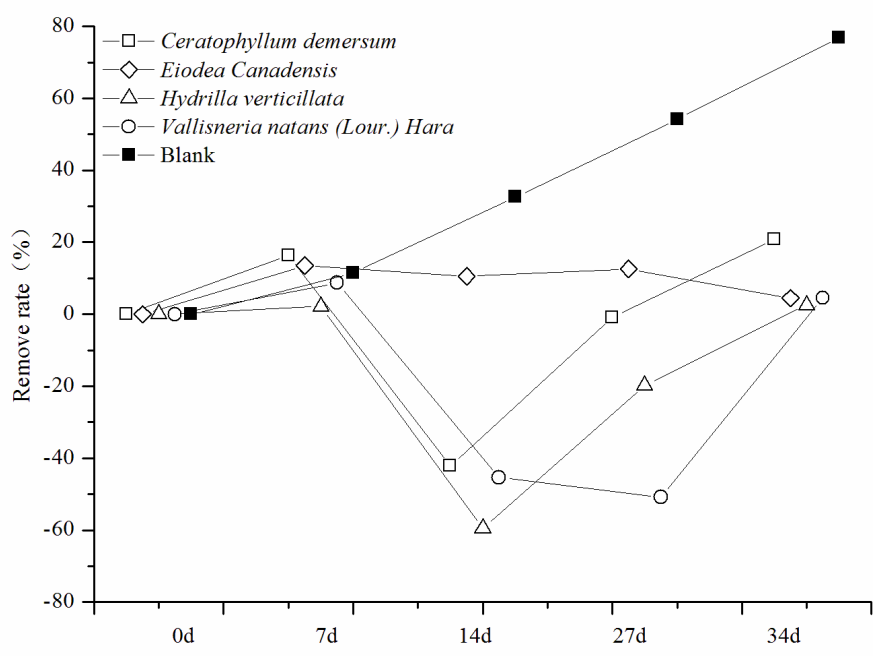

Figure 11. *Removal efficiency of PO_4-P during the experiment*

COD_{Mn} removal efficiency of submerged macrophytes

The submerged macrophytes effectively removed COD_{Mn} from eutrophic water, as shown in *Fig.12*. While water was purified under the submerged macrophyte and control conditions via different ways, the submerged macrophyte conditions were much more effective than the control conditions in the experiment.

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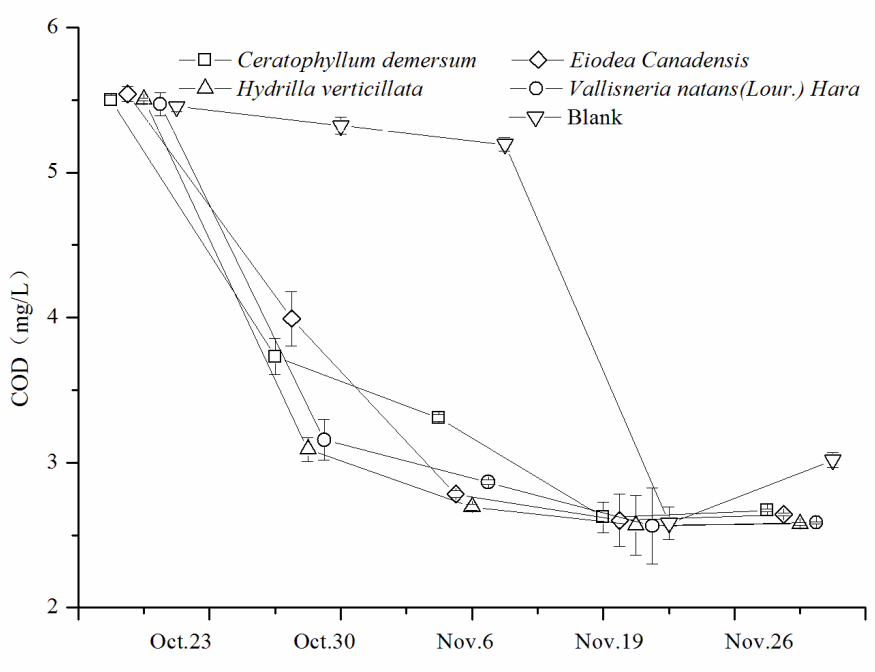

Figure 12. *Variation of COD concentration during the experiment*

Fig.13 presents COD_{MN} removal efficiency levels under the control and submerged macrophyte conditions. Initially, the macrophytes exhibited strong removal capabilitieswith COD_{MN} removal efficiencies increasing until reaching a maximum value on the 14th day. The removal efficiencies of *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 35.11%, 45.06%, 46.30%, and 42.88%. Thus, the removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Hydrilla verticillata, Elodea canadensis, Vallisneria natans* (Lour.) and *Ceratophyllum demersum.* Slight differences in the removal efficiency levels were found between *Hydrilla verticillata* and *Elodea canadensis*. From the 14th to the 34th day of the experiment, all submerged macrophyte removal efficiencies decreased, which was in agreement with the results from Chang-Hua Tong's (2008).

The nutrient removal efficiency of submerged macrophytes

The calculated removal capacities of *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) are shown in *Table 3*.

Table 3. *The nutrient removal capacity characters of four types submerged macrophytes*

Submerged macrophytes	Removal capacity					
	TN	TP	NH_4^+-N	NO_3-N	PO_4-P	COD
Ceratophyllum demersum	0.7169	0.0199	0.1260	0.24098	0.00123	4.57445
Elodea canadensis	1.0853	0.0480	0.1670	0.25517	0.00143	6.00492
Hydrilla verticillata	1.0850	0.0417	0.1325	0.31357	0.00022	10.1771
Vallisneria natans (Lour.)	0.1540	0.0070	0.0162	0.04104	0.00013	1.2980

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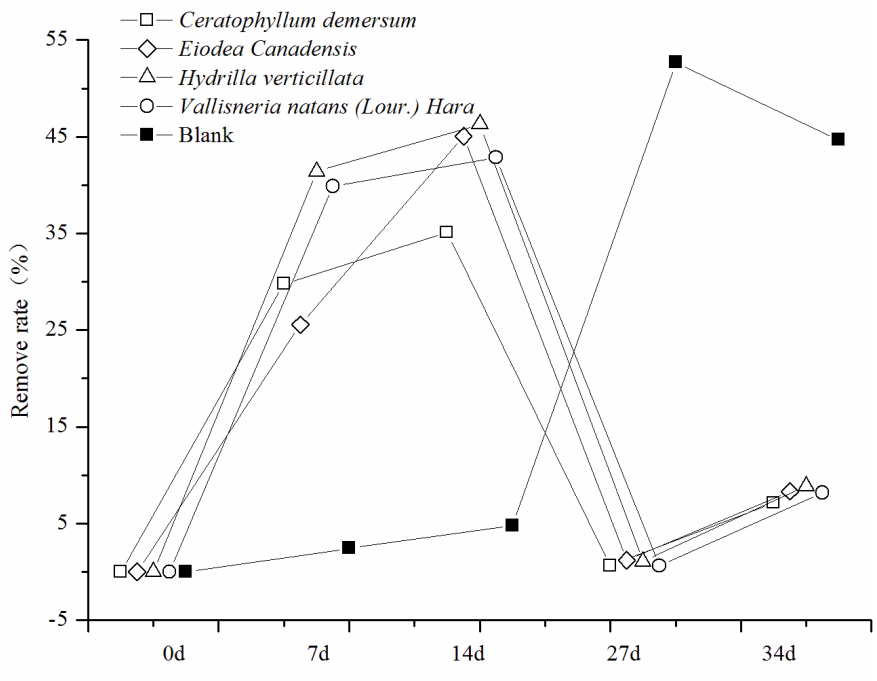

Figure 13. *Removal efficiency of COD during the experiment*

TN-removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Elodea canadensis, Hydrilla verticillata, Ceratophyllum demersum* and *Vallisneria natans* (Lour.).

TP-removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Elodea canadensis, Hydrilla verticillata, Ceratophyllum demersum* and *Vallisneria natans* (Lour.).

NH_4^+-N-removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Elodea canadensis, Hydrilla verticillata, Ceratophyllum demersum* and *Vallisneria natans* (Lour.).

NO_3-N-removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Hydrilla verticillata, Elodea canadensis, Ceratophyllum demersum* and *Vallisneria natans* (Lour.).

PO_4-P-removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Elodea canadensis, Ceratophyllum demersum, Hydrilla verticillata* and *Vallisneria natans* (Lour.).

COD-removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Hydrilla verticillata, Elodea canadensis, Ceratophyllum demersum* and *Vallisneria natans* (Lour.).

Genreally, *Elodea canadensis* and *Hydrilla verticillata* exhibited higher purification capacities than the other two submerged macrophytes. *Ceratophyllum demersum* performed better than *Vallisneria natans* (Lour.), which performed the worst because it was influenced by low temperatures.

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Discussion

These four submerged macrophyte species had various degrees of low-temperature tolerance levels. *Ceratophyllumdemersum L* resisted low temperatures most effectively, followed in descending order by *Elodea canadensis*, *Hydrilla verticillata*, and *Vallisneria natans* (Lour.). Therefor, by the end of the experiments, the *Ceratophyllum demersum* sample had grown considerably, parts of the *Hydrilla verticillata* sample had died, and the *Vallisneria natans* (Lour.) samples had almost died entirely.

Different submerged macrophytes exhibit varying N and P absorption capacities. A study (Shen et al., 2005) showed that *Ceratophyllum demersum, Elodea canadensis,* and *Vallisneria natans* (Lour.) improve aquatic quality in a descending order of *Elodea canadensis, Ceratophyllum demersum*, and *Vallisneria natans* (Lour.). The present study arrived at the same conclusion. This phenomenon also occurred with respect to Nitrogen-Phosphate absorption (Shan and Luo, 2008), which produced various removal rates. According to the results, submerged macrophytes played an important role in removing nitrogen, phosphate, nutrient salts, etc., from eutrophic water. The removal rates were recorded at approximately 35%-70%, except for the case of N and P removal. We present the following findings: 1) The TN removal percentages for *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 67.84%, 68.70%, 64.38%, and 69.13%, respectively. The TN removal efficiency levels for the four submerged macrophyte species a in descending order were as follows: *Vallisneria natans* (Lour.), *Elodea canadensis, Ceratophyllumdemersum L,* and *Hydrilla verticillata*. 2) The TP removal rates for *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 36.59%, 58.60%, 47.94%, and 43.70%, respectively. The removal efficiency levels for the four submerged macrophyte species in descending order were as follows *Elodea canadensis, Hydrilla verticillata*, *Vallisneria natans* (Lour.), and *Ceratophyllum demersum* 3) The NH_4^+-N removal percentages for *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 23.86%, 24.69%, 18.47%, and 22.93%, respectively. Thus the removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Elodea canadensis, Ceratophyllum demersum, Vallisneria natans* (Lour.), and *Hydrilla verticillata.* 4) the NO_3-N removal efficiencies of *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 46.69%, 41.08%, 34.19% and 36.32%, respectively. Thus the removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Ceratophyllum demersum, Elodea canadensis, Vallisneria natans* (Lour.), and *Hydrilla verticillata*. 5) the removal efficiencies of *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 16.41%, 13.52%, 2.18%, and 8.71%. Thus, the removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Ceratophyllum demersum, Elodea canadensis, Vallisneria natans* (Lour.) and *Hydrilla verticillata.* 6) The COD removal efficiencies of *Ceratophyllum demersum, Elodea canadensis, Hydrilla verticillata,* and *Vallisneria natans* (Lour.) were 35.11%, 45.06%, 46.30%, and 42.88%, respectively. Thus the removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Hydrilla verticillata, Elodea canadensis, Vallisneria natans* (Lour.), and *Ceratophyllum demersum*

Submerged macrophyte canopies significantly affected photosynthesis outcomes. Of the four submerged macrophyte species, photosynthesis outcomes generated by

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Ceratophyllum demersum were less significant due to underwater conditions. Due to the low temperatures, *Ceratophyllum demersum* grew slowly and polluted the water sample due to degradation, thus generating the lowest removal capacity result. Under low temperature conditions, different submerged macrophytes exhibited varying removal capacities. Therefore, We conclude the following: 1) The TN removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Elodea canadensis, Hydrilla verticillata Ceratophyllum demersum* and *Vallisneria natans* (Lour.). 2) The TP removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Elodea canadensis, Hydrilla verticillata Ceratophyllum demersum* and *Vallisneria natans* (Lour.). 3) The NH_4^+-N removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Elodea canadensis, Hydrilla verticillata, Ceratophyllum demersum* and *Vallisneria natans* (Lour.). 4) The NO_3-N removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Hydrilla verticillata, Elodea canadensis, Ceratophyllum demersum* and *Vallisneria natans* (Lour.). 5) The PO_4-P removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Elodea canadensis, Ceratophyllum demersum, Hydrilla verticillata* and *Vallisneria natans* (Lour.). 6) The COD removal efficiency levels for the four submerged macrophyte species in descending order are as follows: *Hydrilla verticillata, Elodea canadensis, Ceratophyllum demersum* and *Vallisneria natans* (Lour.).

Conclusion

Based on the growth conditions, nutrient removal rates and removal capacities of the four submerged macrophyte species examined, under low temperature conditions, *Elodea canadensis* and *Hydrilla verticillata* more effectively removed nutrient salts, while *Ceratophyllum demersum* and *Vallisneria natans* (Lour.) were less effective. Therefore, *Elodea canadensis* and *Hydrilla verticillata* could be employed for aquatic system ecological repair and water pollution control in the Poyang Lake basin, especially in aquaculture areas.

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ELECTRONIC APPENDIX

This article has an electronic appendix with basic data.

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LIFE CYCLE ASSESSMENT OF SECONDARY EXTRUDED ALUMINUM PRODUCTION PROCESS IN INDUSTRIAL CITY OF ARAK

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Abstract. In this study, the life cycle assessment model for the secondary extruded aluminum represents a cradle-to-gate process that starts with recycling aluminum scrap and ends in the ultimate product of extruded aluminum transferred to final product manufacturer. In the following, data are mainly collected from the literature, and *ecoinvent3*'s database. The quantitative analysis of the use of the energy and pollutants for production of the secondary extruded aluminum are estimated with eco-indicator-99 methodology and *SimaPro8.0.4* package. The impact of the secondary extruded aluminum production on the environment is studied by evaluating three damage impacts: human health, ecosystem quality and resources. Results indicate that the resources make the highest contribution by 0.58pt (4284.35 MJ) and human health by 0.16pt (0.00136 DALY) is located in the next rank. The endpoint ecosystem quality (44.64 PDF * m^2yr) is insignificant in this process. Also, the total required energy for manufacturing of 600 kg aluminum profile in the model was 8842 MJ. Based on the results, the impact of the secondary aluminum extrusion process on the ozone layer depletion is shown to be insignificant. Also, despite using aluminum scraps, the model incurs significant environmental load. The study is implemented in the industrial city of Arak.
Keyword: *environment, secondary aluminum, scrap, LCA*

Introduction

Since it has been predicted that the aluminum industry is going to be shifted to developing countries (Menzie, 2010; Sevigné-Itoiz et al., 2014), this study is implemented in Iran which is a developing country. Primary aluminum production is one of the most energy-intensive processes in the metals (Ferreira et al., 2014). Meanwhile, compared with other materials, metals have the greatest potential for methodical recycling (Paraskevas et al., 2015). The results of numerous studies have suggested that the emissions and the use of the energy in aluminum production were reduced in the secondary aluminum compared to the primary aluminum (Atherton, 2007; Blomberg and Söderholm, 2009; Ferreira et al., 2014; Green, 2007; Hatayama et al., 2009; Shkolnikov et al., 2011). The environmental credits are a result of reduction in

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raw materials, energy and emissions related to the materials recycling process (Ferreira et al., 2014; Lacarrière et al., 2015). For example, aluminum scrap may be recycled straightly by hot extrusion that needed nearly 10% of energy demand for recycling by re-melt process (Ingarao et al., 2011). Extruded aluminum is widespread used for building sectors.

The secondary aluminum scrap can be categorized as old scrap that is generated following the end-of-life of products, or new scrap that will be created through the casting, semi-finished (such as extruded aluminum profile) and manufacturing production. Hence, new and old scrap (wastes) are converted into the secondary aluminum (Green, 2007). In this case, primary and secondary aluminum may exchange each other into each of extruded aluminum production. Really, it is impossible to determine whether a product is made from bauxite as raw material or from secondary aluminum as recycled material (Frees, 2008). Recycling aluminum from scrap consumes approximately twenty times less energy than the primary aluminum production (Liu and Müller, 2012). The increment in new scrap generation could be related to the increase of semi-finished and final products, as losses throughout their incurred production are classified as new scrap and represented into the production path. The growth in old scrap generation is related to improvements in waste management as a consequence of the presentation of the Packaging waste directive and the end-of-life vehicle directive (Sevigné-Itoiz et al., 2014).

LCA is a principled method that titles the environmental impacts throughout a product's life cycle from raw material acquisition through production, use, end of life treatment, recycling and final disposal (Bertram et al., 2009). A complete LCA study includes four stages: goal and scope definition; life cycle inventory; environmental impact assessment and, finally, interpretation (Chang et al., 2014). The reported LCA in international standard ISO 14040-2006 (ISO, 2006a) and ISO 14044-2006 (ISO, 2006b) may be applied to the environmental aspects. Various studies on aluminum LCA have been done (Detzel and Mönckert, 2009; Ding et al., 2012; Gao et al., 2009; Gatti et al, 2008). Among the aluminum LCA, usual, primary aluminum analysis is used for analyzing the environmental negative effects of products (Liu and Müller, 2012), although some LCA' studies have been done on aluminum alloys, semi-fabricated or finished aluminum products (Detzel and Mönckert, 2009; Hong et al., 2012; Paraskevas et al., 2015; Sevigné-Itoiz et al., 2014). For example, based on LCA model, with the actual situation of aluminum industry in China in 2008, an analysis of the use of energy and pollutants related to aluminum alloy extrusion were carried out (Ding et al., 2012). In what follows, the LCA model is implemented on extruded aluminum. On the other hand, most LCA' studies in aluminum industries don't complete and limit only on a particular process (Liu and Müller, 2012; Norgate and Haque, 2010; Olivieri et al., 2006; Tan and Khoo, 2005). It may be run via grave to gate or gate to gate approach. In addition, it can be semi- finished products. SimaPro software package to be extensively used by scholars to perform LCAs studies (Ferreira et al., 2014). In present study, the LCA model is a cradle-to-gate procedure. The system boundaries determines the unit processes to be contained in the system (Ferreira et al., 2014). In this study, the system boundary of secondary extruded aluminum as a unit process starts with recycling aluminum scrap and ends in the ultimate product of extruded aluminum transferred to final product manufacturer. Once produced, aluminum ingots can undergo numerous fabrication processes,

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normally rolling, extrusion, and casting, to be transformed into different semi-finished products, for example sheet, foil, and profile (Liu and Müller, 2012).

Carbon dioxide emissions increased in the world, more than 2.7 percent over 2011. Meanwhile, the industry plays an important role in a way that almost 40 percent of total energy consumption related to the industrial activities (Ingarao et al., 2011). In extruded aluminum the emission factor, CO2-eq/kg, is about 0.34 kg (Choate and Green, 2003). Also, the published energy quantities of U.S. aluminum rollers and extruders, indicate a broad limit from 2.8 MJ kg $^{-1}$ to 43.2 MJ kg $^{-1}$ (Liu and Müller, 2012). Moreover, it is possible to extrude products without the necessity of additional heat treatments and without dissipation of material. The minimum theoretical energy to extrude a product, in this case, is made of only two components: the energy required preheating the billet to extrusion temperature and the energy required to alter forming the material via a change forming.

Based on the outcomes of a study, the minimum energy intensity is equal to 0.44 kWh/kg of the secondary aluminum, when results are assumed to be 50% for heating and 75% for the hydraulic and electric system (Green, 2007). Considering the fact that no case studies related to the LCA of extruded aluminum have already been done in the Islamic Republic of Iran; a life cycle assessment model is developed using *SimaPro8.0.4* tool to calculate emissions from the production of the secondary extruded aluminum in this country in 2015.

Material and methods

Case study

A case study on aluminum industries in Arak, the industrial area center of the Islamic Republic of Iran, is presented. The unit process includes materials, energy, and environmental releases associated with the secondary aluminum extruding operations. The study is really a cradle-to-gate LCA, thus it ends in the manufactory gate, with the final product of extruded aluminum accessible to transport. The initial material for the secondary extruded aluminum is billet and the result of the process is finished extruded profiles transferred to final product manufacturer. Life cycle inventory (LCI) results are introduced in various impact categories according to characterization factors. The life cycle impact assessment (LCIA) of the secondary extruded aluminum on the environment is studied by evaluating three damage impacts and eleven impact categories. The eco-indicator 99 impact assessment methodology is applied in *SimaPro8.0.4* education package.

Secondary extruded aluminum

Extrusion is the procedure of forcing a metal ingot or billet via a steel die to create a stretched shape of consistent cross section. Extruded products include bars, rods, and defined products called shapes or profiles. Extrusion is significant because aluminum can be readily extruded; this process is difficult for other metals. Extrusion products are more and more used as structural and body components. The secondary aluminum metal is commonly cast into large ingots. The starting material for the secondary extrude aluminum is billet, i.e. a several meters rod in a size 20-50 cm. These billets are mainly produced by direct current casting technology. The ends of the billets are usually sawed at the cast plant for re-melting. The billet may be cut into smaller rod pieces before the

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extrusion process. Before extrusion, the billet is preheated usually to 450 and 500 °C. The billet is then sheared, or cut into length, and deposited right into a hydraulic press. The straightened lengths are cut into finish length multiples and put into an aging furnace to accomplish an ideal operation of tempering. Lengths are then drilled and shaped and placed into a covering process. There are over 40 extrusion plants in Arak. Finally, fabricated extruded profiles transferred to final product plants. The extrusion from cast billet around fabricated profile generates about 320 kg of scrap of 600 kg of extrusion. These scraps are recycled into new ingot through re-melting that is completed either on-plant in integrated cast houses or externally.

Results

The goal and scope

In this study, the LCA model for secondary extruded aluminum represents a cradle-to-gate procedure, beginning at recycling aluminum scrap and ending in the finished product of extrude aluminum that transferred to product plants. The presented model for 600 kg of the secondary extruded aluminum is implemented in Arak.

Life cycle Inventory

Table 1 shows the main material related to environmental impacts of the secondary extruded aluminum. In *Table 1*, the inventories are reported for the secondary extruded aluminum production. The key data were utilized in the *SimaPro8.0.4* model. *Table 1* demonstrates that the air emissions from the production of 600 kilogram of the secondary extruded aluminum were CO_2 (1754000 g), SO_2 (8990 g) and N_2O (2690 g). The emissions to water were Chloride (43860 g), lithium (1220 g), bromide (200 g) and suspended solids (53330 g).Also, this data indicates that the usage of energy resources would be to a considerable extent comprised of electricity (4580 MJ) and natural gas (3440 MJ).

Table 1. *Inputs and outputs for the secondary extrude aluminum production*

Inputs	Unit	Total	Outputs	Unit	Total
Raw materials			Air emissions		
Aluminum scraps	kg	595	Particulates, < 2.5 um	kg	0.09
Natural gas	M^3	286	Particulates, > 10 um	kg	0.08
Electricity	MJ	4580	BTEX[1]	kg	0.07
Water	M^3	1.14	Hydrogen chloride	kg	0.007
Outputs			Water emissions		
Air emissions			BOD_5 [3]	kg	0.63
Carbon dioxide, fossi[1]	kg	1754	COD [4]	kg	0.84
Sulfur dioxide	kg	8.99	Suspended solids	kg	53.33
Nitrogen oxides	kg	2.69	Chloride	kg	43.86
NMVOC[2]	kg	0.58	Magnesium	kg	1.35
VOC, volatile organic compounds	kg	0.20	Lithium	kg	1.22
Particulates, >2.5 um, and < 10um	kg	0.09	Bromide	kg	0.2

[1] Benzene, toluene, ethyl-benzene, and xylene, unspecified ratio, [2] Non-methane volatile organic compounds, unspecified origin, [3] Biological Oxygen Demand, [4] Chemical Oxygen Demand.

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Figure 1 presents a cut from map for 600 kg of the secondary extruded aluminum (LCA-SimaPro model). Also, *Figure 2* shows modeled inventories for 600 kilogram of the secondary extruded aluminum.

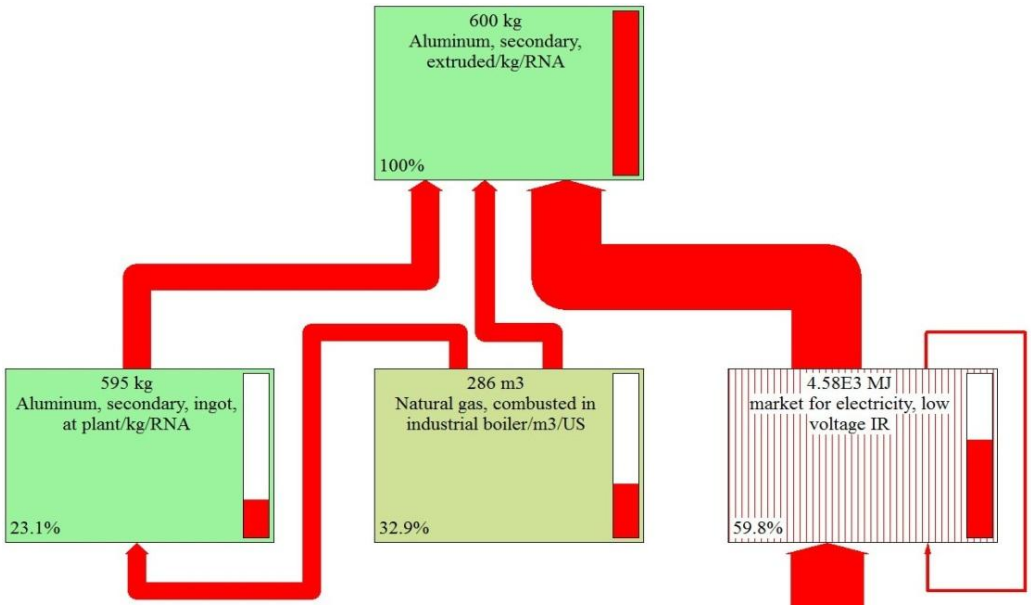

Figure 1. *Cut of the map for 600 kg of the secondary extruded aluminum (LCA-SimaPro).*

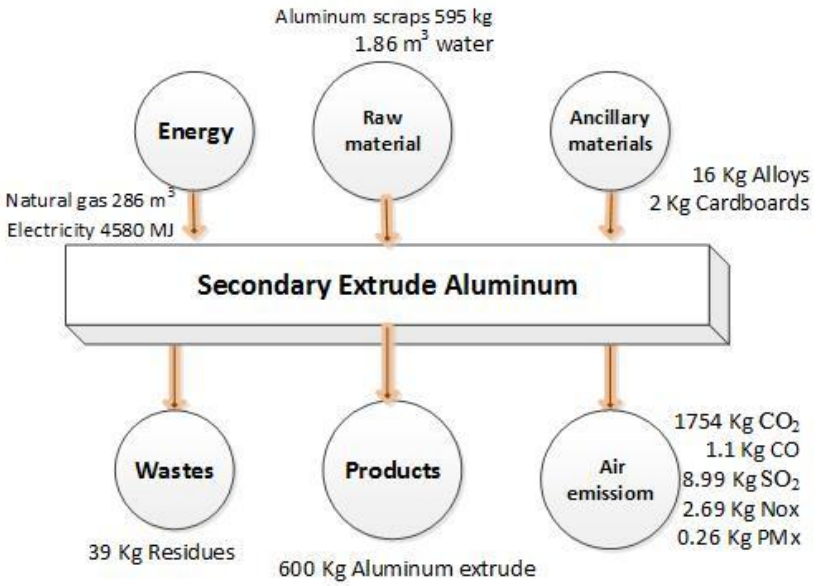

Figure 2. *Inputs and outputs for 600 kg secondary extruded aluminum.*

LCIA stage

In the life cycle impact assessment stage, total inventories, were aggregated into three endpoint categories and eleven impact indicators, according to the eco-indicator 99 method. Endpoint categories are: human health, ecosystem quality and resources.

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Also, midpoint indicators are: carcinogens in DALY (Disability-Adjusted Life Year), respirable organics (DALY), respirable inorganics (DALY), climate change (DALY), radiation (DALY), ozone layer (DALY), eco-toxicity (PDF * m^2yr) (Potentially Disappeared Fraction of species × square meter × Year), acidification /eutrophication (PDF * m^2yr), land use (PDF * m^2yr), minerals (MJ surplus), fossil fuels (MJ surplus)(PRe Consultants, 2013).

Figure 3 present normalized results for environmental impacts of the secondary extruded aluminum, based on damage categories. In *Figure 3*, it is clear that resources exhibit the greatest contribution, by 0.58 pt (4284.35 MJ surplus) and human health by 0.16 t (0.00136 DALY) is located in next rank. The endpoint ecosystem quality (44.64 PDF * m^2yr) is insignificant for this process.

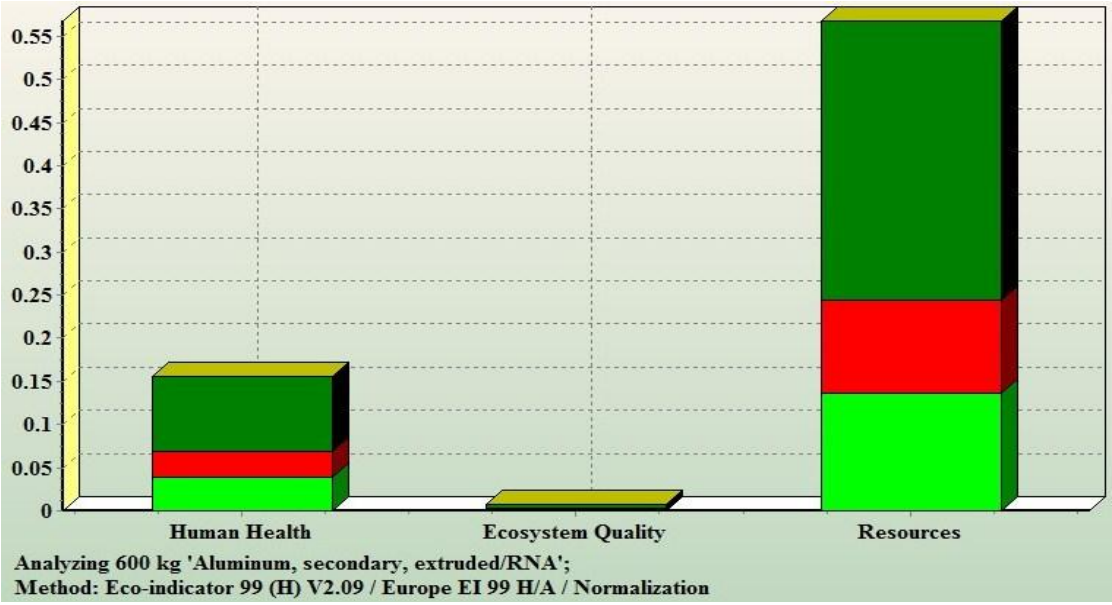

Figure 3. *Normalized results for environmental impacts for the production of 600 kg secondary extruded aluminum, based on endpoint categories.*

Figure 4 presents normalized results for environmental impacts of the secondary extruded aluminum. *Figure 4*, which is based on midpoint indicators, indicates that fossil fuels (4284.35 MJ surplus), respirable inorganic (0.00083 DALY), climate change (0.0004 DALY) and carcinogen (0.00014 DALY) are the most effective indicators.

Figure 5 presents environmental impacts of producing 600 kg of the secondary extruded aluminum, based on single scores (pt). As it can be seen, natural gas (raw material) with 92 pt (286 m^3) is the greatest contributor to environmental impact of 600 kg of the secondary extruded aluminum. The other raw materials (crude oil 92 pt – 132 kg – 822 MJ), emissions from air (SO_2 ((0.000491 DALY), CO_2 (0.000368 DALY), NO_2 (0.000239 DALY) and particles (0.00005 DALY)), and finally, emissions from water (arsenic 0.000056 DALY) contribute to a lesser extent.

Figure 6 illustrates environmental impacts of producing 600 kg of the secondary extruded aluminum, based on human health. *Figure 6* shows that the emissions arising from secondary extruded aluminum which show the highest contribution to human health are SO_2, CO_2 and NO_2 in DALY, respectively.

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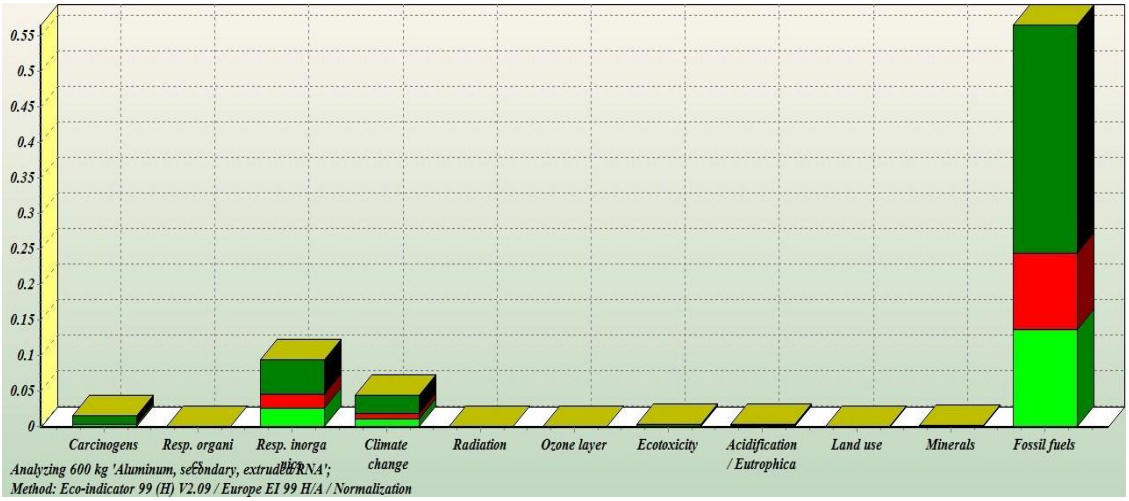

Figure 4. *Normalized results for environmental impacts for the production of 600 kg secondary extruded aluminum, based on midpoint indicators.*

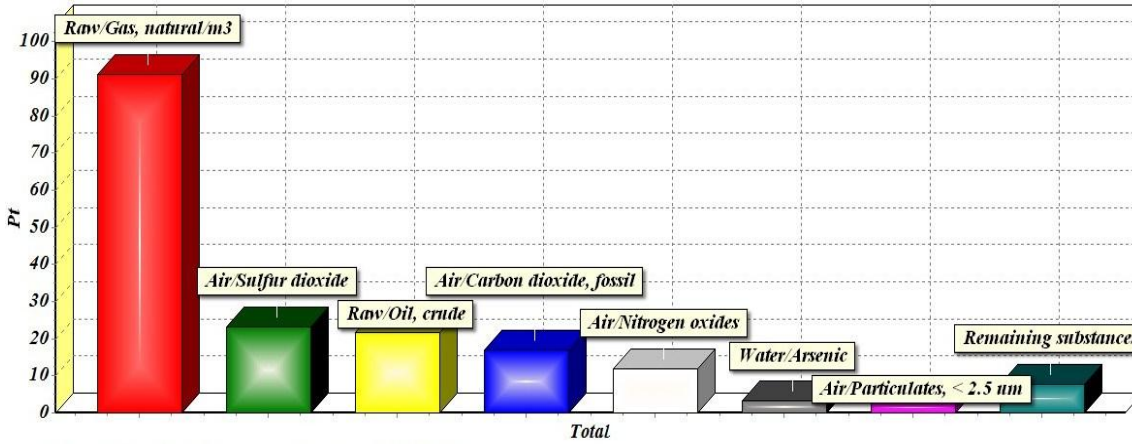

Figure 5. *Environmental impacts for the production of 600 kg secondary extrude aluminum, based on single scores.*

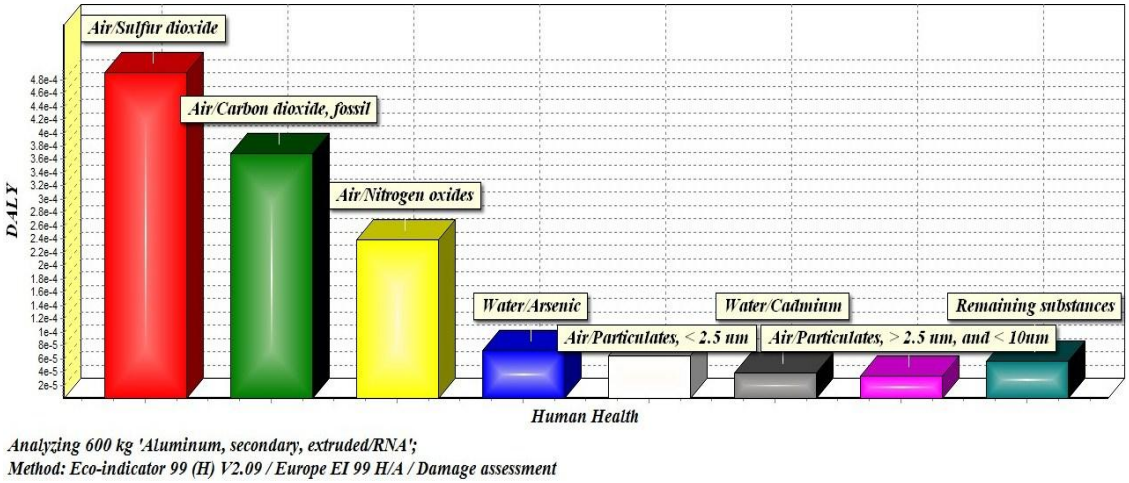

Figure 6. *Environmental impacts for the production of 600 kg the secondary extrude aluminum, based on endpoint human health.*

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Environmental impacts of the production of 600 kg of the secondary extrude aluminum, based on resp.inorganic are presented in *Figure 7*. As can be seen, the total resp.inorganic is 0.00083 DALY. Also SO_2 and NO_2 make up the greatest contributions, with 0.000491 and 0.000239 DALY for 600 kg of the secondary extruded aluminum, respectively.

Figure 8 presents environmental impacts of the production of 600 kg secondary extruded aluminum, based on carcinogen. *Figure 8* demonstrates, the total carcinogen produced from the secondary extruded aluminum is 0.000138 DALY. Pollutants to water (arsenic 0.00007 DALY, cadmium 0.00004, metallic iron 6.97E-06 in DALY) and pollutants to air (cadmium 0.000012, arsenic 0.000005 in DALY) are the causes of carcinogen due to the secondary extruded aluminum process.

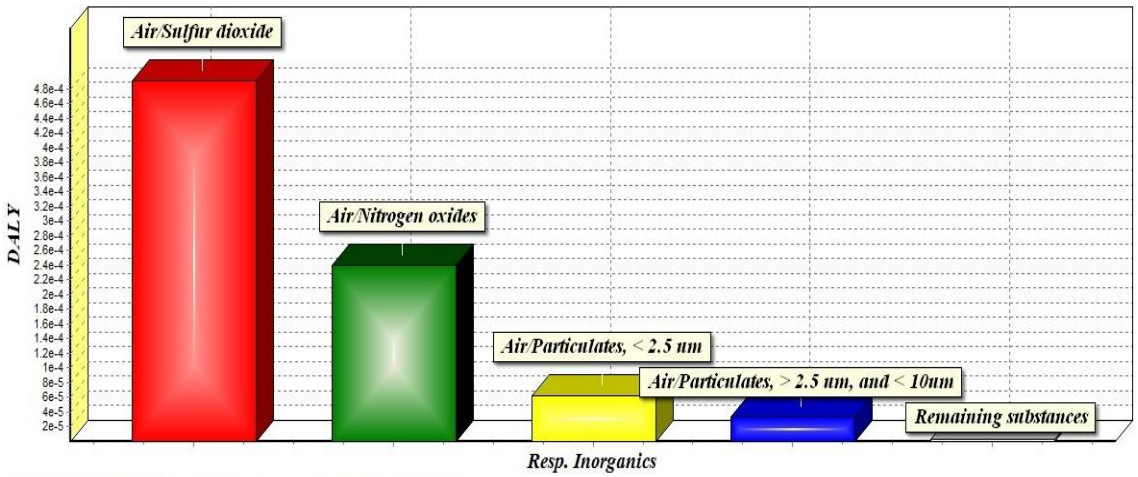

Figure 7. *Environmental impacts for the production of 600 kg the secondary extrude aluminum, based on midpoint respirable inorganic.*

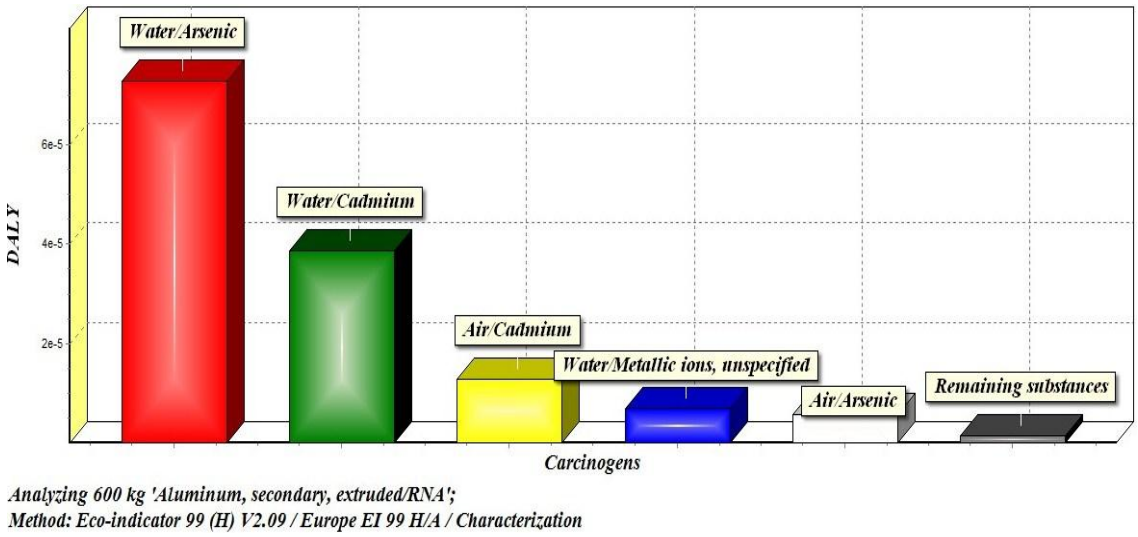

Figure 8. *Environmental impacts for the production of 600 kg the secondary extrude aluminum, based on carcinogen midpoint.*

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Discussion

Life cycle assessment was utilized to evaluate the secondary extruded aluminum process. In this study, data were mainly collected from literature, and *the eco-invent3* database. The present LCA was implemented in the industrial city of Arak in 2015. The model for the secondary extruded aluminum represents a cradle-to-gate procedure, beginning at recycling aluminum scrap and ending in the final product of aluminum extrusion.

The basic objective of the impact assessment stage would be to transform the long list of life cycle inventory output straight into a limited number of indicators. These indicators will show the impact of each operation on the environment. One evaluation was based on endpoint indicators and midpoint indicators. Endpoint indicators tend to be more uncertain and midpoint indicators are apt to have a shorter perspective. In this study, 11 impact categories were applied with average weighting and normalization based on European standards. To accomplish this, the *eco-indicator99* method is used. This method converts the long inventory of outputs in set of indicators which receive the severity to each impact category based on some weighting.

As was seen previously, the usage of aluminum scrap incurs an environmental credit. In spite of this, it is evident that the energy consumption is the highest contributor to negative environmental impacts of production of 600 kilogram of the secondary extruded aluminum. The resources endpoint to the secondary extruded aluminum model accounts for nearly 67% and is the environmental problem area for this product system. Hence, one of the most results read from present research is that natural gas consumption is most responsible for the environmental impacts in this process.

The human health (HH) indicator estimates the negative consequences of a defined process on humans. The total HH from the secondary extruded aluminum production was 0.000138 in DALY. In this case, respirable inorganic, climate change and carcinogen had the greatest share in this category. As was shown, for respirable inorganic, the sulfur dioxide and nitrogen oxides (both emissions to air) resulted from the secondary extrude aluminum model had the highest negative impact on humans. In case of climate change, carbon dioxide with 0.00368 DALY was a large contributor and, in fact, the main contributor to the negative environmental consequences of the secondary extruded aluminum process.

The carcinogens are calculated in DALY. It can be seen that the arsenic and cadmium with 0.00122 DALY (emissions to water) had the highest impact in this midpoint indicator.

The quality ecosystem aims to outline the negative impact of a certain process on the ecosystem. Based on outcomes of proposed research, quality ecosystem category was insignificant in the secondary extruded aluminum process; it was less than 3.28 % for all system.

Based on results, it could be concluded that the main contributors to the negative environmental impacts of the secondary extruded aluminum were commonly energy supply. We think that the perfect solution would be using new heating technologies to reduce energy requirements through more rapid heating in treatment temperature and using the heat of the casting or finishing forming operation method. The recent method requires less energy. Therefore, the implementation of this method reduces energy requirements in extruding aluminum.

Performing such an analysis based on the assumptions made for the study would have proven useful and provides a base for further works. However, research into this

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issue must also be the subject of future works and further contributions to the growing field of LCA in Iran.

Future work

- Comparing the potential environmental consequences of different options of the extruded aluminum production with regarding type or origin of raw material and energy resources will be interesting.
- In our opinion, carrying out a cradle-to-gate LCA study in aluminum production process can be interesting and important in industrial city of Arak. A more detailed LCA method would allow aluminum industries to assess environmental burdens of their activities more practical in order to reduce them.
- In addition, extending MCDM methods such AHP, TOPSIS and ELECTRE based on LCA results in aluminum industry can be interesting. This integrated methodology can be used to make the best decision in different methods of primary and secondary aluminum production.

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ELECTRONIC APPENDIX

Electronic appendix 1: Map for 600 kg of the secondary extrude aluminum (LCA-SimaPro)

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IMPACT OF HYDROTHERMAL CONDITIONS ON COMMON BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH.) PRODUCTIVITY

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Abstract. Investigations were conducted during 2004 – 2014 at the Vokė Branch of the Lithuanian Research Centre for Agriculture and Forestry. Research purpose – to identify changes in the productivity of buckwheat depending on the variations in hydrothermal conditions during the growing season. Studies have shown that all investigated buckwheat varieties demonstrated high yield variation (24.3 – 45.5 %) but somewhat smaller variation of biometric parameters (6.2 – 14.7 %). It was found that the trends of increase in variations are associated with reduction of all assessed parameters. This is confirmed by statistically significant correlation between 1000 grains mass and variation coefficient of this parameter (R^2 = 0.4449*). Hydrothermal conditions during the summer season also influenced variation of all parameters. It was found that, due to wetter than optimal hydrothermal conditions in summer, variation of all parameters (except for 1000 grains mass) was lower. Hydrothermal conditions during summer had no effect on the variation of 1000 grains mass. Hydrothermal conditions of separate summer months produced different impact on buckwheat production formation. Rather wet July (if HTC did not exceed 2.5) was favourable and produced a positive effect on buckwheat biometric parameters (R^2 = 0.3475 and 0.4646*) and grain yield (R^2 = 0.4015*). However, at the beginning of vegetation (June) and during grain ripening (August) buckwheat plants require hydrothermal conditions with optimum moisture.
Keywords: *common buckwheat; yield; climatic conditions*

Introduction

Buckwheat (*Fagopyrum esculentum* Moench) is an annual herbaceous plant of the *Polygonaceae* family originating from Central Asia (Sharma and Jana, 2002; Chauhan et al., 2010). It is one of the most important alternative crops, providing raw materials for food and manufacturing industries (Préstamo et al., 2003; Woo et al., 2010; Wronkowska et al., 2010). Buckwheat proteins are characterised by unique amino acid composition, because they comprise large amounts of essential amino acids (Wei et al., 2003; Kim et al., 2004; Krkoškova and Mrazova, 2005). All parts of a buckwheat plant contain large amounts of phenolic compounds, especially rutin (from 0.48 to 4.97 mg / 100 g) (Park et al., 2000; Sharma et al., 2012; Kreft et al., 2003). In addition, it is a melliferous plant: flowers produce 10.9 – 160.2 kg ha^{-1} of nectar (Racys and Montviliene, 2005).

Buckwheat is a typical low-input crop that is traditionally cultivated on infertile soils. Because of the short vegetation season, buckwheat grows quickly. These plants could very well consume mineral matter present in the soil, because their roots are able to absorb them from the hard-soluble compounds. They were rarely infected by disease or pests, besides buckwheat plants successfully suppress the weeds. All of it makes buckwheat very suitable for cultivation in organic farming, where no industrial

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fertilizers and plant protection products could be used (Radics and Mikóházi, 2010; Loch and Lazanyi, 2010).

Although buckwheat is a valuable crop, in many countries their areas are not large. In 2010-2011 an average of 2.113 million hectares of buckwheat were grown worldwide; the greater part of these areas are situated in China (34.25 %), Russia (32.43 %) and Ukraine (11.46 %) (Popović et al., 2014). Just two decades ago in Lithuania buckwheat crops occupied very small area (4400 hectares), but by 2004 their area has increased by 5 times (up to 21400 ha) (Batulevičiūtė, 2006). Over the past 10 years, in Lithuania buckwheat areas increased by one third (Strakšas and Vaiciukevicius, 2009). The main reason for rather scarce buckwheat cultivation areas is their low productivity compared with other cereals: in Europe it reaches 902 kg ha^{-1}, in Asia - 890 kg ha^{-1}, and in America - 1115 kg ha^{-1} (Popović et al., 2014).

Buckwheat yield is predetermined by the biological properties of these plants and climatic conditions. Buckwheat biology differs from that of other agricultural plants, because buckwheat plants form vegetative and generative organs at the same time. Their flowering is long (about 50 days) and very abundant, but percentage of seed set is very low (Maletić and Jevdjović, 2003). In Belgium the study was carried out with an aim to increase the yield of buckwheat by artificially reducing competition between the flowers and set seed. However, in all cases the seed set reached only 20 – 30 %, and it had only slight effect on the yield (Halbrecq et al., 2005).

Morphological structure of buckwheat flowers allows only cross-pollination. Buckwheat pollination can be performed exclusively by insects, and bees are the main pollinators accounting for 81-95 % of all insects visiting the buckwheat fields (Björkman, 1995; Racy and Montviliene, 2005). Buckwheat pollination efficiency is related with visits by insects pollinators, and these visits directly depend on the meteorological conditions. Studies performed in Ethiopia show that, with the rising temperature, bees visit flowers more frequently ($r = 0.67$, $P = 0.01$), while increasing relative humidity makes these visits less frequent ($r = -0.59$, $P = 0.001$) (Gebremedhn et al., 2014). There is a number of studies about the positive role of bees in buckwheat grain yield formation. It is stated that by way of pollination technological parameters and quality of buckwheat grain improves and the yield increases from 21.7 to 81.0 % (Sim Choi, 1998; Chen and Tsai, 1994; Aryal et al., 2014; Racys and Montviliene, 2005).

Since buckwheat plants are characterised by specific biological properties, their productivity is closely linked with both visiting insects and the hydrothermal conditions during the growing season. Studies carried out in the Balkans revealed that plant height and grain yield of buckwheat mostly depend on rainfall amount during the growing season. It was noted that the hydrothermal conditions had a significant impact on the duration of pollination period, and moisture deficiency induced more rapid grain ripening (Maletić and Jevdjović, 2003). Czech researchers have found that the hydrothermal conditions during buckwheat flowering and seed set period (July) produced a decisive influence on seed endosperm and shell formation. It is argued that extremely high temperatures and moisture deficiency may terminate seed development (Kalinová et al., 2002). In Japan, the research aiming to determine what meteorological factors were dominant in determining buckwheat productivity was carried out. Model research results showed that the vegetative growth of plants and number of flowers positively correlated with air temperature, but the number of pollinated flowers negatively correlated with daily precipitation amount (Inoue et al., 1998).

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Buckwheat is grown in many parts of the world, where climate is very different. In the future, the buckwheat cultivation areas should be increasing due to increasing demand for healthy food and organic products. Therefore, the knowledge of the causes for changing productivity of these plants due to climate factors is important in order to determine the possibilities of productivity enhancement.

Research purpose – to identify changes in the productivity of buckwheat depending on the variation of hydrothermal conditions during the growing season.

Materials and methods

Site and soil

Investigations were conducted in the crop rotation during 2004 – 2014 of the Voke Branch of the Lithuanian Research Centre for Agriculture and Forestry, which is located in Traku Voke (54°63' N, 25°10' E). The experimental plots were established on sandy loam on carbonaceous fluvial-glacial gravel eluviated soil (IDp), according to FAO-UNESCO classification Haplic Luvisols (LVh) (Buivydaitė, 2005). Soil agrochemical characteristics: pH_{KCl} – 5.2–6.2, humus – 2.11–2.18 %, mobile P_2O_5 – 108–152 mg kg^{-1}, mobile K_2O – 150–165 mg kg^{-1}.

Experimental design and management

Investigations were carried out in collection nursery of buckwheat selection trial site. Eleven varieties of buckwheat has been tested: buckwheat variety 'VB Vokiai' of Lithuanian (LT) origin (it is a standard variety), other buckwheat varieties of Belarus (BY) origin – 'Volma', 'Smuglianka', 'Anita Belaruskaya', 'Anika', 'Kvietka', 'Canita', 'Zaleika', 'Mara', 'Zniajarka' and 'Belaruskij determinant'. Soil for buckwheat trials was ploughed in the autumn, two times cultivated and harrowed in the spring. Test field area for varieties trials – 1 m^2. Planting rate – 3 mln ha^{-1} of fertile seeds. Each year buckwheat was sown during the third decade of May. Under climatic conditions of Lithuania buckwheat intensively flowered, set and ripened grain during July and August. Grain was harvested during the first decade of September.

Meteorological conditions

Lithuania is situated in middle latitudes of the temperate zone. A mean annual air temperature of +6.2 ^oC, a mean annual precipitation of 661 mm. (Galvonaite et al., 2007). Climate data (monthly temperature and precipitation) for the years 2004 – 2014 were obtained from the Lithuanian Hydrometeorological Service. According to the standard climatic rate, in Traku Voke average temperature in summer months is 15.7 – 16.9 °C with an average of 68 – 78 mm of rainfall (*Table 1*).In this area the period of 2004 – 2014 was warmer and wetter. Only in June the weather conditions were close to the standard climatic rate. Average temperature in July was by 2 °C, and in August – by 1.3 °C higher than the average. During the mentioned period, the average precipitation during these two months exceeded the rate by 35-44 %. Ratio of precipitation amount and average air temperature helps to better assess the hydrothermal conditions during the growing season of plants, therefore annual meteorological conditions during the period of the experiment are expressed as hydrothermal coefficient (HTC) (*Table 2*). Thermal and irrigation conditions during the summer season could be described by a widely used Selianinov's hydrothermal coefficient HTC = Σp / 0.1 Σt, where: Σp – total

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precipitation (mm) sum during the given period; Σt – total sum active temperatures (°C) of the same period. If HTC > 1.6 – the irrigation is excessive, HTC = 1.0…1.5 – optimal irrigation, HTC = 0.9…0.8 – weak drought, HTC = 0.7…0.6 – moderate drought (arid), HTC = 0.5…0.4 – heavy drought, HTC < 0.4 – very heavy drought (Dirsė and Taparauskienė, 2010).

Table 1. *Meteorological conditions in Traku Voke*

Meteorological index	Month		
	June	**July**	**August**
Long term average (1961 – 1990 period)			
Temperature, °C	15.7	16.9	16.3
Precipitation, mm	77	78	68
Average during 2004 – 2014 period			
Temperature, °C	16.1	18.9	17.6
Precipitation, mm	78	105	98

Table 2. *Hydrothermal conditions during the summer periods in 2004-2014 Traku Voke, 2004 – 2014 years mean data*

Year	Hydrothermal coefficient (HTC)		
	June	**July**	**August**
2004	2.88	0.89	1.73
2005	1.31	1.18	3.88
2006	0.42	0.71	2.79
2007	1.11	3.98	0.45
2008	1.46	1.05	1.01
2009	2.89	1.92	1.34
2010	2.83	3.08	1.91
2011	0.75	2.55	1.88
2012	2.23	1.34	1.62
2013	1.17	1.95	1.12
2014	1.10	1.09	2.12
Long term average	1.63	1.49	1.35

Meteorological conditions varied among experimental years. Nearly each research year (except for 2008 and 2010) normal and less rainy periods in summer (when HTC <1.5) were interchanging with periods of excess moisture (when HTC > 1.5), or vice versa. Based on the standard climatic rate, June is the month of excess moisture (HTC = 1.63). However, over the last 11 years this month was particularly rainy only 4 times (in 2004, 2009, 2010 and 2012). During the remaining years of the research hydrothermal conditions in June were of optimal humidity (except for moisture-deficient 2006 and 2011). When in July and August meteorological conditions are consistent with the standard climate norm, the hydrothermal conditions are of optimal humidity (HTC = 1.49 – 1.35). Hydrothermal conditions in July were much wetter than the climate norm during 5 years out of 11 years of the experiment, and in August – during 7 years out of 11. Only in 2008 favourable weather conditions were recorded throughout the summer season.

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Statistical analyses

The experimental data were statistically processed using analysis of variance and correlation – regression analyses methods employing software *Anova,* software package *Selekcija* (Tarakanovas, 2002). The treatment effect was tested by the least significant differences LSD_{05}. Significance levels: ** - $p < 0.01$ * - $p < 0.05$.

Results

The obtained results showed that over the past 11 years the average grain yield of almost all buckwheat varieties of Belarus origin was higher. Varieties 'Canita', 'Zaleika' and 'Mara' were particularly conspicuous as their grain yield was significantly higher (25.5 - 28.0 %) than of the standard variety 'VB Vokiai' (*Table 3*).

Table 3. *Buckwheat variety yield and biometric parameters of the collection field trials Traku Voke, 2004 – 2014 years mean data*

Variety	Country	Grain yield, t ha^{-1} ± SE	1000 grains mass, g ± SE	Plant height, cm ± SE
VB Vokiai	LT	2.71 ± 0.32	34.2 ± 1.0	100 ± 3.6
Volma	BY	2.83± 0.41	32.7* ± 1.3	105 ± 4.9
Smuglianka	BY	2.80 ± 0.33	31.8* ± 1.5	95 ± 3.5
Anita Beloruskaya	BY	2.71 ± 0.22	31.4* ± 1.1	97 ± 3.9
Anika	BY	2.90 ± 0.22	30.6* ± 1.2	101 ± 3.0
Kvietka	BY	3.06 ± 0.40	31.3* ± 1.2	101 ± 2.9
Canita	BY	3.47* ± 0.41	31.0* ± 1.3	103 ± 3.7
Zaleika	BY	3.40* ± 0.38	31.5* ± 1.3	104 ± 2.6
Mara	BY	3.44* ± 0.38	30.5* ± 1.4	104 ± 2.8
Zniajarka	BY	3.09 ± 0.39	30.8* ± 1.3	100 ± 2.0
Beloruskij determinant	BY	3.12 ± 0.33	32.5* ± 1.2	98 ± 3.3
LSD_{05}		0.562	1.457	5.298

Notes: ± SE – standard error

However, buckwheat variety 'VB Vokiai' formed larger grains, with 1000 grains mass substantially higher (4.4 - 10.8 %) than of other buckwheat varieties. Plants of all tested buckwheat varieties grew to medium height, i.e. height among plants of different varieties ranged from 95 to 105 cm, but no substantial differences were recorded as compared with the plant height of standard variety 'VB Vokiai'.

During the research period, annual variation of yield in all buckwheat varieties was particularly high; coefficient of variation (CV) was 24.3 – 45.5 % (*Fig. 1*). However, the variation of biometric parameters was lower. It was determined that, depending on the variety, variation of 1000 grains mass and plant height was low (CV < 10 %) or medium (CV < 15 %).

The correlation analysis showed that hydrothermal conditions of the summer season produced no effect only on the variation of 1000 grains mass ($R^2 = 0.0012$) (*Figure 2*). However, grain yield and plant height variations were changing simultaneously. The downward trends in the coefficient of variation of grain yield and plant height were noted when summer HTC was increasing from 1.2 to 1.8. More humid summer season (HTC > 1.8) increased the variation of grain yield and plant height.

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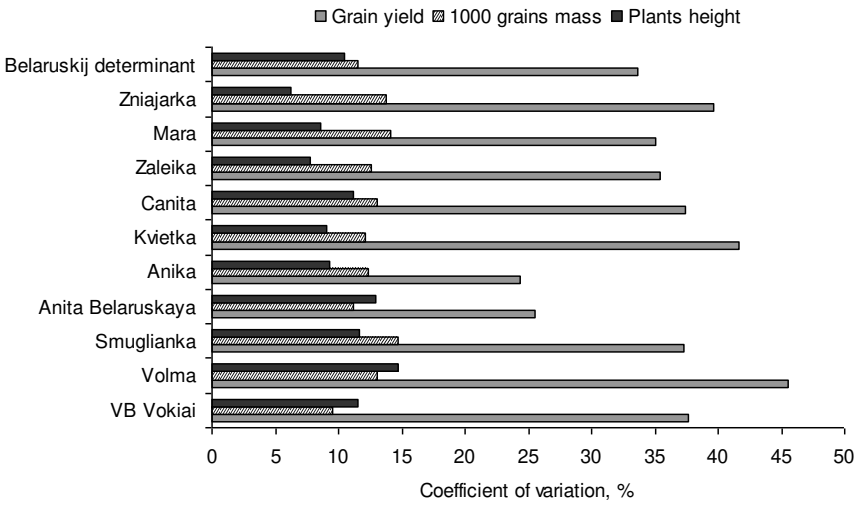

Figure 1. *Variation coefficients of the yield, 1000 grains mass, and plant height of buckwheat varieties (CV, %) (Traku Voke, 2004 – 2014 years mean data)*

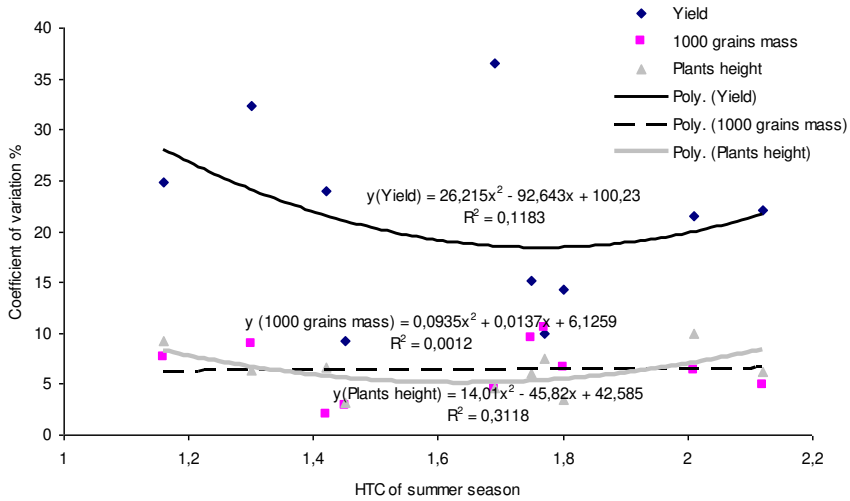

Figure 2. *Correlation between hydrothermal conditions (HTC) of the summer period and coefficients of variation (CV, %) in buckwheat yield, 1000 grains mass and plant height during 2004 – 2014*

These studies showed that wetter than optimal hydrothermal conditions during the summer season had a positive impact on variation reduction in buckwheat plant height and productivity.

It was revealed that changes of average grain yield and biometric parameters of 11 tested buckwheat varieties were associated with changes in their variations, and it was confirmed by correlation. Statistically significant correlation was established between 1000 grains mass and coefficient of variation of this parameter ($R^2 = 0.4449$*) (*Figure 3b*). This shows that in case of higher 1000 grains mass, variation of this parameter among different varieties decreased. Due to decline of grain yield and plant height, upward trends in variations of these parameters were observed.

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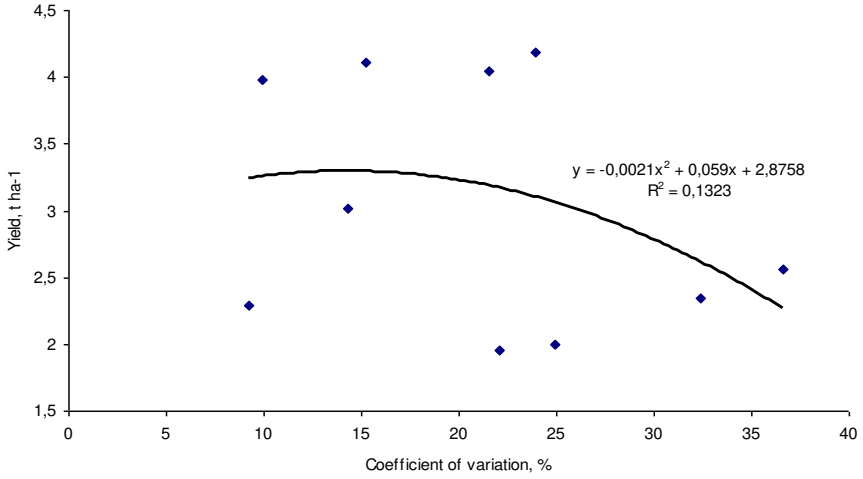

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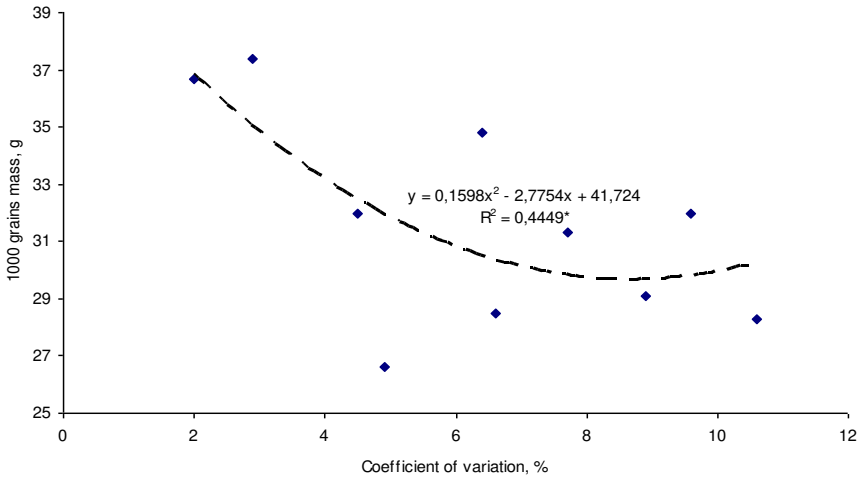

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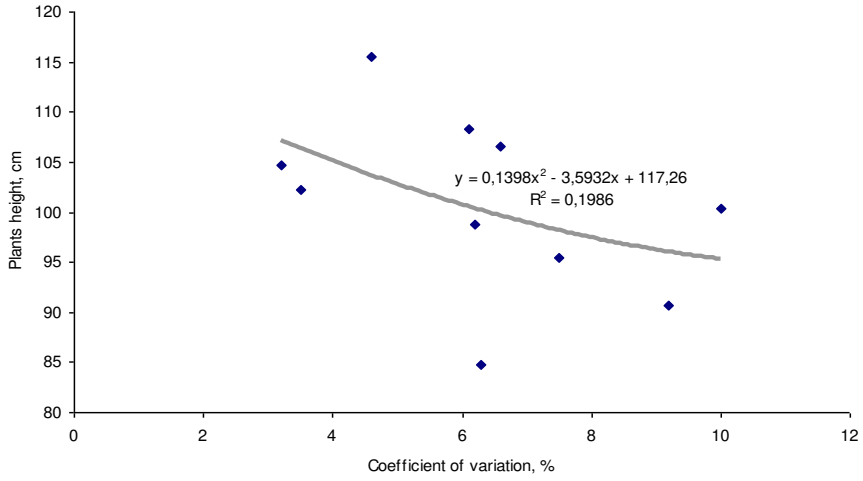

c

Figure 3. Correlation between the buckwheat grain yield (a), 1000 grains mass (b) and plant height (c) and their coefficients of variation (CV, %).

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Hydrothermal conditions of each summer month had a different impact on the formation of buckwheat yield. Correlation analysis showed that the lack of moisture in June, when HTC < 1.5, caused decline in buckwheat grain yield (*Fig. 4a*). Hydrothermal conditions of July affected grain yield stronger than of any other summer month (R^2 = 0.4015*). As HTC of July was increasing up to 2.5, the buckwheat grain yield gradually increased, but further HTC increase caused reduction of the grain yield. During grain ripening period, which coincides with August, excess moisture was unfavourable for buckwheat. Wetter hydrothermal conditions (when HTC > 1.5) resulted in decreased grain yield (R^2 = 0.2645).

Similar patterns concerning the impact of hydrothermal conditions of July and August were determined for changes of 1000 grains mass (*Figure 4b*). Although stronger correlations were determined (R^2 = 0.3475 and R^2 = 0.3636 respectively), but they were not statistically significant. Very weak correlation with hydrothermal coefficient of June indicates that hydrothermal conditions of this month had no influence on 1000 grains mass.

Based on the correlation analysis, hydrothermal conditions of the whole period until August were influencing the buckwheat plant height. Under hydrothermal conditions of optimum moisture content at the beginning of buckwheat growth (June), the buckwheat plant height was increasing. However, the abundance of rainfall in June and HTC above 2.0 adversely affected the buckwheat plant growth, because at HTC > 2.0 downward trend in buckwheat plant height was observed (*Fig. 4c*). The results showed that the buckwheat plant height stronger and statistically significantly correlated (R^2 = 0.4646*) with hydrothermal coefficient of July.

The abovementioned results of correlation analyses indicate general trends for all grain varieties. Correlation analysis of hydrothermal conditions in summer and each grain variety yield has been established for higher yields variation during tests. The results have also confirmed the established trends. Positive correlation coefficients show that in June and July high humidity was favorable for most grain varieties yield. In addition, there was commonly moderate and strong correlation (r = 0.51 - 0.72) between different grain varieties yields and HTC during these months (*Table 4*).

Table 4. *Correlation coefficients between summer hydrothermal conditions (HTC) and tested varieties of buckwheat grain yields*

Variety	Coefficient of correlation (r) between grain yield and hydrothermal conditions (HTC)		
	June	July	August
VB Vokiai	0.40	0.40	-0.71*
Volma	0.37	0.68*	-0.41
Smuglianka	0.51	0.58	-0.56
AnitaBeloruskaya	0.48	0.67*	-0.42
Anika	0.58	0.42	-0.42
Kvietka	0.67*	0.40	-0.25
Canita	0.57	0.72*	-0.61*
Zaleika	0.59	0.63*	-0.44
Mara	0.36	0.61*	-0.46
Zniajarka	0.53	0.55	-0.44
Beloruskijdeterminant	0.34	0.57	-0.39

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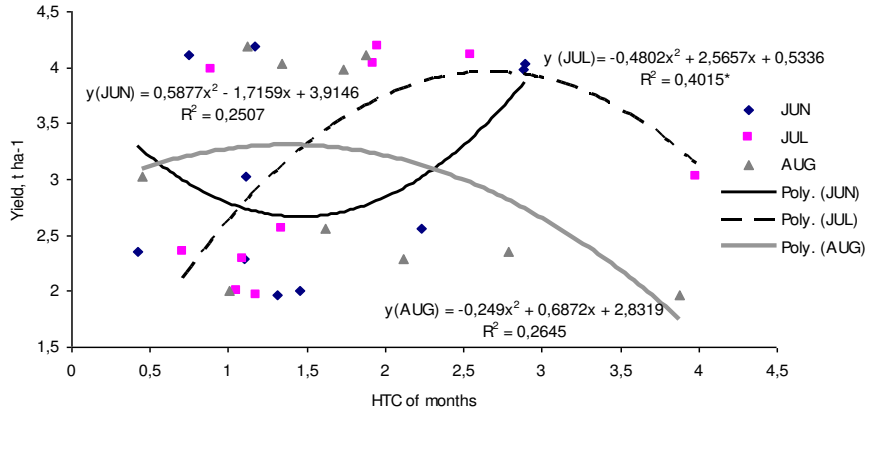
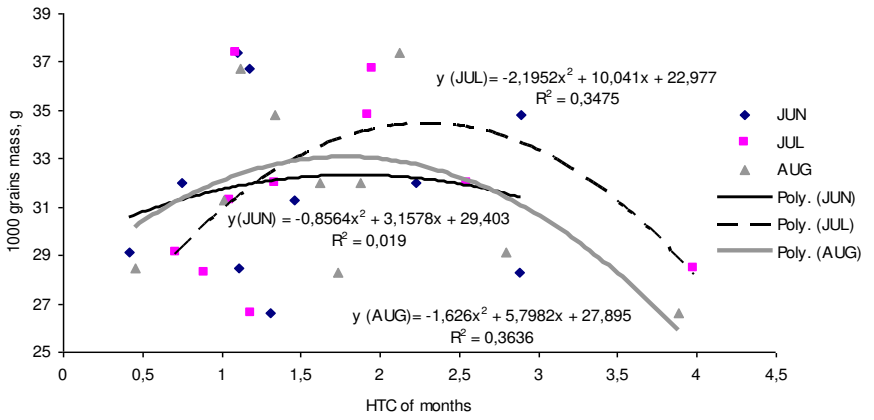


a



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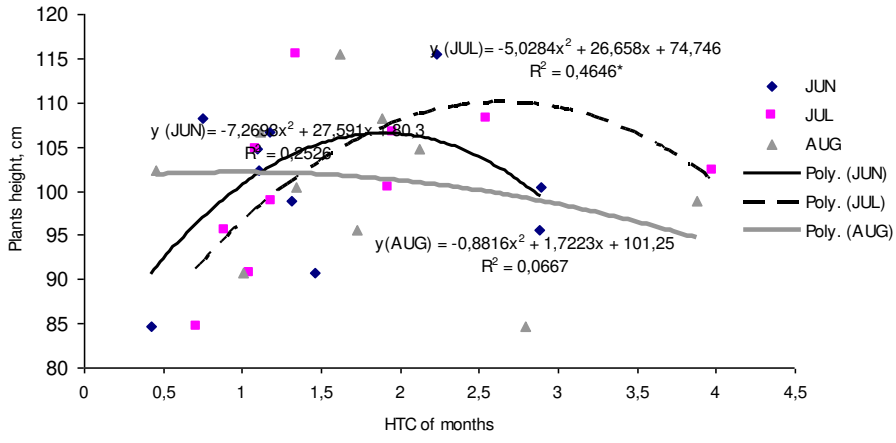

c

Figure 4. *Correlation between hydrothermal conditions (HTC) of summer months and buckwheat grain yield (a), 1000 grains mass (b) and plant height (c)*

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Wet hydrothermal conditions in August reduces the yield of all varieties, especially the following : 'VB Vokiai' (r = -0.71 *), 'Smuglianka' (r = -0.56) and 'Canita' (r = -0.61 *).

Discussion

In Lithuania the buckwheat breeding studies have been carried out since 1999. During the 16-year period the buckwheat variety 'VB Vokiai' was created (Romanovskaja and Razukas, 2006). Until then, buckwheat varieties 'Anita Beloruskaya', 'Smuglianka' and 'Volma', created in Belarus, which is in Eastern Europe in the same climate zone as Lithuania, were widely cultivated. Data analysis of the last decade shows high fluctuations of the buckwheat grain yield in all the tested varieties (coefficient of variation > 20 %) (*Fig. 1*). Average yield of buckwheat variety 'VB Vokiai' did not differ or was by 3 – 4 % lower than of previously popular varieties 'Anita Beloruskaya', 'Smuglianka' and 'Volma' (*Table 3*). However, during the favourable years (2000 – 2001) grain yield of variety 'VB Vokiai' was by 7.7 – 19.1 % higher than of the aforementioned varieties (Almantas, 2002).

In many agricultural systems buckwheat grain yields are low and unstable. It was confirmed by large coefficients of variation determined after statistical evaluation of the results obtained during 2004 – 2014 (*Fig. 1*). Japanese researchers believe that low yield potential is predetermined by allogamy and heterostyly, which make insect pollination indispensable (Ogasahara et al., 1995). Legitimate pollination and the amount of set grain depend on the transfer of suitable pollen on the stigma and favourable meteorological conditions (high temperature - about 20 °C and high radiation - about 30 cal cm^{-2} h^{-1}). It was revealed that the highest number of suitable pollen gets on the stigmas when meteorological conditions are favourable (38.6 – 39.3), but the numbers are greatly reduced in cool (25.7 – 25.8) and windy (17.9 – 11.9) weather (Ogasahara et al., 1995). Small amounts of suitable pollen reaching the stigma or limited activity of pollinators, especially in rainy weather, result in inadequate pollination and low yield potential.

Meteorological conditions of summer months affect not only the process of pollination but the growth of plants as well. In our climate, June is the period of intense buckwheat vegetative growth and biomass formation, therefore wetter hydrothermal conditions (when HTC > 1.5) in June were more favourable that drier conditions and had a positive impact on the productivity of buckwheat (*Fig. 4a*). Physiologically buckwheat flowering and grain formation take place simultaneously and lasts for about 2.5 months (from late June to mid-September). Hydrothermal conditions of this period may affect the formation of flowers, the pollination process, and thus predetermine the buckwheat yield. The model developed in Japan helped evaluate the influence of various factors on buckwheat. Harvest index (HI) was calculated employing the quantities of formed flowers, pollinated flowers and ripened grain, with regard to buckwheat physiology and ecological factors in the course of different growing seasons (Inoue et al., 1998). It was determined that buckwheat vegetative growth and flower formation positively correlated the mean daily temperature, but negatively correlated with the daily temperature range. Meanwhile the quantity of pollinated flowers negatively correlated with both day length and precipitation of each day (Inoue et al., 1998). It means that high temperature during the day had a negative effect on flower formation, and high rainfall negatively affected flower pollination.

According to the results of the research performed in Lithuania, buckwheat yield strongly correlated with hydrothermal conditions of July (r = 0.83**) and August (r =

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0.63**) (Asakavičiūtė et al., 2015). The impact of hydrothermal conditions of this two-month period on the buckwheat yield amounted to 40 - 69 % of all factors. Our research shows that over the past 10 years the impact of hydrothermal conditions in July on grain yield was higher than of any other summer month (R^2 = 0.4015*) (*Fig. 4a*). It was revealed that wet hydrothermal conditions in July (up to the limit of HTC not exceeding 2.5) increased the buckwheat grain yield. In July, hydrothermal conditions corresponding HTC = 2.5 could form when rainfall is about 70 % higher than the climate norm. Based on our results and results published in other countries, we can conclude that higher moisture content during the period of intense flowering (July) is essential for buckwheat, and it has a positive effect on the grain yield. Researchers of Serbia and Montenegro state that buckwheat is more suitable for cultivation in wetter regions. Maletić R. and Jevdjović R. (2003) revealed that higher buckwheat grain yield was obtained in the areas with higher amount of rainfall in July (Maletić and Jevdjović, 2003). Still, it should be noted that our research was performed in light textured sandy loam soil (Haplic Luvisol), where rainfall infiltration regime is intense. During the summer season, in sandy loam soil infiltration amounts to 40.5 % of the atmospheric precipitation (Tripolskaja and Pirogovskaja 2013). Average data of the period of 1987 – 2007 show that higher amount of precipitation percolate in July (16.6 l m^{-2}) and August (15.2 l m^{-2}), less – in June (5.8 l m^{-2}) (Tripolskaja et al., 2014). Therefore, in light textured soils, where in most cases buckwheat is cultivated, even during very wet summers intensive infiltration prevents from excessive moisture. Meanwhile, drier hydrothermal conditions may create unfavourable conditions for buckwheat growth and yield formation. This was confirmed by our data, which showed that the lack of moisture in June caused reduction of buckwheat grain yield (*Fig. 4a*). In June, an intensive buckwheat vegetative growth takes place; it requires a larger amount of water to ensure the normal cause of physiological processes and biomass increase. That is why wetter hydrothermal conditions of June are more favourable than drier conditions and produce stronger impact on grain yield formation. Depending on growth stages, buckwheat requires different hydrothermal conditions during the growing season. It was revealed that after germination wet hydrothermal conditions lasting for about 2/3 of the total growing season were agreeable for their productivity. On the contrary, during the grain ripening excessive rainfall produced no positive effect on grain yield. Our research results indicate that at HTC > 1.5 downward trend in grain yield is observed (R^2 = 0.2645) (*Fig. 4a*). So it be said that during the grain ripening period, which coincides with August, optimal moisture hydrothermal conditions (HTC = 1.0... 1.5) are essential.

Particular feature of different buckwheat varieties is a grain size, which is expressed evaluating the 1000 grains mass. It is a less variable parameter, since grain size is hereditary. According to the results of breeding studies performed in Lithuania, the heredity of grain size accounted for 47.6 % (Romanovskaja and Razukas, 2007). However, studies have shown that over the past 10 years, 1000 grains mass slightly fluctuated depending on meteorological conditions, but variation of the parameter was low (CV = 9.5 –14.7 %) (*Fig. 1*). In most research variety 'VB Vokiai' was distinguished among other varieties by very large grain (Almantas, 2002; Romanovskaja and Razukas, 2006; Asakaviciute et al., 2015). In the period of this research 1000 grains mass of variety 'VB Vokiai' was 34.2 g, i.e. substantially higher than of other buckwheat varieties (30.5 – 32.7 g) (*Table 3*). It should be noted that buckwheat grain size affects technological characteristics and grain yield. It is noted that the fraction of large grain formed higher percentage in the yield of the buckwheat varieties that are characterized by higher 1000

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grains mass (Asakaviciute et al., 2015). Czech researchers have found that with increasing 1000 grains mass, the fraction of large (about 4.5 mm) grain (r = 0:45) and grain yield (r = 0.825**) also increased (Kalinová et al., 2002).

Our research results show that biometric parameters of buckwheat also depended on the hydrothermal conditions, but the impact of hydrothermal conditions during separate months of growing season on the 1000 grains mass and on plant height was different. It was revealed that hydrothermal conditions in June had no effect on 1000 grains mass (R^2 = 0.019) (*Fig. 4 B*), while hydrothermal conditions in August did not affect the height of plants (R^2 = 0.0667) (*Fig. 4c*). Although buckwheat plants are intensively growing in the first half of vegetation, but hydrothermal conditions in June (R^2 = 0.2526) had less impact on their height than hydrothermal conditions in July (R^2 = 0.4646*) (*Fig. 4 C*). Meanwhile relatively wet hydrothermal conditions in July (if HTC did not exceed 2.5) were favourable and had a positive influence on buckwheat biometric characteristics and grain yield.

Conclusions

Over the research period (2004 – 2014) all tested buckwheat varieties were characterised by high yield variation (CV = 24.3 – 45.5 %), but lower variation of biometric parameters (CV = 6.2 – 14.7 %). It was revealed that upward trends in variations are decline of all parameters assess during the study. It is particularly justified by statistically significant correlation between 1000 grains mass and variation coefficient of this biometric parameter (R^2 = 0.4449*). Hydrothermal conditions during the summer season or during separate summer months also influenced variation of all parameters. The research results show that wetter than optimal hydrothermal conditions in summer resulted in lower variation of all parameters (except for 1000 grains mass). However, hydrothermal conditions of the summer season did not affect the variation of 1000 grains mass. Hydrothermal conditions of separate summer months had a different impact on the formation of buckwheat yield. Rather wet hydrothermal conditions in July (if HTC did not exceed 2.5) were favourable and produced a positive influence on biometric parameters of buckwheat (R^2 = 0.3475 and 0.4646*) and grain yield (R^2 = 0.4015*). Still, at the beginning of vegetation (June) and the period of grain ripening (August) buckwheat plants require hydrothermal conditions of optimum moisture (HTC = 1.0...1.5).

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SPATIO-TEMPORAL RESPONSE OF SEDIMENTARY DIATOMS TO WATER LEVEL IN A SHALLOW LAKE

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Abstract. In recent years, human activities have resulted in variations of hydraulic conditions in shallow lakes, thereby affecting interactions between environmental variables and aquatic plants. This feedback of plants on environmental changes is largely recorded in lake sediments. In this study we collected surface sediments in different seasons and a sediment core from a shallow lake Baiyangdian. We investigated the seasonal effects of water level and six physicochemical variables in surface sediments, as well as their impacts on the distribution of diatom assemblages. Therein the response of diatom assemblages to water level and the spatio-temporal effects of water level are highly emphasized. Results indicate that water level significantly changes between seasons, however there is no significant seasonal effects for the composition of grain size. The lake productivity increases with the vigorous growth of aquatic plants. Water level largely determines the distribution of diatom assemblages in surface sediments and the sediment core. Spatial isolation causes the difference between the habitats and alters the relation between water level and diatom taxa. Human activities also affect the relevance of water level with diatom assemblage. The results can provide the helpful information for the local water resource management.
Keywords: *lake sediment; seasonal effects; abiotic and biotic factors; human activities*

Introduction

Lake sediments can be seemed as a key sensor for assessing environmental changes and the fitness of lacustrine ecosystem (Battarbee, 2000). Sediments record variations of biotic factors and the impact of anthropogenic activities on a lake system within a historical period (Velghe et al., 2012). Most of researches have focused on the relation between the biotic factors and environmental changes in the body of water in shallow lakes (Habib et al., 1997; Phiri et al., 2007; Xie et al., 2013), whereas this relation was generally neglected in the sediments. Interactions between surface sediments and the body of water are largely dependent on the flow intensity and temporal fluctuations of water level in shallow lakes. Further variations of the lake underlying surface are closely related to fluctuations of water level. Varying water levels in shallow lakes alter the abundance and the diversity of aquatic plants, especially the phytoplankton community (Shinneman et al., 2010, Wang et al., 2012). Diatoms, as a member of phytoplankton, can be seemed as a reliable indicator to study the relation between the biotic factors in lake sediments and environmental changes (Moos et al., 2005).

Investigating the distribution of diatoms in surface sediments and its relation with physiochemical elements reflects the response of the biotic factors to environmental changes in a spatial scale. Examining the distribution of diatom communities found in

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surface sediments can improve our knowledge of variations in lake productivity (Facca et al., 2005). Spatial heterogeneity should be accounted for in shallow lakes given the difference of microhabitats and diatom assemblages in different lake areas. Koren and Klein (2000) found that surface sediments can be used to assess the spatial distribution of a biotic group in an open lake better than in a closed lake, by calculating the sedimentation rate. The seasonal measurements of indicative elements deposited in surface sediments can lead to an in-depth understanding of anthropogenic effects on the body of water in shallow lakes (Alagarsamy, 2006). The temporal scale of aquatic plants' response to environmental changes could be realized via the investigation of a sediment core. Current studies have put more emphasis upon one sediment core collected from a lake and the reconstruction of environmental variables, ignoring the spatio-temporal distribution of the variables and its effect on the composition of aquatic plants (Witkowski et al., 2009; Liu et al., 2012). Sediment cores are sometimes unreliable when determining the relationships between physiochemical variables and diatom assemblages since compaction, mineralization, and precipitation can weaken their correlation. In contrast, the concentrations of variables are relatively high but temporally dynamic regarding the surface sediments. Water level remains relatively stable over a short period. Using reconstructed and monitoring water levels to analyze the spatio-temporal response of diatom assemblages to environmental changes can help us to understand the spatio-temporal complexity of a lake, and further this might remove doubts that diatoms with different attributes can be found in one field (Weilhoefer and Pan, 2005). Variations of the freshwater diatom assemblage have shown to be closely linked to fluctuations of lake levels (Kingsbury et al., 2012; Leeben et al., 2013). Mean annual water level was a decisive variable affecting the distribution of diatoms in Lake Ossa. However, seasonal distributions of diatom assemblages are less related to water-level fluctuation (Nguetsop et al., 2010). In addition, Showing the seasonal effects of water-level fluctuation on the surface sediments is meaningless in case the lake sedimentation rate is low (< 0.1cm/y) (Tammeorg et al., 2013). Environmental variables indirectly linked to water level similarly affect the diatoms composition. Grain size and wind intensity determined the spatial distribution of diatom assemblages in surface sediments of the shallow lake Peipsi sensu stricto (Puusepp and Punning, 2011). Water quality is an assignable indicator affecting the response of diatoms to water level with respect to some shallow lakes, e.g. in Lake Vortsjarv, the abundance of planktonic diatoms had a significantly positive relation with water level before the 1960s, while this relation was weakened by the eutrophication after the 1960s (Heinsalu et al., 2008).

In this study, we concentrate on the corresponding response of diatom assemblages to water level based on the investigation of surface and core sediments. The studied shallow lake Baiyangdian experienced variations of water levels after the 1950s, since hydraulic projects have been constructed on the upper reach of the lake, which significantly affected hydrological conditions of the lake. Therefore the very surface sediments can be seemed as a product of human disturbance and the sediment core is a useful tool to provide the historical information of the lake. Hereby human disturbance is defined as human activities that interfere with the body of water and then indirectly interact on surface sediments, e.g. variations of water flow and water level due to the construction of hydrological projects, but do not indicate the direct disturbance on the surface sediments, e.g. excavation or pouring external materials. We address the following two points in our study: (1) seasonal effects of the surface sediments and water level (2) spatio-temporal response of diatom assemblages to water level and the effect of human

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activities on this response. The results can provide the useful information for regional water resource management and the protection of aquatic plants.

Materials and Methods

Study area and sampling sites

Baiyangdian is the largest freshwater shallow lake located in the north of China (*Fig. 1*), covering a total area of 366 km2. The lake has a large area of water and a nexus of water channels. These water channels are well connected with no human architecture in between. The body of water is weakly alkaline. An investigation of water quality during the period of June 2009 – April 2010 indicated that mean total phosphorus was 0.14 mg/l and mean total nitrogen was 4.29 mg/l (Zhang et al., 2013). Baiyangdian is located in a warm temperate zone; the regional climate pattern typically displays a strong seasonality with arid windy springs, hot rainy summers, and cold dry winters. The mean annual temperature ranges from 7.3 °C to 12.7 °C. Annual rainfall fluctuates from 350mm to 750mm (Zhang et al., 2013). Water table in the western area is generally higher than that in the eastern area. Water level seems to be a reliable indicator of reflecting hydraulic conditions of the lake. Water level ranges from 6m to 9m based on the record of chorography. Lake Baiyangdian is classified as a natural wetland that has 39 types of aquatic plants, among which Ceratophyllum demersum, a submerged plant, and Phragmites australis, an emergent plant, account for a considerable proportion of the aquatic plants. Severe droughts led to extremely low water levels and heavily destroyed the habitat of the aquatic plants at the beginning of the 1980s.

The sampling of sediments was implemented in November 2009, as well as in January, March, and July 2010. Eight sampling sites were selected to investigate the spatial characteristics of surface sediments (from north to south: SCD, WJZ, YZZ, ZLZ, LWD, QT, DTZ and CPT). It is time consuming to perform a biological analysis of excessive sampling points whereas few sampling points may not stand for the features of a lacustrine system (Weilhoefer and Pan, 2006). We collected surface sediments and four short sediment cores (core length = 20 cm) in the first sampling, in order to determine an ideal place to obtain a long sediment core. One site CPT was found without laminated sediments, implying that physical mixing and bioturbation were relatively weak in the area. We collected the sediment core (core length = 80 cm) and surface sediments representing different seasons in the sequential samplings. Unfortunately, we did not collect the surface sediments representative of the season autumn. The sediment core was collected with the BEEKER sediment corer at a water depth of 1.4m in January. The sediment core was segmented at 2cm interval and separated into 40 layers. The dating results of the sediment core have been introduced in the study of Guo et al. (2012). Surface sediments were collected elaborately to reflect seasonal variations of sediments. Surface sediments were taken repeatedly via a Peterson bottom grab, and passed through a 1mm filter screen with the washing distilled water into a container, in order to remove larger extraneous matters. When filtered grains completely deposited in the bottom of the container, filtered waters in the upper layer were saved in glass bottles and the sediment grains were saved in sealed bags, and then were transferred to a refrigerator with a temperature of -4 °C. Two replicate samples in each sampling site were collected in an area of 5m * 5m to guarantee the samples of surface sediments not being directly disturbed by human activities.

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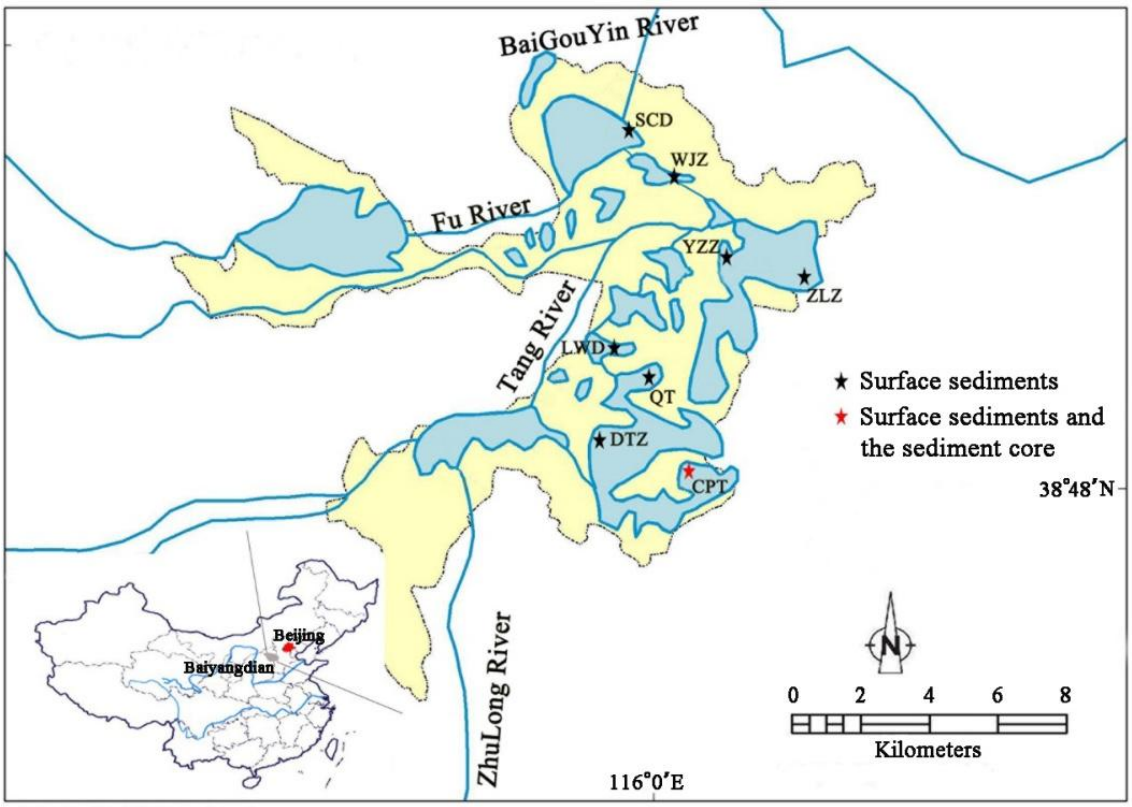

Figure 1. *Location of study area and distribution of surface sediments and the sediment core. Yellow patches denote the land; blue patches denote the body of water; blue lines denote water channels.*

Measurement of sediment properties

Environmental elements such as total organic carbon (TOC), the ratio of organic carbon to nitrogen (C/N), conductivity, pH, available phosphorus (AP), and grain size were selected to investigate the property of surface sediments in spring and summer. In winter, the frozen surface sediments were not easy to filter and the amount of obtained samples was limited. Two variables, namely, loss of ignition (LOI) and grain size, were measured to assess the lake productivity and hydraulic conditions in this season. The distribution of grain size is typically used to determine the intensity and turnover rate of water flow. TOC and LOI can demonstrate the accumulation rate of organic matters. C/N can differentiate the source of sedimentary materials. Conductivity and pH reflect the physiochemical environment of sedimentation. The abundance of phytoplankton may affect the concentration of AP.

We measured the initial wet weight of the surface sediments and dried these samples to determine the water content. TOC was measured using a High TOC-II analyser after a pre-treatment of 10% HCI on 0.5g dry sediments (Huang et al., 2004). Total organic nitrogen (TON) was determined by subtracting total inorganic nitrogen (TIN) from total nitrogen (TN) (Keeney et al., 1970), and then organic C/N was calculated. 5g dry samples were heated to 550 °C in a muffle furnace and were weighted to measure LOI. Conductivity and pH were measured with the 1:3 ratio of sediment-to-distilled water (Freeland et al., 1999). AP was measured in 100ml extractant with 0.2g dry surface sediments after a preliminary digestion (DePinto et al.,

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1981). A Winner 2008 laser particle size analyser was used to measure the grain size. The grain size was classified into clay (< 2 μm), silt (< 20 μm), and sand (< 200 μm). Each environmental element was measured twice. Approximately 0.5g dry samples of the surface sediments and each layer of the sediment core were taken for diatom identification. All sub-samples were placed in a 50 ml tube with H_2O_2 and HCl digestion in a hot water bath at 80 °C. We followed the procedure proposed by Moos et al. (2005). Thereafter all sub-samples were extracted using the floatation with heavy liquid $ZnBr_2$, and then dispersed in $C_{18}H_{29}NaO_3S$ solution to weaken the disturbance of the granule. Diatoms were mounted in synthetic resin mounting medium with a refractive index of 1.65 and were counted using a Nikon Ni50 microscope. More than 300 diatom frustules were enumerated per slide. Identification of diatoms followed the chrysophytax instruction in "The freshwater algae of China" (Qi, 1995; Qi, 2004; Shi, 2004; Li and Qi, 2010). The software C2 was applied to depict the distribution of diatoms (Juggins, 2003).

Seasonal effects of surface sediments

We used one-way ANOVA to show the seasonal effects of surface sediments. TOC and LOI of surface sediments had a significantly linear relation based on the study of Viguri et al. (2002). Therefore the value of LOI for winter samples can be converted into the value of TOC. One-way ANOVA of the variables grain size and TOC was conducted respectively with the MASS package of R. To reveal the spatial information and determine the principle variables in surface sediments, principle component analysis (PCA) was used on the surface sediments in spring and summer. This process was implemented with the pcaMethods package of R (Stacklies et al., 2007).

Response of diatom assemblages to environmental variables

The daily water levels in 2010 for different sampling sites were obtained from the local monitoring station. The seasonal water levels adopted the mean value of the daily water levels corresponding to each season. Historical water levels have been reconstructed using the indicative diatom species in the sediment core (Chen et al., 2013). According to the constructing time of the hydraulic projects on the upper reach of the lake and the dating results, the sediment core was separated into the relatively natural part (1827-1947 yr., lower layer: 80 - 14cm) and the human-disturbed part (1948 – 2008 yr., upper layer: 14 – 0cm) (Guo et al., 2012). The water levels fluctuated from 6.5 m to 9.5m during the period of 180 years (*Fig. A1*). To emphasize the impact of human activities on the response of diatom assemblages in surface sediments to water levels, the diatoms of summer samples were identified since supplementary water flowed into the lake from the reservoir in the upper reach between spring and summer. Ordination methods were used to determine the effect of environmental factors on sedimentary diatom distribution (Yang et al., 2008). Detrended correspondence analysis (DCA) was used to estimate the gradient length of the first axis of the diatom distribution. Linear analysis is generally applied in case the first axis has a gradient length of less than 3(Ter Braak and Smilauer, 2002). Diatom species in one sample with the maximum abundance less than 3% were not accounted for, since rare species had a significant impact on the ordination analysis and then was downweighted. Margalef richness index and Pielou evenness index, jointly reflecting the diversity of diatom assemblage, were calculated for all

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the sediment samples (Mucha et al., 2003). Margalef richness index emphasizes the overall abundance of diatoms, whereas Pielou evenness index effectively indicates the distribution of diatom assemblages. We used the redundancy analysis (RDA) with respect to the response of diatom assemblages to environmental variables. Forward selection and Monte Carlo permutation tests were used to determine the key variables affecting the distribution of diatom assemblages. Constraining one variable to the first axis with treating other variables as covariables was implemented to assess the independent power of each key variable. All the environmental variables involved in the ordination analysis were log-transformed ($log_{10}(x+1)$) to keep a similar scale. The ordination analysis was conducted with CANOCO version 4.5 (Ter Braak and Smilauer, 2002). To compare the results of the diatoms' response to the specific environmental variable in the spatial scale and in the temporal scale, we selected the diatom species simultaneously found in the surface sediments and the sediment core. Further we used grey correlation analysis to differentiate the response of individual diatom species to the specific environmental variable. The description of the method grey analysis can be found in Fu (2001). The species data and the environmental data have been dealt with a normalization in advance. We tested the sensitivity of the grey correlational coefficient ($\propto = 0.3, 0.4, 0.5, 0.6, 0.7$), and used the mean value and standard deviation (SD) to reflect the relation between diatom species and the specific environmental variable.

Results

Seasonal effects of surface sediments

The results show that sand fraction generally has a larger proportion in different seasons (*Table 1*). The percentage of clay ranges from 3.0% to 4.9%, especially for sites YZZ and CPT with values more than 10% with respect to winter samples, whereas this percentage in most areas is zero in spring and summer. The sand content of the eastward sampling sites is higher than that of the westward sampling sites in the northern part, in contrast this trend is opposite in the southern part. Silt content at site ZLZ experiences a dramatic increase of 12.1% from spring to summer, since this site is located near a water channel connecting to the upper reach of the lake. The ratio of silt to sand varies not significantly across seasons ($p_{\text{winter-spring}} = 0.221$; $p_{\text{winter-summer}} = 0.122$; $p_{\text{spring-summer}} = 0.345$). The water levels seem higher in the middle section of the lake than that in the north and south sections. The water levels show significant seasonal difference ($p_{\text{winter-spring}} = 0.017$ *; $p_{\text{winter-summer}} = 0.0074$ **; $p_{\text{spring-summer}} = 0.011$ *). The relation between the ratio of silt to sand and water level is closer in winter ($r^2 = 0.2$), but is weaker in spring ($r^2 = 0.08$) and summer ($r^2 = 0.0004$). TOC varies significantly comparing the spring samples with the summer samples ($p = 0.00769$ **), while this variable in winter indicates no significant difference in comparison with that in spring ($p = 0.68$) and in summer ($p = 0.18$). On the other hand, AP and C/N largely determine the property of surface sediments in spring, with the spindle values of 0.797 and 0.979 respectively. In contrast, AP and conductivity seem to be the key variables affecting the attribute of surface sediments in summer, with the spindle values of 0.904 and 0.930 respectively. The correlation between environmental variables in surface sediments slightly changes comparing the spring samples with the summer samples (*Fig.2*). Vigorous growing

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of algae stimulates a higher AP concentration at several sampling sites in summer than that in spring. Further the relation between C/N and TOC is similar in these two seasons, implying that aquatic plants in the lake, rather than terrestrial plants around the lake, determine the source of organic matter in the surface sediments.

Table 1. *Seasonal distribution of grain size, water level and TOC. Cl = Clay; Si = Silt; Sa = Sand; WL = Water Level*

Site	Winter					Spring					Summer				
	Cl %	Si %	Sa %	WL m	TOC %	Cl %	Si %	Sa %	WL m	TOC %	Cl %	Si %	Sa %	WL m	TOC %
CPT	13.7	60.8	25.6	6.7	0.8	0	20.1	78.9	6.8	2.4	0.7	22.0	73.1	6.1	2.3
DTZ	4.6	35.1	60.3	7.7	1.4	0	17.2	78.2	6.7	4.8	3.0	24.5	68.3	6.8	5
ZLZ	3.9	47.8	48.3	8.6	2.2	0	8.6	85.0	8.2	3.9	0.4	20.7	72.4	7.6	4.6
SCD	4.9	36.7	58.4	7.1	1.7	0	9.0	89.8	7.1	4	0	13.8	78.7	6.7	3.8
QT	3.0	30.1	67.0	7.2	0.9	0.7	18.7	77.4	7.8	2.8	0	12.6	84.1	6.9	2.4
LWD	4.2	41.1	54.7	8.5	1.5	2.2	18.2	78.7	8.6	1.9	0	17.3	80.3	7.9	1.7
YZZ	12.9	58.5	28.6	6.8	2.0	1.2	23.0	74.8	6.9	2.1	2.4	26.5	68.9	7.1	3.3
WJZ	4.1	43.9	52.0	6.8	1.4	0	17.6	81.9	6.9	4.2	0	17.0	80.1	6.2	3.5

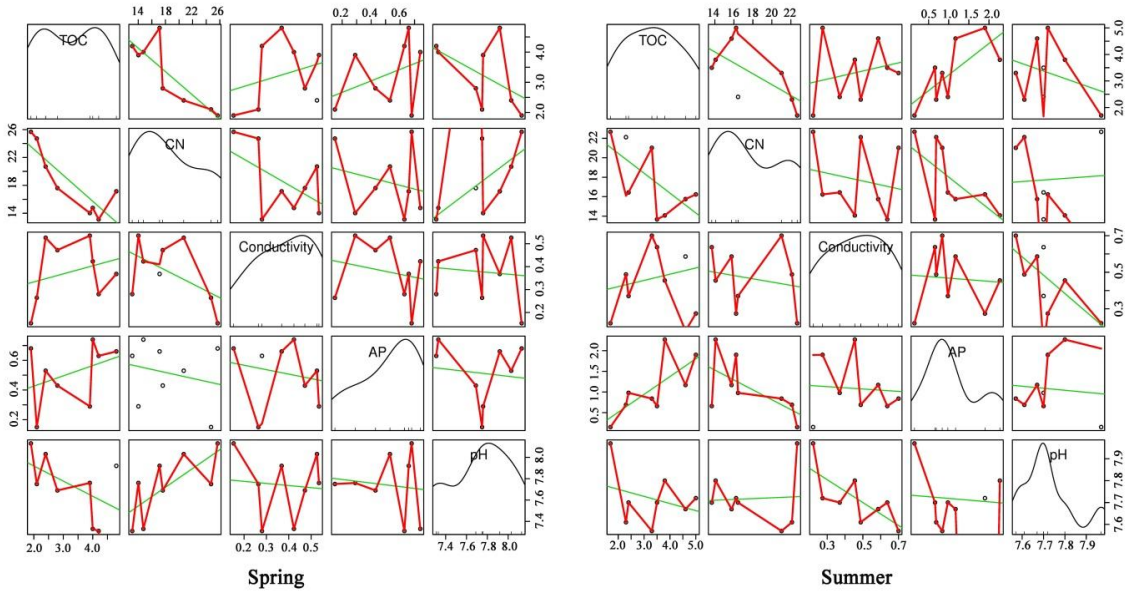

Figure 2. *Correlations between environmental variables in surface sediments in spring and summer. Diagonal line denotes the histogram of environmental variables in eight sampling sites; red dashed line denotes the movement locus of scatter points corresponding to eight sampling sites; green line denotes the fitting curve of two variables.*

Response of diatom assemblages to environmental variables

The dominant diatom species in surface sediments exhibits the disparities in different sampling sites (*Fig. 3*), with the benthic taxa being dominant, such as *Cymbella*, *Navicula*, *Gomphonema and Fragilaria*. The planktonic taxa, such as *Cyclotella meneghiniana* and *Actinocyclus*, account for a relatively large proportion

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of diatoms' total abundance in the sampling sites like SCD and YZZ. DCA ordination of surface sediments indicates that the genus *Gomphonema* prevails in the middle part of sampling sites, while diatom assemblages are dominated by the genus *Cymbella* for the southward sampling sites (*Fig.4*). The first axis of the DCA ordination explains 23.1% of the variation in the diatom distribution. Six environmental variables (water level, TOC, C/N, AP, pH, conductivity) jointly explain 51.2% of the variation of the diatom assemblages based on the RDA ordination. Redundant variables with higher values of the inflation factor (>10) are not accounted for in the further analysis. Water level, TOC and pH finally constitute the environmental group to explain the variance of the diatom distribution in surface sediments. RDA with one constrained axis indicates that pH, water level and TOC account for 13.1%, 9.7% and 8.8% of species variance respectively.

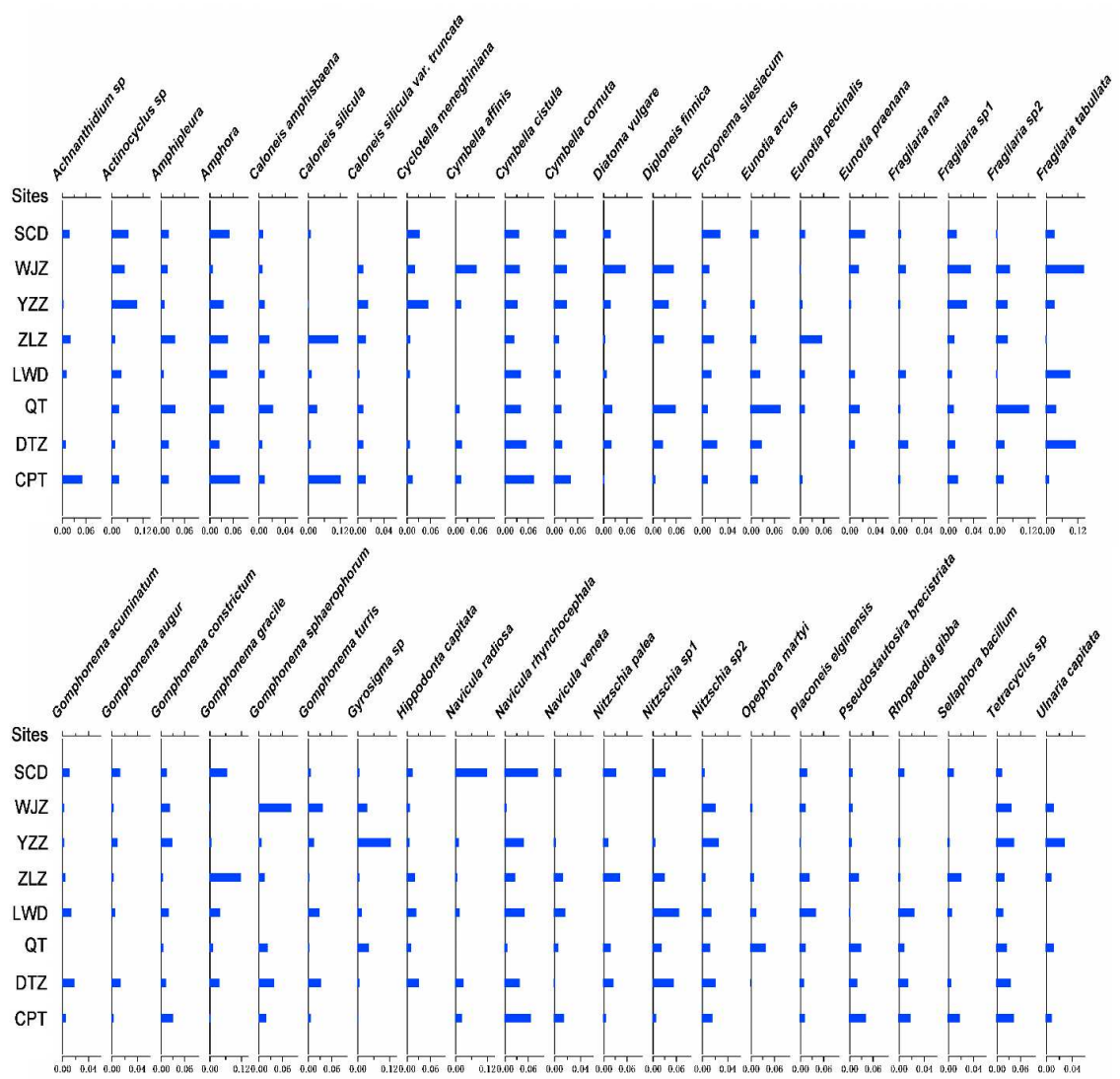

Figure 3. *Distribution of 42 diatom taxa in surface sediments. Abundance of diatom taxa in one sample is at least more than 1%.*

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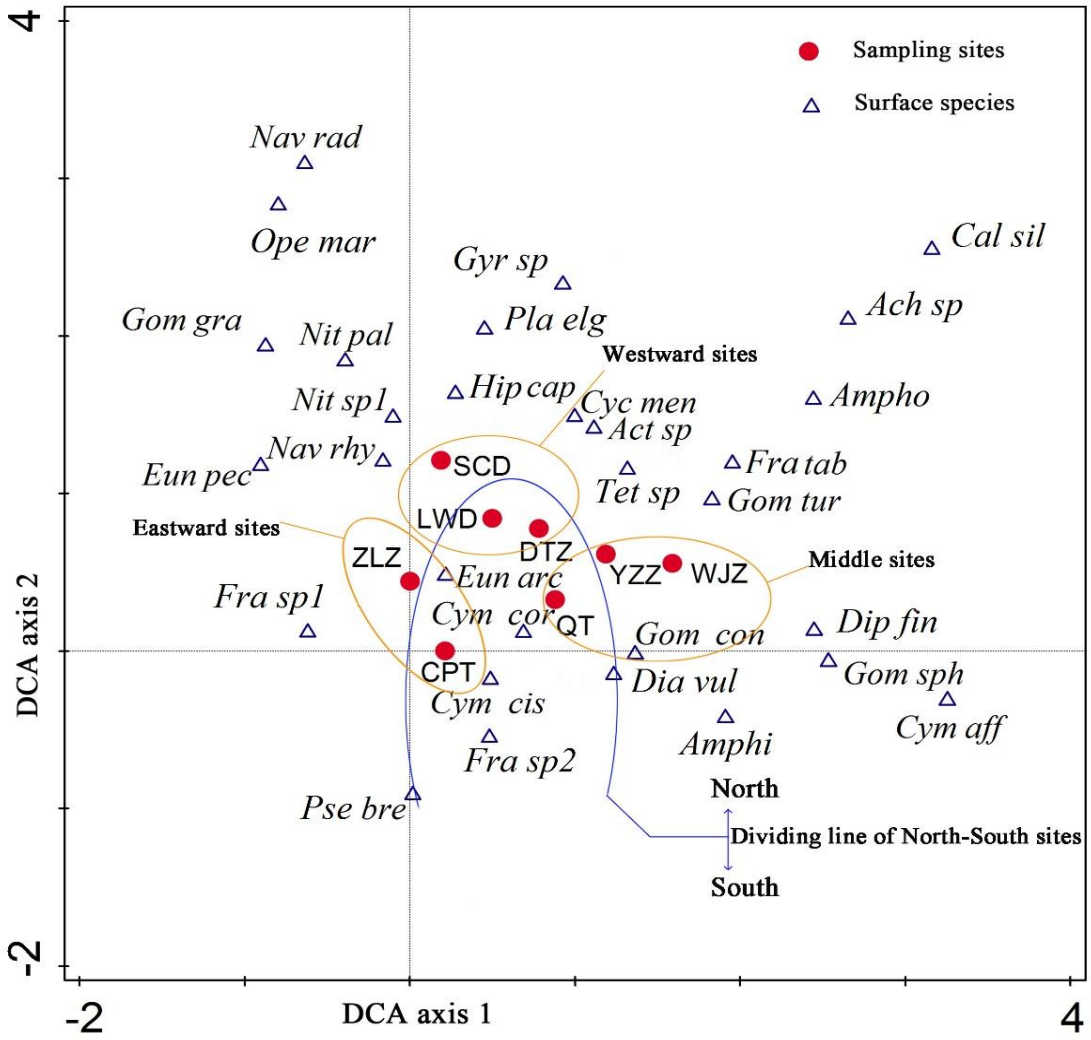

Figure 4. *DCA ordination of diatom assemblages in surface sediments. Abbreviation of the selected diatom species: Nav rad = Navicula radiosa; Ope mar = Opephora martyi; Cal sil = Caloneis silicula; Gyr sp = Gyrosigma sp; Ach sp = Achnanthidium sp; Pla elg = Placoneis elginensis; Gom gra = Gomphonema gracile; Nit pal = Nitzschia palea; Hip cap = Hippodonta capitata; Ampho = Amphora; Nit sp1 = Nitzschia sp1; Cyc men = Cyclotella meneghiniana; Act sp = Actinocyclus sp; Nav rhy = Navicula rhynchocephala; Fra tab = Fragilaria tabullata; Eun pec = Eunotia pectinalis; Tet sp = Tetracyclus sp; Gom tur = Gomphonema turris; Eun arc = Eunotia arcus; Fra sp1 = Fragilaria sp1; Cym cor = Cymbella cornuta; Dip fin = Diploneis finnica; Gom con = Gomphonema constrictum; Gom sph = Gomphonema sphaerophorum; Dia vul = Diatoma vulgare; Cym cis = Cymbella cistula; Cym aff = Cymbella affinis; Amphi = Amphipleura; Fra sp2 = Fragilaria sp2; Pse bre = Pseudostautosira brecistriata.*

The distribution of environmental variables in the sediment core has been introduced in the study of Guo et al. (2012). Three selected environmental variables, TOC, pH, and water level, explain 12.5% of the total variance of the diatom assemblages in the sediment core. The explained variability by environmental variables in the sediment core is smaller than that in surface sediments, since the number of diatom taxa with a zero value in the sediment core is larger, contributing to the relatively poor performance of spe-

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cies transformation. Water level, pH and TOC independently capture 6.4%, 5.9%, 2.4% of the variance of the diatom assemblages in the sediment core respectively. The inflation factor of water level is smaller than those of other two variables, implying that water level is less affected by the collinear effect and more independently accounts for the distribution of diatom assemblages. In addition, the second axis of RDA ordination clearly divides the sediment core into two parts along the water level gradient (*Fig.5*).

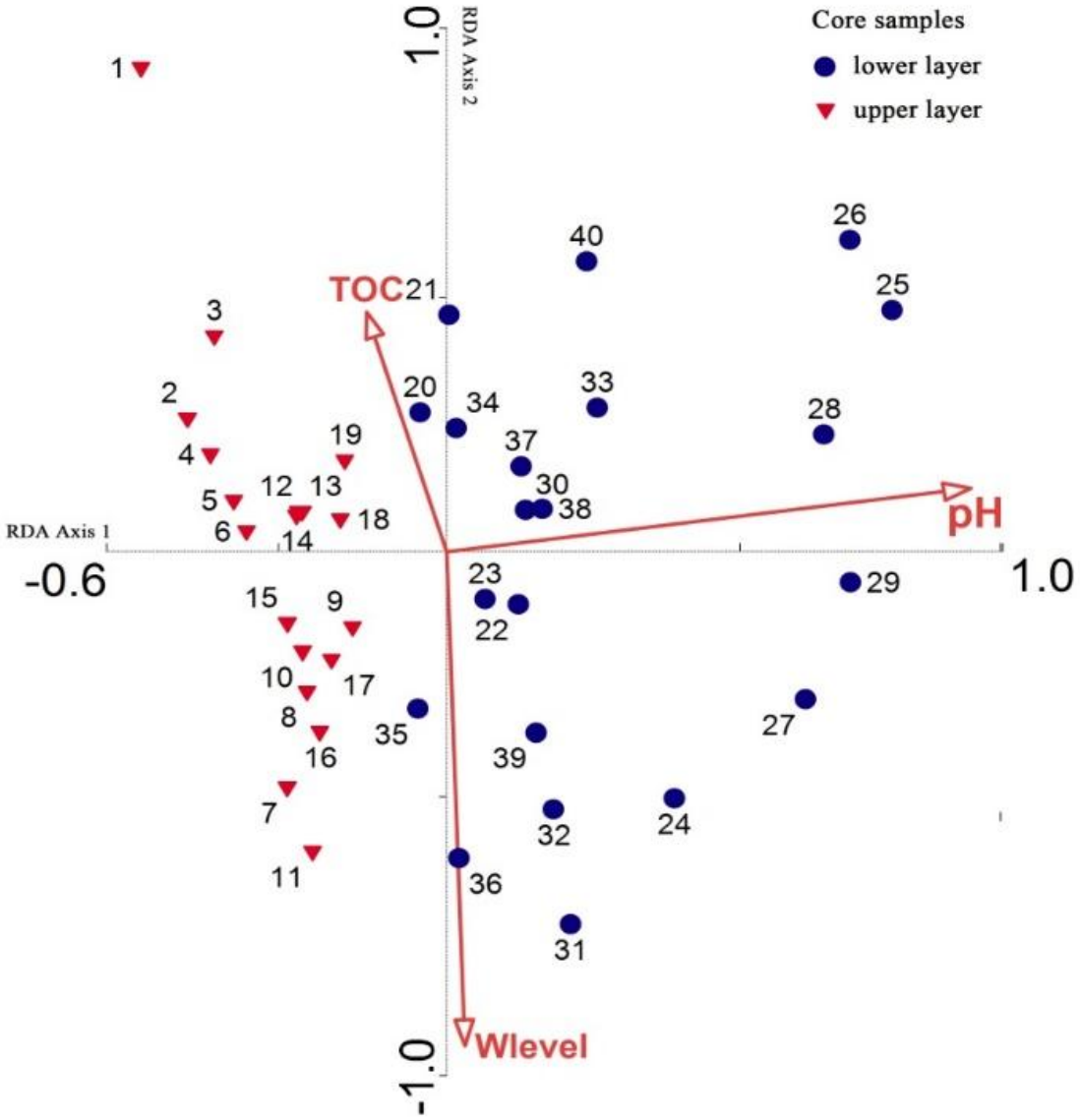

Figure 5. *RDA ordination showing the relation between sample scores and the selected environmental variables in the sediment core*

Spatio-temporal response of diatom assemblages to water level

Thirty-four diatom species are simultaneously found in surface sediments and in the sediment core (*Fig. A2*). Twenty-five diatom species satisfy the principle of ordination. As shown in *Table 2*, water level has a closer relation with the distribution of diatom

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assemblages in the lower layer of the sediment core. Water level accounts for more variability of diatom data in the sediment core compared with surface sediments based on the eigenvalues of the first two axes. The impact of water level on the richness and evenness of diatom species is not significant with respect to surface sediments and the upper layer of the sediment core, but this impact shifts in the lower layer of the sediment core (*Fig.6*). In addition, the distribution of diatom species seems more dispersed in surface sediments and in the upper layer of the sediment core, which may be attributed to the spatial isolation of diatom species and the impact of human disturbance on the sediments. The planktonic taxa, such as *Cyclotella meneghiniana* and *Actinocyclus sp*, closely correlate with water level. In contrast, diatom species, such as *Cymbella cistula* and *Amphora*, show a relatively weak relation with water level. This relation experiences a shift for the diatom taxa, such as *Navicula radiosa, Navicula rhynchocephala* and *Nitzschia palea*, with respect to three sediment scenarios.

Table 2. *RDA ordination on surface sediments, the upper layer and the lower layer of the sediment core*

Scenario Index	Surface	Upper	Lower
Eigenvalue (First axis)	0.156	0.327	0.366
Eigenvalue (Second axis)	0.213	0.303	0.278
Pseudo-F	1.1	2.4	8.5
P	0.344	0.046	0.004

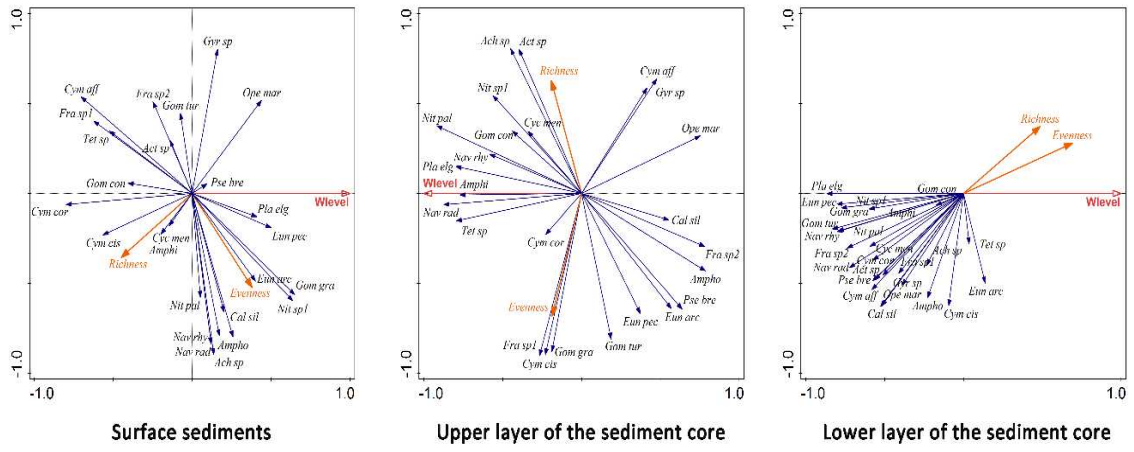

Figure 6. *Response of diatom assemblages to water level in three sediment scenarios. Abbreviation of diatom taxa has been demonstrated in Fig. 4.*

Discussion

Lake sediment, as a supporter registering environmental changes, can provide us with high resolution records of variations in temporal and spatial scales. The multi-proxies analysis of sediments improves our knowledge of the correlation between environmental variables and aquatic plants. The investigation of surface sediments

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shows the seasonal effects of sediment property in Lake Baiyangdian. The results of the ordination indicate that water level largely account for the distribution of diatom assemblages in surface sediments and in the sediment core. Spatial isolation and human activities affect the microhabitat of diatoms in the lake system, thereby altering the diversity of diatoms and the response of the diatom assemblages to water level. Assessing the spatio-temporal feature of water level and its impact on the distribution of diatom assemblages in a single lake can improve our knowledge of environmental changes in the specific lacustrine ecosystem, in comparison with the studies using a group of lakes.

Seasonal effects of surface sediments

The composition of grain size does not show the significantly seasonal effects in Lake Baiyangdian since sand is an essential component accounting for the variations of grain size. Clay and silt content in winter reached its maximum for the entire year. Water flow slowed down to a standstill and human disturbance on the body of water is weak during the frozen period. In contrast, the silt contents in spring and summer are higher since the body of water may experience a strong turbidity as a result of the ice melting and frequent rainfalls. Silt content is typically used to describe the flow intensity and turbidity of the medium (Schmieder et al., 2004). In addition, the distribution of grain size in each season matches the topography of the water table in the lake and potentially indicates the direction of water flow.

Water level has a significant seasonal effect but is weakly related to the composition of grain size, which is contrary to the findings of Dusini et al. (2009) that grain size and water level were closely correlated in shallow lakes. Water level, as a static variable, sometimes does not promptly response to the variations of the flow intensity. Namely higher water levels may not equate with stronger hydrodynamics. Bachmann et al. (2000) proposed a concept of dynamic lake parameters based on the lake area and water depth. The deposition of surface sediments may be driven by wind and wave in case the dynamic lake parameter is more than 0.8. The dynamic parameters of the lake in spring and summer are larger than this value, implying that the seasonal wind and wave movements may determine the water-level fluctuation and the hydrodynamic of the lake.

Lake productivity reflected by the variable TOC shows the significant seasonal effects with the vigorous growth of aquatic plants. This result could be further demonstrated via the higher concentration of available phosphorus in summer. However the value of TOC in winter is relatively low, implying that aquatic plants are weakly active in this season. This result is in line with the findings that organic matter content within surface sediments in shallow lakes is higher in summer owing to the growth of phytoplankton (Yu et al., 2009).

Response of diatom assemblages to water level

Lake Baiyangdian has no definite shallow-to-deep water transects. The water level disparity in modern times is depicted alternatively in a way of spatial distribution. In addition, the spatio-temporal impact of water level on diatom assemblages is implemented in a unique lake, which is different from traditional studies examining the correlation between environmental variables and diatom assemblages based on a group of lakes (Hassan et al., 2009; Yang et al., 2008; Werner and

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Smol, 2005). An integration homogenizes the environmental differences between lakes and may conceal the peculiar interactions between aquatic plants and environmental variables in each lake. Higher water levels increase the abundance of planktonic taxa, e.g. *Cyclotella* and *Actinocyclus*, while benthic types, e.g. *Nitzschia* and *Cymbella*, generally dominate in a shallower water level. This finding is similar with that in other shallow lakes (Shinneman et al., 2010; Laird and Cumming, 2008).

It is found that pH, water level and AP largely account for the distribution of diatom assemblages in surface sediments. Li et al. (2010) found that pH and total phosphorus were the determining variables affecting the distribution of diatom assemblages in the body of water in Lake Baiyangdian. Further the diatom taxa living in the body of water are similar to that found in surface sediments, implying that the interactions between the body of water and surface sediments homogenize the response of diatom assemblages to environmental variables.

The spatio-temporal fluctuations of water level affect the distribution of diatom assemblages in Lake Baiyangdian. On one hand, the spatial partition facilitates the occurrence of new diatom species and species adaption to diverse habitats. On the other hand, the construction of hydraulic projects in the upper reach significantly shifts the hydrodynamics of the lake, thereby potentially altering the interspecific relation between diatom species and the response of diatom assemblage to water level. The impact of human disturbance on the response of diatom assemblages to hydrological processes varies in different spatial scales (Donohue et al., 2010). We explicitly aim at one dimension of human impact, namely the construction of hydraulic projects leading to the changes in water dynamics of the shallow lake. However, we are well aware that another aspect of human impact on the lake ecosystem (eutrophication) also plays an important role in the composition of diatom assemblages, which is not accounted for in this study.

The quantitative analysis of the response indicates that spatial isolation promotes a closer relation of 60% diatom species with water level, comparing the surface sediments with the upper layer of the sediment core (*Table 3*). Human disturbance accounts for 40% enhancive correlations between water level and diatom species, comparing the upper layer with the lower layer of the sediment core. The richness and evenness indices of diatom assemblage relate more closely to water level with respect to the lower layer of the sediment core, implying that spatial isolation (surface sediments) and diverse hydrodynamics (the upper layer of the sediment core) weaken the response of the whole diatom community to water level. Grey correlation analysis can reveal the quantitative relation between diatom species and water level, but may lose some ecological information since this method uniquely concentrates on one environmental variable. We might obtain more using less quantitative but more ecologically meaningful approach (Rioual et al., 2002). Defining an unambiguous breakpoint to differentiate the degree of human disturbance in shallow lakes is also a tough question. This study proposes a criterion of human disturbance on the lacustrine system based on the specific hydrological condition of the lake. The futural expectation is to interpret the data more securely integrating more biotic variables.

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Table 3. *Correlational coefficient between diatom distribution and water level for surface sediments, upper and lower layer of sediment core. Abbreviation of diatom species refers to Fig. 4.*

		Surface sediments		Core (upper layer)		Core (lower layer)	
		mean	SD	mean	SD	mean	SD
Species	*Fra sp2*	0.756	0.038	0.802	0.025	0.613	0.068
	Eun arc	0.604	0.057	0.578	0.057	0.660	0.059
	Act sp	0.687	0.046	0.583	0.066	0.677	0.061
	Cal sil	0.676	0.051	0.632	0.057	0.597	0.069
	Nit sp1	0.590	0.063	0.662	0.051	0.666	0.060
	Eun pec	0.667	0.059	0.628	0.054	0.648	0.057
	Cym aff	0.635	0.054	0.621	0.061	0.607	0.064
	Ampho	0.702	0.054	0.595	0.062	0.685	0.056
	Pse bre	0.710	0.043	0.566	0.064	0.677	0.055
	Cym cis	0.699	0.044	0.643	0.053	0.709	0.056
	Gom tur	0.693	0.047	0.552	0.057	0.658	0.063
	Ope mar	0.509	0.066	0.691	0.042	0.553	0.074
	Nav rad	0.603	0.057	0.662	0.054	0.577	0.068
	Fra sp1	0.632	0.056	0.637	0.054	0.585	0.069
	Cyc men	0.762	0.034	0.582	0.062	0.666	0.062
	Tet sp	0.587	0.057	0.708	0.048	0.657	0.064
	Nit pal	0.702	0.056	0.632	0.054	0.673	0.058
	Cym cor	0.669	0.046	0.616	0.066	0.705	0.057
	Gom gra	0.685	0.050	0.739	0.044	0.662	0.058
	Gyr sp	0.591	0.065	0.733	0.035	0.595	0.066
	Amphi	0.686	0.051	0.693	0.058	0.614	0.068
	Pla elg	0.605	0.067	0.569	0.055	0.599	0.071
	Nav rhy	0.610	0.054	0.593	0.055	0.596	0.067
	Gom con	0.590	0.062	0.643	0.059	0.729	0.054
	Ach sp	0.648	0.054	0.521	0.068	0.636	0.063
Assemblage	Richness	0.620	0.054	0.758	0.031	0.644	0.063
	Evenness	0.584	0.056	0.905	0.011	0.578	0.066

Conclusion

This study assesses the spatio-temporal response of the diatom assemblages to water level fluctuations based on the sedimentary biotic and abiotic variables. Accounting for this response using the historical records allows us to better understand the impact of human activities on lake ecosystems. Therefore, approaches to quantify the relationship between aquatic species and environmental variables deserve further attention, in order to preserve the biodiversity and assess environmental changes in the shallow lakes. A promising way is to investigate the diatom communities in lake water and construct the relation with the sedimentary diatom assemblages.

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APPENDIX

We investigated the diatom assemblages in the sediment core and reconstructed the water levels based on the indicative diatom species (Chen et al., 2013).

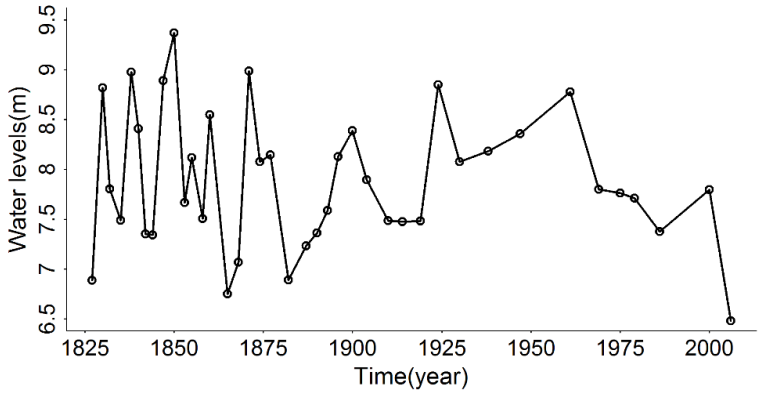

Figure A1. *Water levels in the Lake Baiyangdian from 1827 to 2008*

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Thirty-four diatom species are simultaneously found in surface sediments and in the sediment core. Here we show the distribution of these co-occurred diatom species in the sediment core.

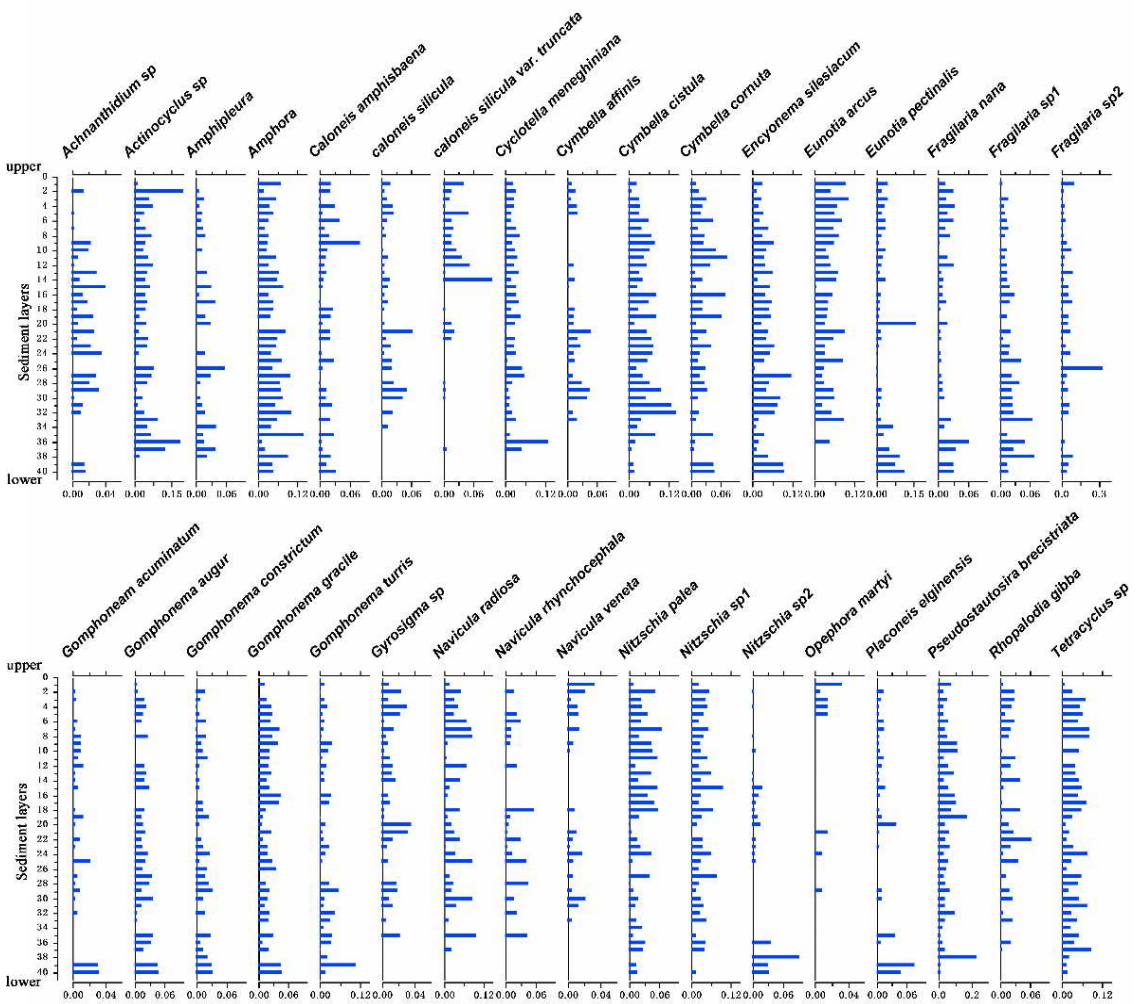

Figure A2. *The distribution of the co-occurred diatom species in the sediment core*

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ASSESSMENT OF CARBON STOCK OF TREE VEGETATION IN THE KOLLI HILL FOREST LOCATED IN INDIA

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Abstract. Forest ecosystems covering approximately 30 % of the terrestrial area of the earth play a significant role in the atmospheric carbon cycle that influence global warming and climate change. Assessment of carbon stock in forest vegetation is necessary for atmospheric carbon mitigation plans. The present study aimed to assess the total carbon stock in tree vegetation of Kolli hill (KH), a reserve forest located in India. The land area of KH (508 km^2) was parted by 6.25 × 6.25 km^2 grids. In each grid, a transect of 0.5 ha (5 m × 1000 m) was laid randomly, and all live trees greater than or equal to 30 cm girth (=9.55 cm diameter) measure at breast height were sampled. Carbon stock of each tree was determined by non-destructive method. The carbon stock estimated for KH forest was 73.7±13.6 tC/ha. Maximum carbon stock was shared by *Alseodaphne semicarpifolia* var. *semecarpifolia* (14 %) among 157 species and Lauraceae (19 %) among 49 families recorded at KH. Statistical analysis revealed that the carbon stock values varied significantly among the eleven tree size classes (ANOVA, $F_{(10,187)} = 4.439$, $p<0.001$) and among the three major forest types ($F_{(2,15)} = 6.101$, $p<0.05$) recognized. Regression analysis was also performed to test the relationship of carbon stock with tree density, species richness and altitude. The present study provides valuable data useful for better management and monitoring of KH forest with regard to tree carbon storage in mitigation of global warming and climate change.
Keywords: c*arbon stock; Kolli hill; India; tropical forest; tree vegetation*

Introduction

Carbon dioxide, one of the greenhouse gases, has been increasing in concentration due to anthropogenic activities worldwide, and elevates the earth's average temperature by greenhouse effect. Increase in temperature causes global warming and climate change. It has been predicted that the mean global temperature will increase by 1.1°C to 6.4°C by the year 2100 (IPCC, 2007). Atmospheric carbon concentration was around 270 ppm at the beginning of industrial revolution, it has crossed 400 ppm by 2015 (NOAA, 2015), and scientists have predicted by 2070 carbon level will reach up to 500 ppm (Jackson et al., 2014). Climate change due to increase in carbon emissions leads to great challenges for carbon mitigation strategies, besides socio-economic, biological problems (Sicard & Dalstein-Richer, 2015) and origin of new catastrophic diseases (WHO, 2015). In recent years, all possible steps have been taken by the developed nations in order to plan for carbon mitigation, management and policy actions. Most industrialized counties have signed the Kyoto Protocol to reduce their carbon outputs, carbon tax and subsidy systems have been developed in support of carbon mitigation targets (Cao et al., 2010).

Forest ecosystems cover approximately 30 % of the terrestrial area of the world (Muraoka et al., 2015), and they are greatly recognized as important elements of global carbon as well as various other greenhouse gases that are believed to considerably affect climate (Teobaldelli et al., 2009). Trees exchange carbon dioxide with the atmosphere through biological processes and act as a major carbon sink by stocking carbon as fixed

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biomass, hence, assessment of tree carbon stock in forest systems is necessary to understand the potential of forests as carbon sinks.

Carbon stock of a tree is determined by its biomass. Tree biomass can be quantified using both destructive and non-destructive sampling methods. Destructive sampling method involves felling of trees is mostly adopted for plantation forests. Non-destructive sampling method, widely used for natural forests, involves estimation of biomass from forest inventory data by using either biomass equations (BE) or biomass expansion factors (BEF), conversion factors (wood density) or biomass conversion and expansion factors (BCEF). BE requires tree level data like diameter at breast height or additionally height, age, etc., BEF needs volume data from forest inventory and BCEF is a combination of the first two factors (Teobaldelli et al., 2009).

Although, many studies have been done to quantify the forest carbon stocks worldwide, there are still some forest systems which remain unexplored in India. The present study was undertaken in Kolli hill (KH) forest with the main objective to assess the carbon stock (tC/ha) of tree vegetation in the forest system. This paper also presents the analysis on carbon stocks by different tree species, families, forest types, and stem size classes. Further, an attempt was also made to discuss the relationship of tree carbon stock with species richness, density and altitude.

Materials and methods

The present study site KH is located in India, between latitudes 11°11.0' - 11°28.0' N and longitudes 78°17.0 - 78°29.0' E *(Fig. 1)*. The study site covering about 508 km^2 of land area falls under national reserve forest category consists of varied metamorphic rocks, and red lateritic soil. The site comes under tropical climate zone with four seasons: summer (late February to June), pre-monsoon (July to August), monsoon (September to November), post monsoon (December to mid February). The mean annual temperature for the past 20 years (data obtained from the regional meteorological center) is 28.3°C, and the rainfall is 1058mm per year.

The complete study site was parted by 6.25 km × 6.25 km grids, and that summed up to 18 grids. In each grid, a transect of 0.5 ha (5 m × 1000 m) was laid randomly, to facilitate sampling each transect was subdivided into fifty 5 m × 20 m (10 sq. m) quadrats, and all live trees greater than or equal to 30 cm girth (9.55 cm diameter) measure at breast height (1.37 m from the ground level) were sampled (Pragasan & Parthasarathy, 2010). Stem girth measure of each tree was noted using measuring tape. A sum of 3824 trees representing 157 species in 49 families was recorded from the 18 transects (900 quadrats) sampled. Tree density, species richness was calculated, and altitude was noted for each transect. Density was determined as the number of individuals per unit area: $Di = ni/A$, where Di is the density of species i, ni is the total number of individuals recorded for species i, and A is the total area sampled. Density for each transect was calculated as the sum of the densities of all the species ($\sum D$), and expressed as individuals/ha. Species richness was determined as the numbers of different tree species recorded per transect and expressed as species/transect. Altitude was noted using global positioning system (GPS Garmin) and expressed as m asl.

Carbon stock of each tree was calculated as 50 % of its biomass following Timilsina et al., 2014 and others (Atjay et al., 1979; Brown & Lugo, 1982; Dixon et al., 1994; Takimoto et al., 2008; Bhat & Ravindranath, 2011; Mohanraj et al., 2011). Tree biomass was calculated as sum of its aboveground biomass and below ground biomass.

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Aboveground biomass was calculated using BE method formulated by Brown et al. (1989), adopted for tropical species: $Y = a - bX + cX^2$, where Y is aboveground biomass in kg, X is stem diameter at breast height in cm, and a, b and c are constant values 34.4703, 8.0671 and 0.6589, respectively. Stem diameter of each tree was calculated from its girth measure at breast height: $d = C/\pi$, where d is stem diameter in cm, C is stem girth measure at breast height in cm, and π is an universal constant value 3.14. Below ground biomass was considered as 15% of the aboveground biomass as adopted by Miria & Khan, 2015 and others (MacDicken, 1997; Alamgir & Al-Amin, 2008).

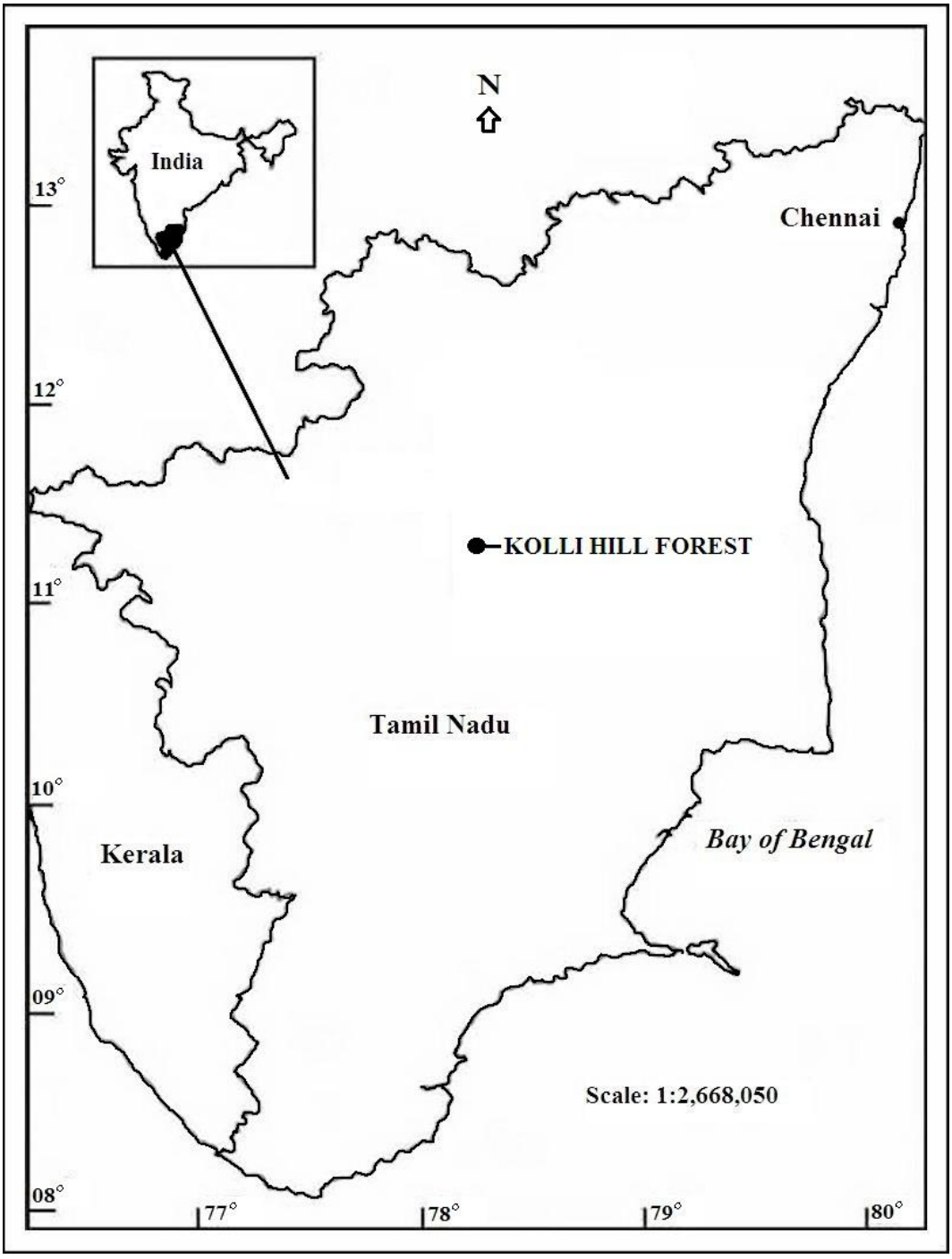

Figure 1. *Map showing the location of the study area, Kolli hill forest in India*

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Transects (n=18) sampled from the study site were categorized into, three forest types semi-evergreen (n=11), mixed deciduous (n=3) and scrub (n=4) forests (based on vegetation characters), three altitudinal ranges low altitude (less than 500 m asl, n=5), medium altitude (500-1000 m asl, n=7) and high altitude (above 1000 m asl, n=6). Trees (n=3824) sampled from the 18 transects were categorized (based on stem girth measure) into eleven stem size classes 30-60cm class, 60-90cm, 90-120cm, 120-150cm, 150-180cm, 210-240cm, 240-270cm, 270-300cm, 300-330cm and greater than 330cm.

Tree carbon stock (tC/ha) for each forest type, altitudinal range, stem size class was determined, and analysis of variance (ANOVA) was used to test the significance of variation in carbon stock among forest types, altitudinal ranges and stem tree size classes. Regression analysis was performed to test the relationship of carbon stock (tC/ha) with tree density (individuals/ha), species richness (species/transect) and altitude (m asl).

Results

Tree density and species richness

Tree density was recorded as low as 278 individuals/ha for Transect17 to a high of 632 individuals/ha for Transect4 *(Table 1)*, and the mean value for the 18 transects was 425±25 individuals/ha (±S.E.). Species richness was recorded minimum (10 species/transect) for Transect17 and maximum (42 species/transect) for Transect2 *(Table 1)*, and the mean value for the 18 transects was 27±2 species/transect.

Table 1. *Tree carbon stock estimated for the eighteen 0.5ha transects sampled from the Kolli hill forest located in India. SE-semi-evergreen; MD-mixed deciduous; SB-scrub*

Transect	Forest type	Altitude (m asl)	Density (individuals/ha)	Species richness (species/transect)	Carbon stock (tC/ha)
Transect1	SE	1128	590	33	141.7
Transect2	SE	973	586	42	204.5
Transect3	SE	1127	414	24	125.6
Transect4	SE	797	632	38	104.9
Transect5	SE	1301	522	31	173.6
Transect6	SE	912	294	42	70.7
Transect7	SE	1274	330	29	48.8
Transect8	MD	587	452	36	26.6
Transect9	SE	1100	370	17	72.5
Transect10	SE	1132	490	18	66.6
Transect11	SE	994	374	29	108.6
Transect12	SE	427	474	14	21.2
Transect13	SB	514	466	27	23.0
Transect14	MD	353	384	30	48.9
Transect15	SB	305	352	23	41.2
Transect16	SB	577	326	17	11.6
Transect17	SB	384	278	10	10.0
Transect18	MD	337	314	27	27.1

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Carbon stock

Tree carbon stock was recorded minimum 10.0 tC/ha for Tansect17 and maximum carbon value 204.5 tC/ha was observed for Transect2 (*Table 1*), and the mean carbon value for the 18 transects sampled was 73.7±13.6 tC/ha.

Forest type

Among the three forest types recognized, carbon stock of semi-evergreen forest (103.5±16.6 tC/ha) was found three folds greater than the mixed-deciduous and almost five folds greater than scrub forests *(Fig. 2)*. The carbon stock (tC/ha) value among the forest types varied significantly (ANOVA: $F_{(2,15)} = 6.101$, $p<0.05$).

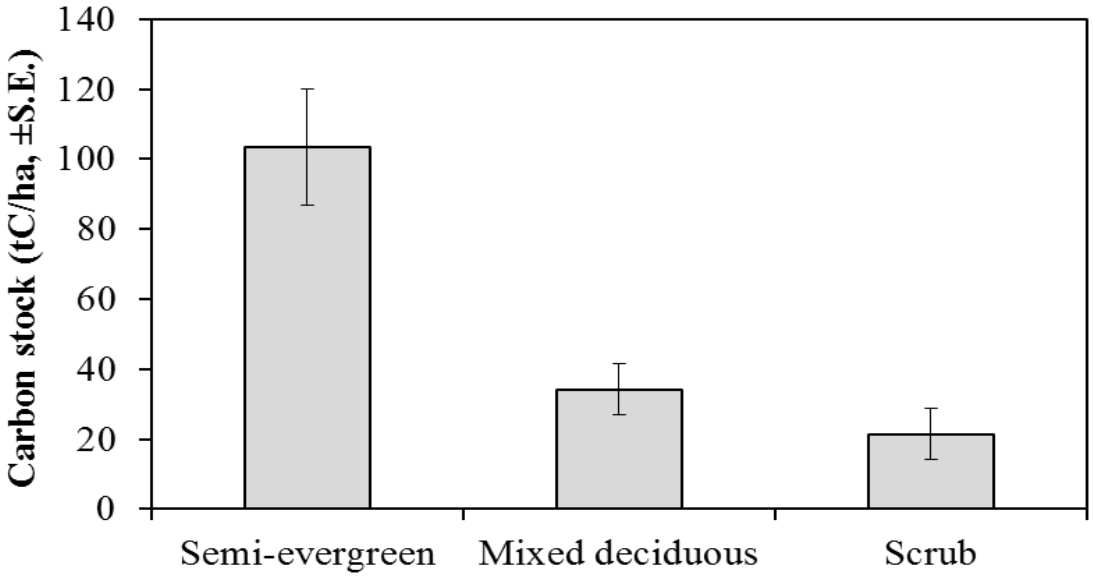

Figure 2. *Comparison of carbon stock between the three major forest types*

Altitudinal gradient

Among the three altitudinal ranges classified, carbon stock was recorded maximum for high altitude range (104.8±20.1 tC/ha) followed by medium (78.6±25.7 tC/ha) and low altitude range (29.7±7.0 tC/ha). The carbon stock (tC/ha) value among the three altitudinal ranges did not vary significantly (ANOVA: $F_{(2,15)} = 2.871$, $p>0.05$).

Stem size class

Of the total eleven stem size classes recognized, 120-150cm class recorded maximum carbon value (5.9±1.2 tC/ha), followed by 90-120cm class, 60-90cm, 180-210cm and 210-240cm class *(Fig. 3)*. ANOVA revealed a significant variation in carbon stock value among the 11 stem size classes ($F_{(10,187)} = 4.439$, $p<0.0001$).

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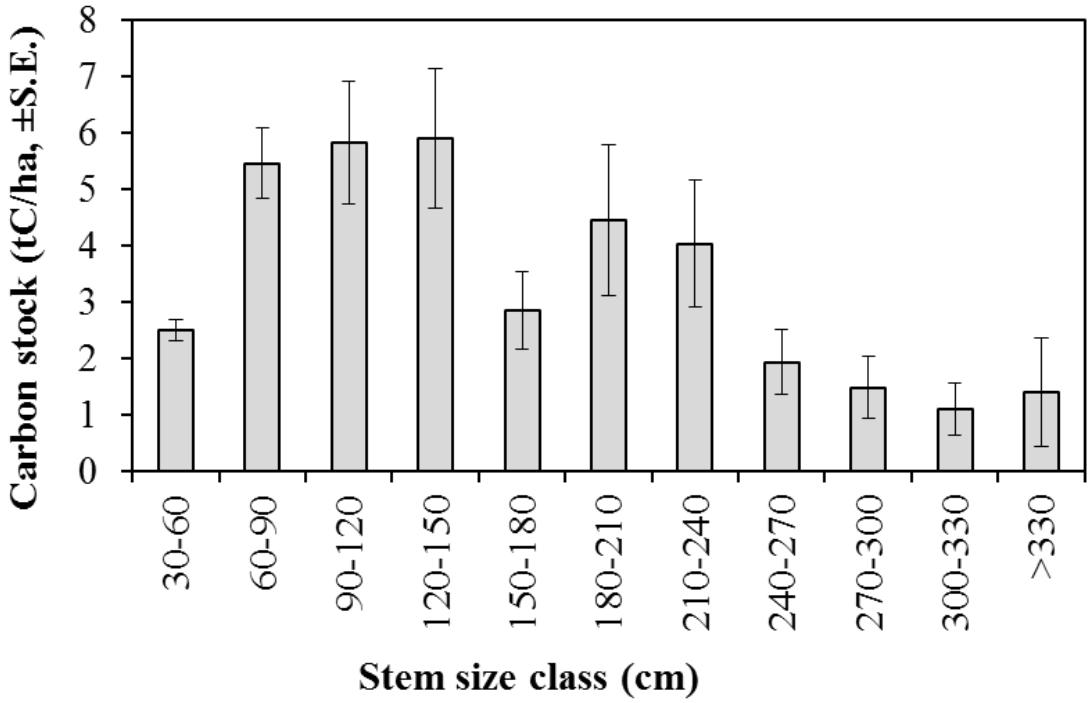

Figure 3. *Distribution of carbon stock among the eleven stem size classes*

Species

Out of the 157 species recorded, the total carbon stock was found maximum (92.34 tC/9ha, 14 %) for *Alseodaphne semicarpifolia* var. *semecarpifolia* followed by *Syzygium cumini, Memecylon edule*, *Canarium strictum* and *Mangifera indica (Table 2)*. The average carbon stock (tC/tree) was recorded maximum for *Sterculia foetida* (1.89 tC/tree) followed by *Ficus amplissima*, *Ficus beddomei*, *Mitragyna parvifolia* and *Ficus benghalensis (Table 2)*.

Table 2. *Carbon stock of tree species recorded from the Kolli hill forest, India*

Species (Family)	Number of individuals (for 9 ha)	Total carbon stock (tC/9ha)	Average carbon stock (tC/tree)
Alseodaphne semicarpifolia Nees var. *semecarpifolia* (Lauraceae)	128	92.34	0.72
Syzygium cumini (L.) Skeels (Myrtaceae)	137	69.36	0.51
Memecylon edule Roxb. (Melastomataceae)	654	44.41	0.07
Canarium strictum Roxb. (Burseraceae)	37	34.08	0.92
Mangifera indica L. (Anacardiaceae)	27	24.27	0.90
Neolitsea scrobiculata (Meisner) Gamble (Lauraceae)	134	22.90	0.17
Prunus ceylanica (Wight) Miq. (Rosaceae)	34	22.78	0.67
Artocarpus heterophyllus Lam. (Moraceae)	24	16.91	0.70
Ficus amplissima J.E. Smith (Moraceae)	9	14.69	1.63
Ficus microcarpa L.f. (Moraceae)	13	14.11	1.09

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Tamarindus indica L. (Caesalpiniaceae)	20	13.46	0.67
Anogeissus latifolia (Roxb. ex DC.) Wall. ex Guill. & Perr. (Combretaceae)	33	12.40	0.38
Myristica dactyloides Gaertn. (Myristicaceae)	31	12.25	0.40
Elaeocarpus serratus L. (Elaeocarpaceae)	44	11.76	0.27
Commiphora caudata (Wight & Arn.) Engler (Burseraceae)	75	11.75	0.16
Moringa concanensis Nimmo ex Gibs. (Moringaceae)	50	11.45	0.23
Ficus beddomei King (Moraceae)	8	11.18	1.40
Vitex altissima L.f. (Verbenaceae)	51	10.33	0.20
Euphorbia antiquorum L. (Euphorbiaceae)	345	9.80	0.03
Mitragyna parvifolia (Roxb.) Korth. (Rubiaceae)	7	9.79	1.40
Diospyros ebenum Koen. (Ebenaceae)	39	9.42	0.24
Bischofia javanica Blume (Bischofiaceae)	21	9.35	0.45
Ficus mollis Vahl (Moraceae)	13	8.75	0.67
Scolopia crenata (Wight & Arn.) Clos (Flacourtiaceae)	37	8.09	0.22
Gyrocarpus asiaticus Willd. (Hernandiaceae)	72	7.59	0.11
Celtis philippensis Blanco (Ulmaceae)	90	6.58	0.07
Nothopegia heyneana (Hook.f.) Gamble (Anacardiaceae)	53	6.43	0.12
Albizia amara (Roxb.) Boivin (Mimosaceae)	175	6.37	0.04
Ficus benghalensis L. (Moraceae)	5	5.60	1.12
Pterocarpus marsupium Roxb. (Papilionaceae)	20	5.58	0.28
Terminalia bellirica (Gaertn.) Roxb. (Combretaceae)	5	5.29	1.06
Memecylon grande Retz. (Melastomataceae)	54	4.94	0.09
Premna tomentosa Roxb. (Verbenaceae)	23	4.88	0.21
Chloroxylon swietenia DC. (Flindersiaceae)	54	4.50	0.08
Litsea deccanensis Gamble (Lauraceae)	25	4.46	0.18
Canthium dicoccum (Gaertn.) Teijsm. & Binn. var. *umbellata* (Wight) Sant. & Merch. (Rubiaceae)	34	4.35	0.13
Sterculia foetida L. (Sterculiaceae)	2	3.78	1.89
Symplocos cochinchinensis (Lour.) Moore (Symplocaceae)	21	3.54	0.17
Chrysophyllum roxburghii G.Don (Sapotaceae)	4	3.44	0.86
Diospyros barberi Ramaswami (Ebenaceae)	38	3.36	0.09
Pleiospermium alatum (Wall. ex Wight & Arn.) Swingle (Rutaceae)	191	3.32	0.02
Pterospermum xylocarpum (Gaertn.) Sant. & Wagh (Sterculiaceae)	24	3.29	0.14
Filicium decipiens (Wight & Arn.) Thw. (Sapindaceae)	10	2.66	0.27
Ficus drupacea Thunb. var. *pubescens* (Roth) Corner (Moraceae)	4	2.64	0.66
Commiphora berryi (Arn.) Engler (Burseraceae)	73	2.53	0.03

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Drypetes sepiaria (Wight & Arn.) Pax & Hoffm. (Euphorbiaceae)	49	2.45	0.05
Cassine glauca (Rottb.) Kuntze (Celastraceae)	16	2.26	0.14
Wrightia tinctoria (Roxb.) R.Br. (Apocynaceae)	29	2.23	0.08
Drypetes roxburghii (Wall.) Hurusawa (Euphorbiaceae)	15	2.21	0.15
Phoebe lanceolata Nees (Lauraceae)	9	2.20	0.24
Remaining 107 species	885	51.40	0.13

Family

Among the 49 families recorded, total tree carbon stock was found maximum (126.10 tC/9ha, 19 %) for Lauraceae followed by Moraceae, Myrtaceae, Melastomataceae and Burseraceae *(Table 3)*.

Table 3. *Carbon stock of 49 families recorded from the Kolli hill forest, India*

Family	Number of species (for 9 ha)	Number of individuals (for 9 ha)	Total carbon stock (tC/9ha)	Average carbon stock (tC/tree)
Lauraceae	11	307	126.10	0.41
Moraceae	14	111	77.35	0.70
Myrtaceae	3	140	69.41	0.50
Melastomataceae	3	709	49.47	0.07
Burseraceae	3	185	48.36	0.26
Anacardiaceae	6	92	32.33	0.35
Rosaceae	1	34	22.78	0.67
Euphorbiaceae	14	498	20.39	0.04
Combretaceae	3	40	17.94	0.45
Verbenaceae	5	91	16.56	0.18
Rubiaceae	11	113	16.06	0.14
Ebenaceae	6	132	15.82	0.12
Caesalpiniaceae	2	25	13.53	0.54
Myristicaceae	1	31	12.25	0.40
Elaeocarpaceae	1	44	11.76	0.27
Moringaceae	1	50	11.45	0.23
Mimosaceae	5	256	9.46	0.04
Bischofiaceae	1	21	9.35	0.45
Papilionaceae	4	39	8.88	0.23
Flacourtiaceae	3	39	8.20	0.21
Sterculiaceae	3	29	7.89	0.27
Hernandiaceae	1	72	7.59	0.11
Ulmaceae	3	97	7.17	0.07
Sapindaceae	5	59	6.33	0.11
Meliaceae	7	51	5.47	0.11
Rutaceae	7	285	5.11	0.02
Flindersiaceae	1	54	4.50	0.08
Apocynaceae	2	35	3.81	0.11

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Symplocaceae	1	21	3.54	0.17
Sapotaceae	2	6	3.49	0.58
Celastraceae	1	16	2.26	0.14
Annonaceae	2	26	1.49	0.06
Loganiaceae	2	38	1.42	0.04
Oleaceae	2	22	1.28	0.06
Bignoniaceae	2	5	1.22	0.24
Meliosmaceae	2	3	0.91	0.30
Asteraceae	1	7	0.84	0.12
Bombacaceae	1	1	0.72	0.72
Cordiaceae	3	18	0.53	0.03
Myrsinaceae	1	5	0.14	0.03
Tiliaceae	1	2	0.10	0.05
Erythroxylaceae	1	4	0.07	0.02
Araliaceae	1	1	0.06	0.06
Alangiaceae	1	4	0.05	0.01
Rhamnaceae	2	2	0.03	0.02
Proteaceae	1	1	0.02	0.02
Arecaceae	1	1	0.01	0.01
Lecythidaceae	1	1	0.01	0.01
Solanaceae	1	1	0.01	0.01

Regression analysis

Regression analysis revealed that there exist no relationship between carbon stock with tree species richness (r^2 = 0.301), density (r^2 = 0.398) and altitude (r^2 = 0.469) of for the 18 transects analyzed *(Fig. 4)*.

Discussion

Currently, international concern on the treat of increased concentration of greenhouse gases particularly atmospheric carbon concentration on global warming and climate change made the super power nations to convene meetings for serious discussions, on reduction of carbon emissions, carbon mitigation policies such as on carbon tax and subsidy. Clean Development Mechanism (CDM) forestry projects began from 2006 in light of carbon offsetting targets, then, the Climate Action Reserve (CAR) projects, Verified Carbon Standard (VCS) and the American Carbon Registry (ACR) came into action (Pearson et al., 2014). Although, several policies and carbon market business were taken, the rise in atmospheric carbon concentration and its consequence are at alarming rate. Carbon storage in terrestrial vegetation is one of the promising natural phenomena in regard to carbon mitigation strategy. Carbon sequestration in vegetation mostly occurs either by expansion of forests or by conserving them (Houghton, 1996), hence, forest expansions and sustainable forest management have a significant role in the protection of environment (Shah et al., 2014). On the other hand, shrinkage of forests may have a strong negative role in achieving carbon targets, and a long term influence and impact (Levy et al., 2004). So, understanding the dynamics of carbon stocks and carbon changes are a key for sustainable management of forest carbon sinks.

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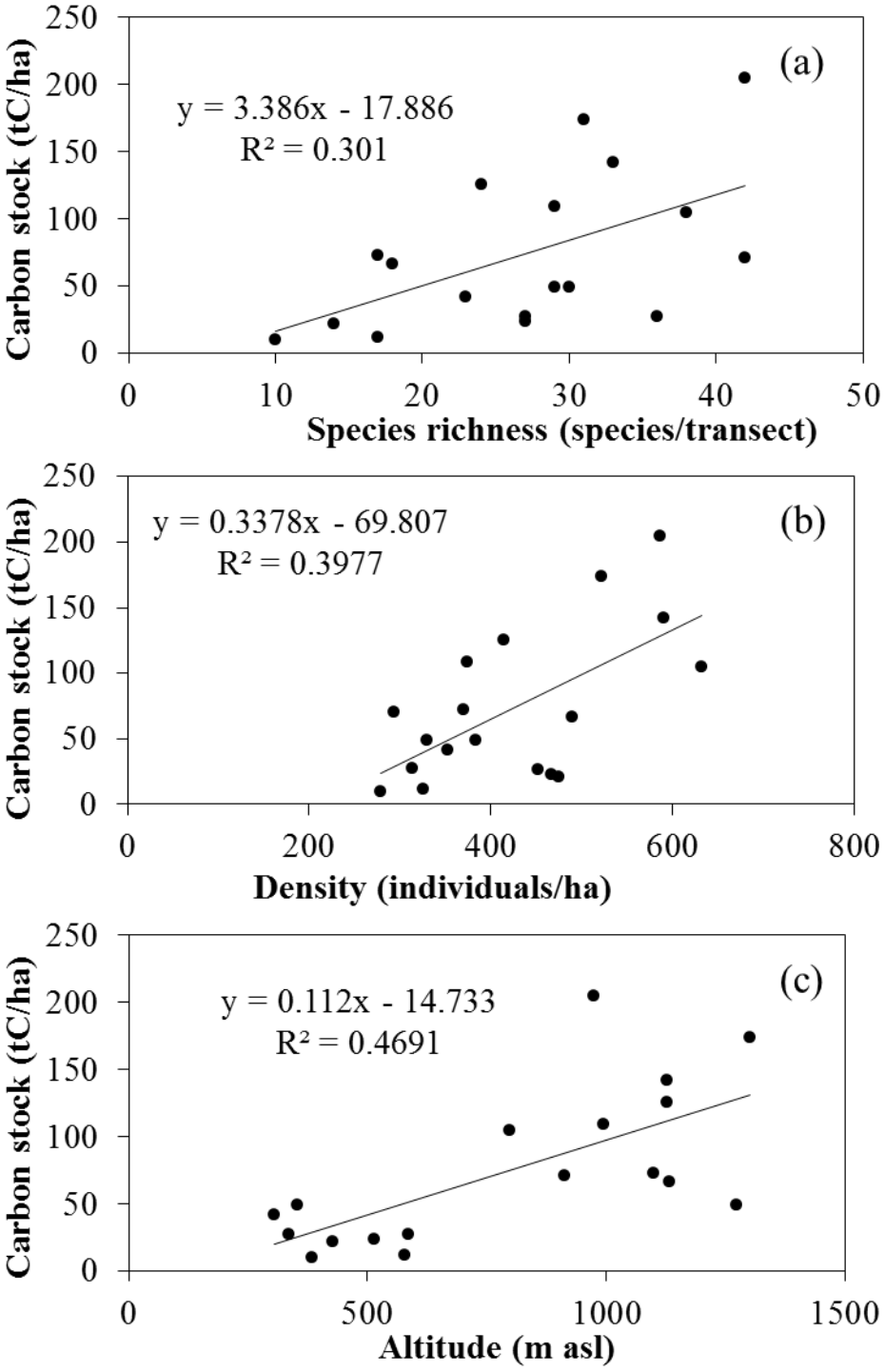

Figure 4. *Relationship of carbon stock with species richness, density and altitude of transect*

Several studies have been carried out to understand the role of forest ecosystem as carbon sink in different parts of the world. The carbon stock (tC/ha) recorded in KH (73.7 tC/ha) is greater than the carbon value reported for tropical forests at Bodamalai hills (10.94 tC/ha, Pragasan, 2015a), mixed species plantation forest (22.25 tC/ha) and Eucalyptus plantation forest (27.72 tC/ha, Pragasan & Karthick, 2013), Kalrayan hills

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(38.88 tC/ha, Pragasan, 2015b), Shervarayan hills (56.55 tC/ha, Pragasan, 2015c), Chitteri hills (58.55 tC/ha, Pragasan, 2014) in India, Nemarket Park in Auckland, New Zealand (45.9 tC/ha, Schwendenmann & Mitchell, 2014) lesser than Lower Montane forests at El Verde, Puerto Rico (134.21tC/ha, Jordan, 1981), tropical seasonal rain forest at Xishuangbanna, China (138.73 tC/ha, Shanmughavel et al., 2001), tropical rain forests at Khade, Ghana (152.84 tC/ha, Greenland & Gowel, 1970), New Guinea (164.45 tC/ha, Enright, 1979), Khado Chang, Thailand (167.10 tC/ha, Kira et al., 1974), Western Ghats, India (263.47 tC/ha, Rai, 1984), Montane rain forests at New Guinea (290.38 tC/ha, Edwards & Grubb, 1977); greater than the range reported for Pine forest at Himachal Pradesh, India (27.65-48.04 tC/ha, Shah et al., 2014); within the range reported for tropical evergreen forests at Myanmar (5.75-115.00 tC/ha, FAO, 1984-85), tropical moist forests at Bangladesh (48.88-118.45 tC/ha, Drigo et al., 1998); and lesser than the range reported for tropical moist forest at Bangladesh (86.25-120.75 tC/ha, Milde et al., 1985), Hardwood forest at Great Lakes, Northern America (96-224 tC/ha, Powers et al., 2011), tropical moist evergreen forests at Sri Lanka (109.25-299.00 tC/ha, FAO/UNDP, 1969), Red pine forest at Great Lakes, Northern America (130-195 tC/ha, Powers et al., 2011), Montane rain forests at Jamaica (131.68-179.40 tC/ha, Turner et al., 1999), tropical rain forest at Malaysia (132.25-166.75 tC/ha, Whitmore, 1975), tropical rain forests at Cambodia (200.10-238.63 tC/ha, Hozumi et al., 1979), sub-tropical Pine forest of Garhwal Himalayas, 203.02-230.84 tC/ha, Sheikh et al., 2012).

The order of carbon stock (tC/ha) by the three forest types recognized in the Kolli hills can be justified as scrub < mixed-deciduous < semi-evergreen forest, and a similar order was observed in Chitteri reserve forest (Pragasan, 2014) and Shervarayan hills (Pragasan, 2015c) located in India. The variation in carbon storage among the three forests types can be influenced by different factors such as leaf traits, microclimate, edaphic characters, etc. Scientists have proved that forest types can alter soil organic carbon stock through several factors, including litter inputs through litterfall, root turnover, litter quality, soil chemistry (Wang et al., 2010). These above factors can indirectly affect vegetation carbon stock that varies in magnitude with varying forest types.

In the present study, carbon stock was contributed maximum (14%) by *Alseodaphne semicarpifolia* Nees var. *semecarpifolia*. While, *Memecylon edule* Roxb. (8%) contributed predominantly to the total carbon stock estimated for the Chitteri reserve forest (Pragasan, 2014), and it was *Syzygium cumini* L. (9%) for the Shervarayan hills (Pragasan, 2015c). The carbon stock sequestered by a single tree was recorded maximum for *Sterculia foetida* L. (1.89 tC/tree) in the Kolli hill forest. While, *Mangifera indica* L. had the highest carbon value 1.73 tC/tree among tree species found in Chitteri reserve forest (Pragasan, 2014), and *Artocarpus heterophyllus* Lam. (2.76 tC/tree) in the Shervarayan hills (Pragasan, 2015c).

A few studies have been reported on relationship of carbon stock with species richness. In the present study, carbon stock had no significant relationship ($r^2 = 0.301$) with species richness *(Fig. 4)*, and similar trend was reported earlier from tropical forests (Pragasan, 2014; 2015c) as well as ago-ecosystems (Nakakaawa et al., 2009). While, some authors argue that conserving species richness increases carbon storage in forest system (Alavalapati, 2002; Kirby & Potvin, 2007). The difference of opinion in the above case may be influenced by the nature of production system and restoration principles adopted in those forest ecosystems.

It is well known that the carbon stock of a tree is directly proportional to its stem size, and hence, total carbon stored in a forest is mostly influenced by the number of

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trees in larger stem size category rather than total tree density. In the present study, no significant relationship (r^2 = 0.398) was observed between carbon stock and density *(Fig. 4)*, and a similar trend was reported for Shervarayan hills (Pragasan, 2015c), while, a strong positive relation was observed between carbon stock and density (r^2=0.689) at Chitteri reserve forest (Pragasan, 2014).

In the present study, carbon stock was greater at high altitude range (104.8±20.1 tC/ha, *Fig. 4*) when compared to low and medium altitude ranges, however, no significant variation was observed in carbon stock (tC/ha) values among the three altitudinal ranges (ANOVA: $F_{(2,15)}$ = 2.871, p>0.05). While, Sheikh et al. (2012), reported high carbon stock (tC/ha) at comparatively low elevation (1300 m asl, 230.84 tC/ha) than the medium (1400 m asl, 218.04 tC/ha) and high elevation (1500 m asl, 203.02 tC/ha). At KH, regression analysis revealed that carbon stock had no significant relationship with altitude of forest location, a similar trend was observed earlier (Pragasan, 2014), while a positive relation (r^2=0.570) was reported at Shervarayan hills (Pragasan, 2015c). According to Shah et al. (2014), the tree felling in higher altitude forests have failed to regenerate and lead to serious disturbances in the ecosystem functioning, particularly in forest moisture retention and local ecology, and this phenomenon emphasize strengthening protection for forest at high altitude range in KH, for maintaining the sustainability of carbon storage in vegetation particularly at high altitudes.

The results of the present study conclude that 1) Carbon stock of KH is 73.7 tC/ha, 2) Among the three forest types in KH, carbon stock is recorded maximum for semi-evergreen forest (103.5±16.6 tC/ha), 3) Among the three altitude ranges categorized in KH, carbon stock is found maximum for high altitude range (104.8±20.1 tC/ha), 4) Among the eleven stem size classes recognized in KH, 120-150cm class has recorded maximum carbon value (5.9±1.2 tC/ha), 5) Out of the 157 species recorded in KH, total carbon stock is found maximum (92.34 tC/9ha) for *Alseodaphne semicarpifolia* var. *semecarpifolia*, 6) Among the 49 families recorded in KH, total carbon stock is found maximum (126.10 tC/9ha) for Lauraceae, 7) Regression analysis reveals that there is no significant relationship of carbon stock (tC/ha) with tree species richness, density and altitude of forest location. The study provides valuable data on carbon stock of tree vegetation of KH forest useful for better management and monitoring of tree carbon stock in study site. Creating awareness on forest carbon sinks to the local inhabitants is at most the prime need for forest protection from illegal timber extraction that causes irreversible reduction in carbon stock, which negatively affects atmospheric carbon capture process for mitigation of global warming and climate change.

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REVEGETATION OF FLY ASH – A REVIEW WITH EMPHASIS ON GRASS-LEGUME PLANTATION AND BIOACCUMULATION OF METALS

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Abstract. Uninterrupted generation of fly ash by the coal based thermal power plants and its dumping has lead to steady encroachment of useful land in India. The deleterious effects of fly ash on the nearby environment are inevitable due to its fine texture and presence of toxic metals. Thus, proper revegetation programme of the sites are highly desirable due to their continuance in being the part of landscape. This paper conglomerates all the issues which should be taken into account to prevent groundwater contamination and increase phytostabilization of metals. An insight to the past and the prevailing restoration scenario will help in selecting of plant species for biomass production. Primarily, an integrated approach towards revegetation is necessary which comprises native and exotic grass-legume species, readily available composts, green manure, and mulches. The paucity of studies in relation to the long term changes in fly ash due to vegetation is to be permeated through regular analysis of substrate nutrient status, extent of nutrient loss, bioavailable toxic metals, in restoration sites. The range of methodologies and indexes discussed here will benefit the future management approaches of fly ash with emphasis on phytoremediation of trace metals, development of aesthetically pleasant landscape and productivity.
Keywords: *bioaccumulation factor, translocation factor, maximum allowable limit, available metals, amendments.*

Introduction

Coal based thermal power plants generate fly ash (FA) as the main industrial waste product, approximately 70 – 75% (Belyaeva and Haynes, 2012) and it has been recognized as an environmental hazard across the globe. India ranks third among the list of countries which generate high amount of FA, namely China, United States, Europe, South Africa, Australia, Japan, Italy, and Greece (Ram et al., 2008). The total capacity of TPPs in India had been 110232 MW at the end of 2012 and it has been estimated that an extra capacity of 53400 MW is likely to be added by 2017. Conceding the fact that 1MW thermal power results in production of 1800t of ash FA generation is likely to surpass 300mt by the year 2017 (end of 12th plan). In this context moderate utilization of FA has been marked by various industries while the rest is disposed on sites (landfills, FA basins and abandoned mines near TPPs) encroaching a land area of more than 40000 Ha (Jain and Gaggar, 2013). Moreover disposal in slurry form requires 1040 Mm^3 of water annually which is an extra consumption of water resources (Paliwal, 2013). This dumping activity has deleterious effects and contaminates nearby aquatic and terrestrial ecosystems. During summers, strong winds lead to blowing of fine FA

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particles into atmosphere over long distances causing health hazards. Air blown particles <10 μm size remain suspended in the air for a long time often leading to atmosphere invisibility. Local people residing in nearby villages of the thermal power plants have been found to suffer from cancer, heart disease, genetic and respiratory disorders (USEPA, 2007). Moreover, Bryan et al. (2012) studied the effects of FA on birds nesting around coal FA basins and observed adverse results due to accumulation of Se, As, Cd, and Sr in their offspring.

FA management is therefore an important environmental perspective which requires management of mainly 4 aspects: (1) a safe FA disposal method (2) land requirement (3) control of air pollution by suspended particulate matter and (4) control of soil and water pollution through curbing movement of heavy metals into ground water and through food chain. The third and fourth constraints can be managed through vegetation establishment. Phytomanagement is the most cost-effective and eco-friendly approach as it would stabilize the ash dumps, oversight wind and water erosion and incur gradual restoration of the site. Several other functions include reduction in leaching of water and solutes, stabilization or bioaccumulation of metals, carbon sequestration in ash soil–plant system and creation of shelter for wildlife. Furthermore proper rehabilitation programmes also serve the purpose of generation of bio-resource for local villagers and thus elevate their socio-economic status. Thirdly these would help in buildup of an aesthetically pleasing landscape and places for tourist attraction (Belyaeva and Haynes, 2012; Pandey, 2012b). In a nutshell, an engineered sustainable ecosystem can be developed with the help of tolerant plant species which would also alleviate the problems.

The initial cover development is generally done by establishment of grasses and legumes. Grass-legume cover has become the most efficient choice as they can readily colonize the area and develop a thick vegetation mat in a short period of time. Consecutively it enhances, fertility of the area, curtail erosion, air pollution and also phytoremediates the metal contaminated substrate. This paves the pathway for future long term management and gradual restoration of the site. Inspite of this choice, revegetation of existent abandoned fly ash dumps, landfills and also those which are being created require prior consideration of various factors. These are the characteristics of the material, its effect on the nearby ecosystem and probable future effects. This paper addresses all these crucial issues which should be considered before revegetation. Further, retrospection of the earlier studies done here will also enlighten the research needs and paradigms which should be focused. A special emphasis has been given to the bioaccumulation of metals by the various plant species and various indexes to conjecture metal pollution level. Conglomeration of all this information in this paper would help in selection of the best strategy for restoration of fly ash dumps for long term management.

Fly ash utilization and disposal

The high rate of FA generation in India is due to the fact that Indian coal has very high ash content (35–45%) and is of lower quality (Mathur et al., 2003). Albeit thermal power producers are now exploring methods for 100% FA utilization, a target set by environment ministry, but with a slow success according to a report by Central Electricity Authority, 2012. 163.56 million ton of ash was produced in India in the year 2012-13 and corresponding utilization amounted to 63% which is

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branched in cement sector (41.33%), reclamation of low lying areas (11.83%), roads and embankments (6.02%), mine filling (10.34%), bricks and tiles (9.98%), agriculture (2.5%), and others (6.41%) (*Figure 1*) (Central Electricity Authority, 2012). FA has been extensively used as amendments in agricultural soils to boost crop growth and yield for example in Lettuce (Lau and Wong, 2001); *Zea mays, Medicago sativa, Phaseolus vulgaris* (Wong and Wong, 1989); *Brassica parachinensis* and *Brassica chinensis* (Wong and Wong, 1990); *Brassica oleracea* (Kim et al., 1997); *Brassica campestris* (Jayasinghe and Tokashiki, 2012); *Oryza sativa* (Lee et al., 2006) and many more. Nevertheless, utilization of FA for agricultural purposes is not always beneficial for crops. Further in a study carried by Singh et al., 2008 it was recommended not to grow green leafy vegetables with FA as an amendment. In the experiment it was observed that metal pollution index (MPI) of both roots and shoots of *B. vulgaris* plants was showing significant negative relationships with the yield. Although earlier reports show that small application of FA as amendment can bring success and proves it as an efficient additive but the carryover of toxic metals from FA to plants may produce hazardous effects in the long run or in future and also in the higher trophic levels of the food chain (Singh et al., 2008). Therefore use of FA in agriculture has to take into account the possible toxic effects of toxic heavy metals which may be present. As per the data acquired, country wise FA generation and utilization of 5 nations in the world scenario is also shown in *Figure 2* (Ram and Masto, 2014). The worldwide average utilization of FA is only about 25% (Wang, 2008). A huge amount of FA, approximately 63 million tonnes was dumped in India even after its utilization in major sectors (Central Electricity Authority, 2012). FA is disposed off in two ways, i.e. dry disposal in ash mounds and wet disposal in ash ponds in the form of slurry. Dry disposal incurs air pollution by blowing of fine particles by wind. It has also been reported that power plants are missing the mark of proper disposal of FA as stated by the ministry (Central Electricity Authority, 2012).

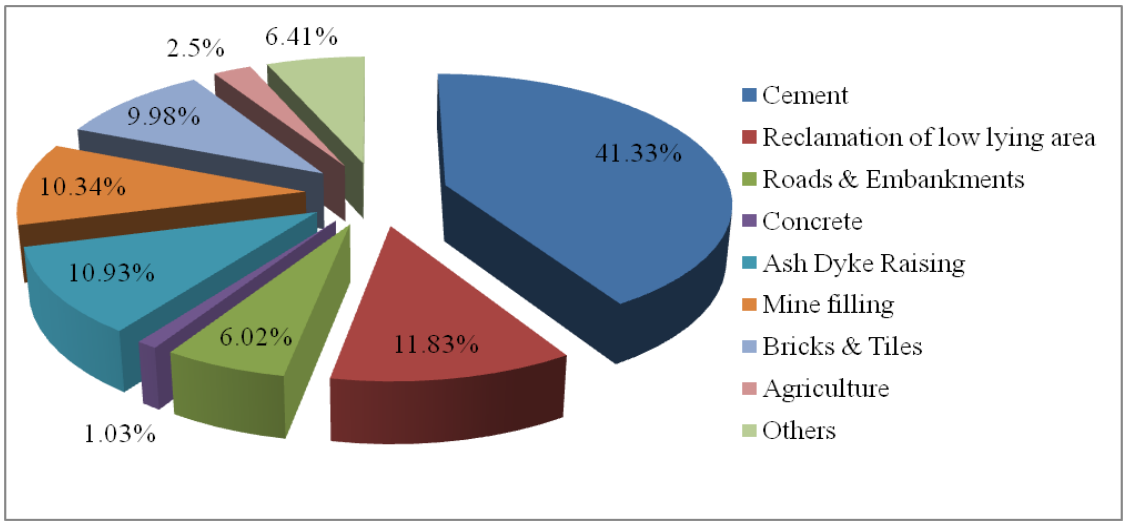

Figure 1. *Fly ash utilization in Indian scenario, in different sectors/industries in the year 2012-2013. Modified from Central Electricity Authority, 2012.*

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From all these information it is evident that a major part of the FA is unutilized and needs urgent strategies for potential utilization. FA utilization in backfilling would be of some help in this case but is still in infancy. The next major area for FA utilization apart from construction is in biomass production which covers agriculture, forestry, and floriculture. FA has been used as an amendment for clay soil (Adriano et al., 1980) while alkaline type of FA has proven to be useful in agriculture for neutralizing acidic soils (Taylor and Schuman, 1988) thus facilitating revegetation of degraded lands. Very few economically important trees such as pulp and paper tree, biodiesel crops, firewood, timber wood and plywood trees are being grown in forestry. There are several issues responsible for mismanagement in utilization of FA, which includes lack of awareness, regulation, and easy availability of land. Thus a challenge stands at the forefront towards a sound management of FA and its utilization as it will save precious topsoil; reduce land requirement, degradation, and water consumption as well as quality.

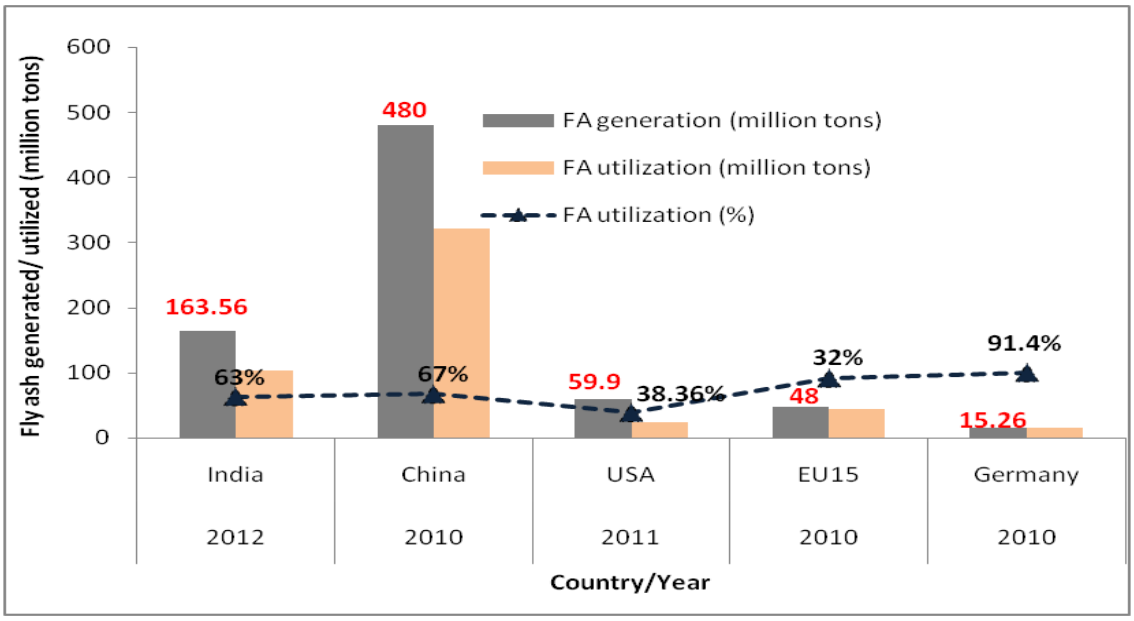

Figure 2. *Fly ash generation and utilization in different countries in the world. Modified from Ram and Masto, 2014.*

Fly ash characteristics and constraints in vegetation establishment

Physico-chemical properties

The prevalent factors which influence mineralogical, physical and chemical properties of FA are nature of parent coal, the conditions of combustion, type of emission control devices and storage or handling methods. Higher temperature during combustion may lead to volatilization of many mineral elements. FA is generally a residue after burning of coal and also enters flue gas stream. It consists of glass-like spherical particles ranging in size from 0.01 to 100 mm (Pandey et al., 2009b) which can be easily airborne (El-Mogazi et al., 1988). Some physical properties of FA are shown in *Table 1* and have been compared to the natural soil. The glass-like spherical particles are hollow spheres called cenospheres, as shown in *Figure 3* and may be filled with smaller amorphous crystals called pelospheres. Some authors have also considered

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FA to be predominantly composed of ferroaluminosilicate elements containing both amorphous and crystalline phases. The smaller particle size increases the specific surface area in the range from 2500 - 4000 cm^2 $g^{-1,}$ (Alonso and Wesche, 1991) which further explains its high sorption capacity. Therefore FA is used as a sorbent to clean flue gas of SOx, NOx, toluene vapors and wastewater of Cu, Pb, Cd, Ni, Zn, Cr, Hg, As, Cs, F, B, dyes and pigments (El-Mogazi et al., 1988). Various studies done in literature have also shown the capability of FA to be used as zeolite for treatment of metal contaminated water (Prasad and Mortimer, 2011). Specific gravity of FA ranges from 2.1 - 2.6 g cm^{-3} and has a low to medium bulk density. It is generally observed to be whitish or yellow-orange to deep red or black-grey in color which depends on iron oxide and carbon contents. LOI (loss on ignition) can range from 0.5 to 12% which corresponds to the unburnt coal content in FA (Alonso and Wesche, 1991). FA is generally of silt loam texture (Nyambura et al., 2011) and is of finer quality if produced from bituminous coal when compared to lignite coal. Size of particles present in FA also impacts its chemical composition which generally contains oxides, hydroxides, carbonates, silicates, and sulfates of calcium, iron, aluminium, and other metals in trace amounts (Adriano et al., 1980) (*Table 2*).

Table 1. *Physical properties of fly ash and natural soil.*

Parameters	**Fly ash[a]**	**Soil[b]**
Particle diameter	0.01 - 100 μm	-
Texture	Silt loam	Sandy-clayey-silty loam
Specific surface area	2500 to 4000 cm^2g^{-1}	-
Specific gravity	1.6 - 2.6 g cm^{-3}	2.5 - 2.8 g cm^{-3}
Bulk density	0.9 - 1.3 g cm^{-3}	1.3 - 1.8 g cm^{-3}
Water holding capacity	40 – 60 %	40 %
Color	White/yellow-orange/black	Yellow/orange-brown/black

[a]El-Mogazi et al., 1988; Nyambura et al., 2011; Alonso and Wesche, 1991
[b]Kabata-Pendias and Sadurski (2004)

Table 2. *Chemical properties of FA from lignite, bituminous as well as anthracite coal (Ram and Masto, 2010).*

Parameters	**Lignite ash**	**Bituminous/sub bituminous ash**	**Anthracite ash**
pH	11.00	4.50 – 11.0	4.5
SiO_2 (%)	48.40 ± 0.99	38.0 – 63.0	51.7 – 54.4
Al_2O_3 (%)	29.80 ± 0.81	27.0 – 44.0	21.5 – 23.8
Fe_2O_3 (%)	5.40 ± 0.68	3.3 – 6.4	6.09 – 7.18
CaO (%)	7.90 ± 0.53	0.2 – 0.8	0.29 – 0.47
MgO (%)	2.60 ± 0.30	0.01 – 0.5	0.92 – 1.18
K_2O (%)	0.20 ± 0.02	0.04 – 0.9	2.80 – 2.99
Na_2O (%)	0.40 ± 0.03	0.07 – 0.43	0.22 – 0.35
SO_3 (%)	2.80 ± 0.22	0.03 – 0.16	0.05 – 0.27
P_2O_5 (%)	0.40 ± 0.03	-	-
TiO_2 (%)	1.40 ± 0.09	0.4 – 1.8	-
LOI (%)	5.70 ± 0.22	0.2 – 3.4	9.55 – 12.92
Cl^- (%)	0.04	-	-
NO_3^- (%)	0.004	-	-

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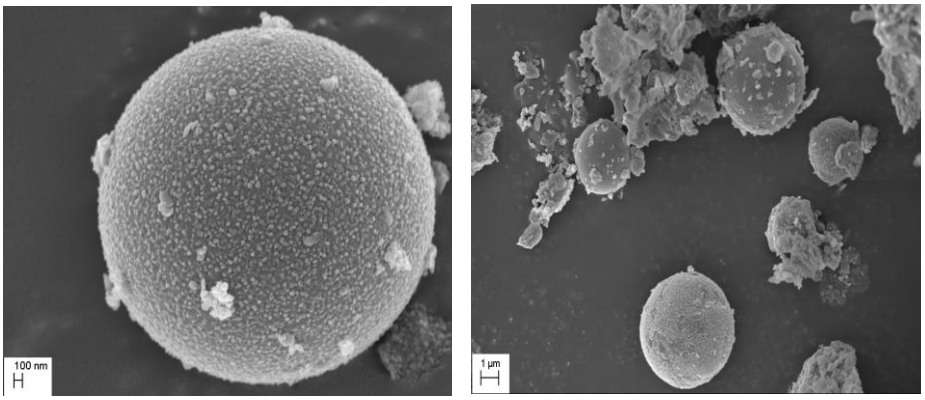

Figure 3. *Cenospheres of fly ash as seen under scanning electron microscope.*

The pH of FA varies widely from 4.5 to 11.0 and mainly depends on S and CaO content of the parent coal (Ram and Masto, 2010) (*Table 2*). Anthracite coals of eastern U.S. produce acidic ashes and lignite coal of western U.S. produce alkaline ashes (Page et al., 1979). It has high moisture retention capacity and low electrical conductivity (EC); and lower cation exchange capacity (CEC) than normal soil. Different types of coal such as anthracite, bituminous, sub-bituminous (class F FA containing less than 7% CaO, high S, low pH) and lignite (class C FA containing up to 30% CaO, low S, high pH) produce ashes of different compositions as shown in *Table 2* (Wang and Wu, 2006). XRD analysis of FA contributes direct information about the mineralogical composition of the FA sample as shown in *Table 2*. It is based on the principle that each crystalline compound produces a unique diffraction pattern.

Phase identifications are done by comparing the diffraction patterns to a database of pure phase reference patterns (Stutzxna and Centeno, 1995). Lignite ash has high SiO_2, CaO, MgO, Al_2O_3, and SO_3 in compared to others whereas anthracite ash has high SiO_2, Al_2O_3, with a considerable amount of K_2O. In humid conditions weathering may take place on the stored ash in landfills or dumps and solubilise constituents which get leached (Adriano et al., 1980). Leaching of the salt compounds and metals may contimanate the ground water in a long term scenario. Therefore, disposal to ash should be accompanied by prior application of geoliners on the surface of landfills or mines.

Elemental composition

There exists a wide variation in elemental composition of fly ashes and usually contain considerable amounts of plant nutrients (such as Ca, K, and Mg) when compared to soils, and as shown in *Table 3*. Various cations in FA are present in the form of oxides, hydroxides, carbonates and bicarbonates which dissolve at different rates (Ulery et al., 1993) and their availability depends on the pH of the system and its microbial activity during gradual plant growth on the substrate. Micronutrients such as Cu, Fe, Mn, Mo, Zn, B are also present in similar quantities as in soils and constitute a considerable pool for nutrient source for plants. Phosphorus concentration in FA is quite high compared to soils in some cases; however it is generally present in unavailable forms which are unusable by the plant (Page et al., 1979). P mostly remains occluded in aluminosilicates or is present in the form of weakly soluble aluminum phosphate (Erich, 1991). Similarly, FA also lacks nitrogen supply which is an essential constituent for

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plant growth. Tripathi et al., 2008 showed the presence of 0.676% total nitrogen in FA when compared to soil which had 1.2% nitrogen. Initial establishment of vegetation on FA sites would require high rate of fertilizer application. During gradual succession of vegetation the available nitrogen in the FA landfills increase at a steady rate. This has also been reported in various studies (Pandey et al., 2014, 2015).

Besides micronutrients various toxic metals such as Cd, Pb, Ni, Se, and Hg are also present in FA and may enter food chain through vegetable crops growing on it. A study by Patra et al., 2012a through particle induced X-ray emission spectroscopic technique confirmed that K, Ca, Ti, Fe are present as major elements in FA samples while V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Rb, Sr and Pb are present in trace amounts. Dumping of FA on land contaminates soil and water through the presence of potentially toxic elements mostly in water soluble form. High levels of pH and toxic metals, lack of microbial activity as well as natural compaction of FA particles inhibits water infiltration and root growth and this restricts vegetation establishment to some extent (Haynes, 2009). Heavy metals induce oxidative stress within plant systems leading to production of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet}$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2). These ROS readily react with lipids and proteins leading to cellular damage (Pandey et al., 2010; Sinha and Gupta, 2005). Plants can also encounter ROS through various enzymatic and non-enzymatic defense systems which involve production of cysteine (Grill et al., 1991). Revegetation with efficient FA tolerant plants ensures faster stabilization of the area. Plant tolerance to these stresses can be examined through analysis of a group of enzymes or compounds and thus candidate species for the phytoremediation of FA landfills can be identified.

Plant species used for revegetation

Establishment of a vegetation cover on FA basins helps to increase organic matter content of the substrate through inputs of litter and fine roots. Organic matter is further decomposed and mineralized by microbial communities through enzyme activities and this regulates nutrient cycling.

Fly ash tolerant, native and non-native species

Rehabilitation programmes should be designed in such a way to conjointly incur both ecological restoration of sites and also involve significant economical outputs. This can be done through an effective eco- engineering technology which would constitute the selection of both native invaders and economically useful species as primary and secondary colonizers. Natural succession on FA basins at regional scale has been studied at different parts of the world. Native species have inherent adaptability to resist adverse conditions, increase soil fertility, have faster establishment and provide a sustainable micro-climate for establishment of commercially useful species. This in turn brings out an economical value from the rehabilitation programs (Pandey and Singh, 2011). Recently, Pandey et al., 2014 and Zolnierz et al., 2016 have enumerated various naturally growing species on FA deposits at during initial colonisation and 11 years after vegetation establishment. Some examples of naturally growing species are *Saccharum munja* (Pandey et al., 2012); non-nodulated species *Cassia siamea Lamk*; nodulated species of chickpea (Pandey et al., 2010) and *Pteris vittata weredone*, a fern, (Srivastava et al., 2005). *S. munja* has been called as an "ecological engineer" due to properties such as; firm ash–soil binding capability and stabilizes the ash dump surface.

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This indirectly controls suspended particulate matter generation in the air. It is also useful in various rural applications and is thus economically viable. Some naturally colonizing species over FA dump and those used for pot scale studies along with their metal accumulation tendencies has been enumerated in *Table 4*. Pandey (2013) suggested *Ricinus communis* L., naturally growing on FA land fill sites to be a suitable plant species for revegetation in tropical and sub-tropical regions. Apart from being an industrially valuable oil yielding crop, it has the properties of metal accumulation and stabilization in root parts. Moreover it is unpalatable in nature and also provides benefits such as carbon sequestration, substrate quality enhancement, aesthetically pleasant landscape, and biodiversity conservation. Ferns like *P.vittata* L., *Ampelopteris prolifera* (Retz.) Copel., *Diplazium esculentum* (Retz.) Sw. and *Thelypteris dentata* (Forsk.) E. St. John also grow well on FA and does not show toxicity symptoms from heavy metals which is unavoidable in case of crop plants (Haynes, 2009). Kumari et al., 2013 reported that the fern *T. dentata* has high tolerance potential against heavy metals in FA and can be used efficiently to revegetate/stabilize FA landfills.

Studies have been carried out to revegetate FA disposal sites with tree species selected on the basis of economic importance which would fulfill wood demands of forest based industries. *Leucaena leucocephala, Dendrocalamus strictus* and *Eucalyptus* sp. established on FA dumpsites may be used in pulp and paper industry (Pandey et al., 2009b). Some timber and plywood yielding plants have also been used successfully in studies are *Shorea robusta, Tectona grandis*, *D. sissoo*, *Bombex ceiba, Populus euphratica* and *Eucalyptus tereticornis* (Juwarkar and Jambhulkar, 2008; Ram et al., 2008). Fuel wood tree species recommended by some workers, such as *Albizia lebbek, Acacia auriculiformis, Acacia nilotica, Casuarina equisetifolia, Cassia siamea, Prosopis juliflora* and *Dalbergia sissoo* have nitrogen fixing characteristics, excellent growth characteristics in nutrient poor conditions in addition to their economic importance. Trees which yield both fuel as well plywood are *Tamarindus indica, Melia azedarach, Populus deltoides, Eucalyptus hybrid, Eucalyptus globulus* and *Syzigium cumini* (Pandey et al., 2009b). In a study by Carlson and Adriano (1991) it was depicted that a new ecosystem can been created on alkaline and acidic FA dump sites with *Platanus occidentalis* (Sycamore) and *Liquidambar styraciflua*, (Sweetgum) which are important timber trees. Gradual development of a productive forest ecosystem also provides habitat for biotic communities, establishes food-chain trophic levels and bio-geochemical cycles. Despite of the useful properties of this tree species initial establishment in the harsh conditions of fly ash is a tedious process. Therefore, strategies such as additions of amendments, spreading of grass legume fodder seeds, forage legumes, tuft or hardy grasses are an efficient choice.

They help in development of a vegetation mat in a small period of time when compared to the tree species. Moreover, a reclaimed site can then be used as per the destined land use.

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Table 3. *Ranges in elemental composition of fly ash, soil, farm yard manure, plants in India and worldwide along with maximum allowable concentrations (MAC) for trace metals in soils and plants.*

Metals (mg kg^{-1})	Soil[a]	Fly ah				FYM[d]	MAC[e]	Plants[f]	MAC for plants[f]
		Range[a]	India[b]		Worldwide[c]				
			Total	Available					
Fe	7 - 550	10 - 290	68	10 - 15	0.31 - 36.6	3040	-	-	-
Al	40 - 300	1 - 17.3	4.8 - 312	0.1 - 822	0.5 - 108.5	-	-	-	-
Ca	7 - 500	1.1 - 222	0.029 - 34	460 - 4400	1.84 - 86.4	-	-	-	-
Mg	0.6 - 6	0.4 - 76	0.017 - 1.4	0.8 - 179	0.02 - 11.5	-	-	-	-
K	0.4 - 30	1.5 - 35	10.8	32 - 8900	24.5	-	-	-	-
B	2 - 100	10 - 618	17 - 38	0.5 – 3	0.4 - 50	52	-	-	-
Mn	100 - 4000	58 - 3000	500 - 739	0.9 – 19	100 - 679	53.1	1500 - 3000	20 - 1000	300 - 500
P	0.05 - 2	0.4 - 8	10.8	6.2	2.1	24	-	-	-
Co	1 - 40	7 - 520	21.1 - 58	0.05 – 0.15	7 - 26	0.85	20 - 50	0.02 - 1	15 - 50
Cu	2 - 100	14 - 2800	40 - 80	0.5 – 11	19 - 57	44.1	60 - 150	5 - 20	2 – 100
Zn	10 - 300	10 - 3500	52 – 203	0.4 – 4.6	39 - 167	24.7	100 - 300	1 - 400	100 - 400
Mo	0.2 - 5.0	7 - 160	4.0 - 33.3	0.1 – 0.6	3 - 4.2	4.5	4 - 40	0.03 - 5	10 - 50
Ni	10 - 1000	6 - 4300	50 - 204.8	0.15 – 3	15 - 88	39.4	20 - 60	0.02 - 5	10 - 100
Se	0.1 - 2	0.2 - 134	0.6 - 2.6	0.1 – 0.4	8 - 10	0.56	3 – 10	0.001 - 2	5 - 30
As	0.1 - 40	2 - 6300	1 - 4	0.1 - 16	20.4	0.62	15 - 20	0.02 - 7	5 – 20
Cd	0.01 – 0.5	0.7 - 130	5 - 43	0.03 – 0.07	0.03 - 1.3	<0.002	1 - 5	0.1 – 2.4	5 - 30
Cr	5 - 3000	10 - 1000	38.2 - 330	0.3 – 1.3	15 - 148	<0.002	3 – 25	0.03 -14	5 – 30
Hg	Up to 1	0.02 - 1.0	BDL	BDL	0.18 - 0.4	<0.001	0.5 - 5	0.005 – 0.17	1 - 3
Pb	2 - 100	3 - 5000	20 - 70	<0.1	16 - 97	<0.002	20 - 300	0.2 - 20	30 - 300

-: Not available, FYM: farmyard manure; BDL: below detection limit,

[a]Page et al., 1979, [b]Pandey et al., 2009, [c]Adriano et al., 1980, [d] Tripathi et al., 2004, [e]Kabata-Pendias and Sadurski (2004), [f]Alloway, 2013.

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Table 4. *List of plant species found growing efficiently on fly ash dumpsites as well pot studies alongwith the detailed description of the, BAF, BCF and TFs.*

Species	Place	Native/ introduced	Metals studied and technology involved	Experimental setup/ treatment used	Periods of plant harvest	Plant part and bioacccumulation of metals	Reference
T. dentata	Lucknow UP, India	Native fern	Fe, Si, As, Cd, Pb; phytostabilization	Grown in pots 25, 50, 75 and 100% FA treatments	30 and 45 days	BAF values of Si, As, Cd and Pb was >1; TF<1 except for Pb (1.16) and As (1.07) in 25% and 100% FA respectively	Kumari et al., 2013
R. communis L.	India	Native, Bio-energy crop	Cu, Ni, Zn, Cd, Pb; phytostabilization	Growing naturally in FA landfill sites	-	Conc in R>S	Pandey, 2013
S. munja	Uttar Pradesh, India	Native grass	Fe, Cd, Cr, Cu, Mn , Ni, Pb, Zn; phytostabilization and phytoextraction for Fe, Mn	Growing naturally in FA lagoons	-	All BAF values in R and L<1 (excluder species); TF = 0.6 - 1.52 except Mn, Fe; excluding order= Zn>Cd>Cr>Ni>Pb>Cu	Pandey et al., 2012
Azolla caroliniana	Uttar Pradesh, India	Native fern	Cu, Pb, Mn, Ni, Zn, Cr, Cd, Fe; phytostabilization	Ferns growing naturally on FA ponds	-	Metal in R were 175 to 538 mg kg^{-1} and in S was 86 to 753 mg kg^{-1}	Pandey, 2012a
I. carnea	Uttar Pradesh, India	Invasive	Cd, Pb, Cu, Cr, Mn and Ni; phytoextraction for Cd, Cr and phytostabilization for other metals	Naturally grown on FA deposits	-	BCF values of Cd, Pb, Mn, and Ni in R and S were >1; TF for Cd and Cr >1	Pandey, 2012b
S. virgata	Argentinean Pampas	Native	Cu, Zn, Cr; phytostabilization	Pot experiment, Cu, Zn, Cr added	-	Conc in R>S; BCF was in order Zn>Cr>Cu	Branzini et al., 2012

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				individually and in binary mixtures			
A. indica, *C. siamea,* *E. hybrida,* *E. officinalis,* *T. grandis, D.strictus,* *D. sissoo* *P. pinnata*	Khaperkheda thermal power plant, Nagpur, India	Native	Fe, Mn, Ni, Zn, Cu, Cr, Pb; *C. siamea* has been found as a hyperaccumulator	Field experiment (10 ha) FA dump with organic amendments and biofertilizers	-	BCF was in order Fe>Mn>Ni>Zn>Cu>Cr>Pb;	Jambhulkar and Juwarkar, 2009
S. cannabina	Uttar Pradesh, India	Green manure crop	Fe, Mn, Zn, Cu, Pb, Ni; phytostabilization	Pot experiment with mixtures of FA + GS from 10 - 100%	30 and 90 days	BCF of metals in order, Fe > Mn > Zn > Cu > Pb > Ni and TF<1	Sinha and Gupta., 2005
P. juliflora	Uttar Pradesh, India	Native tree	Fe, Mn, Zn, Cu, and Cr; phytostabilization	Pot experiment with mixtures of FA + GS + FYM + PM + BGA	15, 30 and 45 days	BCF was in order Fe>Mn>Cu>Zn>Cr in the treatments.	Rai et al., 2004
Cassia seamea	Uttar Pradesh, India	Native tree	Cu, Zn, Ni, Fe; phytostabilization for all metals except Ni	Pot experiment; GS, FA, FA+GS (1:1 w/w), FA+FYM (1:1 w/w), FA + PM (1:1 w/w)	20, 40 and 60 days	TF<1 for all metals except Ni	Tripathi et al., 2004

FA: fly ash, GS: garden soil, BAF: bioaccumulation factor, BCF: bioconcentration factor, TF: translocation factor, E_f: enrichment factor, Root: R, Shoot/stem: S, Leaves: L conc.: concentration, Max.: maximum, Min.: minimum, FYM: farm yard manure, PM: press mud, BGA: blue green algae

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Role of grasses and legumes

Grasses are considered for initial vegetation cover as they are mostly drought tolerant and can grow in even nutrient poor conditions. Their gradual growth to develop a massive fibrous root network helps in slowing down erosion, increasing soil shear strength and conserving soil moisture (Tengbeh, 1993). Eventual drying of the vegetative shoots at the end of the life cycle, form mulches which also prevents erosion, facilitates water infiltration, improves soil moisture, ameliorates soil temperature and enhances nutrient supply. Stabilizing steep slopes by developing a grass cover is a better soil conservation practice and works faster than trees (Brindle, 2003). Aromatic grasses are a better option for in situ management of FA as they yield environmental and societal benefits. They are stress tolerant crops and can flourish in adverse conditions.

Earlier reports by several workers show that wild aromatic grasses are found to grow profusely and producing high biomass on FA disposal sites, for e.g. *S. munja* and *S. spontaneum* (Pandey et al., 2015). Roots of *S. munja* have been found to grow up to 10–15 ft deep in FA (Pandey et al., 2012). Various other aromatic grasses like *Cymbopogon martinii*, *Vetiveria zizanioide*s, *Cymbopogon flexuosus* and *Cymbopogon winterianus* can also be grown extensively on FA deposits to earn profits (Verma et al., 2014). Very few studies have been done in past on the growth of these grasses on FA except some reports which studied the growth pattern with high rate application of FYM (Kumar and Patra, 2012). The oil released from the leaves (*C. flexuosus* and *C. winterianus*), inflorescence (*C. martinii*), and roots (*V. zizanioides*) of these grasses is used in perfumery, pharmaceuticals and cosmetics. Heavy metal toxicity in the oil extracted can be avoided due to hydro distillation (Khajanchi et al., 2013). Moreover, their growth is supported due to porous nature of FA which assists better root growth. The unpalatability, minimal water requirement and perennial nature of these grasses make them more suitable to be used in restoration studies (Gupta et al., 2013).

Legumes have also been found to be very effective in the revegetating FA landfills (Jambhulkar and Juwarkar, 2009). They exhibit rapid growth as well as enhance substrate characteristics by increasing organic carbon and total nitrogen content. In this context, drought resistant, fast growing species are chosen which readily produce decomposable nutrient rich litter for soil (Madejon et al., 2006). Turnover of their fine roots as well as nodules also have dramatic effect on soil fertility (Singh et al., 2002). The amount of nitrogen (N) fixed by a legume can be calculated by multiplying its biomass by a factor of 0.8 (Thomas et al., 1997) whereas approximately 13 - 682 kg N ha^{-1} yr^{-1} can be fixed in a legume-grass plantation. Persistence of legumes, soil N status and competition with the associated grasses are the factors which influence N fixation in a mixed plantation. Use of N fertilizers as well as dry conditions favor increase in soil inorganic N which in turn reduce nitrogen fixation.

On the other hand, uptake of soil N by grass conjointly with competition from grasses increases N fixation by legumes (Lenka et al., 2012). Fixed N in the range of 3 - 102 kg N ha^{-1} yr^{-1} is transferred from legume to grass through a complex pathway including contact between roots, mycorrhizal fungi, release of N in exudates and turnover of roots (Spehn et al., 2002). The amount of N transferred figures out to 2 - 26% of the total nitrogen fixed (Milcu et al., 2008). Lazzarotto et al., (2009) developed a dynamic plot-scale model (PROGRASS) with respect to parameters such as plant dry biomass yield, leaf area index, uptake of soil N and biological N fixation in a grass-clover plantation. This model simulated seasonal and inter-annual dynamics of plantation and the role of root development in lowering substrate C: N ratio and favoring carbon allocation to the shoot. Mixed plantation

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of grasses and legumes produce sufficient aboveground biomass which prevent formation of gullies during soil erosion (Maiti and Maiti, 2015; Normaniza and Barakbah, 2011) by obstructing raindrops and increasing surface roughness. Above ground prostrate parts also reduce flow velocity of water during heavy rain (Gray and Sotir, 1996). In a nutshell this mixed plantation creates N balance in soil. Generally, legumes can be used in combination with grasses, to revegetate FA disposal sites by the following ways (Tripathi et al., 2004):

1. Sowing of pasture legumes with grassy species as initial colonizers to cover the ash surface. This technique requires minute amounts of fertilizer input and facilitates nodulation in legumes. A similar type of revegetation strategy has also been done in a study conducted by (Maiti and Maiti, 2015).
2. Sowing of legumes with non-legumes. This technique requires high rate of fertilizer application to help the growth of non-legumes. For example application of sewage sludge can supply huge amounts of mineral N (Maiti and Maiti, 2015).
3. Seeding the site with rapidly growing tolerant grass species to cover the ground in a short period with the aids of fertilizers. The establishment of the grassy cover can be followed by growing leguminous forbs, shrubs or trees with the withdrawal of N fertilizers (Maiti and Maiti, 2015).

Some commonly used efficient and highly recommended legume-grass species for the above vegetation strategies are as follows (Maiti and Maiti, 2015):

Stylosanthes sp., commonly called Stylo legume, is widely grown in tropic-agricultural areas with acidic soils (Liu et al., 1997), mainly as a cover crop to suppress weed growth (Ramesh et al., 1997). It restores soil fertility by establishing thick vegetation cover in a short period of time and thus considered as a pioneer crop for plantation on waste lands, mine sites as well as watersheds (Pathak et al., 2004; Maiti and Maiti, 2015). Some species such as *S. humilis*, yields high biomass, uptakes P (limiting nutrient) very efficiently from deficient soils and incurs biological nitrogen fixation (BNF) through nodules (O'Hara et al., 1988).

Crotalaria juncea L. is also an herbaceous, annual, fast growing tall legume plant which fixes nitrogen in nodules present in its long taproot system. Propagation of *C. juncea* occurs through seeds with a germination time of 2 - 3 days after sowing in presence of adequate soil moisture of 6.4% (Maiti and Maiti, 2015). A hectare land can be revegetated with *C. juncea* with approximately 5 kg seeds in regular spacing of 0.5 m. The plant dies to generate a nitrogen rich litter and mulch for the substrate. Moreover the bark of the plant gives valuable fibre and is the earliest recorded fiber crops in history (Chaudhury et al., 1978). It is a pioneer species for nitrogen fixation and restoration of degraded lands as it improves soil quality, adds organic matter to soil, suppresses weeds and recycle plant nutrients. It is widely grown in the tropics as a summer cover crop and green manure because of its fast growth to produce more than 5.4 ton dry matter ha^{-1} and 1.1 kg ha^{-1} nitrogen in 9 – 12 weeks. Nitrogen concentration in leaves ranges between 2 – 5% and 0.6 – 2% in roots and stems (Treadwell and Alligood, 2008).

Cymbopogon citratus, also called lemon grass is a tall, aromatic, perennial grass with deep roots and linear leaves (Akhila, 2010). It is propagated by planting old tillers through. Tops of well grown culms are generally harvested after 5.5 - 6.5 months after growth and cut till 20-25 cm above ground to divide the plant into slips containing 2-3 tillers. A single plant can produce approximately 40 - 50 tillers after 6 months. The growth of tillers begins at the apical meristem followed by production of axillary buds and subsequent emergence of new tillers. Increase in the number of tillers during the growth phase of lemon grass follows a sigmoid-shaped curve to reach a peak point after

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which tillers start to die. It is difficult to distinguish the main culms from new grown tillers when a maximum growth is reached (Linares et al., 2005). Oil extracted from lemon grass is used as an insecticide, as main constituent in perfumery, and as a raw material in the synthesis of aromatic substances. In Europe, leaves are used in tea and in Mexico, it is traditionally used as a sleep aid, tranquilizer, digestive, anti-influenza and antispasmodic (Rauber et al. 2005). It has been widely used for reclamation of degraded lands and can be used as barrier to control runoff and erosion. The root structure permits water to pass while holding soil particles (Sugumaran et al., 2005; Maiti and Maiti, 2015).

In addition, to the above advantages of grasses and legumes various studies in the past have proved the metal phytoremediation potential of these plants. Mitrovic et al., 2008 reported spontaneous colonisation of fly ash deposit by a grass *Calamagrostis epigejos* after 13 years. It was found to exhibit phytoextracting property towards metals such as B and As. In recent study Pandey et al., 2012 reported metal levels within the toxic limit for plants in *S. munja*. More studies on the phytoremediation potential of legumes and grasses growing on fly ash should be done to explore newer species for restoration of the degraded areas.

Addition of amendments to fly ash for efficient establishment of plants

FA landfill sites provide a hostile environment, unfavorable for plant growth, which can be made suitable through application of amendments. Amendments may be added into the surface layer of ash, preceding vegetation to favorably improve chemical and physical conditions of the substrate and sustain plant growth. This helps in boosting up vegetation establishment and thus support rehabilitation programmes (Haynes, 2009).

Topsoil, organic and chemical amendments

Covering of waste material with topsoil or subsoil typically of 5 – 10 cm depth has been the most successful method of restoration. Topsoiling furnishes favorable physicochemical conditions for plant growth, builds up nutrient supply and curtails detrimental toxic effects of ash. In general topsoil and FA are mixed considering their heterogeneous nature, i.e. clayey FA with sandy topsoil for much beneficial effects. This procedure has some limitations as it can be put into use only when there is a ready supply of locally available topsoil. In absence of topsoil, dewatered biosolids, composted chicken manure or green waste and other organic amendments can be used. In addition, organic mulches can be spread over the surface, to mimic a temporary soil cover. This combination is exceptionally useful during preliminary stages of establishing vegetation on fly ash lagoons (Jusaitis and Pillman, 1997). Mulches have following major roles in reclamation processes: (i) curtailing water loss through evaporation and lessening surface temperature, (ii) diminishing erosion by wind and rain thus adding to soil stability, (iii) improving infiltration and water holding capacity of the soil, (iv) accretion of soil organic matter, (iv) and reducing weed germination (Haynes, 2009). Chemical amendments, such as EDTA, limestone although enhance phytoremediation process but are generally toxic (Evangelou et al., 2007) to both plants and beneficial soil microorganisms. Organic amendments provide a source for slow-release of nutrients, improve soil physico-chemical biological conditions, increase water retention capacity and help to establish a self-sustaining microbial community in the substrate (Haynes 2009). One of the mostly used and upcoming amendments in recent times is biochar, a charcoal like residue leftover after pyrolysis of biomass. It is

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relatively stable and inert in nature and its inclusion into substrate is a pathway for C sequestration. Addition of biochar greatly increases water retention capacity and helps developing the soil microbial community of the soil (Belyaeva and Haynes 2012).

A great number of studies have been carried out by blending sandy topsoil/farmyard manure/mill mud/compost/biosolids/sewage sludge with ash dams, fly ash landfills to ascertain the positive effect of organic amendments on revegetation of degraded lands (Juwarkar and Jambhulkar, 2008; Schwab et al., 1989). Cheung et al., 2000 used 30% vermiculite as well as sewage sludge compost (w/w) in pot scale studies with lagoon ash. However field studies provide more valuable information about potential revegetation of ash. Studies have shown that field practices of revegetation on FA deposits require utilization of high rate of fertilization and addition of NPK in absence of topsoil. This is essential for initial establishment of green cover. According to agronomic techniques mineral fertilizer containing NPK is used in the ratio 15:15:15 or 19:19:19 for initial fertilization to a root tilled depth of 8 cm, which can be made by using 228 kg Urea, 90 kg P_20 and 90 kg K_2O per ha. Amendments are usually applied in 5 cm layers and then root tilled to depth of 15 cm (Punshon et al., 2002).

Microbial amendments

FA being toxic in nature to living organisms is desolated of microbial activity and microbes, usually comprised of heterotrophic microflora, N_2-fixing bacteria and mycorrhizal fungi. Despite of the fact, that these microbes are gradually brought into the site from neighboring soils, predominantly in the form of air-borne inoculums, FA makes it a tough medium for their survival and colonization (Sinha and Gupta, 2005). Notwithstanding all this, different species of mycorrhiza as well as *Rhizobium* have been found to possess varying range of survival potentials on FA medium (Juwarkar and Jambhulkar, 2008). The tolerant fungi and bacteria get the advantage of being selected during progressive vegetation establishment, forming symbiotic associations with existing and invading plant species leading to beneficial outputs. Mycorrhiza plant consortium increase the effective root volume of plants by more than eighty times and help them to scavenge nutrients and water. In augmentation with this mycorrhiza also protect plants against heavy metal-induced oxidative stress. Leguminous plants fix atmospheric nitrogen in association with *Rhizobium* bacteria to increase soil N (Juwarkar and Jambhulkar, 2008). Furthermore, there exists specificity in symbiotic association of legumes and strains of *Rhizobium* and accordingly leguminous seeds or plants are often inoculated with appropriate *Rhizobium* strain. In some studies, rhizobial and mycorrhizal inoculums had been isolated from an already revegetated site in view of their tolerance to the FA medium (Juwarkar and Jambhulkar, 2008). Some non leguminous trees have also been found to be symbiotically associated with N_2-fixing organisms (e.g. *Alnus* spp), and can also aid in forming vegetation cover on ash deposits. Sometimes, locally available organic wastes (eg. press-mud, sewage sludge, municipal solid waste etc.) can also be blended or inoculated with FA-tolerant bacteria (Pandey et al., 2009b). Long term carbon sequestration in soil can be mediated through the priming effect of fungi (Fontaine et al., 2011). In a study carried out in south-eastern USA it has been reported that fungal endophyte infection increases carbon sequestration potential of tall fescue stands (Iqbal et al., 2012).

Amendments also help in reduced leaching of harmful trace metals from the substrate for example Zhou et al. (2014) showed decreased heavy metal concentrations in plants compared to control soil which contained no amendments. Percentage of

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inhibition increases with increasing doses of amendments. They help plants sequester heavy metals and assist in phytoremediation. Recently, microbe mediated processes of metal uptake by plants are being studied (Ma et al., 2011). Microbial metabolites in the rhizosphere alter metal mobility and bioavailabity in turn being biodegradable, less toxic and can be easily produced in situ in rhizospheric soils. Plant-associated microbes also produce some growth promoting substances such as siderophores, growth hormones; 1-amino cyclopropane-1-carboxylic acid (ACC) deaminase which further improve vegetation growth (Babu and Reddy, 2011).

Trace metal availability and uptake by plants from fly ash

Bioavailability of metals

Heavy metals are one of the toxic pollutants present in FA which cannot be degraded and hence persist in the substrate. Bioavailable metals is the fraction of the total metal contaminant in the substrate which is actually available to the receptor organisms, such as plants, microbes or humans and the extent to which these chemicals may become involved in the metabolism of the organism. Dynamics of metals is most active in surface layers due to their interaction with diverse microbes, higher organic matter content and cation exchange capacity. Microorganisms sequester/bioaccumulate trace metals from even very low concentrations in the substrate. The accumulation is the result of either (i) biosorption or (ii) physiological uptake through metabolic processes. Trace metals, once deposited in soil also interact with the soil minerals and organic constituents and this depends on soil properties (pH, nature of organic as well as inorganic anions) and environmental factors. The charged metal ions get attached to the charged soil surface by electrostatic or other specific bonds (Ma et al., 2011). A pH value >6 lowers the concentration of free metal ions in the soils due to increased surface charge on oxides of Fe, Al and Mn and chelation by organic matter. Higher pH also causes precipitation of metals and causes metal immobilization in the presence of anions such as sulfate, carbonate, hydroxide and phosphate (Adriano, 2001). Formation of complexes by trace metals and the general order of their affinity to get complexed with organic matter are in the following sequence (Adriano et al., 2004): $Cu^{2+} > Cd^{2+} > Mn^{2+} > Fe^{2+} > Zn^{2+} > Pb^{2+} > Ni^{2+} > Co^{2+}$. In the natural environment, deposited metals undergo natural attenuation or remediation through transformation of their labile or bioavailable fraction (i.e., ion pair, those weakly adsorbed on exchange surface or complexed with humic ligands) (National Research Council, 2003; Campbell, 1995).

The natural remediation and uptake by plant roots has been depicted in *Figure 4*. The whole process is divided into four phases. Metal partitioning between solid and liquid phase is guided by processes such as adsorption, precipitation, complexation and redox reactions. This completes phase A which encompasses the base for bioavailability of metals. Phase (B, B') involves the transport of metal to the organism in soluble or colloidal form. This phase can also become an exposure pathway to grazing livestock through ingestion of soil particles while feeding on contaminated pastures. Colloidal form of metal transportation involves organic matter and is highly reactive. It contains higher metal concentration in comparison to those in the solution. Phase C involves passing of the metals through a biological membrane or root membranes. Roots also serve as a biofilter for contaminants. Phase D or the last phase involves circulation and assimilation of metals in the metabolic machinery of the organism and resulting into a biological response which may include growth and biomass of the organisms (Adriano

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et al., 2004). Transfer of metals from soil to plant is a very complex process and is governed by natural as well as anthropogenic factors (Rodriguez et al., 2011). Baker and Walker (1990) suggested that uptake, translocation and bioaccumulation mechanisms differed for various heavy metals and for the plant species.

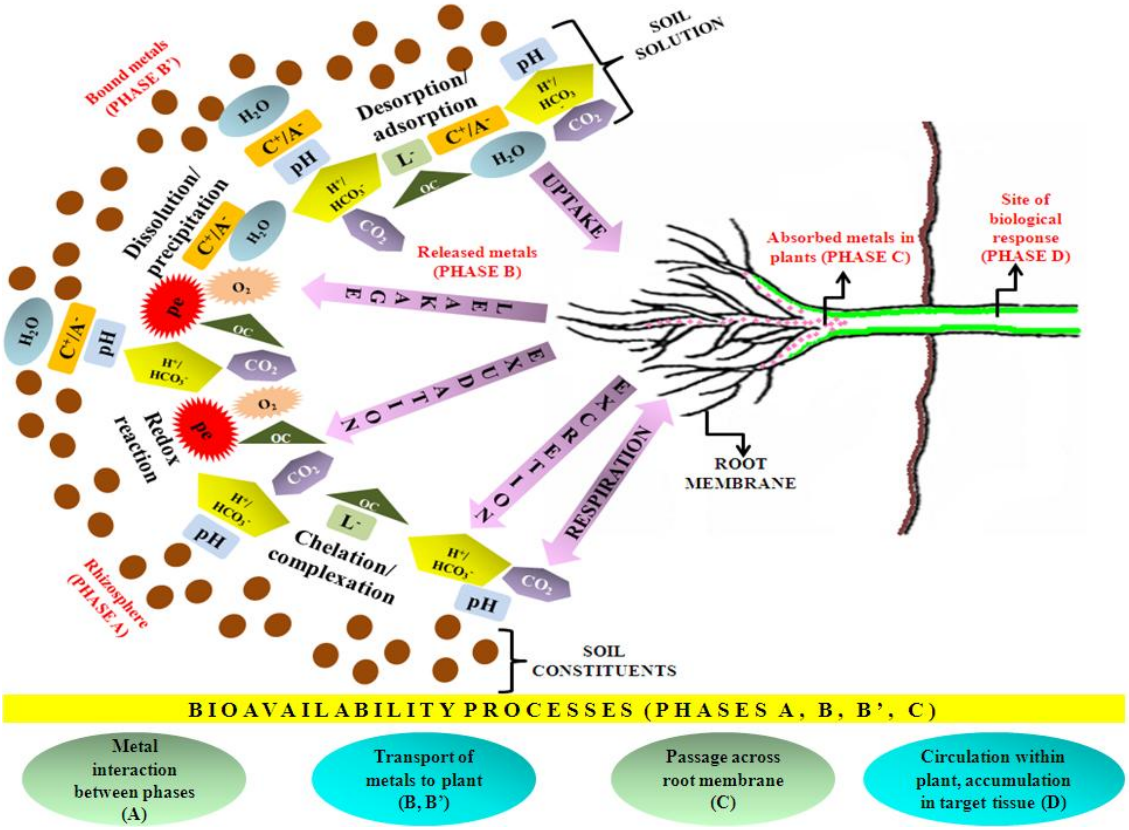

Figure 4. *Bioavailability processes for metals in the rhizosphere of plants, emphasizing the mechanisms in soil solution interface. Legends: OC = organic carbon; C+ = cation; A+ = anion; L- = ligand; pe = redox potential. Modified from Adriano et al., 2004.*

Trace metal extraction procedures

Bioavailability tests are generally conducted to examine the effects of toxic metals leached from ash on living organisms (Shim et al., 2005). Generally various single extractions as well as sequential extraction procedures are used for estimation of and distribution of various chemical forms of a metal in soils/sediments. They include mineral acids (e.g., 1N HCl or 1N HNO_3), salt solutions (e.g., 0.1M $CaCl_2$), buffer solutions (e.g., 1M NH_4OAc) and chelating agents (e.g., DTPA) which help in estimation of the bioavailable fraction of trace elements in soils (van der Watt et al., 1994). In a study, Zhou et al. (2014) prepared the extracts by suspending soil in 1M $MgCl_2$ solution in 1:10 ratio w/v and shaking at 150 rpm at room temperature for 2 h followed by separating the extracts by centrifuging at 4000 rpm for 10 min and filtering out the solid. Phytoavailable metals in FA and soil are also determined by DTPA extraction protocol (Lindsay and Norvell, 1978). Mehlich I and III are also being used to extract soil micronutrients in 1:4 and 1:8 ratio of w/v respectively. Mehlich I is composed of 0.0125M H_2SO_4 + 0.025M HCl while Mehlich

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III is made by mixing 0.2N CH_3COOH + 0.25N NH_4NO_3 + 0.013N HNO_3 + 0.015N NH4F + 0.001M EDTA (Mylavarapu et al., 2002).

On the other hand, leaching tests are a group of protocols which are carried out to find the rate of leaching and release of metals as a function of pH (Shim et al., 2005). Shim et al 2005 used distilled water and 1N HNO_3 for leaching test and pH dependent leaching test respectively. The toxicity characteristic leaching procedure (TCLP) is also the method of choice accepted by the USEPA for determining the amounts of potential toxic materials that could potentially leach from the soil and fly ash samples by 0.57 % glacial acetic (USEPA, 1992). TCLP was also followed in various studies (Xenidis et al., 2003; Zhou et al., 2014) to monitor the release of trace metals from contaminated soil by extracting 5 g of sample with 100 mL of extractant on a rotary extractor at 30 rpm for 18 h. The extracts are filtered and processed for further analysis. *Table 5* depicts the range of metals released by various reagents with values below and above the general regulatory limits given by different countries which are also called soluble threshold limit concentration.

Table 5. *Available concentration of metals (mg kg-1) in fly ash, obtained by extracting with various reagents by different workers. The concentrations are compared to soluble threshold limit concentration.*

Metals (mg kg^{-1})	**Extractant used**					**STLC[#]**
	Deionized water/ distilled water[a]	**0.05M DTPA-$CaCl_2$[b,e]**	**1N HNO_3[b,e]**	**Mehlich I[c]**	**Acetic Acid + NaOH (TCLP)[d,e]**	
Sample: solution (w/v)	1:10	1:2	1:10	1:4	1:20	1:20
pH	5.8 – 6.3	7.3	3.5 - 4	2.5	4.93	4.93
Period of extraction	6h	2h	2h	4h	18h	18h
Fe	0.01 - 1.7	8.2 – 21.9	22.8	161	5.4 - 8.2	NA
B	0.1 – 1.1	25	4	3	2.9	NA
Mn	0.03 - 0.13	0.41 – 3.5	23.1	19	2.1	NA
Co	0.02 - 0.06	<0.05	1.9	1.07	0.11	80
Cu	0.01 - 0.02	0.85 – 6.2	4.5	11	0.48	25
Zn	0.02 – 4.8	1.2	7.4	4.6	2.4	250
Ni	0.01	0.09 - 0.56	1.4	2.3	0.25	20
Se	0.1	1.1	0.84	0.35	0.12	0.3 - 1
As	0.1	0.6 - 4.7	0.91	16	0.29	0.3 - 5
Cd	0.01 – 0.71	0.14 - 0.34	0.14	0.12	0.02	0.3 - 1
Cr	0.03 – 0.86	0.94 – 2.1	1.6	1.3	0.05	5
Hg	BDL	0.04	0.07	0.07	0.08	0.2
Pb	0.07 - 1.10	0.38 – 1.8	2.2	<0.1	0.004	0.3 - 5
Ba	0.04	0.2 - 15	0.2 - 15	0.63	1.7	100
Ag	BDL	NA	NA	<0.25	BDL	5

NA: not available; BDL: below detection limit; STLC: soluble threshold limit concentration.

[a,e] Shim et al., 2005

[b] Lindsay and Norvell, 1978; Nayak et al., 2014

[c] Punshon et al., 2002

[d] USEPA, 1992; Zhou et al; 2014

[e] Ward et al., 2003; Pandey et al., 2016

[#] Shim et al., 2005

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Usually, if the amount of trace metals released from the FA exceeds the regulatory limits it is grouped under hazardous waste (Shim et al., 2005). All the toxic elements are generally found within the STLC levels except Se and As. It has been reported that leachability of Se and As is variable in different leaching conditions (Ward et al., 2003). Leachability of As is significantly guided by the pH of the extractant which peaks at the middle of the pH range and lowers at the ends (Ward et al., 2003).

Factors assessing phytoremediation potential of plants and pollution level in FA

Decontamination/detoxification processes mediated through plants also called "phytoremediation" can render contaminated substrate harmless. This further improves soil biological activity, structure, and fertility (Salt et al., 1998). Fe, Si, As, Cd, Pb is some examples of the various trace elements found in FA. Studies have reported that treated FA generally has lower quantities of toxic metals compared to untreated ones. It has also been observed that concentration of metals decreases during gradual weathering and revegetation processes (Kumari et al., 2013). Phytoremediation of heavy metals involves two basic processes:

1. Phytoextraction - metal accumulation by hyperaccumulator plants from soil in their harvestable parts, which after a certain time period are harvested, disposed or incinerated.
2. Phytostabilization is captivating metals and preventing their leaching with the help of adventious roots along with associated rhizospheric microbes. Phytostabilization has become the most successful and well approved process.

Differing metal contents within various plant parts are due to different cellular mechanisms which control their translocation in plant systems. Hyper-accumulation of a metal by a plant is judged by two dimensionless parameters (Gonzaga et al., 2006):

1. Bioaccumulation factor (BAF) is the ratio of metal concentration in roots to that in substrate. BAF values greater than 1 indicate the potentiality of the plant to be used for phytoremediation (Kumari et al., 2013). This factor can be calculated in the similar way for stems and leaves (Pandey, 2012a). Various plant species with different values of BAF have been enumerated in *Table 4*.
2. Bioabsorption coefficient (BAC) is metal content in shoot/metal content in soil (Varun et al., 2012).
3. Translocation factor (TF) is the concentration of metal accumulated in above ground part (shoot) by that accumulated in below ground part (root) of a plant. Various plant species with different values of TF have been enumerated in *Table 4*.
4. Metal pollution Index (MPI) finds out the relationships between metal load in roots and shoots and can be calculated by the following formulae (Singh et al., 2008), where C_f is the concentration factor or metal concentration of n metals in a sample. In this context concentration factor of a metal can be determined by dividing a particular metal concentration in the sample by the concentration in the background.

$$MPI = \sqrt[n]{(Cf1 \times Cf2 \times Cf3 \times \ldots . Cfn)}$$

5. Enrichment factor (E_f) is determined by comparing accumulated trace metals with background concentration (Sinex and Helz, 1981) and can be calculated by

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the below formulae. E_f and MPI do not involve threshold values of metals in samples during calculation, which is a drawback.

$$\text{Enrichment factor} = \frac{\text{TM}}{\text{Background}}$$

6. Enrichment Index (EI) is calculated by averaging the ratios of element concentration to a threshold or permissible level. The permissible levels of metals in soil and FA are given in *Table 3,* which shows the maximum allowable limit of metals in the substrate to be safe for growth of food crops (Das and Chakrapani, 2011). For example the formulae for 5 metals will be as follows, where metal symbols represent metal concentration in the sample.

$$EI = \frac{1}{5}\left(\frac{Cr}{25} + \frac{Cu}{150} + \frac{Ni}{60} + \frac{Zn}{300} + \frac{Pb}{300}\right)$$

7. Geoaccumulation index (I_{geo}) (Ruiz, 2001) is another index similar to enrichment index and calculated for quantification of metal in the substrate in relation to the background. It is expressed by the following formulae where C_n is the metal concentration in the sample and B_n is the background value whereas 1.5 is the background matrix correlation.

$$Igeo = \log_2 \frac{Cn}{1.5 \times Bn}$$

I_{geo} can be classified into six grades according to the values obtained, such as:

- Grade 1: unpolluted when $I_{geo} < 1$
- Grade 2: very lightly polluted when $1 < I_{geo} < 2$
- Grade 3: lightly polluted when $2 < I_{geo} < 3$
- Grade 4: moderately polluted when $3 < I_{geo} < 4$
- Grade 5: highly polluted when $4 < I_{geo} < 5$
- Grade 6: very highly polluted when $5 < I_{geo} < 6$

Background values in the above formula can be substituted by the maximum allowable concentration or the threshold limit.

The above indexes can be used to determine the metal pollution level in plants as well as the substrate. They are very helpful in performing comparisons between differently reclaimed FA dumps. Moreover, intial studies for species selection for a future restoration programme can also be done on the basis of these indexes. Growing of hyperaccumulator species in restoration programmes is another aspect in which heavy metals contamination can be remediated from FA sites (Mendez and Maier, 2008). Pandey (2012a) reported that naturally growing *Azolla caroliniana* (water fern) on metal enriched FA ponds can be beneficial due to its toxitolerant characteristics such as high bioconcentration factor (BCF). *A. caroliniana* had high BCF values for all metals in roots and fronds in the range from 1.7 to 18.6 and 1.8 to 11.0, respectively while TF ranged from 0.37 to 1.4 for various heavy metals (Pandey, 2012a). Biomass of this fern doubles in 3–9 days, depending on habitat conditions and success of

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phytoremediation depends on growth rate of the plant. Physicochemical and biological factors such as pH, soil mineralogy, texture, salinity, amount of humic acids, and presence of organic chelators are responsible for metal availability and bioaccumulation in the plants (Pandey et al., 2010). Pandey (2013) studied about the potentiality of *Ricinus communis* L. to be used as a vegetation cover on metal enriched FA site and found that it has a BCF value greater than 1 and TF less than 1. *T. dentata*, is another a fern species which accumulates more metals in their roots/rhizome than the fronds (Kumari et al., 2011). Pandey et al. (2010) also found higher metal concentrations in root parts of chickpea growing on FA. The tendency of plants to accumulate metals in their aboveground and below ground parts is directly proportional to amount and time of exposure to the fly ash. Metals are generally sequestered in the root cell vacuoles to diminish its toxicity. Roots often act as a barrier against heavy metal translocation and are more tolerant to toxic metal concentrations, thus explaining higher accumulation when compared to shoots (Shanker et al., 2005).

Conclusion

In conclusion, this paper emphasizes on an efficient reclamation strategy of FA disposal sites which are a foremost challenge nowadays to check land and environment degradation. Above discussion on FA characteristics, enrichment and geo-accumulation index of toxic metals can be considered before initiating technical restoration on the site. A prior survey on the FA properties such as pH, texture, metal leaching should be carried out before selecting the type of amendment application, rate of application and type of plant species to be used. This would also help in reducing ground water contamination. Use of geotextiles is also recommended in certain cases to curtail erosion of FA during monsoon.

Plant species such as fast growing legumes act as green manure whereas hardy tuft grasses act as mulches. They can reduce the use of costly topsoil and also require less manure. Knowledge of the phytodiversity of old FA deposits and also the inventory discussed in this paper will help in selecting a right combination of native and exotic species for gradual restoration of the sites. In addition, rapidly growing perennial cover species, mixed plantation of grasses and legumes ameliorates the substrate, stabilises toxic metals, produces sufficient aboveground biomass, prevents erosion and can also lend a future source of income for local people. Biomass production is a new prospect in FA landfills management and can be initiated at later stages of restoration. Economically important trees which generate pulp, paper, biodiesel, firewood, timber wood, plywood, aromatic grasses yielding essential oils, fast growing legumes producing non timber forest products should be practised efficiently in these areas.

Above all this, a regular monitoring schedule is of prime importance in each restoration programme. Time to time analysis of substrate nutrient status, extent of nutrient loss, bioavailable toxic metals, and their accumulation in the vegetation will help in guiding the future steps to improve the status of these sites. This type of monitoring data emphasizing on the long term change in FA properties due to vegetation and metal leaching into ground water will benefit the future approaches to be used in management of FA deposits.

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TESTING PLANT PHENOPHASE AS PROXY: SENSITIVITY ANALYSIS OF FIRST FLOWERING DATA FROM THE 19TH CENTURY

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Abstract. Eco-climatological studies recognise plant phenophases as high-confident climate indicators, since they are strongly dependent on heat conditions. We investigated the first flowering response of numerous plant species to inter-annual fluctuation of seasonal temperatures (e.g., heat sensitivity of the phenophase), also the rate of these species-specific sensitivities in order to test their applicability as proxy. From the few available data sources recorded in the Carpathian Basin during the 19th century, the first flowering data sets of 16 plant species and time series of monthly mean temperature (site: Hermannstadt; period: 1851-1891), furthermore the North Atlantic Oscillation (NAO) were selected for the analysis. We found that the first flowering dates of different plants fluctuated significantly synchronously, however, temporal trends were not detected in any of the time series. Based on the main heat sensitivity characteristics the species were ranked as phyto-thermometers to select the best heat indicator plants. The first flowering data of these indicators were applicable to estimate temperature data. The accuracy of different plants as proxies varied in the range of 1.0 °C and 1.5 °C. Therefore our procedure is of interest in order to better understand past climates of periods at locations where no instrumental records are available.
Keywords: *flowering onset, effective temperature, moving window technique, heat sensitivity, proxy*

Introduction

The Earth is already experiencing human induced global scale climatic changes, which affect the whole biosphere. Evidences are increasing according to the biological responses documented (Walther et al., 2002; Parmesan and Yohe, 2003; Root et al., 2003; Bartholy et al., 2012) in plant and animal populations. The most easily detectable and widely reported changes can be worldwide seen in the timing of phenological events (Miller-Rushing and Primack, 2008). Several study have been gathered from the past half-century about spatial and temporal shifts of plant phenophases associated with

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global warming trends. Evidences of plant phenological responses are known across the globe (Badeck et al., 2004; Cleland et al., 2007; Elzinga et al., 2007), from the Northern (Schwartz et al., 2006) to the Southern Hemisphere (Chambers et al., 2013), towards Europe (Fitter et al., 1995; Ahas and Aasa, 2006; Menzel et al., 2006), Russia (Ovaskainen et al., 2013) and China (Ge et al., 2015). Thus, one of the most appropriate indicator of climatic changes are phenophases of living beings. Phenology, the science of natural recurring events (Demarée and Rutishauser, 2011) analyses the timing of periodic life-history events (i.e. phenophases) such as budburst or first flowering of plants (Pau et al., 2011). Specifically, the first definition by Lieth (1974) says: 'Phenology is the study of timing of recurrent biological events, the causes of their timing with regard to biotic and abiotic forces, and the interrelation among phases of the same or different species'.

In moderate and cold climatic zones, phenological stages occurring in the spring season are particularly sensitive to their environment. Their adaptation to interannual day length can cause detectable changes in their growth activity if reinforced by increasing temperature (Rutishauser et al., 2007). Atmospheric teleconnection patterns, e.g. the North Atlantic Oscillation, influence both temperature and precipitation conditions of the Northern Hemisphere (Trigo et al., 2002; Hurrell et al., 2003; Bartholy et al., 2009; Mandl, 2009), and thus, indirectly the phenological patterns too (Menzel, 2003; Stenseth et al., 2003). Precipitation cannot be considered as a major driving factor at the mid-latitudes (Buermann et al., 2003), because it usually does not significantly explain variances of the spring plant development (Rutishauser et al., 2007). However, it is more important in arid and semi-arid regions (Lima and Rodal, 2010).

Eco-climatological studies referring for plant phenophases can often be used as bio-indicators of climate change or proxies for temperature (Menzel, 2002, 2003; Miller-Rushing et al., 2008), especially when the seasonal timing of the phenological event is closely related to specific climatic conditions during plant development (Sparks et al., 2000; Aono and Kazui, 2008). The so-called climate proxies are preserved physical characteristics of the past that stand for direct measurements and can be utilized for climatological reconstructions (Rutishauser et al., 2007). Furthermore, vice versa, future climate projections can be used for the prediction of the proxy based on the strong relationship between the variables. Numerous studies reconstructed temperature conditions using different phenophases from available phenological data series (e.g., Holopainen et al., 2006; Lavoie and Lachance, 2006; Rutishauser et al., 2007; Aono and Kazui, 2008; Kiss et al., 2010). Although phenological data series compiled from historical records allow climatic reconstructions on shorter time-scale compared to other proxies, such as tree ring, pollen or ice core data, they are also important sources for analysing the past climate and prepare cross-validation independently (Dickinson and Bonney, 2012). Detailed analyses of heat sensitivity of different phenophases were carried out in Germany, Switzerland and UK (Rutishauser et al., 2009; Schleip et al., 2009), but not yet for the Carpathian Basin.

There are numerous endemic and climatic-endangered plant species living in the Pannonian biogeographical region. The enhanced protection of these species and their habitats under climatic changes is substantial, otherwise they might face to severe consequences and even extinction (Root et al., 2003; Estes et al., 2011). In order to understand and predict the impact of current climatic changes on plant phenophases, it is necessary to analyse phenological time series as a reference from the period when recent anthropogenic warming effect did not influence the local climate conditions.

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Unfortunately, most of the phyto-phenological data series recorded in the 19th century, suffer lacks both in time and space for the Carpathian Basin (Szalai et al., 2008). The available studies from this region (e.g. Walkovszky, 1998; Varga et al., 2009a,b, 2010; Szabó et al., 2016) rely on phenological data series recorded at the second half of 20th century, which period is already significantly influenced by the warming spring (Pongrácz et al., 2011; Cramer et al., 2014).

In the present study, we investigated the first flowering response of 16 wild plant species to interannual fluctuation of local seasonal temperatures (i.e., heat sensitivity of the flowering onset), also the rate of these species-specific sensitivities in order to test their applicability as proxy. The analyses were accomplished using first flowering data series, recorded in the second half of 19th century, in Hermannstadt and Mediasch located in Transylvania (nowadays in Romania). The following issues were addressed using different statistical methods: (i) characterization of the effect of mean temperatures in various time periods (monthly, bi-monthly, tri-monthly, etc) on flowering onset dates using a moving-window technique; (ii) determination of the effective temperature values (T_{eff}) estimated from the responses of each species; (iii) calculation of the temporal shifts of first flowering date as a response to T_{eff}. Furthermore (iv) the plant species were ranked based on the temperature sensitivity of their first flowering dates; and (v) the accuracy of use of plant phenophases as proxy estimations was evaluated.

Materials and methods

Phenological data

The analyses are accomplished using flowering onset data sets of 16 wild plant species (*Table 1*) recorded in the second half of 19th century. The observations were carried out in the period 1851-1891, near Hermannstadt (45° 48' N, 24° 9' E, named Sibiu today, located in Romania), by Ludwig Reissenberger, a local teacher deeply interested in natural science. The data recording is considered reliable and the documentation is precise due to the unchanged observer.

Table 1. *Flowering onset data characteristics of the observed 16 plant species near Hermannstadt in the period 1851-1891. (SD= standard deviation; *herbaceous plants)*

	Species names		**Flowering onset (FO)**		
			Mean FO date	**Days after 1 January**	**± SD [day]**
Sp-1	*Tussilago farfara* L.	Coltsfoot*	02 March	62	15.7
Sp-2	*Scilla bifolia* L.	Two-leaf squill*	25 March	85	10.0
Sp-3	*Taraxacum officinale* W.	Common dandelion*	05 April	96	12.0
Sp-4	*Caltha palustris* L.	Marsh marigold*	07 April	98	9.1
Sp-5	*Salix fragilis* L.	Crack willow	16 April	106	9.9
Sp-6	*Ribes rubrum* L.	Red currant*	20 April	110	9.0
Sp-7	*Fragaria vesca* L.	Woodland strawberry*	23 April	113	8.9
Sp-8	*Orchis morio* L.	Green-winged orchid*	01 May	122	8.2
Sp-9	*Syringa vulgaris* L.	Common lilac	02 May	123	8.8
Sp-10	*Aesculus hippocastanum* L.	Horse chestnut	04 May	125	8.5

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Sp-11	*Euonymus europaeus* L.	European spindle	07 May	128	8.1
Sp-12	*Salvia pratensis* L.	Meadow sage*	10 May	130	8.7
Sp-13	*Dianthus carthusianorum* L.	Carthusian pink*	24 May	144	9.3
Sp-14	*Robinia pseudoacacia* L.	Black locust	25 May	145	9.1
Sp-15	*Sambucus nigra* L.	Black elder	26 May	146	9.3
Sp-16	*Vitis vinifera* L.	Common grape vine	13 June	165	7.3

In order to test the accuracy of flowering dates as proxy, data have also been involved into analyses from Mediasch (46° 10' N, 24° 21' E, named Mediaş today, located at cc. 50 km distance from Hermannstadt), for the period 1854-1865. (All the data mentioned above available in the Austro-Hungarian and Hungarian Meteorological Yearbooks.) At both sites, the date of flowering onset was defined as the date when some individuals from the whole plant population are totally flowering as it was given by the protocol of phenological observation in the 19th century (see in Meteorological Yearbooks). At Hermannstadt 24 plant species were observed by Reissenberger, however for detailed analyses 16 species were selected based on two criteria: (i) the plant was required to be common, widespread and possibly wild, in order to identify them by the observer easily, (ii) the average first flowering date was required to occur in the period from late-winter/early-spring until early-summer to enable comparisons of species-specific responses to different seasonal temperatures. According to similar investigations (Menzel, 2002, 2003; Fitter and Fitter, 2002), these early flowering species are more sensitive to climatic variations than the later (summer and/or autumn) flowering ones. In addition, half of the selected 16 species were herbaceous plants and the others were woody. Date of phenophase was given as the 'day of the year', i.e., the number of days elapsed since 1st January of a given calendar year.

Climatological data

The time series of monthly mean temperatures were also obtained from the mentioned Meteorological Yearbooks, and covered the same period (1851-1891 and 1854-1865) as the phenological observations originated from the two observational sites. The monthly means of air temperature were calculated from daily data. These daily time series were averaged and corrected from three daily measurements, recorded in the yearbooks. The meteorological measurements were carried out by standard devices of the Austrian weather service. Detailed descriptions of the measuring methods, conditions, devices, and applied corrections can be found in the yearbooks. After transforming the Réaumur degrees into standard Celsius degrees, and completing quality control, the monthly averaged data sets were considered as local homogeneous time series. The teleconnection pattern of North Atlantic Oscillation (NAO) has also been involved into our analysis as winter NAO index (Jones et al., 1997; Climatic Research Unit database), since several studies (e.g., Menzel, 2003; Gordo and Sanz, 2010; Szabó et al., 2016) confirmed the indirect effect of winter NAO on the timing of plant phenophases.

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Statistical methods

Both phenological and temperature data sets can be characterized by normal distribution, which was checked with Kolmogorov-Smirnov statistical test using 95% confidence interval.

Linear regression analyses were applied to describe the possible long-term trends in the time series and possible relations between temperature and phenological data. The goal was to identify linear trend via regression of the observed time series against time and test the estimated slope coefficient of the linear regression equation for significance (Haan, 2002). The well-known least squares method was used for parameter estimation.

Cross correlation function (CCF) was calculated between the two time series (y_t: phenological and x_t: climatic) for identifying lags of the x-variable that might be useful predictors of y_t. CCF was defined as the set of sample correlations between x_{t+l} and y_t for $l = 0$. Cross correlation values reflect the degree of linear relationship between the two data sets. Significant negative values for r_0 show if there was a negative correlation between the x-variable and the y-variable at time t with 0-lag (confirmed by t-test with 0.95 level of significance).

In phenological analyses, climatic variables are usually aggregated into averages over a month or more. Despite the loss of information due to aggregation, this aggregating method was applied in order to avoid both numerical problems and difficulties with interpretation arising from the high dimensional and correlated nature of daily weather data (Roberts, 2010). In this study bi-, tri-, and tetra-monthly mean temperatures were calculated from the monthly mean data to examine the relationships between the timing of first flowering and temperature data.

The effective temperature (T_{eff}) is a nominal temperature that represents the heat conditions of the period, which is considered to possess the highest impact on the timing of flowering onset of a plant species. So, the T_{eff} values represent different heat conditions due to different length of aggregating periods. The effective temperature periods were found by a 'moving window' technique: bi-, tri-, and tetra-monthly temperatures were calculated from the monthly means by shifting 1-month-steps. As a result of this method, newly aggregated time series were obtained such as T_{FM}, T_{MA}, T_{AM}, T_{MJ}; T_{JFM}, T_{FMA}, T_{MAM}, T_{AMJ}; and T_{JFMA}, T_{FMAM}, T_{MAMJ}. The heat conditions of the winter-spring period prior to the time of flowering (even the previous summer and autumn conditions) can significantly influence as well as determine the date of flowering onset (Miller-Rushing and Primack, 2008). Hence the average temperatures of these periods can be considered as rough representation of the cumulative amount of heat. We determined the most effective temperature period for the phenophase of each species, by calculating serial CCF values. T_{eff} was selected by the highest absolute value of the CCF at r_0.

The temporal shifts of first flowering as a response to T_{eff} and the heat sensitivity were described after applying linear regression. These characteristics were determined from the slope of the regression equations between the flowering and temperature time series. The regression coefficients indicate the effect (shift of flowering onset in days) of 1°C change in temperature in the certain period. Negative value of the regression coefficient indicates the advancement of flowering in response to increasing temperature.

To describe the species-specific relative response of flowerings to relative changes in T_{eff}, both data series were converted into relative measures (expressed in percentages). These were obtained as follows: (i) determination of the anomalies of

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time series compared to the average of time series, (ii) sum of these anomalies without signs (this sum means the 100%), and finally (iii) expression the anomalies with signs as a percent of the previously calculated amount of 100%. The obtained relative responses of flowerings were considered as rough indicators of heat sensitivity characterising the plant species. By this indicator the plants were ranked and compared in terms of possible utilization as proxy.

In order to test the flowering onset of the selected plants as proxy data for local average seasonal temperatures (assuming relatively constant heat sensitivity in at least 50 km vicinity of the Hermannstadt site), phenological and temperature data of Mediasch (period: 1854-1865) were involved into the analysis. In case of 14 plant species observed at both places, by replacing the phenological data of Mediasch into the regression equation established on the relation between the phenological and temperature data of Hermannstadt, a robust estimation of local effective temperature was gained. The statistical analysis was carried out with codes written in FORTRAN language and with the *Statistica* software package (version 6.1, StatSoft Inc., USA).

Results

Characteristics of studied time series

Observed temperature data

According to the completed trend analysis, significant temporal trend was not detected in any of the temperature time series of Hermannstadt (1851-1891) and Mediasch (1854-1865), except mean temperature of April at Hermannstadt ($p < 0.05$), which was detrended for further analyses. After comparing monthly temperature data of the two locations, Mediasch was warmer than Hermannstadt by 1.04 °C on a yearly basis. Such difference could be resulted from the microclimates caused by differences in topographical conditions. Nevertheless, the general temperature conditions are quite similar at both places. As preliminary analysis showed, the early spring temperature series were significantly synchronously fluctuating at the two sites in the same period.

Overview of flowering onset data

Plants were selected from species observed at Hermannstadt flowering from late winter to early summer. The means of flowering onset dates with their standard deviations are listed for each species in *Table 1*. In the first part of the flowering onset temporal rank the herbaceous plants, in the second part the woody plants appear typically, which is reasonable when considering plant physiology.

Standard deviation (SD) of the first flowering time was decreasing from the earlier to later flowering plants due to the higher variability of mean temperatures in cooler months (January - March) (*Fig. 1*). The earliest spring flowering plant was *Tussilago farfara*, which was characterized by relatively high SD (15.7 days) and total range (75 days). In contrast, the early summer flowering *Vitis vinifera* had the lowest SD among the examined species and significantly lower range (47 days) than the others. The group of early May flowering plants (i.e., *Orchis morio, Syringa vulgaris, Aesculus hippocastanum* and *Euonymus europaeus*) as well, as the group of late May flowering species (i.e., *Dianthus carthusianorum, Robinia pseudoacacia* and *Sambucus nigra*) were characterized by similarly high minimum, maximum and SD values within the groups (*Table 1*).

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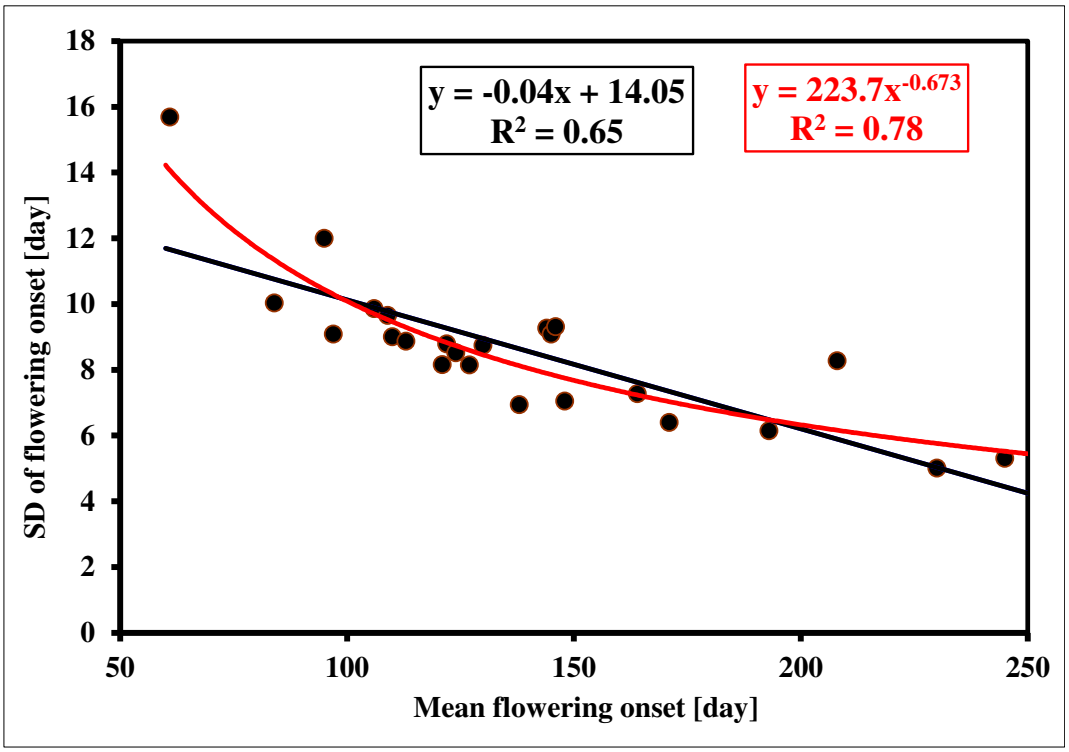

Figure 1. *Relationship between mean flowering onsets and their standard deviations (SD) in case of plants observed (1851-1891) near Hermannstadt. Both linear and exponential regressions clearly show significant decrease of SD towards the late flowering plant species.*

Significant temporal trend was not detected in any of the time series. Based on the CCF values, flowering time series significantly synchronously fluctuated not just intralocally (between species), but interlocally (between locations) as well. In order to illustrate this synchrony, the temporal patterns of FO of four plants are drawn in *Fig. 2*. The sharp yearly fluctuation of *Tussilago farfara* (Sp-1) – as the earliest flowering plant – is conspicuous, indicating a strong sensitivity to late-winter temperatures.

Impact of temperature on flowering onset

In order to determine the strength of the relationship between the timing of flowering onset and temperature data, correlation coefficients (r_0) at 0 lag CCFs were calculated (*Table A; Appendix*). The signs of r_0 were mostly negative in case of winter-spring months, indicating that plants responded to higher temperatures with earlier flowering onsets (*Fig. 3*).

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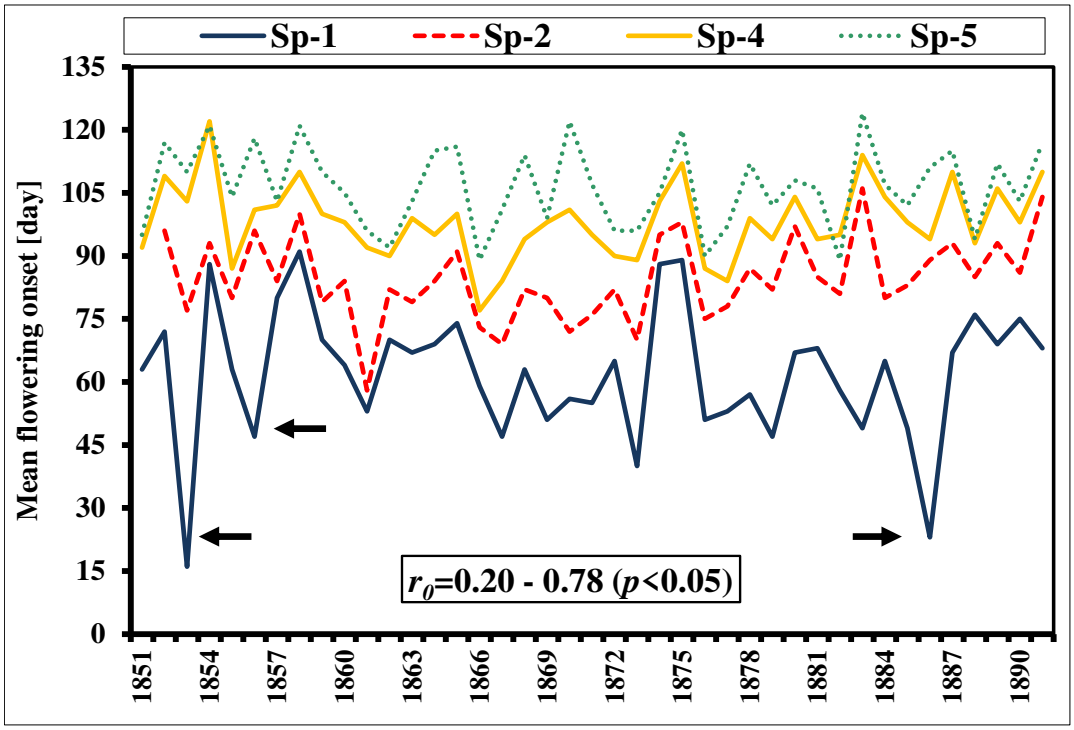

Figure 2. *The synchronous fluctuations of four early flowering plants near Hermannstadt (1851-1891). The value of correlation coefficient (r_0) was higher between the later ones, Scilla bifolia (Sp-2), Caltha palustris (Sp-4) and Salix fragilis (Sp-5), than the earliest flowering Tussilago farfara (Sp-1). The black arrows point at the marked deviations of T. farfara due to late winter heat waves.*

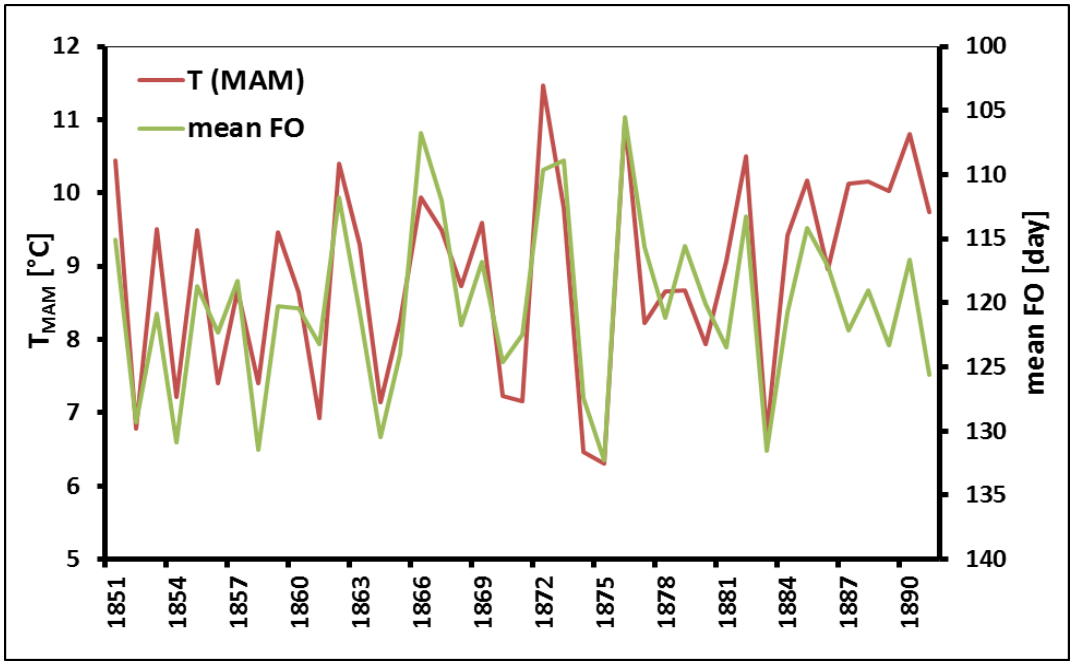

Figure 3. *Mean flowering onset (FO) of 16 plant species and tri-monthly mean temperature of the period March-May recorded near Hermannstadt (1851-1891). The high negative value of the correlation coefficient indicates strong reverse relation between the timing of the phenophase and heat conditions of spring.*

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In late winter – spring seasons, strong correlations ($p < 0.05$) were found between the flowering onset data series and monthly, multi-monthly mean temperature time series in case of most species (indicated in bold in *Table A; Appendix*). Effective temperatures of 16 plant species were determined by serial correlations using 'moving window' method described above (Materials and methods). The effect of mean monthly, bi-monthly, tri-monthly, tetra-monthly temperatures on the first flowerings was determined in the first step. Then, from the obtained different strength of FO responses, each species-specific effective temperature value (T_{eff}) was estimated. The most effective temperature period for a certain plant was selected by the highest absolute value of r_0 (*Fig. 4*).

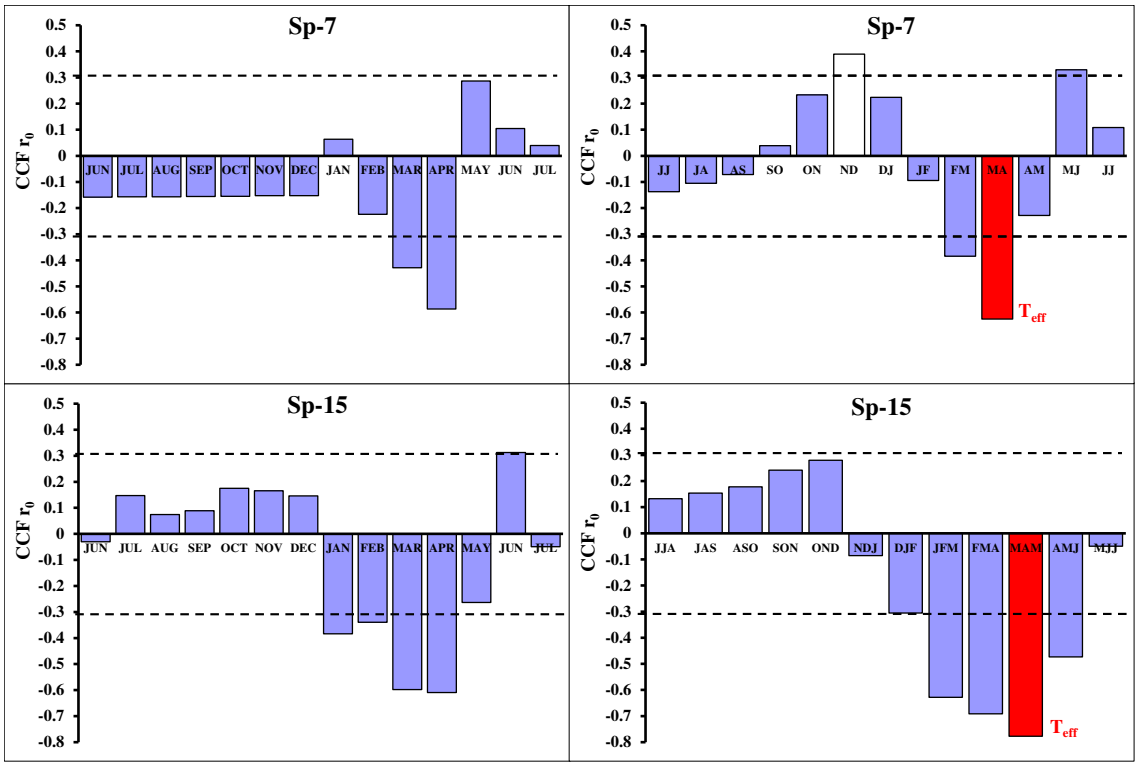

Figure 4. *Two examples (Sp-7: Fragaria vesca and Sp-15: Sambucus nigra) for finding the most effective temperature (Teff) of flowering onsets using serial cross correlation functions (CCF) and moving window technique with different number (1, 2, 3 and 4) of months. Scattered lines on the graphs indicate the threshold of significant ($p < 0.05$) correlation coefficient values (r0).*

Periods of the effective temperature (T_{eff}) and periods with high FO-T correlation ($r_0 > 0.5$) are given in *Table 2.* for the 16 examined plant species. The majority of plants expressed the highest correlation with the bi-monthly mean temperature preceding the flowering onset, however there were some examples, which produced the highest r_0 with tri-monthly period (e.g. *Tussilago farfara* – JFM) or even longer, tetra-monthly period (e.g. *Vitis vinifera* – MAMJ). In case of almost all plants the DJFM and the JFMA periods were the first 'negative-effect' (i.e. causing advanced FO) periods, while for *Tussilago farfara* and *Taraxacum officinale* the mean temperature of the late autumn – winter (ONDJ, NDJF) period found to be also significantly effective on the timing of subsequent flowering onset (*Table A; Appendix*).

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Table 2. *The effective temperature (T_{eff}) periods and the 1-month periods of temperature with the highest influence ($r > 0.5$; $p < 0.05$) on the timing of flowering onset (FO) of the 16 studied plant species observed near Hermannstadt (1851-1891).*

	Period of T_{eff} (multi-months)	**Correlation FO-T_{month} ($r > 0.5$)**
Sp-1	JFM	Feb
Sp-2	FM	Feb, Mar
Sp-3	FM	Mar
Sp-4	FMA	Mar
Sp-5	MA	Mar, Apr
Sp-6	MA	Mar, Apr
Sp-7	MA	Apr
Sp-8	MA	Mar, Apr
Sp-9	MA	Apr
Sp-10	MA	Apr
Sp-11	MA	Mar, Apr
Sp-12	A, MA	Apr
Sp-13	MAM	Apr
Sp-14	AM	Apr, May
Sp-15	MAM	Mar, Apr
Sp-16	MAMJ	Apr, May

For half of the species a 'positive effect' (i.e. causing delayed FO) by the multi-monthly mean temperatures of previous years in summer-autumn season was observed. In case of eight plants, significant ($p < 0.05$) positive values of r_0 were found, associated with relation to bi-, tri-, tetra-monthly summer – autumn mean temperatures and the mean flowering onset. The FO of *Scilla bifolia* was influenced by the mean temperature of late summer – early autumn period; similarly the FO of *Salix fragilis, Syringa vulgaris, Dianthus carthusianorum* and *Robinia pseudoacacia* by the mean temperature of autumn period; and FO of *Fragaria vesca* by the mean temperature of late autumn – winter period were affected as well. Finally, for *Aesculus hippocastanum* the FO seemed to be influenced by the temperature conditions of the entire June to December period.

Species-specific heat sensitivity of flowering onset

Flowering sensitivities of the selected plants in response to their effective temperatures were different. Based on results of the regression analysis (RA) the 16 plants species were ranked. The rank was created by (i) the correlation coefficients between the first flowering dates and the T_{eff} temperatures (*Fig. 5* and *Fig. 6*), (ii) the temporal shifts (expressed in day/°C) of first flowering as a response to a unit change in T_{eff} (*Fig. 5*), (iii) the relative response of first flowering to a relative change in T_{eff}. The negative value of slope (a) referred to the straightforward feature that higher mean

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temperature of previous periods of phenophase caused advanced flowering onset. These responses of the flowering onsets were species-specific and significantly ($p < 0.05$) measurable (*Figures 5-6*).

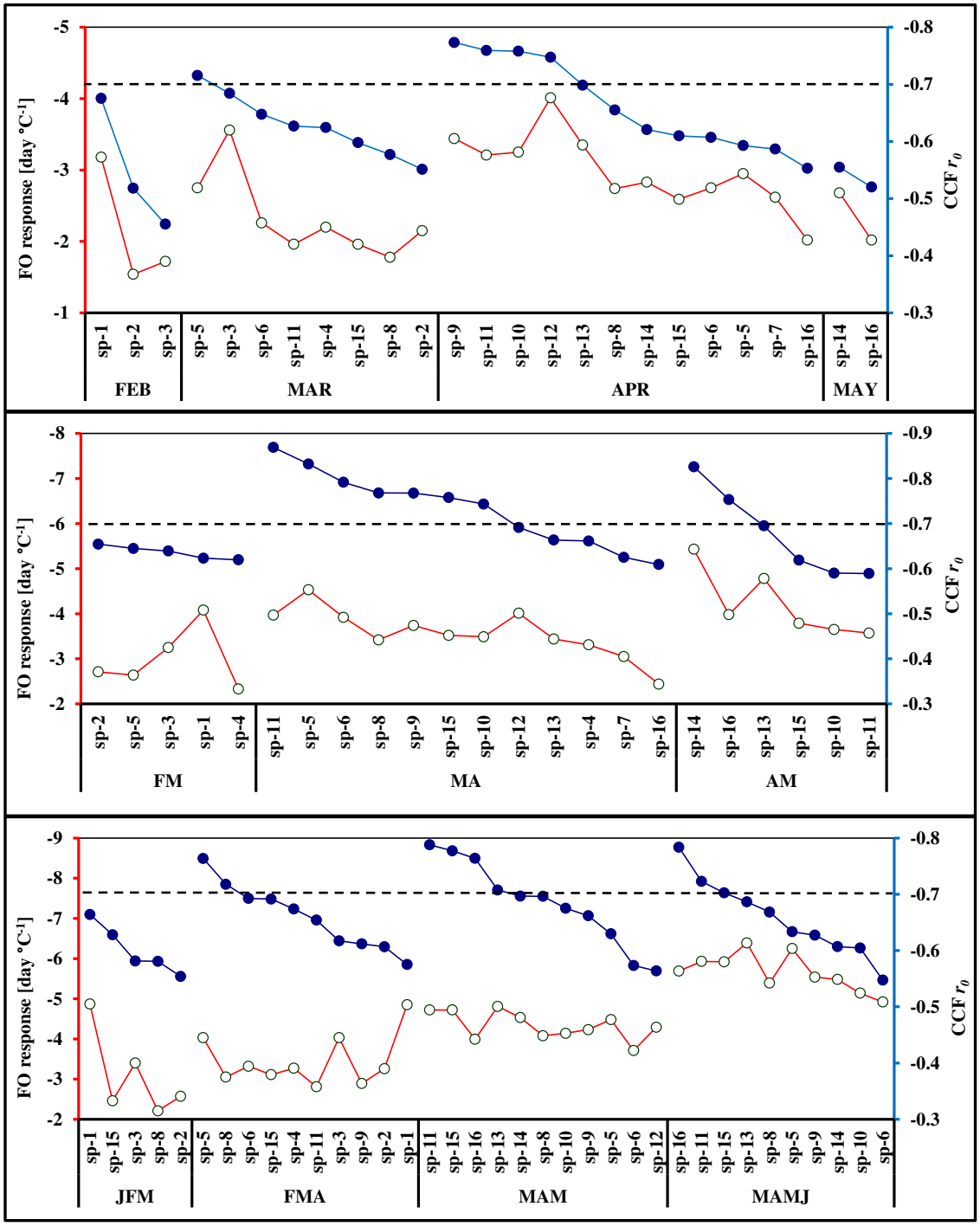

Figure 5. *Rank of 16 plant species by significant ($p < 0.05$) correlations (vertical axis on the right, filled markers) and response of flowering onsets (expressed in the value of slopes originated from regression equations; vertical axis on the left, empty markers) given to the mean temperatures of various time periods based on observations near Hermannstadt from 1851-1891. The straight dashed lines indicate the threshold of the strongest relations between the phenophase and the mean temperature.*

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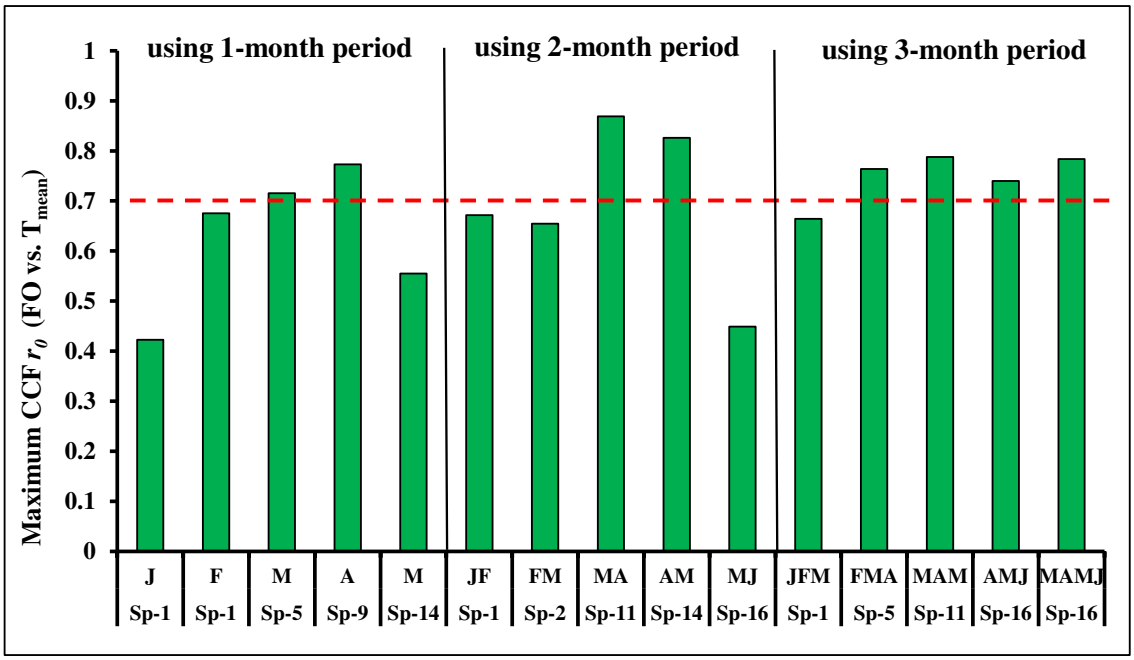

Figure 6. *The highest significant CCF r0 ($p<0.05$) values found by the moving window method between flowering onset (FO) and mean temperature (T_{mean}) for periods of different length based on observations near Hermannstadt, 1851-1891. The red straight dashed line indicates the threshold of the strongest relationships between the phenophase and the mean temperature.*

Plants related to the same T_{eff} period were compared and ranked by the strength of the relation (r_0) between FO and T_{eff}. Then the magnitude of the response to a unit change in T_{eff} (a) was considered. In *Fig. 5* the strongest relationships ($r_0 > 0.5$) of plants belong to different monthly and multi-monthly effective temperature periods are shown.

The strongest correlation and the highest response to 1°C change in temperature were found in the following cases. Correlation coefficients (r_0) and slopes (a) in cases of the mean temperature of February (T_{FEB}), March (T_{MAR}), April (T_{APR}) and May (T_{MAY}) were considered. For T_{FEB} the highest reaction was shown by *Tussilago farfara* (r_0=-0.68; a=-3.18); for T_{MAR} by *Salix fragilis* (r_0=-0.72; a=-2.75) and *Taraxacum officinale* (r_0=-0.68; a=-3.56); for T_{APR} by *Syringa vulgaris* (r_0=-0.77; a=-3.44) and *Vitis vinifera* (r_0=-0.75; a=-4.01); and for T_{MAY} by *Robinia pseudoacacia* (r_0=-0.56; a=-2.68) as it is drawn in *Fig. 5*.

In case of bi-monthly temperature means, for T_{FM} the relationships of the five earliest flowering plants were nearly the same (r_0=-0.62-0.65), but in terms of the FO response, *Tussilago farfara* seemed to be the most 'sensitive' (a=-4.08). T_{MA} influenced 12 plants effectively, in which case *Euonymus europaeus* was at the first place of the ranked series. T_{AM} showed the strongest correlation with the late spring flowering plants, the highest response to 1°C change in temperature was expressed by *Robinia pseudoacacia* (r_0=-0.83; a=-5.43).

In terms of the tri- and tetramonthly effective temperature periods in the ranked plant series belonging to T_{JFM}, the first was again *Tussilago farfara* (r_0=-0.66; a=-4.87) and a late spring plant, *Sambucus nigra* (r_0=-0.63; a=-2.46) occurred in the ranked series, too. The strongest relationship was detected and the highest reaction of FO was given to 1°C change in T_{FMA} by *Salix fragilis* (r_0=-0.76; a=-4.03); and in T_{MAM} by *Euonymus*

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europaeus (r_0=-0.79; a=-4.72) and *Sambucus nigra* (r_0=-0.78; a=-4.72). Finally, in case of the tetramonthly T_{MAMJ} *Vitis vinifera* (r_0=-0.78; a=-5.69) showed the strongest relation between T_{eff} and FO.

Taking into consideration the FOs climatological utilization (e.g. as a proxy), the highest CCF r_0 values per period are shown for each monthly, multi-monthly 'time-window' in *Fig. 6*. Interestingly, to all investigated time periods a total of 7 plants expressed the strongest response as 'thermal indicators'. These species (*Tussilago farfara, Scilla bifolia, Salix fragilis, Syringa vulgaris, Euonymus europaeus, Robinia pseudoacacia, Vitis vinifera*) were mostly characterized by the highest FO responses as well.

In order to determine a rough but comparable indicator of heat sensitivity of FO, regression analyses were carried out on the time series of relative changes of FO and monthly, multi-monthly temperatures. *Fig. 7* is an illustration for the regression slope assessment of sensitivity using relative monthly temperature and flowering changes. From a geometric aspect, heat sensitivity is the higher, the regression line fits the better to the 45° line, namely to the theoretic, perfect phyto-thermometer. Thus, in the example of the figure, *Euonymus europaeus* (Sp-11) (a=-0.90; R^2=0.69) responded more sensitively to 1°C change in mean temperature of MA period, than *Fragaria vesca* (Sp-7) (a=-0.60; R^2=0.39).

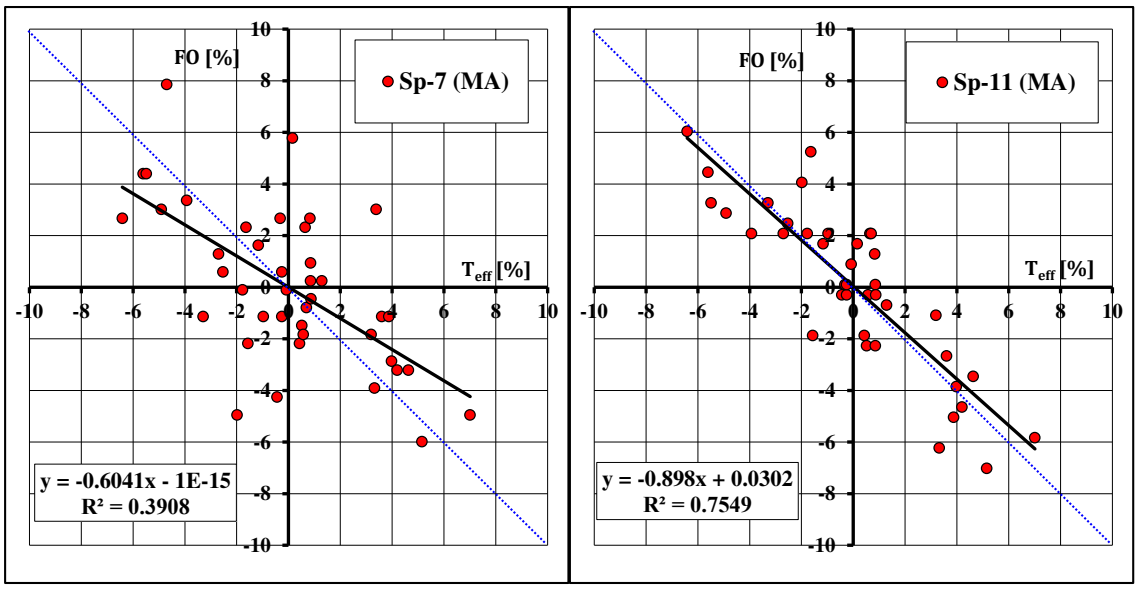

Figure 7. *Example of regression slope assessment of heat sensitivity using relative bi-monthly effective temperature (T_{eff}) as explanatory variable and relative changes of flowering onset (FO) as dependent variable. In this way the plants are comparable as thermometers for the same period. (Sp-7: Fragaria vesca; Sp-11: Euonymus europaeus; MA=T_{eff} period; solid line=linear regression line; dotted line=line with a = -1 as a 45° slope)*

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Testing flowering onset as a proxy

The phenophase onset was tested as temperature proxy using datasets from Mediasch (*Fig. 8*). Results of the regression analyses on temperature and phenological data of Hermannstadt were applied to estimate the effective temperature of 14 plant species observed at both places. According to the proxy-testing, the later the plant begins to flower, and the longer the period of effective temperature is (i.e. multi-monthly mean temperature was the most effective), the more accurate the estimation of T_{eff} by the FO.

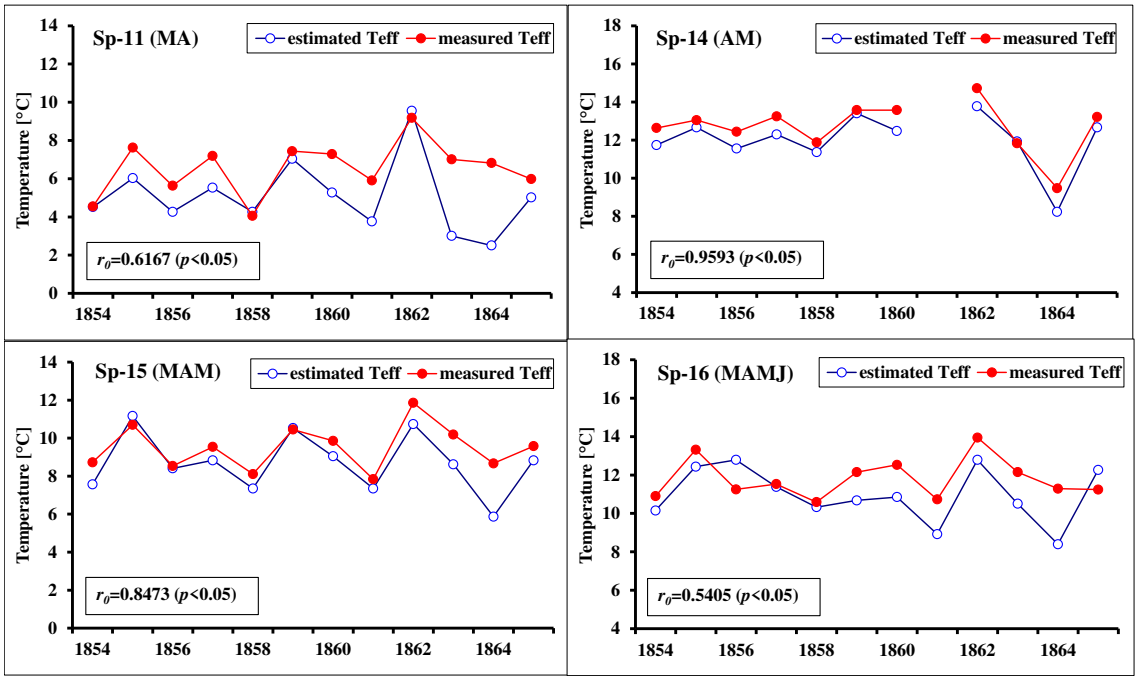

Figure 8. *Four examples of testing the accuracy of the proxy by estimating the effective temperature (T_{eff}) from the flowering onset data of Mediasch based on the linear regression equations on temperature and phenological data of Hermannstadt in case of 14 plant species observed (1854-1865) at both places. (Sp-11: Euonymus europaeus; Sp-14: Robinia pseudoacacia; Sp-15: Sambucus nigra; Sp-16: Vitis vinifera; T_{eff} periods: MA, AM, MAM, MAMJ)*

In *Fig. 8* the four most accurate indicator plants are shown, which were selected by considering the previous results of heat sensitivity rankings. These were mostly late spring – early summer flowering species, namely *Euonymus europaeus* (Sp-11), *Robinia pseudoacacia* (Sp-14), *Sambucus nigra* (Sp-15), and *Vitis vinifera* (Sp-16). In case of *Robinia pseudoacacia* the average difference between measured and estimated T_{eff} (=T_{AM}) was 0.50 °C, and SD was 0.41 °C. For *Sambucus nigra* concerning T_{MAM} the same values were 0.34 °C and 0.84 °C, respectively. Finally, in terms of *Vitis vinifera* these values appertain to T_{MAMJ} were found as 0.32 °C and 1.24 °C, respectively. In summary, FO data of the most sensitive heat indicator plants were applicable to estimate the T_{eff} data – as a first guess. The accuracy of estimation was between 1.0 °C and 1.5 °C.

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Discussion

In the first part of this study the main characteristics of the flowering phenological and climatological time series, as well as their relationships were analysed. Since several previous studies (e.g., Hurrell et al., 2003; Menzel, 2003) confirmed that the winter/early spring temperature variability in the 20th century is significantly influenced by the teleconnection pattern of North Atlantic Oscillation (NAO), we also involved the winter NAO index into our phenological analyses. Our results are consistent with other studies (Auer et al., 2001; Böhm et al., 2001) found for climatological conditions in the second part of the 19th century, namely, the temperature time series did not contain any increasing or decreasing trend in this part of Europe. Furthermore, other studies focusing especially on NAO (Jones et al., 1997; Hurrell et al., 2003; Osborn, 2006) did not find any significant trend in winter NAO time series, either. Our climatological results strengthen these findings and call for attention to discover more historical time series, the importance of reconstructions and the need for further research.

Considering the quantification of the FO – T – NAO impact-system, we have also found that the flowering onset (FO) is primarily influenced by the heat conditions of the preceding period of flowering (Menzel, 2003; Miller-Rushing and Primack, 2008; Szabó et al., 2016), and the impact of winter NAO was negligible. Based on our findings the majority of plants are affected most strongly by the mean bi-monthly or tri-monthly temperatures prior to the date of flowering. In addition, several plants (such as the flowering onset of *Scilla bifolia*) were also influenced by the heat conditions in late summer – autumn of the previous year, as similar conclusion was drawn by Gordo and Sanz (2010) for the Mediterranean region.

The main aim of this paper was to analyse the species-specific heat-sensitivity of flowering onset characteristics of different plant species. Only a few studies (e.g. Root et al., 2005; Aono and Kazui, 2008; Rutishauser et al., 2009) focused on this topic using this perspective so far. According to studies of 20th century data major synchronous break was found in phenological time series during the 1980s in Europe (Dose and Menzel, 2004; Schleip et al., 2006). Furthermore significant earlier shift in flowering onset dates (1952-2000) of common dandelion, black elder, as well as in case of the black locust (1951-1994) were shown by Szabó et al. (2016) and Walkovszky (1998) among our examined species. In contrary their findings in the neighbourhood country, Hungary, we did not find linear trend in the flowering onset data – probably because our data were recorded during the 19th century when the impacts of human induced climatic changes were not yet as influential as in the late 20th century.

Our central addressed issue of testing flowering onset as proxy variable for temperature was based on our heat sensitivity results. According to the validation tests on data from Mediasch, the flowering onsets of *Robinia pseudoacacia* and *Vitis vinifera* proved to be the most accurate phyto-thermometers. Hence, these two plants can be utilized to provide data with highest confidence as proxy for estimating the mean temperature of their effective temperature periods (*Robinia pseudoacacia* – April-May; *Vitis vinifera* – March-April-May-June) in the examined time period and region. Overall, the 14 tested plant species estimated their effective temperature with 1-1.5 °C accuracy. Taking into account the general climatological differences of the two sites (Mediasch is warmer in yearly average by 1.04 °C compared to Hermannstadt), the average bias of proxy estimations could be slightly reduced by applying a simple additive correction. Therefore, this method in first approach is appropriate as a robust estimation of mean temperature from flowering data. The estimation is robust which

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originate from the uncertainty of geographical factors, which can explain the spatial variance of flowering dates (Wang et al., 2015). Furthermore, this uncertainty also comes from the rough resolution of the temperature records, since the monthly and multi-monthly averages of temperature time-series are good representatives of the spring heat conditions, but not as accurate as if the effective temperatures were obtained based on daily data (e.g. degree-day calculation; see Schwartz, 2013). If more detailed data series (either temporally or spatially) are available, the method can be refined and resulted in a more accurate estimation, which is of interest in order to better understand past climates of periods or locations where no instrumental records are available.

Conclusions

- Decomposing the total climatic impacts, the temperature proved to be the main determining variable for the timing of flowering onset, whereas the impact ofthe winter NAO was negligible in the second part of the 19th century in Transylvania. The time series of flowering onset and effective temperature fluctuated significantly synchronously, nevertheless, temporal trends were not detected in the datasets (1851-1891).
- The species-specific effective temperature values obtained from the flowering onset response to monthly, bi-monthly, tri-monthly average temperatures were calculated and applied for ranking the plant species. The species-specific heat sensitivities were determined via examining the temporal shifts of first flowering date as a response to effective temperatures. According to the species-specific heat sensitivities the most accurate phyto-thermometers (*Robinia pseudoacacia* and *Vitis vinifera)* were selected.
- The beginning of flowering phenophase was tested as proxy for the effective temperature, and the accuracy of different plant proxies ranged between 1.0 °C and 1.5 °C. Thus our method is appropriate for climatological utilization as a robust estimation of heat conditions, when no other records are available.

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Appendix

Table A. *Four examples (Sp-1: Tussilago farfara, Sp-2: Scilla bifolia, Sp-4: Caltha palustris, and Sp-5: Salix fragilis) for finding the period of most effective temperature (T_{eff}) of flowering onsets using serial cross correlation functions (CCF) and moving window technique with different number (1, 2, 3 and 4) of months. Legend: bold numbers: significant correlation coefficient value (r0) ($p < 0.05$); pale yellow cell: the highest correlation coefficient in the column; orange cell: the highest correlation coefficient for the plant, so it reflects to the effective temperature period; blue cell: significant influence of the previous year. To determine the T_{eff} period the moving window method was applied on monthly data from the previous June until the July of the actual year regarding the occurrence of phenophase.*

1-monthly mean	r0	2-monthly mean	r0	3-monthly mean	r0	4-monthly mean	r0	1-monthly mean	r0	2-monthly mean	r0	3-monthly mean	r0	4-monthly mean	r0
JUN	-0.178	JJ	-0.0173	JJA	0.0116	JJAS	-0.0031	JUN	0.1106	JJ	0.1557	JJA	0.1721	JJAS	0.1883
JUL	0.1745	JA	0.06	JAS	0.0278	JASO	0.0324	JUL	0.1112	JA	0.1833	JAS	0.2026	JASO	0.2348
AUG	0.1921	AS	0.0127	ASO	0.0227	ASON	-0.0387	AUG	0.1118	AS	0.2151	ASO	0.2576	ASON	0.2531
SEP	-0.0721	SO	-0.0097	SON	-0.0928	SOND	-0.1858	SEP	0.1121	SO	0.281	SON	0.2608	SOND	0.2939
OCT	0.0606	ON	-0.0966	OND	-0.2101	**ONDJ**	**-0.3647**	OCT	0.1132	ON	0.2214	OND	0.2588	ONDJ	0.2155
NOV	-0.1765	ND	-0.2818	**NDJ**	**-0.4143**	**NDJF**	**-0.6294**	NOV	0.111	ND	0.1564	NDJ	0.1192	NDJF	-0.0947
DEC	-0.2064	**DJ**	**-0.3595**	**DJF**	**-0.5935**	**DJFM**	**-0.6507**	DEC	0.1129	DJ	0.0888	DJF	-0.1307	**DJFM**	**-0.3203**
JAN	**-0.3561**	**JF**	**-0.6427**	**JFM**	**-0.6642**	**JFMA**	**-0.6328**	JAN	0.0345	JF	-0.2286	**JFM**	**-0.444**	**JFMA**	**-0.5132**
FEB	**-0.6754**	**FM**	**-0.6232**	**FMA**	**-0.5752**	**FMAM**	**-0.544**	**FEB**	**-0.4133**	**FM**	**-0.6196**	**FMA**	**-0.6738**	**FMAM**	**-0.5619**
MAR	-0.2894	MA	-0.2661	MAM	-0.2205	MAMJ	-0.2552	**MAR**	**-0.6243**	**MA**	**-0.6613**	**MAM**	**-0.4567**	**MAMJ**	**-0.4849**
APR	-0.112	AM	-0.0632	AMJ	-0.1032	AMJJ	-0.0475	**APR**	**-0.3992**	AM	-0.1079	AMJ	-0.1243	AMJJ	-0.0505
MAY	0.0244	MJ	-0.0265	MJJ	0.0312	-	-	MAY	0.2639	MJ	0.2211	MJJ	0.2579	***Caltha palustris* (Sp-4)**	
JUN	-0.0735	JJ	0.0164	-	-	***Tussilago farfara* (Sp-1)**		JUN	-0.0269	JJ	0.0798	-	-		
JUL	0.1144	-	-	-	-			JUL	0.155	-	-	-	-		
1-monthly mean	**r0**	**2-monthly mean**	**r0**	**3-monthly mean**	**r0**	**4-monthly mean**	**r0**	**1-monthly mean**	**r0**	**2-monthly mean**	**r0**	**3-monthly mean**	**r0**	**4-monthly mean**	**r0**
JUN	-0.0049	JJ	0.1133	JJA	0.1764	JJAS	0.1664	JUN	0.1858	JJ	0.1836	JJA	0.1772	JJAS	0.1957
JUL	0.0139	JA	0.2797	JAS	0.2477	**JASO**	**0.3351**	JUL	0.1862	JA	0.1759	JAS	0.2016	JASO	0.2365
AUG	0.0108	AS	0.1598	ASO	0.2906	ASON	0.257	AUG	0.1856	AS	0.2001	ASO	0.2492	ASON	0.2581
SEP	0.0037	SO	0.249	SON	0.2191	SOND	0.1089	SEP	0.1872	SO	0.2949	SON	0.2891	**SOND**	**0.3604**
OCT	0.0218	ON	0.2137	OND	0.0987	ONDJ	0.01	OCT	0.1886	ON	0.2524	**OND**	**0.3365**	ONDJ	0.3058
NOV	0.009	ND	-0.0138	NDJ	-0.0819	NDJF	-0.2989	NOV	0.1874	ND	0.2467	NDJ	0.2134	NDJF	-0.0075
DEC	-0.0114	DJ	-0.131	**DJF**	**-0.3497**	**DJFM**	**-0.507**	DEC	0.1908	DJ	0.1723	DJF	-0.0532	DJFM	-0.2706
JAN	-0.1174	**JF**	**-0.3915**	**JFM**	**-0.554**	**JFMA**	**-0.5367**	JAN	0.0983	JF	-0.1666	**JFM**	**-0.4273**	**JFMA**	**-0.5521**
FEB	**-0.5182**	**FM**	**-0.6544**	**FMA**	**-0.6068**	**FMAM**	**-0.5077**	**FEB**	**-0.3795**	**FM**	**-0.6448**	**FMA**	**-0.7637**	**FMAM**	**-0.6659**
MAR	**-0.5514**	**MA**	**-0.4594**	MAM	-0.2922	MAMJ	-0.2591	**MAR**	**-0.7155**	**MA**	**-0.8321**	**MAM**	**-0.6297**	**MAMJ**	**-0.6334**
APR	-0.1191	AM	0.0772	AMJ	0.1447	AMJJ	0.1519	**APR**	**-0.5927**	AM	-0.2848	AMJ	-0.2582	AMJJ	-0.2424
MAY	0.2363	MJ	0.3014	MJJ	0.2867	-	-	MAY	0.209	MJ	0.2334	MJJ	0.1717	***Salix fragilis* (Sp-5)**	
JUN	0.1309	JJ	0.1503	-	-	***Scilla bifolia* (Sp-2)**		JUN	0.066	JJ	0.0179	-	-		
JUL	0.0724	-	-	-	-			JUL	-0.0521	-	-	-	-		

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STRUCTURE, COMPOSITION AND DIVERSITY OF FOREST ALONG THE ALTITUDINAL GRADIENT IN THE HIMALAYAS, NEPAL

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Abstract. The aim of this study was to assess the structure (stem density, height, and basal area), composition and diversity in relatively undisturbed forests along an altitudinal gradient from 2000 m asl to 3900 m asl of Langtang National Park in Central Himalaya. The forest stands along the altitudinal gradient were studied on 20 sampling plots of two sub zones of the Temperate and Subalpine zone. *Tsuga dumosa* was the ecologically most important species in the Upper and the Lower Subalpine zone with high important value index (IVI = 124.31). *Quercus semecarpifolia* and *Lithocarpus elegans* were the ecologically most important species in the Upper and the Lower Temperate zone with IVI of 66.64 and 46.39 respectively. Similarly, indicator species' analysis was performed to know the preferences of tree species within the vegetation zones. *Rhododendron campanulatum* is highly significant ($p < 0.001$) and *Tsuga dumosa* significant ($0.05 < p < 0.01$) in the Upper Subalpine zone, whereas *Rhododendron anthopogon* prefers the Lower Subalpine zone with significant value ($0.01 < p < 0.001$). Only the *Quercus semecarpifolia* prefers the Upper Temperate zone. There was no specific trend in the structure along the altitudinal gradient. The Shannon diversity index ranged from 1.10 to 2.34 with the highest value in the Lower Temperate zone. Both Simpson index (0.89) and Evenness (0.86) were high in the Lower Temperate zone. From this study it is concluded that the contribution of forest composition in terms of species richness and Shannon diversity is significant in Lower Temperate zone. The Upper Subalpine zone has less diversity and may also be less endangered by human impact but may profit from climate change in the future.

Key words: *vegetation zone, density, basal area, diversity, altitudinal gradient, Nepal*

Nomenclature of tree species WCSP (2015).

Introduction

The integrated knowledge of the structure, composition and diversity of plant species is the basis for sustainable management strategies in different forest ecosystems (Gutierrez and Huth, 2012). Forest ecosystems (structure, composition and diversity of the species) play also an important role in the global carbon budget as they sequester carbon dioxide from the atmosphere and act as huge C-pools (Canadell et al., 2007; Denman et al., 2007). Forest stands, forest structure and diversity are also important for

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canopy community (Adhikari et al., 2012a; Adhikari et al., 2015). Covering about one third of the earth's land surface, forest ecosystems are a major component in the proposed strategies for the mitigation of atmospheric CO_2 emissions (Richards and Stokes, 2004; Neilson et al., 2006). In this context non-managed forests are of specific interest as they include high carbon stocks. Consequently, Weber (2001) highlighted the need for the protection of natural forests as carbon reservoirs. However, so far the characteristics of forest ecosystems of the world's protected area network remains poorly studied (Campbell et al., 2008). In this study we present data of protected forests along an altitudinal gradient in the Langtang National Park, Nepal.

In Nepal approximately 29 % of the total land is covered by forest (DFRS, 1999) consisting of 35 forest types (Stainton, 1972) along the elevational gradient from 60 m asl to tree line at around 4000 m asl. Protected areas constitute about 20 % of Nepal´s total land area (Bhuju et al., 2007) and represent 47 types of vegetation (Shrestha, 2001). Mountain ranges have a special status due to its characteristic to increase geodiversity and harbour at least a third of terrestrial plant species diversity (Barthlott et al. 1996). Researchers around the world for centuries have extensively studied mountains as steep ecological gradients and hotspots of biodiversity (Körner, 2007). Alexander von Humboldt was the first to describe the latitudinal gradient of vegetation and the corresponding altitudinal gradient of vegetation that led to basic understanding of species composition and diversity along an altitudinal gradient (Fischer, 2011).

Altitudinal zonation of vegetation is one of the most striking gradational patterns of vegetation (Oshawa, 1977, and 1984) and much information is accumulated on its local patterns. Theoretically, the division of vegetation zones should be based on the climax vegetation which refers to the forest structure. Research to describe the vertical and horizontal distribution of the vegetation of the Himalayas is still ongoing considering the spatial differences in forest canopy (Adhikari and Fischer, 2011; Adhikari et al., 2016) and diversity of the plant species between particular sections of this mountain range (Polunin and Stainton, 2000). Several scientists have studied the altitudinal climax vegetation zonation on the slopes of Himalaya (e.g., Schweinfurt, 1957; Stainton, 1972; Dobremez, 1976). The basic pattern of vegetation distribution along altitudinal gradients is controlled by solar radiation, temperature and humidity. However, these factors can vary on small scales due to varying environmental factors like aspect, slope, altitude (here we focused on) and exposure. The altitudinal distribution and the main tree species composition of the forest communities in Nepalese Himalayas show an extreme complexity attributed to bioclimatic diversity.

Protected areas have been implemented to maintain natural forest structures and biodiversity by restricting direct anthropogenic impacts. Consequently, knowledge on the structure and composition of the protected forests along the high altitude can be important as a reference in the context of the initiatives to reduce emissions from deforestation and forest degradation (REDD) (UNFCCC, 2007). It also provides input to the development of strategies for the selection of taxa in reforestation, particularly for the conservation of canopy community, especially flora (Adhikari et al., 2012a; b; Adhikari et al., 2015). However, to assess the role of existing protected areas and forest stands in REDD, a comprehensive assessment of their potential elements is needed (Scharlemann et al., 2010).

In this study, we generate knowledge on the structure, composition and diversity of the forest in the Langtang National Park (hereafter LNP) in central Himalayas. The study is based on a thorough floristic tree inventory of four sub vegetation zones of two

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zones. The research aims to answer the question of whether elevation gradient affects composition, structure and diversity of tree species along altitudinal gradient by (i) comparing composition and structure of stands in different zones and (ii) comparing diversity patterns of forest stands along altitudinal gradient.

Materials and Methods

Study area

The study was conducted in the Langtang National Park, Rasuwa district, Nepal. Field work was carried out from December 2013 to February 2014. The National Park (28° 10' 26'' N 85° 33' 11'' E) extends from 32 km north of Kathmandu to the Nepal-China (Tibet) border in the Central Himalayan region of Nepal (*Fig. 1*). We selected the National Park as our study area, because i) it has a clear altitudinal gradient, ii) it is a relatively less disturbed area, and iii) it has typical forest structure which we couldn't find outside the National Park.

It is the second largest national park established in 1976 to preserve this unique ecosystem of significant value to the world's biodiversity. The study area has cool temperate monsoon climate with annual precipitation of 2078 mm (total). The annual mean temperature at 2000 m asl is 15.3°C, and there is a 6 °C drop in temperature for every 1000 m rise in altitude (Government of Nepal, Meteorological Service Office).

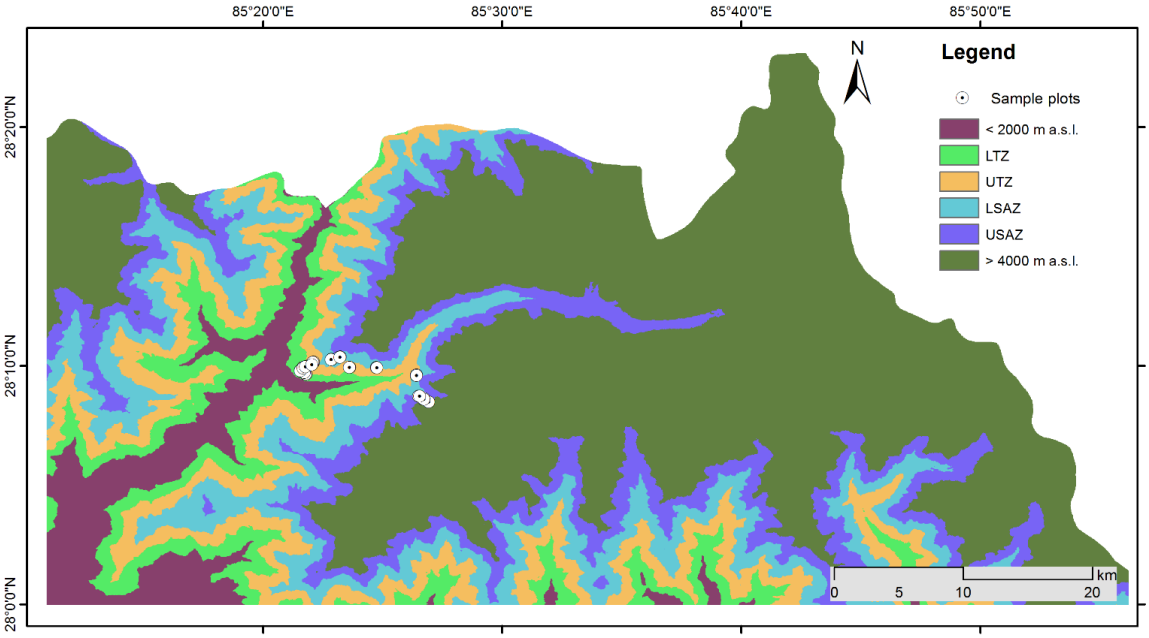

Figure 1. *The study area: Dots in the map represent the sample plots. Abbreviation in legend are (LTZ=Lower Temperate zone; UTZ= Upper Temperate zone; LSAZ= Lower Subalpine zone; USAZ=Upper Subalpine zone).*

Forest vegetation of study area

The diverse topography, geology, and climatic patterns have resulted in a great variety of vegetation types. LNP comprises 18 forest types from Subtropical (1000-

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2000 m asl) to Nival (above 5000 m asl) vegetation zones found in Nepal (Shrestha, 2001). The total forest area of LNP is about 25% of the total area. The composition of all vegetation zones has been already mentioned in the Langtang National Park management plan (1977) and Dobremez et al. (1976), however, we have described here structure, IVI and diversity of the species in Upper and Lower Subalpine zone, and Upper and Lower Temperate zone respectively. For this study, we categorized vegetation zones within 2000 m to 3900 m asl equally in four zones. The first zone is Upper Subalpine zone (3500-4000 m asl), the second zone is Lower Subalpine zone (3000-3500 m asl). Similarly, the third and fourth zones are the Upper Temperate (2500-3000 m asl) and Lower Temperate zone (2000-2500 m asl) respectively.

Field sampling

A systematic sampling design for the selection of sampling plots along the elevation gradient was used. The sampling plots were surveyed with a square shaped at each interval of 100 m altitude above 2000 m up to the tree line around 3900 m (*Fig. 1*). The sampling plots size (20 × 20 m) were identified using Geographic Positioning System (elevation, latitude, and longitude) as suggested by Mueller-Dombois and Ellenberg, 1974; Chytry et al., 2003. Altogether 20 sampling plots for trees (≥ 5 cm diameter at breast height i.e. dbh) were laid down. Because there were only very few possibilities to enter the area (very steep slopes, rugged terrain), the trekking trail from Ghatte river to Lauribinayak section was used as the basis for the elevation gradient. In order to eliminate anthropogenic influences on the forest, all plots were laid at least 100 m away from trekking trails. In each sampling plot, we recorded name of tree species, height and dbh. Height was measured with the Sunnto Clinometer as described by Pretzsch (2009).

Data analysis

Composition of tree species

For each species, values of frequency, density and dominance were calculated following the methods of Curtis and McIntosh (1951). A species IVI is a summation of three values: (1) the species' relative dominance or percent basal area coverage relative to other species in the stand; (2) relative density or percent occurrence per unit area relative to other species; and (3) relative frequency or percent probability of occurrence in a sample plot relative to other community species.

Density, frequency, and dominance

Density, frequency, and dominance and their relative values were calculated by using following formulae:

$$\text{Density (per hectar)} = (\text{D}) = \frac{\text{Total no.of individuals of a species found}}{\text{Total area examined}} \times 10{,}000 \quad \text{(Eq. 1)}$$

$$\text{Relative Density of species A} = (\text{RD}) = \frac{\text{No.of individulas of species A}}{\text{Total no.of individuals of all species}} \times 100 \quad \text{(Eq. 2)}$$

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$$\text{Frequency (\% of plot)} = (F) = \frac{\text{No.of quadrats in which species occurs}}{\text{Total no.of quadrats examined}} \times 100 \quad \text{(Eq. 3)}$$

$$\text{Relative Frequency of species A} = (RF) = \frac{\text{Frequency of species A}}{\text{Sum of frequency values of all species}} \times 100 \quad \text{(Eq. 4)}$$

$$\text{Dominance} = (Do) = \frac{\pi\,(dbh)2}{4} \quad \text{(Eq. 5)}$$

$$\text{Relative dominance} = \frac{\text{Basal area of a species}}{\text{total basal area of all species}} \times 100 \quad \text{(Eq. 6)}$$

Indicator values were calculated according to Dufrêne and Legendre (1997) with PCord version 6 (McCune and Mefford, 2011).

Structure of forest stands

The structure of the forests along the altitudinal gradient was described based on three structural features: diameter size class distribution, basal area and height. Trees in each sampling plot were categorized in three diameter classes (<10, 10-29.5 and ≥ 30 cm dbh) and the percentage of trees in each diameter class was analyzed for each vegetation zone. The total basal area of each tree species inside each plot was calculated and then average basal area for each vegetation zone was obtained. Similarly, the height of each species inside the sampling plots was measured and then average between the plots was taken to obtain an average height for vegetation zones.

Diversity of tree species

Simpson's diversity index (Equation 7) is one of the most meaningful and robust diversity measures available. The index is calculated as proportion of species *i* relative to the total number of species (pi) (Simpson, 1949).

$$D = 1 - \frac{\sum_{i=1}^{s} n_i.(n_i-1)}{N.(N-1)} \quad \text{(Eq. 7)}$$

Where n_i is the number of individuals in the species *i*
s is the number of species
N total individuals (species).

Shannon's index is calculated from the equation:

$$H' = -\sum_i^s p_i . log p_i \text{ Where } p_i = \frac{n_i}{N} \quad \text{(Eq. 8)}$$

Where p_i proportional abundance of species *i*

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s is the number of species
As a heterogeneity measure, the Shannon index takes the degree of evenness in species and abundance into account. Evenness E is calculated as proportion of observed and maximal diversity (Pielou, 1969):

$$E = \frac{H'}{H_{max}} \quad \text{(Eq. 9)}$$

Where E = evenness (range 0-1)
H' is Shannon index
H_{max} = (ln s) = maximum possible diversity
s is the number of species.

Results

Forest composition

Altogether 25 species belonging to 16 families were recorded. The identified species represent the flora of the majority of patches forming the forest along an altitudinal gradient from 2000 to 3900 m asl. On the basis of relative density, relative basal area and relative frequency, the four vegetation zones differed in the most important species. All field inventory sheet, recorded tree species, their average height and dbh in each vegetation zone is synthesized in (*Appendix Table A1*).

In the Upper Subalpine zone (3500-3900 m asl), only four species were recorded (*Table 1*). Among them *Tsuga dumosa* was the ecologically most important species for this zone with an IVI value 124.31. The second important tree species was *Juniperus recurva* with an IVI value 99.10. The other associated tree species *Rhododendron campanulatum* and *Betula utilis* had IVI values of 67.94 and 8.65 respectively. In the Lower Subalpine zone (3000-3500 m asl), eight species were recorded (*Table 1*). As in the upper zone *Tsuga dumosa* was the ecologically most important species with an IVI value of 97.25. The second important tree species was *Rhododendron anthopogon* with an IVI value of 86.45. Among the other six associated tree species, *Viburnum spp.* was the least important species.

Table 1. *Recorded tree species and their composition in four altitudinal zones of the study area, ranked by importance value index (IVI)*

Rank	Species	Stem density		Basal area (m^2/ha)		Frequency		IVI
		no/ ha	RD (%)	BA	RBA (%)	F	RF (%)	
Upper Subalpine zone								
1	*Tsuga dumosa*	305	36.31	36.65	49.54	1	38.46	124.31
2	*Juniperus recurva*	335	39.88	32.43	43.83	0.4	15.38	99.10
3	*Rhododendron campanulatum*	195	23.21	4.64	6.27	1	38.46	67.94
4	*Betula utilis*	5	0.60	0.27	0.36	0.2	7.69	8.65

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Lower Subalpine zone								
1	*Tsuga dumosa*	175	23.49	94.23	52.71	0.8	21.05	97.25
2	*Rhododendron anthopogon*	305	40.94	43.73	24.46	0.8	21.05	86.45
3	*Rhododendron arboreum*	135	18.12	15.47	8.65	0.8	21.05	47.83
4	*Quercus semecarpifolia*	105	14.09	24.38	13.64	0.4	10.53	38.26
5	*Acer species*	5	0.67	0.29	0.16	0.4	10.53	11.36
6	*Betula utilis*	10	1.34	0.26	0.14	0.2	5.26	6.75
7	*Pyrus species*	5	0.67	0.40	0.23	0.2	5.26	6.16
8	*Viburnum species*	5	0.67	0.01	0.01	0.2	5.26	5.94
Upper Temperate zone								
1	*Quercus semecarpifolia*	305	35.26	15.97	13.99	0.8	17.39	66.64
2	*Tsuga dumosa*	120	13.87	31.62	27.71	0.6	13.04	54.63
3	*Rhododendron arboreum*	155	17.92	8.54	7.48	0.6	13.04	38.45
4	*Lyonia ovalifolia*	95	10.98	15.74	13.79	0.6	13.04	37.82
5	*Lithocarpus elegans*	70	8.09	18.71	16.39	0.2	4.35	28.83
6	*Pinus wallichiana*	25	2.98	9.80	8.58	0.6	13.04	24.52
7	*Lindera pulcherima*	60	6.94	3.39	2.97	0.4	8.70	18.60
8	*Rhododendron anthopogon*	10	1.16	8.84	7.75	0.2	4.35	13.25
9	*Quercus lantana*	10	1.16	0.93	0.82	0.2	4.35	6.32
10	*Betula species*	10	1.16	0.49	0.43	0.2	4.35	5.94
11	*Symploccus species*	5	0.58	0.09	0.08	0.2	4.35	5
Lower Temperate zone								
1	*Lithocarpus elegans*	60	13.04	17.10	24.65	0.4	8.70	46.39
2	*Pinus wallichiana*	60	13.04	10.55	15.22	0.4	8.70	36.95
3	*Lyonia ovalifolia*	70	15.22	4.75	6.85	0.6	13.04	35.11
4	*Alnus nepalensis*	30	6.52	15.70	22.63	0.2	4.35	33.50
5	*Rhododendron arboreum*	95	20.65	1.65	2.38	0.4	8.70	31.72
6	*Lindera pulcherima*	40	8.70	5.83	8.41	0.6	13.04	30.15
7	*Berberis species*	10	2.17	1.10	1.58	0.4	8.70	12.45
8	*Quercus lantana*	15	3.26	2.68	3.86	0.2	4.35	11.47
9	Galechop*	25	5.43	0.62	0.89	0.2	4.35	10.67
10	*Pyrus pashia*	15	3.26	1.92	2.76	0.2	4.35	10.37
11	*Juglans regia*	5	1.09	3.25	4.69	0.2	4.35	10.12
12	*Elaegnus species*	5	1.09	2.81	4.04	0.2	4.35	9.48
13	*Lagerstroemia parvilora*	15	3.26	1.08	1.56	0.2	4.35	9.17
14	*Rhus species*	10	2.17	0.23	0.34	0.2	4.35	6.86
15	*Quercus glauca*	5	1.09	0.10	0.14	0.2	4.35	5.58

Abbreviations are: RD relative density; BA Basal area; RBA Relative basal area; F frequency; RF Relative frequency; IVI importance value index. Galechop* (local name) broadleaf unidentified species.

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In the Upper Temperate zone (2500-3000 m asl), eleven species were recorded (*Table 1*). Here, *Quercus semecarpifolia* was the ecologically most important species for this zone with an IVI value of 66.64. The second was *Tsuga dumosa* with an IVI value of 54.63. Among the other nine associated tree species, *Symploccus spp.* was the least important species. In the Lower Temperate zone (2000-2500 m asl), altogether fifteen species were recorded (*Table 1*). Among them *Lithocarpus elegans* was the ecologically most important species for this zone with an IVI value of 46.39. *Pinus wallichiana, Lyonia ovalifolia, Alnus nepalensis, Rhododendron arboreum and Lindera Pulcherima* were also found to be ecologically important species in this zone. *Quercus glauca* was found to be the least important species.

The species composition of trees of each vegetation zone was analyzed. The recorded tree species and their corresponding vegetation zones, indicator value (IV) and *p* value are shown in (*Table 2*). Out of 25 tree species, only four species showed a significant preference to a specific zone. Species *Rhododendron campanulatum* and *Tsuga dumosa* preferred the Upper Subalpine zone, similarly *Rhododendron anthopogon* and *Quercus semecarpifolia* preferred Lower Subalpine zone and Upper Temperate zone respectively (*Table 2*).

Table 2. *Indicator species analysis of all recorded tree species in an elevation gradient of each vegetation zone*

Species	Vegetation zones	Observed indicator value (IV)	Randomized groups mean	S. Dev	*P* value
Rhododendron arboreum	LSA	28.1	28.4	10.19	-
Lindera pulcherima	LT	24	25.1	12.61	-
Pinus wallichiana	LT	28.2	24.3	11.95	-
Lyonia ovalifolia	UT	34.5	26.1	11.79	-
Quercus lanata	LT	12	17.6	11.24	-
Lithocarpus elegans	LT	18.5	20.6	11.81	-
Pyrus pashia	LT	20	20	0.28	-
Juglans regia	LT	20	20	0.28	-
Berberis species	LT	20	20.3	0.28	-
Elaegnus species	LT	20	20	0.28	-
*Galechop**	LT	20	20	0.28	-
Lagerstroemia parvilora	LT	20	20	0.28	-
Alnus nepalensis	LT	20	20	0.28	-
Rhus species	LT	20	20	0.28	-
Quercus glauca	LT	20	20	0.28	-
Tsuga dumosa	**USA**	**50.8**	**31.7**	**9.48**	*
Rhododendron anthopogon	**LSA**	**77.5**	**25.4**	**12.6**	**
Quercus semecarpifolia	**UT**	**59.5**	**30.8**	**14.31**	*
Symploccus species	UT	20	20	0.28	-
Acer species	LSA	20	20	0.28	-

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Viburnum species	LSA	20	20	0.28	-
Betula utilis	LSA	13.3	19.3	11.11	-
Pyrus species	LSA	20	20	0.28	-
Rhododendron campanulatum	**USA**	**100**	**23.6**	**10.83**	***
Juniperus recurva	USA	40	17.1	11.75	-
Averages	-	29.86	22.01	6.18	-

* Monte Carlo test of significance of observed maximum indicator value for variable 4999 permutations, random number seed: 1146. Proportion of randomized trials with indicator value equal to or exceeding the observed indicator value. p = (1 + number of runs >= observed) / (1 + number of randomized runs). Vegetation zones = Group identifier for group with maximum observed IV. Bold Significant species. "-"not significant, *Significant at 0.05 < p < 0.01, ** Significant at 0.01 < p < 0.001, and ***Significant at p < 0.001. Abbreviation for zones; USA = Upper Subalpine, LSA = Lower Subalpine, UT = Upper Temperate, and LT = Lower Temperate.

Forest structure

The total number of stems, total basal area and average tree height in each elevation is presented in *Fig.2*. It shows the trend of stand features along the altitudinal gradient. Within the sampling plots, total number of stems (≥5 cm dbh) inventoried per plot fluctuated from 275 per hectare at 2100 m asl to 1700 trees per hectare at 2800 m asl. The number of stems was highly fluctuating among the plots along elevation, beyond 3700 m asl, it is decreasing. The total basal area on the plots varied from 10.43 m^2 ha^{-1} at 3900 m asl to 248.41 m^2 ha^{-1} at 3200 m asl. It was found to be increasing to an elevation about 3200 m asl and getting lower in higher elevation. The average tree height was maximum at 2100 m asl (30.03 m) and minimum in the highest plot at 3900 m asl (2.44 m). The average height of tree stands in each plot was found to be slightly decreased with increasing altitude. However, the high value in the plots situated at 2900 m asl and 3400 m asl was because of high number of *Tsuga dumosa* (13 out of 19 individuals) and (17 out of 24 individuals) respectively (*Appendix Table A1*).

The forest structure parameters were measured per vegetation zones. Stem density was found to be maximum in Upper Temperate zone (865 trees ha^{-1}) and minimum in Lower Temperate zone (460 trees ha^{-1}) (*Table 3*)**.** The analysis of size class distribution of tree stems inventoried in the entire sampling plots showed that the Upper Subalpine zone, Upper Temperate zone and Lower Temperate zone had maximum percentages of trees in the 10 - 29.9 cm DBH range. In contrast to this, the maximum percentages (55.1 %) of trees were recorded in ≥30 cm dbh range in the Lower Subalpine zone. The average tree height (>5 cm dbh) by vegetation zones is shown in *Table3*. The highest average tree height was found in the Lower Subalpine Zone (20.04 m) and the lowest in the Upper Temperate zone (13.81 m). The more dispersed values were observed for Upper Subalpine and Lower Temperate zone as indicated by their elevated standard deviation (SD). The average basal area of tree species was found to be maximum in the Lower Subalpine zone (22.43 m^2 ha^{-1}) and minimum in Lower Temperate zone (4.56 m^2 ha^{-1}). The vegetation zone with higher basal area (Lower Subalpine zone) showed high variability which is also denoted by the elevated standard deviation encountered compared to other zones (*Table 3*).

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Table 3. *Number of trees per hector (%) by diameter size and average tree height by vegetation zones*

Vegetation zones	% of Stems/ha by diameter classes			Total stems (no/ha)	Average tree height		Average basal area	
	<10 cm	10-29.9 cm	≥30 cm		m	± SD	m^2/ha	± SD
Upper Subalpine	12.5	51.1	36.3	840	14.06	9.42	18.49	18.69
Lower Subalpine	8.7	36.2	55.1	745	20.04	7.82	22.43	33.01
Upper Temperate	6.9	53.8	39.3	865	13.81	5.88	10.37	9.63
Lower Temperate	6.5	48.9	44.6	460	14.06	9.14	4.56	5.48

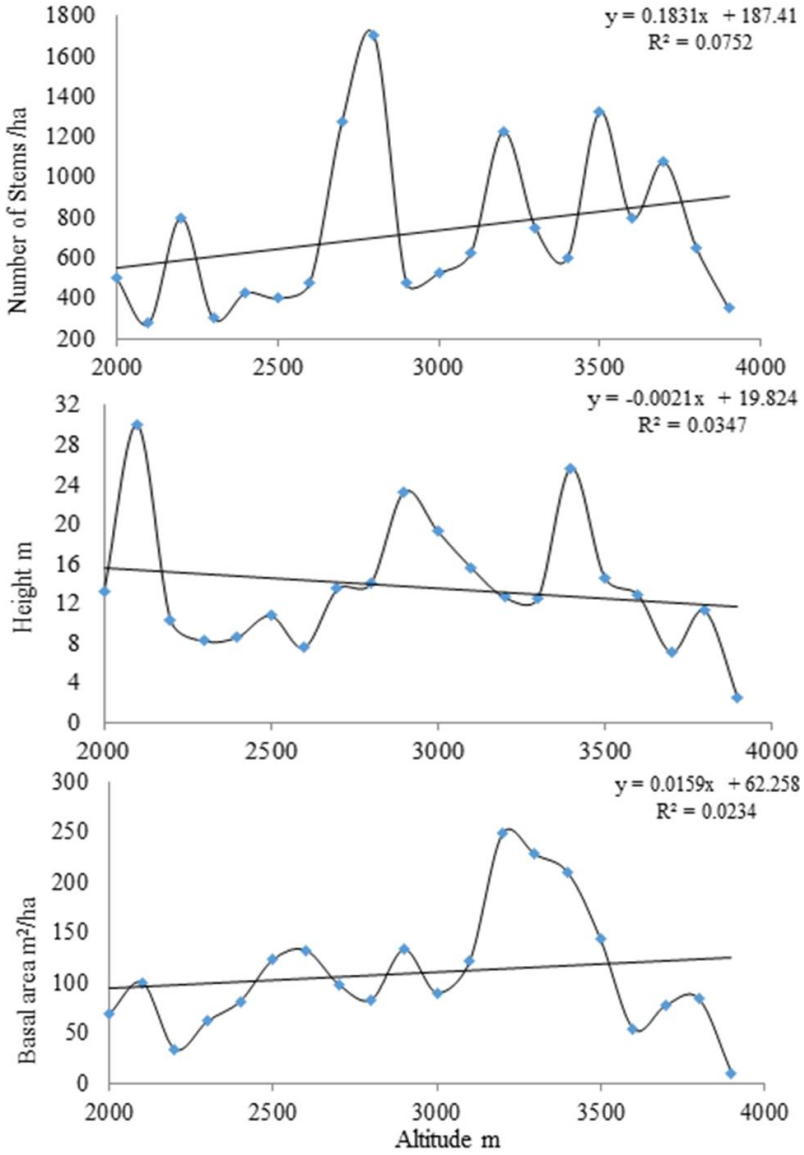

Figure 2. *The tendency of no. of stems, height and basal area ha^{-1} in each 20 plots along the elevation gradient (2000 m -3900 m asl). Figure shows the trend of stand features (total no. of stems, total basal area and average tree height along the altitude.*

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Species diversity

Tree species diversity was calculated for the four vegetation zones separately (*Fig. 3*). The values of Shannon index, Simpson index and Evenness index showed variations among the vegetation zones. The Shannon index which measures diversity in categorical data ranged from 1.10 to 2.34. The peak value along the elevation zone centered around 2500-3000 m asl in the Lower Temperate zone while it was found to be least in the Upper Subalpine zone. The values of this index were found to be gradually increasing with decreasing altitude.

The Simpson index which measures the probability that two randomly selected individuals from a sample will belong to the same species, ranged from 0.65 to 0.89 The Lower Temperate zone exhibited the maximum value while the Upper Subalpine zone constituted the least value.

There was no distinct trend for this index with increasing altitude. The evenness index which measures the degree of equal distribution of individuals within the whole species pool ranged from to 0.69 to 0.86. The Lower Temperate zone exhibited the maximum value while the Lower Subalpine zone constituted the least value.

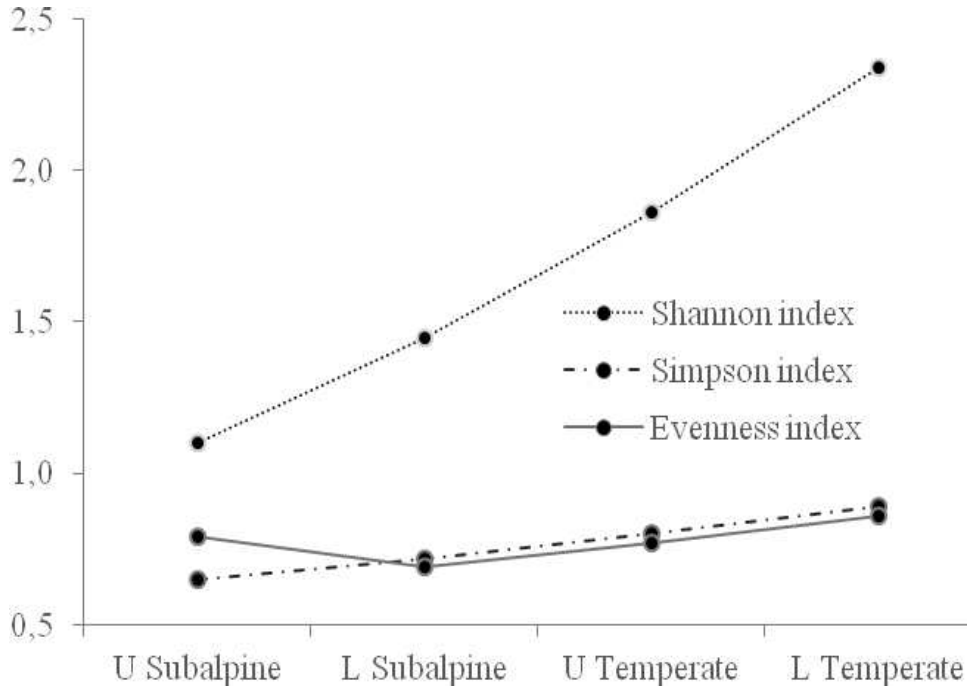

Figure 3. *Tree diversity along the altitudinal gradient (U= Upper; L=Lower)*

Discussion

This study attempted to assess the structure, composition and diversity of natural forest stands in forests in the LNP of Nepal in Central Himalaya. The results derived from this study provide baseline information regarding potential of the protected area to secure conservation of species along an altitudinal gradient. The results are further discussed in the following sections.

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Forest composition

Altitude is one of the most important determinants of tree distribution due to its direct impact on the microclimate of the habitat (Adhikari et al., 2012a). In the present study importance values of species differed along the altitude. This reflects the relative importance of each species in a spectrum of climax communities established in the park (Pandey, 2015). The variability of the distribution of plant species in the sampling plots at each vegetation zones could be attributed to the effect of co-factors like topography, aspect and exposure within the same altitudinal range. Climatic variations influenced by the complex topography in a predominantly monsoonal area most often determine the dominant type of vegetation in the park. The vegetation zones are partly obscured by factors of aspect, drainage, soil and human impact (Neilson et al., 2006).

The floristic composition of tree species differs at different altitudinal zones based on indicator value. *Rhododendron campanulatum* is highly significant ($p < 0.001$) and *Tsuga dumosa* is significant ($0.05 < p < 0.01$) in the Upper Subalpine zone respectively. *Rhododendron anthopogon* indicates the Lower Subalpine zone with significant value ($0.01 < p < 0.001$). Similarly, the *Quercus semecarpifolia* prefers the altitudinal range of the Upper Temperate zone. However, other species show their appearance in their respective zones but without significant preference.

Forest structure

Structural characteristics such as stem densities, basal area, dbh and height of tree are a function of the forest type, edaphic conditions, and the age and degree of disturbance of each stand. Here, the results derived from this study are compared and contrasted with the results found in similar kind of forests outside LNP. The vegetation zones in LNP showed average tree density (≥5 cm dbh) values from 460 to 865 per hectare which is consistent with the density values of 295 and 850 tree ha^{-1} (≥10 cm dbh) for major types of protected forests in Indian forests reported by Sharma et al. (2010). Tree density of our study is comparatively higher than the values in the Moist Temperate Conifers zone (90 ha^{-1}) and in Central Himalayan Moist Temperate Forest (170-283 ha^{-1}) (Chaturvedi and Singh, 1986; Shaheen et al., 2012). The range of stem density values observed in other studies ranged between 420-1300 trees ha^{-1} in Kumaun Himalaya (Saxena and Singh, 1982), and 990-1470 trees ha^{-1} in western Himalaya (Gairola et al., 2011) in 1200 to 2500 m asl range. In this study, the average height of tree species recorded was maximum in the Lower Subalpine zone (3000-3500 m asl). In contrast to this, some of the tallest and largest trees in the Himalaya were reported between 2500 and 3000 m by Singh and Singh (1987). They had also revealed that with further rise in elevation, in response to a sudden decline in the rainfall, and in severely cold and windy conditions, tree height of Himalayan forests were found to be reduced drastically. In this study, canopy height was also found to be slightly decreased with the elevation in LNP. In our study, the total basal area for sampling plots in LNP ranged from 10.43 to 248.41 m^2 ha^{-1} which is higher than the findings from other studies in Himalayan region such as 78-92 m^2 ha^{-1} in lesser Himalayan Moist Temperate forests (Ahemed et al., 2006); 90- 152 m^2 ha^{-1} in trans Himalayan forests of Nepal (Kunwar and Sharma, 2004); 86-129 m^2 ha^{-1} in Garwal Himalayas (Pandey, 2001).

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Tree diversity

In Nepalese Himalaya, the number of species in 100 m altitudinal bands increases steeply with altitude until 1,500 m above sea level. Between 1,500 and 2500 m, little change in the number of species has been observed, but above this altitude, a decrease in species richness is evident (Vetaas and Grytnes, 2002). The results of this study also reflect this general trend found in plant diversity of mountain forests in Himalaya. Oommen and Shanker (2005) provided evidence for different mechanisms along spatial scales and explained a mechanism based on the investigations on woody plant diversity in the Indian Western Himalaya. They revealed that the species with high tolerance to climatic variability had followed mid-domain model predictions, and showed a nonlinear relationship with temperature, whereas tropical species richness tracked temperature and area. The Shannon diversity values recorded (1.10 to 2.34) in our study lies close to the reported range of 1.16 to 3.40 for the Himalayan range (Pandey, 2001, Kunwar and Sharma, 2004**).** Maximum value of evenness in Lower temperate zone represented homogeneous communities having similar species distribution. Low evenness values of forest communities in Lower Subalpine zone and Upper Temperate zone can possibly be due to suppression and out-shading of associated species by the dominant conifers obtaining maximum amount of light and water. A similar result was found in Colombian fragmented forest by Murcia (1995).

Limitation of the study

This study has attempted to explore the structure, composition, and floristic diversity of forest vegetation of the Langtang National Park along the altitudinal gradient. However, it has some limitation as follows: this study is limited to i) sample replications, ii) important site characteristics such as: slope, edaphic factors, iii) elevation limited (2000-3900). Finally, we will consider these short comes of the research and formulate some hypotheses and test on an experimental fieldwork in the future.

Outlook

The understanding of forest structure, composition and diversity in natural forests is the basis for sustainable management of any forest ecosystem. Suwal et al. (2014) have reported that people overused the forest products (timber and non-timber) from LNP despite its legal protection. This combined with potential negative implications of future climate change on ecosystem function in the Himalayan forests, highlights the urgent need for conservation attention. The knowledge derived from this study can be useful to identify priorities for management and biodiversity conservation in such forests in the future. With the increasing focus on ecosystem services, protected areas are now also seen as a crucial component of global climate change mitigation efforts (Soares-Filho et al., 2010; Watson et al., 2014). The vegetation zones with distinct forest structure, composition and diversity in LNP may facilitate gains for both adaptation of climate and key of forest conservation in the future. The information and monitoring of ecological indicator species in particular zones of the study area can provide hints about adaptability of species in typical forest ecosystem, which will play important role in forest management strategies especially post-earthquake situation.

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APPENDIX

Table A1. *Number of individuals, average height and dbh of each species inventoried per plot (plot one is the highest elevation and plot 5 is the lowest elevation) in each vegetation zone*

Vegetation zones	Species	Plots						Height (m)	dbh (cm)
		1	2	3	4	5	Total		
Upper Subalpine zone (3500-3900)	*Rhododendron campanulatum*	13	3	7	8	8	**39**	4.40	14.32
	Tsuga dumosa	1	22	6	24	8	**61**	13.87	34.99
	Betula utilis	0	1	0	0	0	**1**	5	26
	Juniperus recurva	0	0	30	0	37	**67**	11.92	27.66
Total no. of individuals in each plot		**14**	**26**	**43**	**32**	**53**	**168**		
Lower Subalpine zone (3000-3500)	*Tsuga dumosa*	17	6	4	8	0	**35**	30.69	74.89
	Acer species	0	0	0	1	0	**1**	6	27
	Rhododendron anthopogon	7	21	30	0	3	**61**	9.26	33.90
	Rhododendron arboreum	0	2	13	10	2	**27**	9.17	29.67
	Viburnum species	0	1	0	0	0	**1**	6.50	6
	Betula utilis	0	0	2	0	0	**2**	11.50	18
	Quercus semecarpifolia	0	0	0	6	15	**21**	22.77	45.57
	Pyrus species	0	0	0	0	1	**1**	7	32
Total no. of individuals in each plot		**24**	**30**	**49**	**25**	**21**	**149**		
Upper Temperate zone (2500-3000)	*Tsuga dumosa*	13	1	10	0	0	**24**	28.11	51.54
	Rhododendron anthopogon	2	0	0	0	0	**2**	3.75	11.50
	Rhododendron arboreum	0	11	12	8	0	**31**	7.39	30.61
	Quercus semecarpifolia	2	53	5	1	0	**61**	15.40	21.31
	Lindera pulcherima	2	0	10	0	0	**12**	8.86	24.17
	Betula species	0	2	0	0	0	**2**	17	25
	Pinus wallichiana	0	1	0	3	1	**5**	19.76	53
	Lyonia ovalifolia	0	0	13	5	1	**19**	8.44	34.84
	Symplocus species	0	0	1	0	0	**1**	9	15
	Quercus lanata	0	0	0	2	0	**2**	6	32.5
	Lithocarpus elegans	0	0	0	0	14	**14**	9.04	49.82
Total no. of individuals in each plot		**19**	**68**	**51**	**19**	**16**	**173**		
Lower Temperate zone (2000-2500)	*Rhododendron arboreum*	0	0	18	0	1	**19**	6.18	14.05
	Lindera pulcherima	2	0	0	4	2	**8**	15.41	38.69
	Pinus wallichiana	0	0	5	0	7	**12**	24.74	42.96
	Lyonia ovalifolia	0	0	6	1	7	**14**	7.26	24.21
	Quercus lanata	0	0	3	0	0	**3**	19.33	41.33
	Lithocarpus elegans	4	8	0	0	0	**12**	8.26	50.42
	Pyrus pashia	3	0	0	0	0	**3**	9	38.17
	Juglans regia	1	0	0	0	0	**1**	31.80	91
	Berberis species	1	1	0	0	0	**2**	5	36.75
	Elaegnus species	1	0	0	0	0	**1**	9	84.50
	Galechop	5	0	0	0	0	**5**	6.70	17.20
	Lagerstroemia parviflora	0	3	0	0	0	**3**	7.33	30
	Alnus nepalensis	0	0	0	6	0	**6**	36.50	79.67
	Rhus species	0	0	0	0	2	**2**	6.25	17
	Quercus glauca	0	0	0	0	1	**1**	6.50	16
Total no. of individuals in each plot		**17**	**12**	**32**	**11**	**20**	**92**		

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THE EFFECT OF AL^{3+} AND HG^{2+} ON GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM *CAPOETA UMBLA* KIDNEY

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Abstract. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49; G6PD) is an important enzyme found in all mammal tissues, and produces NADPH in the metabolism. NADPH provides a reductive potential to maintain a balanced redox state within the cell. The aim of this study was to purify G6PD from *Capoeta umbla* kidney and determination of inhibition or activation effects of aluminium and mercury on enzyme activity. In this purpose, glucose 6-phosphate dehydrogenase was purified from *Capoeta umbla* kidney by using preparation of homogenate, ammonium sulphate precipitation and 2',5'-ADP Sepharose 4B affinity chromatography. Molecular weight of the enzyme was determined on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the purified enzyme showed a single band on the gel with a molecular weight of 75 kDa. Moreover, K_i constants of Al^{3+} and Hg^{2+} were found as 0.98 ± 0.084 and 0.57 ± 0.019 mM, respectively. In conclusion, affinity of the Hg^{2+} to the enzyme was higher than Al^{3+}

Keywords: *Capoeta umbla*, *fish, glucose 6-phosphate dehydrogenase (G6PD), purification, metals, inhibition.*

Introduction

Heavy metals are readily released to agricultural ecosystem causing an opposite effect due to widespread human activities. Agricultural ecosystem has a close relation with human health; hence, heavy metal pollution of agricultural ecosystem has been of attention throughout the world (Pandey and Pandey, 2008; Bermudez et al., 2012). Heavy metals are extremely toxic, non-degradable and bio-accumulative. Although some heavy metals such as zinc (Zn) and copper (Cu) are necessary elements for plants and humans as catalytic components of proteins and enzymes, a great majority of them do not have any useful physiological function, and their extreme accumulation in the human body can cause many diseases (Godt et al., 2006). For instance, accumulation of cadmium (Cd) in the human body can give rise to kidney, bone and pulmonary damage (Godt et al., 2006); lead (Pb) can harm the central nervous system, kidneys and blood system (Tong et al., 2000), etc.

Heavy metal pollution is constantly caused by waste water irrigation, solid waste disposal, vehicular exhaust, fertilisation, industrial activities, etc. (Khan et al., 2008; Liu et al., 2012). Among these pollution sources, industrial activities are the dominant

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sources of heavy metals near factories. Kabala and Singh (2001) reported that, in the vicinity of a Cu smelter in Poland, the concentrations of Cu, Pb and Zn in the surface soils were significantly higher than their concentrations in the subsurface soils. It has reported that industrial waste can give rise to heavy metal pollution of the surrounding soils and water.

Mercury (Hg) is found in comestible seafood, such as fish. Hg is a neurotoxin which can give rise to numerous effects in humans such as memory loss, disrupted coordination, sight disturbances, cardiovascular problems, etc. It also influences the thyroid gland, digestive system, liver and skin (Nigam et al., 2009). The toxicity of Hg exposure is partly a function of enhanced oxidative stress (OS). Enhancement of OS probably arises from the inhibition of antioxidant enzymes and the consumption of thiol compounds (especially GSH) (Franco et al., 2007) giving rise to cell injury, damage to biomolecules, and lipid peroxidation (Leonard et al., 2004).

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49; G6PD) is the first enzyme of the pentose phosphate metabolic pathway (Beydemir et al., 2003). It catalyses the conversion of glucose 6-phosphate into 6-phosphogluconate (Ciftci et al., 2007). The most important function of this enzyme is the production of ribose 5-phosphate and NADPH which are necessary for membrane lipids synthesis, reductive and nucleic acid synthesis (Kuo et al., 2000; Ceyhun et al., 2010). In addition, NADPH is a coenzyme which is involved in the synthesis of some amino acids, fatty acids and steroids, protecting the cells against the oxidant and detoxification of xenobiotics through the glutathione reductase peroxidase system (Ceyhun et al., 2010; Guler et al., 2013).

The genus *Capoeta* of Cyprinids is distributed in southern China, northern India, Turkmenistan, Lake Aral, the Middle East and Anatolia (Turkmen et al., 2002). The species diversity of *Capoeta* was last revised by Karaman (1969). While textbooks such as Geldiay and Balık (2007) recorded 7 species in *Capoeta* (plus five subspecies) from Turkey, Ozulug and Freyhof (2008) recorded 17 species from this area. In the last years, five new *Capoeta* species have been described from Turkey (Turan et al., 2006; Ozulug and Freyhof, 2008; Turan et al., 2008). Turkey is clearly the centre of diversity of this genus which comprises about 23 species (Kucuk et al., 2009). One of the well known species is *Capoeta umbla* (*C. umbla*). *C. umbla*, Transcaucasian barb, inhabits Euphrates-Tigris River Systems. It is also known as "lake fish or stream fish" locally and it is the most commercially valued fish for the local people (Coban et al., 2013) around Murat River. Murat River is one of the most important large and long (722 km) tributary of the Euphrates River in South East Anatolia of Turkey. The distribution area of Murat River is upper basins systems of the Euphrates and Tigris River (Koyun, 2011).

In the present study we have purified G6PD from *C. umbla* kidney and determination of inhibition or activation effects of aluminium (Al^{3+}) and mercury (Hg^{2+}) ions on enzyme activities.

Materials and Methods

Chemicals

2',5'-ADP Sepharose 4B was purchased from Pharmacia. $NADP^+$, glucose-6-phosphate, protein assay reagent, and chemicals for electrophoresis were purchased from Sigma. All other chemicals used were of analytical grade and were purchased from Merck.

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Animal and experimental procedure

Ten *C. umbla* (healty, adult fish-weighing 150-250 g) were caught from Murat River (Turkey, Bingöl). All procedures were conducted in strict compliance with the guidelines established by the Animal Care and Use Committee. The fish were decapitated and their kidneys were extracted and stored at -80°C.

Preparation of the homogenate

For analyses, the frozen kidney was thawed and cut into small pieces by using a scalpel. Kidney simples (10 g) were washed three times with 0.9% sodium chloride solution. These samples were homogenized gently for about 45 sec. and suspended in standard homogenizator buffer, containing 50 mM KH_2PO_4, 1 mM PMSF, 1 mM EDTA and 1 mM DTT. The homogenates was centrifuged for 2 h at 13.500 rpm. The supernatant was collected and kept for analysis.

Activity determination

In accordance with the Beutler (1971) method, enzyme activity was spectrophotometrically measured at 37°C. This method is based on the fact that NADPH, which is formed as a result of reducing $NADP^+$, yields absorbance at 340 nm. One enzyme unit was described as the enzyme amount reducing 1μmol $NADP^+$ per minute.

Ammonium sulphate fractionation and dialysis

G6PD enzyme homogenate was exposed to ammonium sulphate precipitation at 0–20, 20–30, 30–40, 40–50, 50–60, 60–70, 70–80% ranges; and the precipitation range of the enzyme was determined. During each precipitation process, centrifugation was carried out at 13.500 rpm for 15 min. After ammonium sulphate, the precipitate was obtained and dissolved in 50 mM KH_2PO_4 (pH 7.2) buffer. Enzyme activity was measured in the precipitate and supernatant for each time. Then, the enzyme solution was dialysed at 4°C in 10 mM KH_2PO_4 including 1 mM EDTA (pH 7.2) for 2h with two changes of buffer (Ninfali et al., 1990).

2',5'-ADP Sepharose 4B affinity chromatography

For 10 ml of bed volume, 2 g of dry 2',5'-ADP Sepharose 4B was washed several times in 400 ml of distilled water. During several washings, the impurities were removed and the gel was conditioned. After the removal of the air in the gel, it was resuspended in the buffer (0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0) at a ratio of 25% buffer to 75% gel and was packed in a column (1 x 10 cm). Precipitation of the gel, it was equilibrated with the same buffer by means of a peristaltic pump (flow rate: 50 ml/h). After the dialyzed enzyme solution was loaded on the column which was equilibrated with buffer (0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0) and the flow rate was regulated to 20 ml/h. The column was respectively washed with 25 ml of 0.1 M K-acetate + 0.1 M K-phosphate (pH 6.0) and 25 ml of 0.1 M K-acetate + 0.1 M K-phosphate (pH 7.85). Eventually, the enzyme was eluted with a solution of 80 mM K-phosphate + 80 mM KC1 + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.8). The enzyme activity was measured, and the activity-containing tubes were collected together (Ninfali et al., 1990).

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Protein determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method, with bovine serum albumin used as a standard (Bradford, 1976).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The control of enzyme purity was carried out using Laemmli's procedure (Laemmli, 1970) in 3% and 8% acrylamide concentrations for running and stacking gel, respectively. Gel was stained with Coomassie Brilliant Blue R-250.

In vitro effects of metal ions

Al^{3+} (5, 8, 10, 12.5 and 20 mM) and Hg^{2+} (0.5, 1, 2, 5 and 8 mM) were used as inhibitors. Assays were carried out under standard conditions with varying concentrations of Al^{3+} and Hg^{2+} metal ions. The inhibition of enzyme by Al^{3+} and Hg^{2+} was further examined by varying G6-P concentrations at a fixed NADP concentration and at six different constant concentrations of each metal ion. The activity of control cuvette in the absence of an inhibitor was taken as 100%. All compounds were tested in triplicates for each concentration. For each inhibitor, an activity %-[Inhibitor] graph was drawn. Metal ions concentrations that produced 50% inhibition (IC_{50}) were calculated from the regression graphs.

To determine the K_i values, three different inhibitor concentrations (Al: 7, 10 and 12.5 mM; Hg: 0.5, 2 and 5 mM) were tested for each metal ion. In these experiments, G6-P was used as substrate at five different concentrations (0.03, 0.06, 0.09, 0.15 and 0.27 mM, respectively). Inhibitor (metal ions) solutions were added to the reaction medium, resulting in three different fixed concentrations of inhibitors in 1 ml of total reaction volume. All assays were repeated three times. Lineweaver–Burk graphs (1934) were drawn by using 1/V vs. 1/[S] values. K_i constant and the inhibitor type were calculated from these graphs.

Results and Discussion

In this study, G6PD was purified from *C. umbla* kidney tissues by using ammonium sulphate precipitation and 2',5'-ADP Sepharose 4B affinity gel chromatography. As a result of the two consecutive steps, the enzyme was purified 402.14-fold in a yield of 22.7% with a specific activity of 11.26 U/mg (*Table 1*). Purity of the enzyme was determined by SDS-PAGE and showed single band on the gel (20 µl of the sample was loaded onto SDS-PAGE gel) (*Fig. 1*). Rf values were calculated for standard proteins and G6PD according to Laemmli's procedure from Rf–Log MW graph and molecular weight of protein was 73.8 kDa. For each metal, Lineweaver-Burk graphs were drawn and are shown in *Figs. 4 and 5*. K_i constants were determined as 0.98 ± 0.084 and 0.57 ± 0.019 mM from the graphs for Al^{3+} and Hg^{2+}, respectively (*Table 2*).

In addition, [Metal] vs. activity % graphs were drawn for the metals and are shown in *Figs. 2 and 3*. IC_{50} values were calculated as 7.22 and 3.12 mM from the graphs for Al^{3+} and Hg^{2+}, respectively (*Table 2*). Both Al^{3+} and Hg^{2+} inhibited the G6PD activity *in vitro* and showed competitive inhibition (*Figs. 2-5*). Hg^{2+} was a stronger inhibitor than Al^{3+}. Furthermore, Hg^{2+} had higher affinity for G6PD than that of Al^{3+}.

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Table 1. *Purification scheme of G6PD from C. umbla kidney*

Purification Step	Activity (U/ml)	Protein (mg/ml)	Total Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg protein)	Purification Factor	Yield (%)
Hemolysate	0.274	9.78	31	8.494	303.18	0.028	1	100
Ammonium Sulphate Precipitation and Dialysis	0.462	14.2	6	2.772	85.2	0.033	1.18	32.6
2',5'- ADP Sepharose 4B Affinity Chromatography	0.642	0.057	3	1.926	0.171	11.26	402.14	22.7

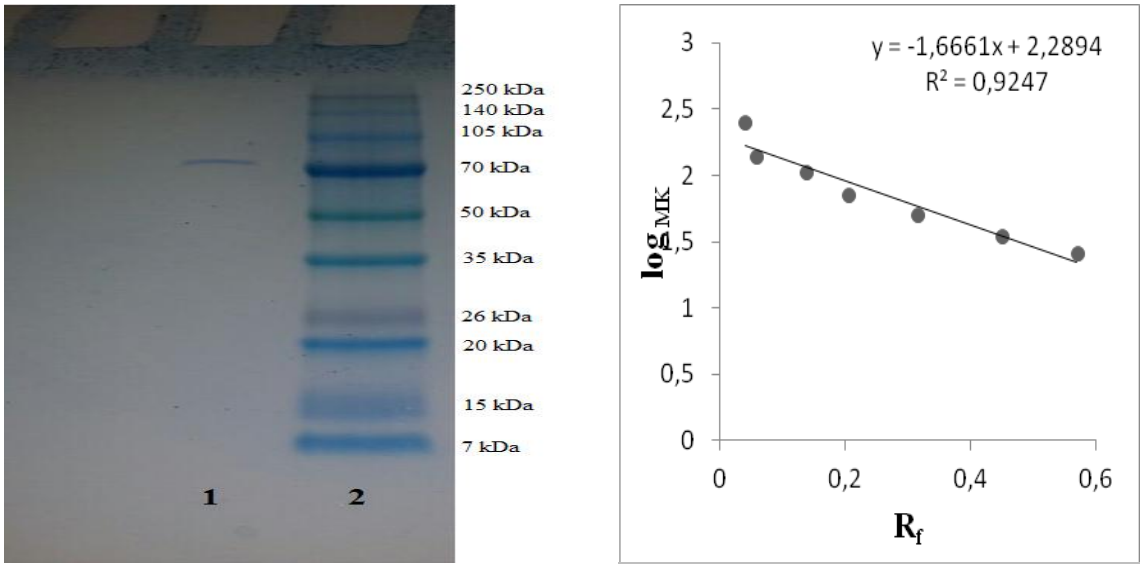

Figure 1. *SDS–polyacrylamide gel electrophoresis of purified G6PD. Lane 1: standard proteins and Lane 2: C. umbla kidney G6PD.*

G6PD has been purified previously with chromatographic methods from many different sources, such as, humans (Yoshida, 1966; Cho and Joshi, 1990; Ozmen et al., 2005), animals (Beydemir et al., 2003; Erat, 2005), plants (Coban et al., 2002; Esposito et al., 2005; Wei-Fu et al., 2007) and microorganisms (Heise and Opperdoes, 1999; Ibraheem et al., 2005). Inhibitory effects of many metal ions on G6PD enzyme activities in different animal species have been reported in many investigations (Velasco et al., 1994; Comaklı et al., 2013; Hu et al., 2013).

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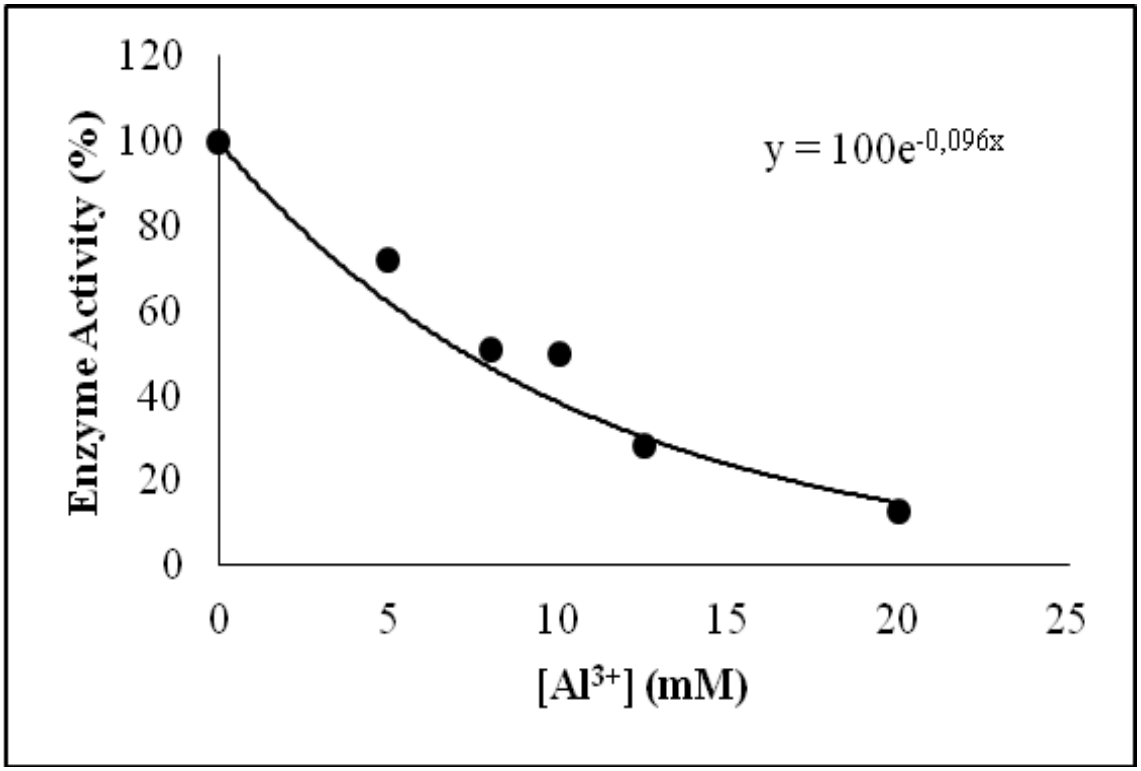

Figure 2. *Effect of Al^{3+} at five different concentrations on C. umbla G6PD activity*

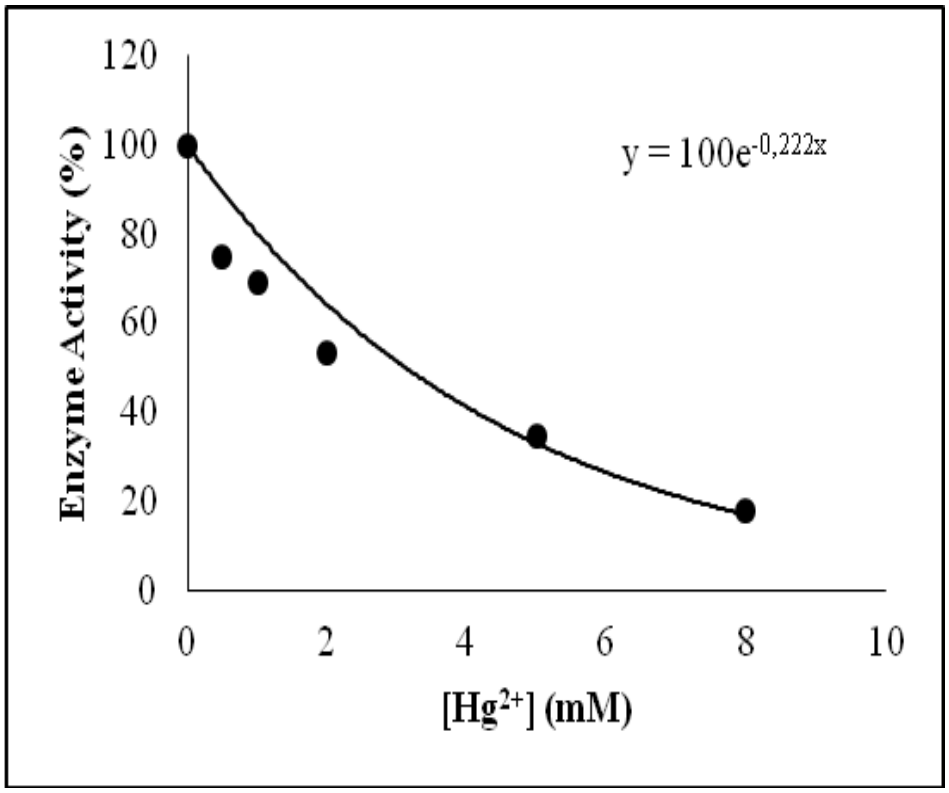

Figure 3. *Effect of Hg^{2+} at five different concentrations on C. umbla G6PD activity*

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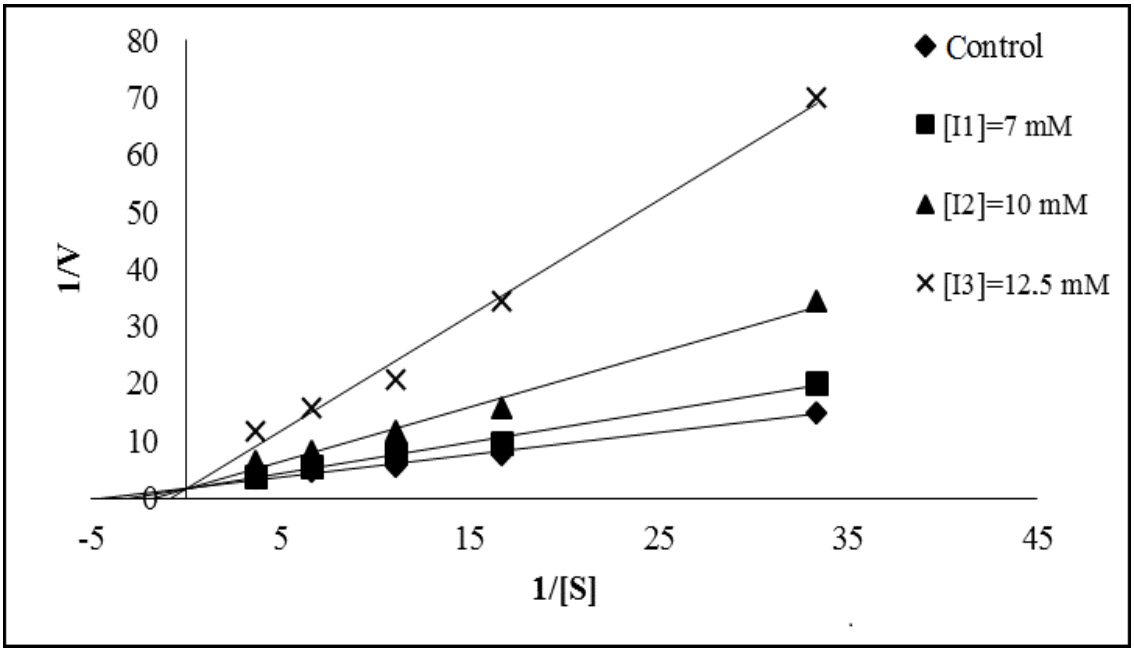

Figure 4. *Lineweaver–Burk graph in five different substrate concentrations and in three different Al^{3+} concentrations for the determination of K_i.*

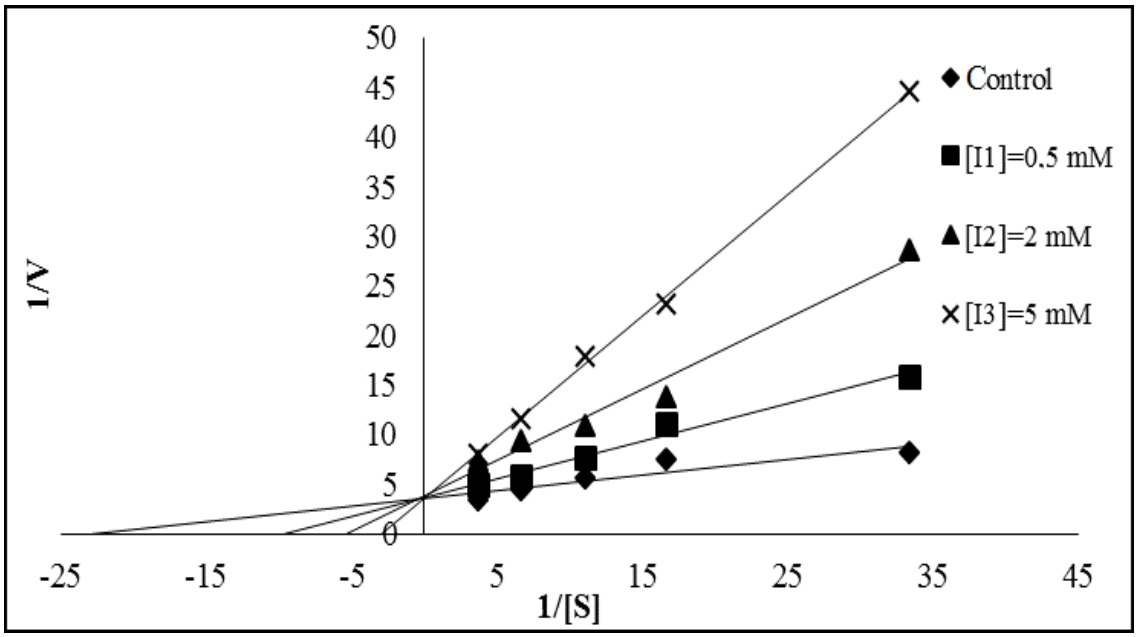

Figure 5. *Lineweaver–Burk graph in five different substrate concentrations and in three different Hg^{2+} concentrations for the determination of K_i.*

In the present study, specific activity of the enzyme was determined as 11.26 U/mg protein, which was lower than those in chicken erythrocytes (20.86 U/mg protein, Yilmaz et al., 2002), rainbow trout liver (36.25 U/mg protein, Cankaya et al., 2011), rat kidney (32 U/mg protein, Adem and Ciftci, 2012), grass carp (18 U/mg protein, Hu et al, 2013), but higher than that in sheep lens (0.15 U/mg, Charlton and Heyningen, 1971)

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and bovine lens (2.64 U/mg, Ulusu et al., 1999). The observation of different specific activities for G6PD from different sources was not uncommon, depending on several factors such as NADP, salt, etc.

Table 2. *The results of the activity of G6PD; K_i, I_{C50} values and inhibition types*

Metals	IC_{50} (mM)	K_i (mM)	Inhibition Type
Al^{3+}	7.22	0.98 ± 0.084	Competitive
Hg^{2+}	3.12	0.57 ± 0.019	Competitive

A molecular weight of G6PD was 75 kDa with SDS-PAGE in this study, which was similar to that reported in chicken erythrocytes (73.2 kDa, Yilmaz et al., 2002), but higher than bovine lens (69.2 kDa, Ulusu et al., 1999), human placenta (54 kDa, Ozer et al., 2001), dog liver (52.5 kDa, Ozer et al., 2002), buffalo erythrocyte (67.6 kDa, Ciftci et al., 2003), rainbow trout (60 kDa, Erdogan et al., 2005), rainbow trout liver (48.5 kDa; Cankaya et al., 2011), rat kidney (68 kDa, Adem and Ciftci, 2012) and grass carp (71.85 kDa, Hu et al., 2013).

Inhibition of some significant enzymes, which play a key role in a metabolic pathway, may give rise to pathologic conditions or disorders. In the literature, effects of various drugs and chemical substances on the catalytic activity of the G6PD enzyme were investigated. K_i values of these substances are higher than the values calculated for the coumarin derivatives. Ki values of isepamicin sulphate, omeprazole, morphine sulphate, vancomycin, magnesium sulphate, metamizol and granisetron hydrochloride were reported as 1.7 mM, 8.2 mM, 25.9 mM, 2.71 mM, 13.2 mM, 6.3 mM, 4.5 mM, respectively (Ozmen and Kufrevioglu, 2004; Ozmen et al., 2005).

Hopa et al. (2014) investigated the inhibition effects of IC_{50} and K_i parameters of coumarin derivatives for G6PD. IC_{50} values of OPC (6,7-Dihydroxy-3-(2-methylphenyl)-2H-chromen-2-one), MPC (6,7-dihydroxy-3-(3-methylphenyl)-2H-chromen-2-one) and PPC (6,7-dihydroxy-3-(4-methylphenyl)-2H-chromen-2-one) were 0.305, 0.769 and 0.790 mM and the K_i constants were 1.37 mM, 0.734 mM 0.269 mM, 0.835 mM, respectively.

Hu et al. (2013) purified G6PD from grass carp (*Ctenopharyngodon idella*) and determined the inhibition effects of Zn, Mn, Al, Cu and Cd on G6PD activity *in vitro*. They found IC_{50} values as 0.42, 0.54, 0.94, 1.20, and 4.17 mM, respectively. K_i constants were determined as 0.52, 1.12, 0.26, and 4.8 mM, respectively. Cankaya et al. (2011) reported that the IC_{50} values of Fe, Pb, Hg, Cu, Zn, and Cd on the purified G6PD activity of trout liver was 0.39, 0.78, 0.87, 1.19, 1.97, 2.16 mM and the K_i constants were 0.197,0.213, 0.542, 1.721, 2.034, 2.770 mM, respectively.

Fish meat is a precious food of animal source for human depletion. Accumulation of metals in fish may be considered as an important warning signal for fish health and human consumption. Metal ions accumulated in fish as a toxic concentration will be hazardous for human health. For this reason, great efforts and cooperation between different authorities are need to protect the aquatic resources from metal pollution. To avoid the aquatic life loss there is need to use the advanced technologies generating less metal pollution to environment. Briefly, concentration of metal ion in contaminated lakes and rivers must be decreased.

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CARBON OCCLUSION POTENTIAL OF RICE PHYTOLITHS: IMPLICATIONS FOR GLOBAL CARBON CYCLE AND CLIMATE CHANGE MITIGATION

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Abstract. Phytoliths are silica bodies produced by plants through bio-mineralization process. During such process, occlusion of carbon (C) also takes place within the phytoliths called phytolith occluded C (PhytOC). The PhyOC is highly stable and may substantially contribute to long-term terrestrial C sequestration. The present study aimed to investigate the phytolith and PhytOC contents variability in rice cultivars and its potential for long-term terrestial C biosequestration.The results indicate that dry matter yield of rice cultivars varied from 15.52 to 28.82 g plant^{-1}and, the PhytOC contents of straw, root, husk, and grains range from0.22-0.68%, 0.09-0.22%, 0.43-0.82% and 0.002-0.024%, respectively. The PhytOC content of rice depends on the content of phytoliths and the efficiency of C occlusion within the phytoliths. The C sequestration rates of rice cultivars are approximately 0.05 - 0.12 Mg of C dioxide equivalents (Mg-e-CO_2) ha^{-1} year^{-1}. Assuming maximum phytolith C biosequestration rate of 0.12 Mg-e-CO_2 ha^{-1} year^{-1}, the global annual potential sink rate of PhytOC in soils through rice phytoliths would approximately be 16.4 Tg-e-CO_2. Therefore rice crop may play a significant role in long-term C sequestration through PhytOC.
Keywords: *carbon biosequestration, plant silica, phytolith occluded carbon, rice cultivars*

Introduction

Green house gases (GHGs) mediated global warming and climate change is one of the major global environmental issues of recent decades. Recently it is estimated that global CO_2 emission rate had increased to 3.11×10^{11} Mg per year by 2014. Therefore all mechanisms of carbon (C) sequestration should be explored in order to reduce the concentration of CO_2 in the atmosphere. Land use systems like forest, grassland, croplands, and shrub lands substantially contributes to terrestrial C storage as a major part of C sequestered is again returned to soil as plant residues. Therefore, a minor change in such a big terrestrial C pool may significantly affect the C flux. In this context phytolith occluded C (PhytOC) of plants that has long resident time (millennia) and resist decomposition, fire, animal digestion, etc. can be considered for long-term terrestrial C storage. The presence of silica phytoliths within cereal crops is well documented. But information on C sequestration potential of phytoliths for cereals is meager. Therefore, it is aimed to study the phytolith and PhytOC content variability in rice, a hyper silica accumulator and widely cultivated food crop across the world, to explore its C biosequestration potential within the phytoliths.

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Review of Literature

The PhytOC is an organic C fraction, where C is entrapped within recalcitrant silicified structure called phytoliths. Phytoliths or opal silica is deposits of silica within plant tissues (e.g. cell walls, cell lumina and inter cellular spaces) during the process of biomineralization (Siever and Scott, 1963; Piperno, 1988; Parr and Sullivan, 2005). Compared with other organic fractions PhytOC fraction is very stable and highly resistant against decomposition and may accumulate in soil for several thousands to millennia of years after plant decomposition (Wilding et al., 1967; Wilding and Drees, 1974; Mullholland and Prior, 1993; Prasad et al., 2005; Parr et al., 2010). For example it has been reported that the age of phytoliths in volcanic soils and peat land sediments ranges from 0 to 8000 years (Parr and Sullivan, 2005) and also radio C date of phytolithsindicate13300±450 years old (Wilding, 1967). The C flow in soil plant atmospheric continuum and long-term stability of PhytoC in soil is depicted pictorially in *Fig. 1*. PhytOC plays a major role in soil C cycle (Parr and Sullivan, 2005; Oldenburg et al., 2008) and many researchers emphasized the importance of soil C in climate change (Gifford, 1994; Kosten et al., 2010). Moreover PhytOC can be easily measured in standing vegetation bypassing many of the current issues associated with standard soil C measurement (Parr and Sullivan, 2011).

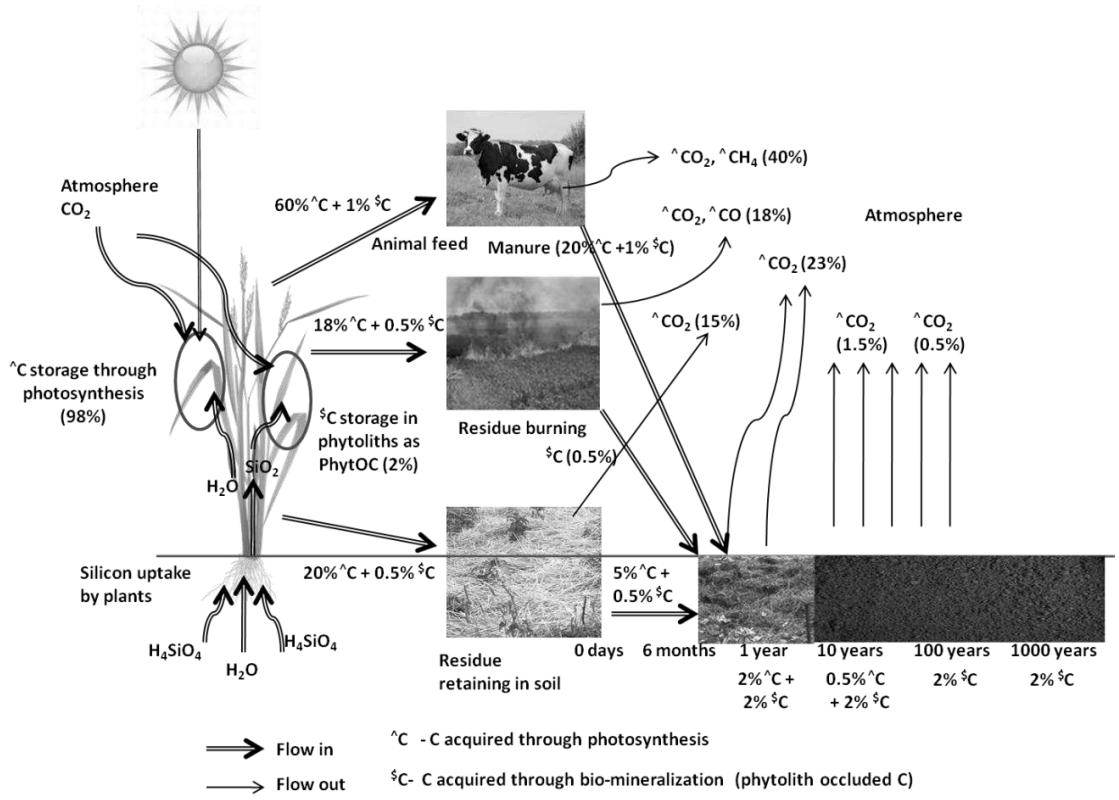

Figure 1. *Carbon flow of soil plant atmosphere continuum and long-term stability of PhytOC in the soil (the ^C fraction which acquired through photosynthesis return back to atmosphere within a short time span of 100 years whereas the $C fraction that entrapped in phytolith can stay in the soil for more than 1000 year)*

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The soil C sequestration rate due to PhytOC under natural vegetation with varying climatic condition varies between 0.4 to 0.9 g C m^{-2} $year^{-1}$ (Parr and Sullivan, 2005). It contributes 15 to 37% of the long term global soil C sequestration rate indicating that PhytOC plays a major role in long term soil C sequestration processes under natural vegetation systems. Also it was demonstrated that the PhytOC yields of crops can be far higher than those of native vegetation (Parr et al., 2009). The PhytOC content and variability in wheat, sugarcane, millet and some bamboo cultivars were extensively studied by many researchers (Parr and Sullivan, 2005, 2011; Parr et al., 2009, 2010; Zuo and Lu, 2011). For example, the phytolith C biosequestration fluxes from millet, wheat, sugarcane and bamboo range up to 0.04, 0.25, 0.36 and 0.71 Mg-e-CO_2-ha^{-1} $year^{-1}$, respectively (Parr and Sullivan, 2005, 2011; Parr et al., 2009, 2010; Zuo and Lu, 2011) and also Song et al. (2013) estimated that global PhytOC sink of croplands is 26.35±10.22 Tg CO_2 $year^{-1}$ and the major contributors of this cropland sinks are rice, wheat and maize, as they are widely cultivated across the world, and play significant role in global cropland C balance (Rajendiran et al., 2012). Bamboo leaf litter has PhytOC yields of upto 0.7 t e-CO_2 ha^{-1} $year^{-1}$ and PhytOC from bamboo and sugarcane grasses has a global potential to bio-sequestrate approximately 1.5 billion t e-CO_2 $year^{-1}$, equivalent to 11% of the current increase in atmospheric CO_2 (Parr et al., 2010). Simply by growing high PhytOC yielding cultivars over low PhytOC yielding cultivars resulted in additional sequestration of C in soil by ~ 5 million t e-CO_2 $year^{-1}$ and 53 million t e-CO_2 $year^{-1}$, for sugarcane (20 million hectares) (Parr et al., 2009)and wheat (214 million hectares) (Parr and Sullivan, 2011), respectively. Hence, this process offers the opportunity to use the plant species that yield high amounts of PhytOC to enhance terrestrial C sequestration.

Rice is currently cultivated on around 164.1 million hectares and it is the stable food source for more than half of the world's population. India has long history of rice cultivation and is accounting for 22% of world production. The current area of rice production in India is around 44 million hectares. Rice is cultivated two to three times in a year because of variability in temperature and irrigation facilities. The presence of silica phytoliths and C sequestration potential of major cereal crop like rice (Li et al., 2013; Guo et al., 2015;), millet (Zuo and Lu, 2011), sugarcane (Parr et al., 2009) and wheat (Parr and Sullivan, 2011) have been reported. Also the process of C accumulation in phytoliths are affected by many factors like location, climate, varieties, pest and disease resistances, fertilizers etc. (Ma et al., 2002; Ding et al., 2005; Meena et al., 2013). Keeping in view of all the above, the objectives of the present study are:

1. To study the phytolith and PhytOC content variability in rice cultivars that cultivated in sub-tropical conditions
2. To screen high phytolith and phytolith occluded C yielding rice cultivars, and
3. To explore the potential of C bio- sequestration within the phytoliths of rice cultivars.

Materials and Methods

Collection of planting material

The seeds of 15 commonly grown rice cultivars of central India were collected from the Department of Plant Breeding and Genetics, Jawaharlal Nehru Krishi Vishwa Vidhyalaya, Jabalpur (India) (*Table 1*).

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Table 1. *Rice cultivars and organs, dry matter yield of rice organs (g per plant), phytolith content of rice organs by dry weight basis (mg g^{-1}), C content of rice phytoliths (%), PhytOC content of organs (%), total dry biomass of rice plant (g) and total estimated PhytOC production in rice phytoliths on the basis of Mg-e-CO_2 ha^{-1} $year^{-1}$ (considering optimum crop stands per ha as 330000 plants by assuming crop spacing with 20 cm x 15 cm as well as 3 season per year) and PhytOC sink rate in soil (Mg-e-CO_2 ha^{-1} $year^{-1}$).*

Rice cultivar	Name of the organ	Dry weight of organ (g per plant)	Phytolith content (mg g^{-1})	C content of phytolith (% by weight)	PhytOC content of organs (% by weight)	Total dry biomass of rice plant (g)	Total estimated PhytOC sequestration per plant (g-e-CO_2)	Total estimated PhytOC sequestration in rice (Mg-e-CO_2 ha^{-1} $year^{-1}$)	PhytOC sink rate in soil (Mg-e-CO_2 ha^{-1} $year^{-1}$)
MR - 219	Straw	11.89	12.47	3.37	0.420	23.87	0.28	0.094	0.084
	Root	3.55	8.30	2.13	0.177				
	Husk	2.64	14.06	5.67	0.797				
	Grain	5.79	0.26	1.00	0.003				
P - 1121	Straw	9.20	23.62	1.87	0.442	19.97	0.24	0.078	0.070
	Root	4.27	10.70	1.87	0.200				
	Husk	2.21	21.60	3.20	0.193				
	Grain	4.29	0.61	0.87	0.005				
WGL - 32100	Straw	8.71	21.32	2.23	0.475	17.80	0.22	0.074	0.066
	Root	3.06	11.40	1.90	0.217				
	Husk	2.09	24.41	2.40	0.585				
	Grain	3.95	1.42	1.07	0.015				
MTU - 1081	Straw	11.14	22.75	2.20	0.500	24.20	0.28	0.095	0.085
	Root	3.13	7.05	1.70	0.120				
	Husk	3.04	22.06	2.80	0.618				
	Grain	6.73	0.32	0.80	0.003				
JGL - 3844	Straw	8.95	19.24	2.00	0.385	19.06	0.19	0.063	0.057
	Root	3.31	9.17	1.50	0.138				
	Husk	2.05	18.30	3.33	0.610				
	Grain	4.76	1.46	0.83	0.012				

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SUREKHA	Straw	14.62	20.98	2.27	0.476	28.82	0.36	0.118	0.106
	Root	3.95	8.26	2.03	0.168				
	Husk	4.07	19.66	2.63	0.517				
	Grain	6.18	0.14	1.23	0.002				
PRATIKSHYA	Straw	7.30	26.39	2.53	0.668	16.89	0.26	0.086	0.077
	Root	2.96	6.54	2.27	0.148				
	Husk	2.04	21.49	3.80	0.817				
	Grain	4.59	1.94	1.23	0.024				
KAVYA	Straw	8.53	23.33	2.53	0.590	17.60	0.24	0.079	0.071
	Root	2.79	8.45	1.97	0.166				
	Husk	1.98	21.18	2.37	0.502				
	Grain	4.29	1.17	0.93	0.011				
VARALU	Straw	7.61	23.13	1.93	0.446	15.52	0.17	0.055	0.049
	Root	2.42	5.5	1.63	0.090				
	Husk	1.84	15.46	3.21	0.495				
	Grain	3.64	0.41	0.73	0.003				
KRANTI	Straw	11.46	15.72	1.40	0.220	23.21	0.17	0.056	0.051
	Root	2.98	7.2	1.13	0.081				
	Husk	2.73	16.01	4.20	0.672				
	Grain	6.05	1.04	0.70	0.007				
MTU - 1010	Straw	11.88	18.48	2.30	0.425	24.83	0.30	0.099	0.089
	Root	3.26	7.26	1.43	0.104				
	Husk	3.27	13.13	6.30	0.827				
	Grain	6.43	1.15	1.13	0.013				
OR - 1912-24	Straw	9.58	16.33	2.53	0.413	19.96	0.23	0.075	0.068
	Root	2.87	8.51	2.13	0.181				
	Husk	2.49	14.27	4.80	0.685				
	Grain	5.02	0.49	1.30	0.006				
JAGTIAL SANALU	Straw	8.25	20.76	2.27	0.471	16.31	0.19	0.063	0.056
	Root	3.46	10.4	1.77	0.184				
	Husk	1.49	20.3	2.13	0.433				

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	Grain	3.10	0.23	1.07	0.002				
JGL - 3828	Straw	9.11	16.59	2.43	0.403	20.29	0.23	0.075	0.068
	Root	3.47	7.44	1.77	0.132				
	Husk	2.48	13.96	5.90	0.824				
	Grain	5.56	0.73	1.33	0.010				
BPT - 5204	Straw	9.01	14.47	2.90	0.420	17.64	0.22	0.072	0.064
	Root	3.15	8.43	2.20	0.185				
	Husk	1.90	14.52	5.50	0.799				
	Grain	3.99	0.65	1.43	0.009				

Note: Values in the table are a mean of triplicate observations

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These cultivars are raised in the pots under same environmental conditions to eliminate factors that might influence silica uptake and deposition. The greenhouse study was conducted in a "black cotton soil" (Typic Haplusterts) in the Division of Environmental Soil Science, ICAR-Indian Institute of Soil Science (23°18' 33.6" and N, 77°24' 27.2" E and 504 m above mean sea level), Bhopal (Madhya Pradesh), India. It is located in central part of India and has semi-arid and sub-tropical dry summer and cold winter climate with a mean annual air temperature of 25°C and annual average rainfall of 1146 mm. The planted rice cultivars are used for analysis of phytolith and PhytOC content in different organs (straw, husk, grain and root; *Table 1*). The current study compares the PhytOC production variability among the rice cultivars and also contribution of different organs towards this C fraction. After harvest of the crop, plant parts like straw, root, husk and grains were separated and washed with distilled water to remove the dusts accumulated on the plant material. It was allowed to air dry for 2-3 days and then placed in hot air oven at 60°C for 24 hours. The dried plant parts were grounded and passed through 0.02 mm sieve Dry matter yield of straw, root, husk and grain were recorded and these data were used for calculating PhytOC production of each rice cultivar.

Phytolith extraction and occluded C analysis

The phytolith in plant material was extracted by dry ashing and acid extraction method using muffle furnace (Rovner, 1983; Bowdery, 1989). The carbonates and organic materials in the acid extracts were removed with the help of 10% HCl and 15% H_2O_2, respectively. About 1.0 g of dry plant material was placed in a silica crucible and was heated in muffle furnace at 500°C for 8 hours. Then it was cooled and transferred to test tubes. About 20 ml of 10% HCl was added to test tubes and heated in water bath at 70 °C for 20 minutes. Then it was centrifuged at 3500 rpm for 5 minutes and the supernatant solution was removed. The remaining stuff was rinsed with distilled water and was centrifuged again at 3500 rpm for 5 minutes and the supernatant was decanted. Further 20 ml of 15% H_2O_2 was added and heated in water bath at 70°C for 20 minutes. Then the content was centrifuged at 3500 rpm for 5 minutes and was decanted. Then rinsed with distilled water and centrifuge at 3500 rpm for 5 minutes and decanted, this process was repeated twice. And finally 1 ml of 100% ethanol was added and left overnight to dry. Then dried material was weighed and phytolith was calculated. The separated phytolith was mounted onto microscopic slides in Canada balsam and was observed in microscope at 400x magnification (*Fig 2*). The C content of phytoliths, which extracted from plant materials, was analyzed in CHN analyzer (model FLASH 2000 organic elemental analyzer). The organic C data were monitored with standard soil samples (NC Soil Standard 338 40025 Certif. 133317) and the precision is better than 80%. The C sequestered in different plant parts and the total C sequestered by the rice cultivars were calculated and expressed in CO_2 equivalent basis. The correlation between phytolith content and C content of phytoliths in different plant parts like straw, root, husk and grain was studied.

Estimation of PhytOC production and PhytOC Sink

The production of PhytOC in any plant species is depends primarily on the PhytOC content and dry weight of the material. The PhytOC content of a plant parts is a product of C content in the extracted phytoliths and phytolith content of the plants parts. The

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PhytOC production rate of a plant in an area is estimated from PhytOC content and dry weight of the organs as follow:

$$PhytOC\ production\ rate = \sum_{i=1}^{n} PhytOC\ content_i \times Dry\ biomass_i \times \frac{44}{12} \quad \text{(Eq. 1)}$$

Where, PhytOC production rate is the PhytOC production by a particular plant per hectare per year (Mg CO_2 $ha^{-1}year^{-1}$), PhytOC $content_i$ is the concentration of PhytOC in an organ (wt %), and Dry $biomass_i$ is the dry weight of the crop organs per hectare (Mg ha^{-1} $year^{-1}$), and i =1 to n (number of crop organs). From single plant to per hectare conversion the optimum plant population of 330000 was used in this study by assuming with crop spacing of 20 cm x 15 cm.

The PhytOC sink rate is controlled by the PhytOC production rate and the stability of phytolith in environments. The PhytOC sink rate can be estimated from phytolith production rate and phytolith stability factor as mentioned by Song et al. (2013):

$$PhytOC\ sink\ rate = PhytOC\ production\ rate \times phytolith\ stability\ factor \quad \text{(Eq. 2)}$$

Where, PhytOC production rate may be obtained from *Eq. 1* and the phytolith stability factor is assumed to be 0.9 as most of the phytolith have been proved stable for thousands of years (Meunier et al., 1999; Parr and Sullivan, 2005).

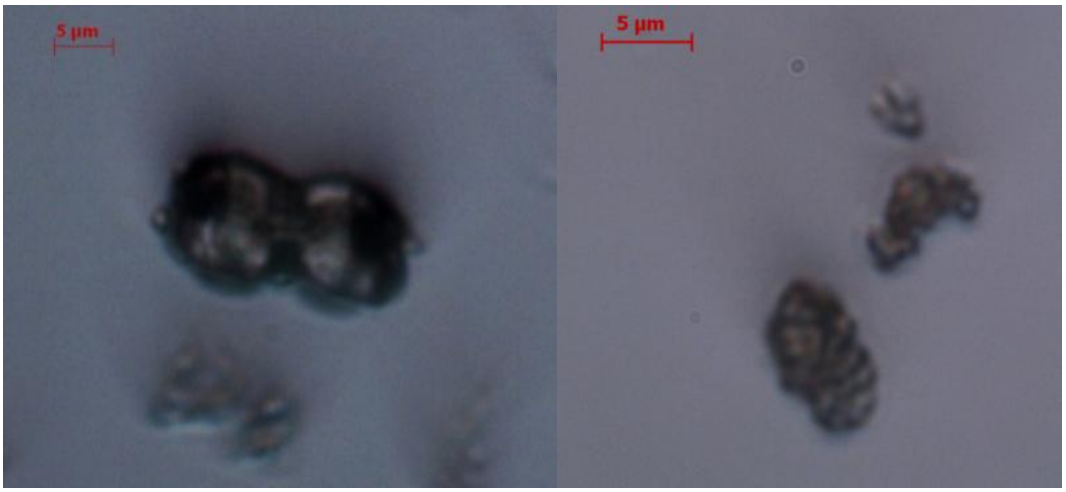

Figure 2. *Morphology of rice phytoliths*

Results

Dry weight of various plant parts straw, root, husk and grain, and total dry matter yield (DMY) of the 15 rice cultivars is presented in *Table 1*. The dry matter yield of rice cultivars vary from 15.52 – 28.82 g per plant. Also the dry biomass of different rice organs such as straw, root, husk and grain range from 7.30 to 14.62 g, 2.42 to 4.27 g, 1.49 to 4.07 g and 3.1 to 6.73 g per plant, respectively. Results indicate that rice straw

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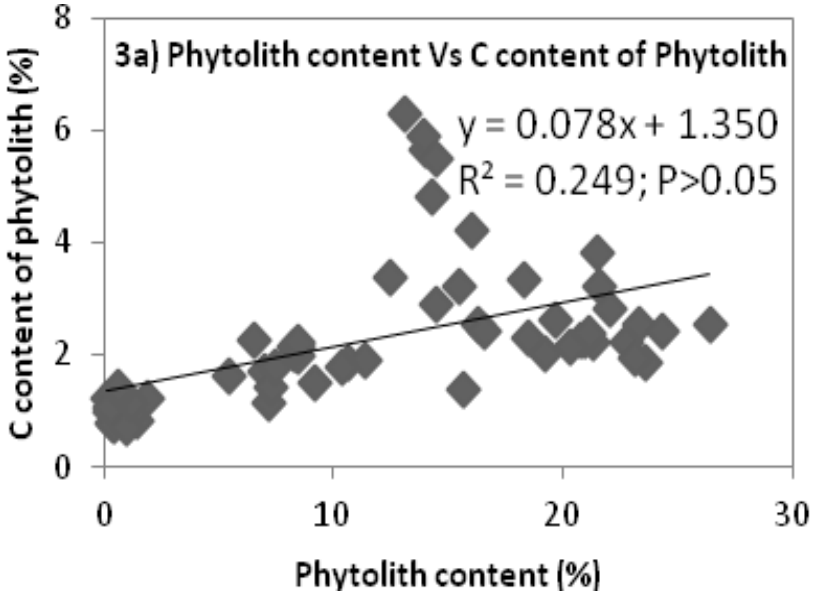
contributes more towards the total dry biomass. The order of rice organs that contributes towards dry matter accumulation in rice cultivars was straw>grain>root>husk.

The microscopic observation of rice pytoliths is very clear and avoid of other impurities (*Fig. 2*). The content of phytoliths in different rice organs varied from 0.14-26.4 mg g^{-1} and there was no clear trend with respect to phytolith content in different plant parts of rice. In some cultivars straw portion has higher phytolith content and in some other cases phytolith content was greater in husk. However in most of the cases straw portion records the highest phytolith content followed by husk, root and grain for rice cultivars (*Table 1*). Also C content of phytoliths of rice organs differs from 0.7-6.3% (*Table 1*). It is observed that C content of phytoliths in sheath is higher than that of straw, root and grain (*Table 1*). Further, substantial variations in PhytOC content were observed in different rice organs ranging from 0.002 to 0.82 mg g^{-1}.

There is weaker positive correlation prevails between phytolith content and C content of phytoliths (R^2 = 0.249, P>0.05) (*Fig.3a*), However there is strong positive correlation exists between phytolith content and PhytOC content (R^2 = 0.694, P<0.05) (*Fig.3b*) as well as C content of phytoliths and PhytOC content of organs (R^2 = 0.760, P<0.01) (*Fig.3c*). Similarly the relationship of phytolith content and C content of phytoliths in different organs show weaker negative correlation in straw (R^2 = 0.139, P>0.05) and grain (R^2 = 0.000, P>0.05), stronger negative correlation in husk (R^2 = 0.721, P<0.05) and weaker positive correlation in root (R^2 = 0.028, P>0.05), respectively (*Fig. 4a*), but correlation between phytolith content and PhytOC content of different organs show strong positive correlation in straw (R^2 = 0.490, P<0.05), root (R^2 = 0.0.662, P<0.05) and grain (R^2 = 0.897, P<0.05) and weak negative correlation in husk (R^2 = 0.245, P>0.05), respectively (*Fig. 4b*). However the correlation between C content of phytoliths and PhytOC content of different organs is positive (*Fig.4c*) for all the organs but stronger for root (R^2 = 0.500, P<0.05) and husk (R^2 = 0.746, P<0.05) and weaker for straw (R^2 = 0.147, P>0.05) and grain (R^2 = 0.072, P>0.05), respectively.



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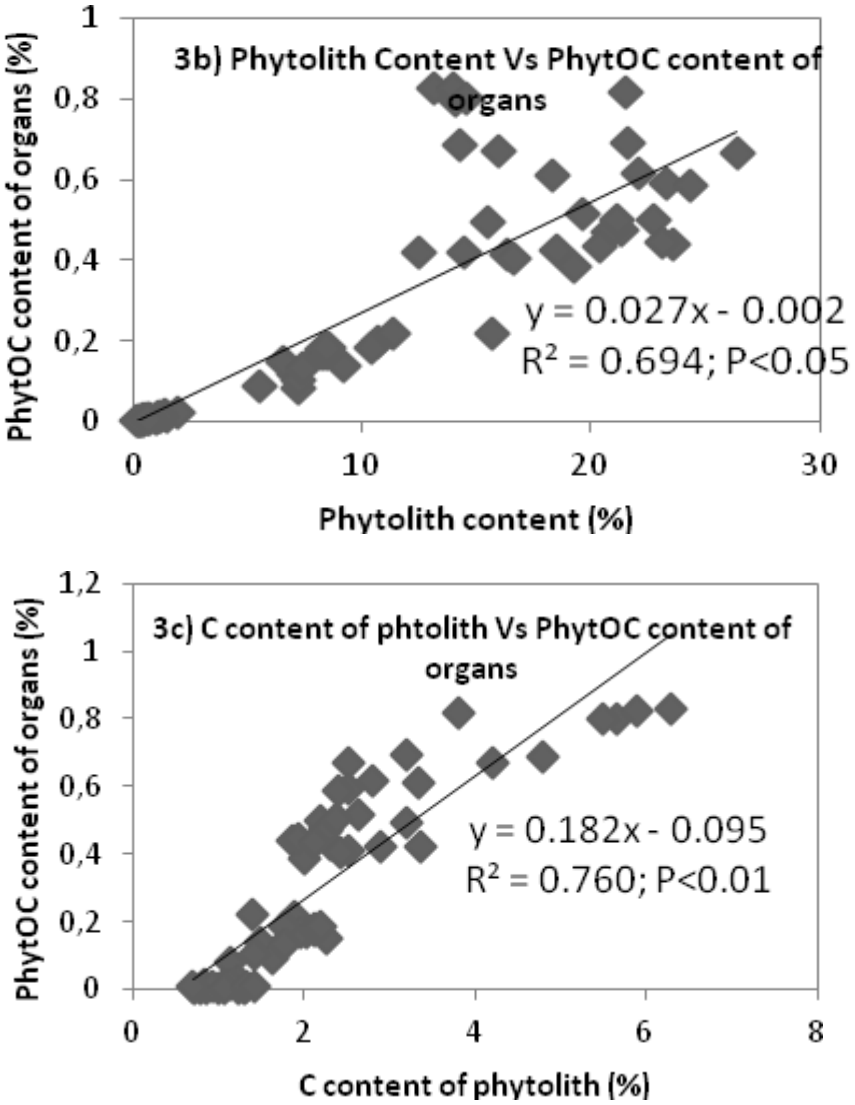
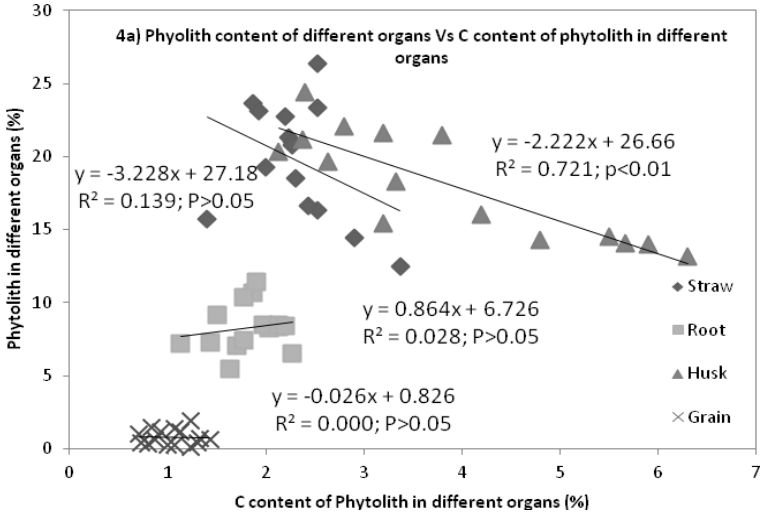


Figure 3. *Relationship between 3a) phytolith content and C content of phytolith; 3b) PhytOC of organs and phytolith content; and 3c) PhytOC content of organs and C content of phytolith*



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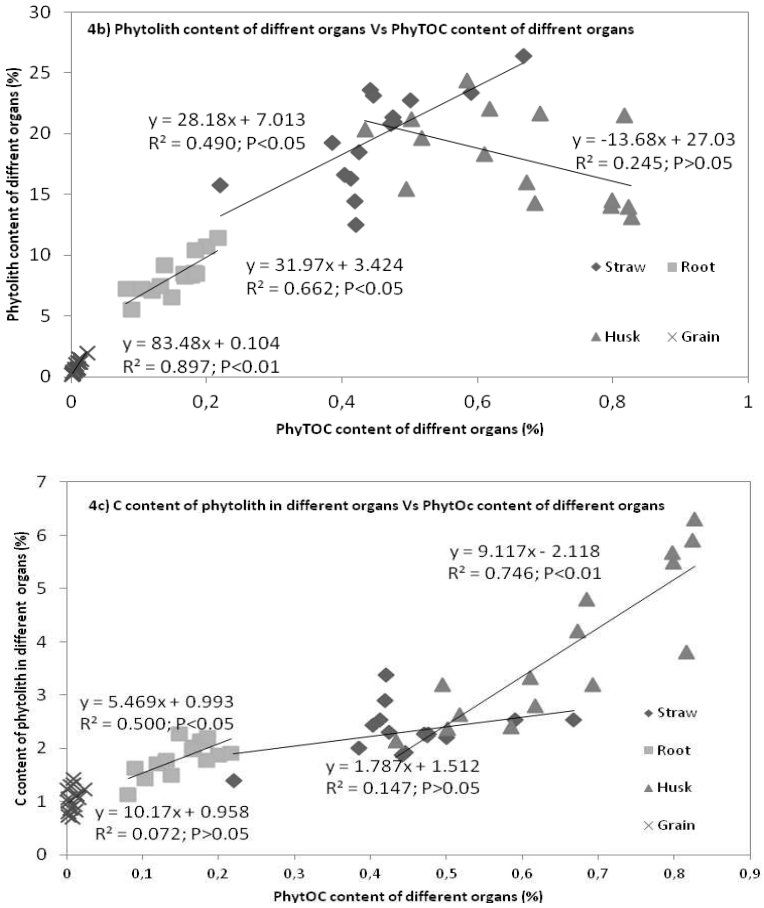

Figure 4.*Correlation of 4a) phytolith content of different organs and C content of phytolith in different organs; 4b) phytolith content of different organs and PhytOC content of different organs; and 4c) C content of phytolith in different organs and PhytOC content of different organs.*

Discussion

Variability of DMY, phytolith and PhytOC content in rice cultivars

Many researchers documented that genotype and environment factors influence DMY of cultivated arable crops (Ying et al., 1998; Khush et al., 2001; Horie et al., 2002; Katsura et al., 2007). In our study, genotypic factor substantially influenced the DMY and significant variations in DMY among the cultivars were observed and similar kinds of results were reported by many workers (Khush et al., 2001; Katsura et al., 2007). The contribution of rice straw towards the total DMY was higher followed by grains, root and husk. The result was in accordance with the results observed by Sun et al. (2008) in rice crop under hydroponic condition.

The distribution patterns of phytoliths in different plant organs show that straw portion contains most of the phytoliths (60%) followed by husk, root and grain. The similar results and trends were reported by many workers (Li et al., 2013; Guo et al., 2015). In the current investigation, the phytolith content of rice straw varied from 12.46 mg g^{-1} to 26.39 mg g^{-1}. Guo et al. (2015) and Li et al (2013) reported the phytolith

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content of rice leaf varies from 26.85- 37.12 mg g^{-1} and 55.45- 79.27 mg g^{-1}, respectively. When compared to other studies, it gives the impression of low phytolith content, but stem and sheath also parts of rice straw, they contain lower phytolith than that of leaf (Li et al., 2013; Guo et al., 2015). Further the process of phytolith accumulation in a plant is affected by many factors like location, climate, soil type, varieties, management practices, etc. (Parr et al., 2010; Parr and Sullivan, 2011; Meena et al., 2013; Zhao et al., 2015; Guo et al., 2015). The high DMY and silica accumulation in the straw might have resulted in higher phytoliths accumulation in the rice straw compared to other organs (Ding et al., 2005). The high phytolith accumulation in rice straw has additional advantage of being recycled and incorporate into the soil through proper crop residue management may substantially buildup of the phytolith content of the soil (Parr et al., 2010).

It was reported that the PhytOC in bamboo, wheat, sugarcane and millet has no direct relationship with the phytolith content of the plants and mainly depends on the efficiency of the C occluded within the phytoliths during biomineralization process (Parr and Sullivan, 2005, 2011; Parr et al., 2009, 2010; Zuo and Lu, 2011). However the results on C occlusion in the phytoliths from this study are quite different from the above studies. The strong correlation between the phytolith content and the PhytOC content of organs (R^2= 0.694, P<0.05) (*Fig. 3b*), and between the C content of phytoliths and the PhytOC content of organs (R^2= 0.760, P<0.01) (*Fig. 3c*) were observed in the 15 rice cultivars tested. In case of individual organs, between the phytolith content and the PhytOC content each organs (R^2= 0.245- 0.897) (*Fig. 4b*), and between the C content of phytoliths and the PhytOC content of individual organs in 15 cultivars (R^2= 0.072- 0.746) (*Fig. 4c*) show positive correlation. It indicates that the PhytOC content in rice plants might depend on both the phytolith content and C occlusion efficiency of biomineralization process within phytoliths during plant growth. Similarly Li et al. (2013) and Guo et al., (2015) observed the strong positive correlation between the content of phytoliths and the PhytOC content of rice organs, implying that PhytOC content of organs depends not only on the C content of phytoliths but also on phytolith content. Thus all the factors influencing the content of phytoliths and the content of silica occluding C within the phytoliths could result in significant variation of the phytolith and PhytOC content in plants. The findings of Li et al. (2014) were also in concurrence with the findings of the current investigation.

Screening of PhytOC yielding rice cultivars and its application

The estimate shows that the PhytOC flux of rice cultivars is 0.05-0.12 Mg-e-CO_2 ha^{-1} $year^{-1}$ (*Table 1*). Researchers have emphasized that the PhytOC sequestration rate can be improved by selecting cultivars which produce high PhytOC rather than low PhytOC yielding cultivars for cultivation (Parr et al., 2010; Parr and Sullivan, 2011; Song et al., 2013). This study also shows that by selecting high PhytOC yielding cultivar over low PhytOC yielding cultivar for cropping may additionally sequester 0.07 Mg-e-CO_2 ha^{-1} $year^{-1}$ through PhytOC. However cultivars cannot be selected only on the basis PhytOC production, but rather other desirable traits like biomass and yield, climatic factors, location and taste preferences to be considered (Parr et al., 2009). Also in this study it is demonstrated that there is a strong correlation between phytolith content and PhytOC production (*Fig. 3 and 4*), and the DMY also influences the PhytOC production of crops. Hence there is possibility to increase C occlusion potential of phytolith through improving the phytolith content and the DMY of crops by adopting simple management

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practices like nutrient management, regulating silicon (Si) supply, etc. For instance, many reports clearly reveal that the content of phytolith in crops can be enhanced through addition of Si and N fertilizers, straw, organic fertilizer, calcium-magnesium phosphate fertilizers and slags to crops (Bao et al., 1996; Chen et al., 2008; Zhang et al., 2008; Meena et al., 2013; Zhao et al., 2015). In fact phytolith accumulation in plants also may yield some additional advantages to the crop growth such as enhancement of growth and yield, resistance to pest and disease, and increase in shoot rigidity and hardness, etc. (Epstein, 2001) by silicon supplementation. Further Guo et al. (2015) reported that applying Si through basalt powder amendment in rice ecosystem can significantly enhance the phytolith concentration in different rice organs, in turn, substantially improve phytolith carbon sink. Rice is mostly cultivated in resource poor condition in India, as 60% of rice area is under rainfed and grown under poor crop management practices. Therefore, it is likely to enhance PhytOC content through regulating silicon supply, optimization of cropping and fertilization management under various agro-ecosystems across the country (Song et al., 2013; Song et al., 2014).

Moreover the plant phytoliths that present in straw and root are returned directly to soil through plant litter fall or root decomposition and some extent through rice straw as crop residue (Seyfferth et al., 2013; Ngoc Nguyen et al., 2014) and indirectly through burning of crop residues in the field as biochar (Houben et al., 2014; Liu et al., 2014) that is commonly practiced in India by the growers (Venkataraman et al., 2006; Pathak et al., 2010). In contrast, a substantial proportion of phytolith produced in crops is taken from the site during harvest (Meunier et al., 2008). Even some harvested phytoliths are transformed to human and animal waste after food/fodder consumption and returned to soil as amendment or disposed off as a waste into sewage plant systems and surface water bodies (Song et al., 2011). Therefore sustainable management of crop residues may further enhances the phytolith mediated C sequestration potential of terrestrial ecosystems.

Estimated C biosequestration within the phytoliths of rice

Based on the available records for planted area of rice in India from 1950 to 2010 (*Fig. 5a*), we have estimated the trend of possible lowest and highest CO_2 sequestration of rice phytoliths though PhytOC from 1950-2010 (*Fig. 5b*). It shows that between 1.94×10^6 to 4.66×10^6 Mg-e-CO_2 year^{-1} is occluded within the rice phytoliths in India. Considering the largest flux of PhytOC sequestration (0.12 Mg-e-CO_2 ha^{-1} year^{-1}) in this study, our results estimate that the rice phytoliths have potentially occluded around 2.84×10^8 Mg-e-CO_2 during the past 60 years. In 2010, the rice planted area of the world was around 155 million ha (IRRI, 2011). Applying the largest flux of PhytOC in rice phytoliths, the global annual potential sink rate of PhytOC in soils through rice phytoliths would approximately be 1.64×10^7 Mg-e-CO_2. The annual CO_2 occlusion within the rice phytoliths per unit area is comparatively lower than that of other plants like bamboo, wheat and sugarcane (*Table 2*), based on the total area of rice production, the global CO_2 sink rate (1.64×10^7 Mg-e-CO_2 year^{-1}) in rice phytoliths is greater than that is reported for bamboo leaf litter (1.40×10^7 Mg-e-CO_2 year^{-1}) (Parr et al., 2010), and sugarcane (0.65×10^7 Mg-e-CO_2 year^{-1}) (Parr et al., 2009) (*Table 2*). When rice residues returned to soil, phytoliths are released into soil after rice straw decomposition. The phytoliths from rice is very stable and can be stored in soil for more than thousands of years (*Fig. 1*) (Cao et al., 2006, 2007; Zheng et al., 2003). For example, rice phytoliths could be found intact in ancient paddy soils for 6280 years (Cao et al., 2007).

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Hence, the PhytOC can be considered as an important soil organic C fraction and play an important role in global C cycle and long-term C sequestration (Parr and Sullivan, 2005; Parr et al., 2010) and also in mitigation of climate change (Parr and Sullivan, 2005, 2011; Parr et al., 2009, 2010, Zuo and Lu, 2011). Therefore, it is very important to further estimate the potential of PhytOC in rice and other cultivated arable crops under various climatic, soil and management conditions.

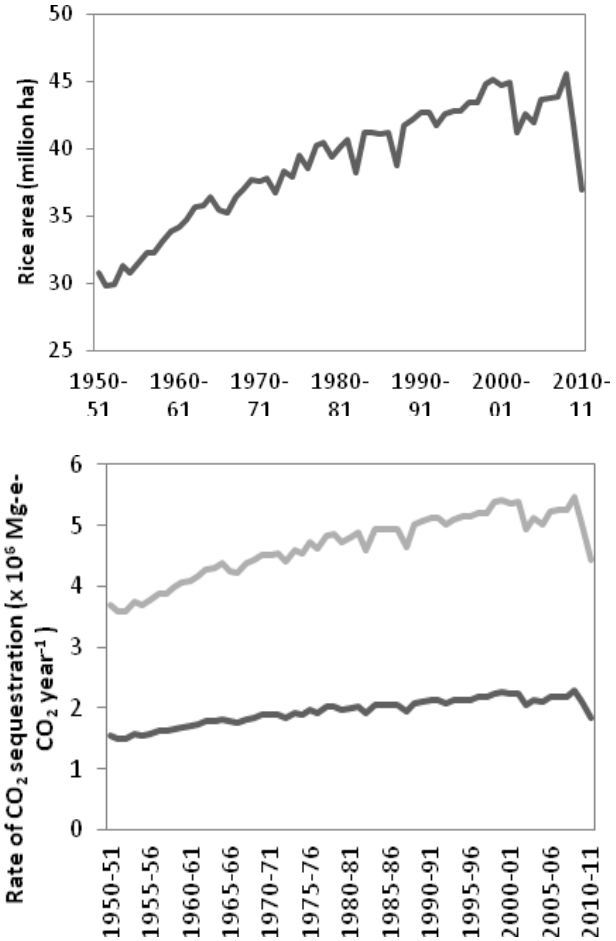

Figure 5. *5a) The fluctuation trend of rice cultivated area during 1950-2010 in India; 5b) Estimated lowest and highest CO_2 sequestration rate through phytoliths of rice plants from 1950 to 2010 in India.*

Table 2.*Comparison of PhytOC content in plant tissues, estimated PhytOC storage fluxes (Mg-e-CO_2 ha^{-1} $year^{-1}$) and global PhytOC sink rate (Mg-e-CO_2 $year^{-1}$) in different plants.*

Plant species	PhytoC content of dry material (%)	PhytOC storage fluxes (Mg-e-CO_2 ha^{-1} $year^{-1}$)	global PhytOC sink rate (Mg-e-CO_2 $year^{-1}$)	References
Rice	0.02-0.68	0.05-0.12	1.64×10^7	This study
	0.04-0.28	0.03-0.13	1.75×10^7	Li et al. (2013)
Bamboo	0.24-0.52	0.01-0.71	1.40×10^7	Parr et al. (2010)
Sugarcane	0.31-1.54	0.12-0.36	0.65×10^7	Parr et al. (2009)
Wheat	0.06-0.60	0.01-0.25	4.77×10^7	Parr and Sullivan (2011)
Millet	0.04-0.27	0.01-0.04	0.24×10^7	Zuo and Lu (2011)

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To sum up, the PhytOC, an environmentally stable C fraction, can be retained for several hundreds or thousands of years and has the potential to contribute terrestrial C sequestration. Rice, a well known silica accumulator, is a widely cultivated crop across the world and occludes C within their phytoliths. The C sequestration rates of rice phytoliths are approximately 0.05-0.12 Mg-e-CO_2 ha^{-1} $year^{-1}$depending upon the variation in DMY, Phytolith and PhytOC contents among the rice cultivars. The estimated global annual potential sink rate of PhytOC in soils through rice phytoliths would approximately be 1.64 x 10^7 Mg-e-CO_2. Therefore rice crop may play a significant role in long-term C sequestration and climate change through the PhytOC. The C sink potential of the rice phytoliths can be further enhanced by optimization of cropping and fertilizer management practices including regulation of silicon supply for crops. Also plant breeding and biotechnological tools can be applied to improve the traits which are responsible for the higher occlusion of C in the phytoliths of rice.

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INFLUENCE OF NEMATODE-BACTERIAL INTERACTIONS ON N AND P MINERALISATION IN SOIL AND ON DECOMPOSITION OF CROP RESIDUES DURING AEROBIC COMPOSTING

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Abstract. The chemical composition of the crop residues such as the N and lignin content is one of the important factors that influence microbial activity, including their efficiency and contribution to the decomposition process. The objective of the present study was to compare the effects of organic substrates of different carbon to nitrogen (C:N) ratios viz; cabbage (*Brassica oleracea*) leaves, Soybean (*Glycine max*) stover and paddy (*Oryza sativa*) straw on population densities of a bacterivorous nematode, *Cephalobus persegnis* and its effect on N and P mineralization in soil microcosms and rate of organic matter decomposition in aerobic composting. The bacterivorous nematode, *C. persegnis* enhanced the release of ammonium nitrogen (NH_4^+-N) in presence of soybean (*Glycine max*) stover and paddy (*Oryza sativa*) straw in soil microcosms. Sampling at 15-day intervals revealed a progressive increase in the population densities of *C. persegnis* up to 45 days, followed by a decline. The bacterial densities in soil were significantly low in presence of nematodes on all days of sampling indicating the grazing effect of the nematodes. The microbial biomass carbon (MBC) was significantly higher in the presence of nematodes, on all days of sampling. A second trial on the rate of decomposition of the above crop residues during aerobic composting revealed enhanced reduction in C:N ratios in presence of nematodes on 75 and 90 days of incubation, in paddy straw treatments.
Keywords: *Cephalobus persegnis, carbon-to-nitrogen ratio, mineralisation, cabbage, paddy, soybean*

Introduction

The crop residues are incorporated into the soil as a soil amendment or used as composting material. The chemical composition (carbon-to-nitrogen ratio) of the crop residue and their rate of decomposition in soil is influenced by soil moisture, temperature and microbial interactions. The biotic and abiotic interactions resulting in nutrient dynamics in the soil also influence the rate of decomposition of organic matter in the soil. Such interactions are poorly understood as limited investigations have been carried out on this aspect (Alphei et al., 1996; Schutter and Dick, 2001; Chigineva et al., 2009; Carrillo et al., 2011; Ball et al., 2014). Among the biotic interactions, bacterivorous nematodes are an important component of the soil fauna and it needs to be established if they cause a significant effect in enhancing the rate of nutrient mineralization in the soil and accelerating the process of organic matter decomposition.

The carbon-to-nitrogen (C:N) ratios in organic matter incorporated in soil are variable and range from 20:1-30:1 in legumes and farm yard manure to as high as 100:1 in certain cereal straw residues. Incorporating organic matter of wide C:N ratio (more than 50:1) into the soil under favourable conditions for decomposition results in an increase in heterotrophic microbial populations with production of

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large amount of CO_2 (Das, 2008). Incorporation of wheat straw is reported to increase CO_2 fluxes from 0.30 to 1.30 kg CO_2 $ha^{-1}d^{-1}$ (Curtin et al., 1998). When carbon is abundant, an increase in microbial biomass may result in immobilisation of N. Under such circumstances, mineralization may be increased by organisms such as bacterivorous nematodes that graze on soil bacteria and enhance soil nutrient availability (Ferris et al., 1997).

Bacterivorous nematodes constitute 20-50% of the total number of nematodes present in soil. Sometimes their proportion reaches 90-99% at sites of high microbial activity (Griffiths, 1989). Their interactions with microbes in the soil resulting in enhanced nutrient release have been reported by several workers (Coleman et al., 1984; Freckman, 1988; Griffiths, 1994).

Bacterivorous nematode, *Cephalobus persegnis* Bastian 1965, was found to be one of the most abundant rhabditid in the Indo-gangetic rice-wheat growing regions of India (Singh, 2007). The basal threshold temperature of this nematode is 4.2 °C and the upper threshold is 42.2 °C with an optima of 32.2 °C (Venette and Ferris, 1997). This thermal adaptation is possibly responsible for the predominance of this species in the soil. Further, *C. persegnis* is reported to enhance the release of NH_4^+–N in soil microcosms (Kamra et al., 2003).

The present study was undertaken to compare the effects of three crop residues with different C:N ratios, viz cabbage (*Brassica oleracea* L.) leaves, soybean (*Glycine max* L.) stover and paddy (*Oryza sativa* L.) straw on population densities of *C. persegnis* and its effect on N and P mineralization in soil microcosms for a period of 75 days. In a second trial, the rate of decomposition of the above three substrates during aerobic composting was investigated in the presence and absence of the bacterivorous nematode for the period of 90 days.

Material and methods

Isolation of nematodes

The soil samples for isolation of nematodes were collected row-wise in a zig zag pattern, using an auger, from the upper 30 cm layer of soil in a field under rice-wheat cropping system, located at the Indian Agriculture Research Institute (IARI), New Delhi, India. The composite samples were prepared by collecting 50 samples of 500 cm^3 soil, from 1 hectare field area. The nematodes were extracted using ten samples, each of 500 cm^3 soil, by Cobb's decanting and sieving technique (Cobb, 1918) followed by modified Baermann funnel technique (Schindler, 1961). The nematode suspension collected after 48 h was examined for presence of the dominant bacterivorous nematode. A single gravid female of the predominant bacterivorous nematode, *C. persegnis*, was handpicked and placed on water agar plate supplemented with bacteria isolated from the same soil samples. After egg laying, the juveniles were allowed to develop and multiply on the plates for 4 weeks at 32 °C. Thereafter the nematodes were extracted from the plates by Schindler's method. Nematodes so collected were distributed on fresh agar plates. This process was repeated for mass multiplication of the nematodes. They were processed by the Seinhorst method (Seinhorst, 1959) and identified at the Division of Nematology, IARI, New Delhi, India. The gnotobiotic culture plates of the nematode were maintained in a BOD incubator at 32 ± 2 °C for experimental trials.

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Isolation and identification of bacteria

The soil samples used in the isolation of nematodes were also used for the isolation of bacteria in order to retain the native soil bacteria. Colony forming units (CFU's) of bacteria were estimated using 10 cm^3 of air dried soil. The soil was thoroughly vortexed using 100 cm^3 of sterile distilled water for 5 minutes. One mL of the aliquot was drawn from this suspension to make a serial dilutions upto 10^{-16} of which 10 microlitre was plated on nutrient agar plates (Wollum, 1982). Plates were incubated at 25 ± 1 °C for 24 h and colonies were identified under a stereoscopic microscope at 6.7× based on colony morphology and standard biochemical tests (Benson, 2002). Colonies developed on the plates belonged to the genera *Bacillus*, *Pseudomonas,* and *Enterococcus*.

Soil and substrate Sterilisation

The sandy loam soil from the field of IARI was used for all the experiments. The soil was steam sterilized at 120°C, 103421.35 Pa pressure for 1 h. Three random samples of sterilized soil were processed by Cobb's decanting and sieving technique (Cobb, 1918) followed by modified Baermann funnel technique (Schindler, 1961) and examined to ensure that the sterilized soil was free from nematodes. The substrates were chopped and put in autoclavable bags for steam sterilization at 120°C, 103421.35 Pa pressure for 30 min. each. This sterilized soil and crop residue substrates were used for preparing microcosms.

Experiment 1: Nitrogen and phosphorus mineralization in soil

The experiments were conducted in soil microcosms using 150 g sterilised soil in 200 cm^3 plastic cups inoculated with sterilised substrates, bacteria and nematodes and incubated for a period of 75 days. All the three substrates, viz cabbage leaves (C:N ratio: 30:1), soybean stover (C:N ratio: 56:1) and paddy straw (C:N ratio: 90:1) were shredded to about 1 cm size and mixed thoroughly with sterilized soil at the rate of 1 g residue carbon (C) per 100 cm^3 soil before filling in microcosms. The microcosms were inoculated with freshly prepared bacterial suspension at the rate of 1.69×10^{16} CFUs g^{-1} soil and kept for two days at 32 ± 2 °C for 48 h for the bacteria to establish, prior to the nematode inoculation (5 g^{-1} soil). The treatments thus comprised of each of the three substrates with and without nematodes, and respective no-substrate controls. For each treatment, four replications were maintained. The moisture was adjusted to field capacity and maintained by replacing the weight loss with distilled water at 5 day intervals during incubation period. Four sets of microcosms were established to allow destructive sampling at 15 day intervals for determination of nematode and bacterial densities, microbial biomass carbon (MBC), ammoniacal N (NH_4^+–N), nitrate N (NO_3^-–N) and available phosphorus (P) The nematode population density was estimated after extraction from 90 g soil by Cobb's decanting and sieving technique (Cobb, 1918) followed by modified Baermann funnel technique (Schindler, 1961) and CFU's of bacteria were estimated using 1 g soil on nutrient agar plates by serial dilution method (Wollum, 1982). The MBC was estimated by fumigation extraction method (Voroney et al., 1993), and NH_4^+–N and NO_3^-–N were analysed by Kjeldahl distillation method by adding MgO and Devarda alloy,

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respectively (Keeney and Nelson, 1982). The available phosphorus (P) was estimated by Olsen's method (Olsen, et al., 1954).

Experiment 2: Organic matter decomposition in aerobic composting

Plastic trays (45×35×15 cm), containing organic substrates (cabbage leaves, paddy straw and soybean stover), denematised cow dung, soil and composting starter were mixed in the ratio of 8:1.0:0.5:0.5 on weight basis. Finally, each tray contained 800 g substrate, 100 g cow dung, 50 g soil and 50 g composting starter. The treatments comprised of each of the three substrates or no substrate with and without nematodes. In the treatments with no substrate, 800 g soil is used instead of the 800 g substrate. Each treatment was replicated four times. The inoculation of bacteria and *C. persegnis* were carried out as in the experiment described above. The trays were incubated at 32 ± 2 °C for 90 days. The nematode population densities were estimated after extraction from 60 g composting material by Cobb's decanting and sieving technique (Cobb, 1918) followed by modified Baermann funnel technique (Schindler, 1961), the total carbon and total N was estimated by wet oxidation (Snyder and Trofymow, 1984) and Kjeldahl distillation (Bremner, 1965), respectively, at 15 days interval.

Statistical analysis

The data on various parameters i.e., nematode, bacterial counts, NH_4^+–N, NO_3^-–N, available phosphorus (P) and microbial biomass carbon (MBC) were analysed using 'analysis of variance' (ANOVA) technique (Gomez and Gomez, 1984) using DSAASTAT, version, 1.1 statistical package (Onofri, 2007) available at http://www.unipg.it/~onofri/DSAASTAT/DSAASTAT.htm. The data on nematode population were square root transformed prior to analysis to meet the assumptions of ANOVA and conclusion drawn in the transformed scale. However, only untransformed arithmetic means of all data are presented. The differences at $P < 0.01$ and $P < 0.05$ level were considered statistically significant using the least significant difference (LSD) test.

Results and discussion

Experiment 1

The population densities of *C. presegnis* increased significantly up to 30 days of incubation in soybean stover treatment, while in cabbage and paddy straw treatments, an increase was observed up to 45 days followed by a progressive decline in both (*Fig. 1*). The bacterial densities were significantly low in the presence of the nematode than in their absence, in all three substrate treatments, on days 15, 30 and 45, indicating the grazing effect of the nematode (*Fig. 2*). Such a decline in bacterial densities due to grazing effect of bacterivorous nematodes has been reported by several other workers (Gould et al., 1981; Anderson et al., 1983). However, a decline in bacterial CFUs does not necessarily indicate a reduced bacterial activity (Djigal et al., 2004).

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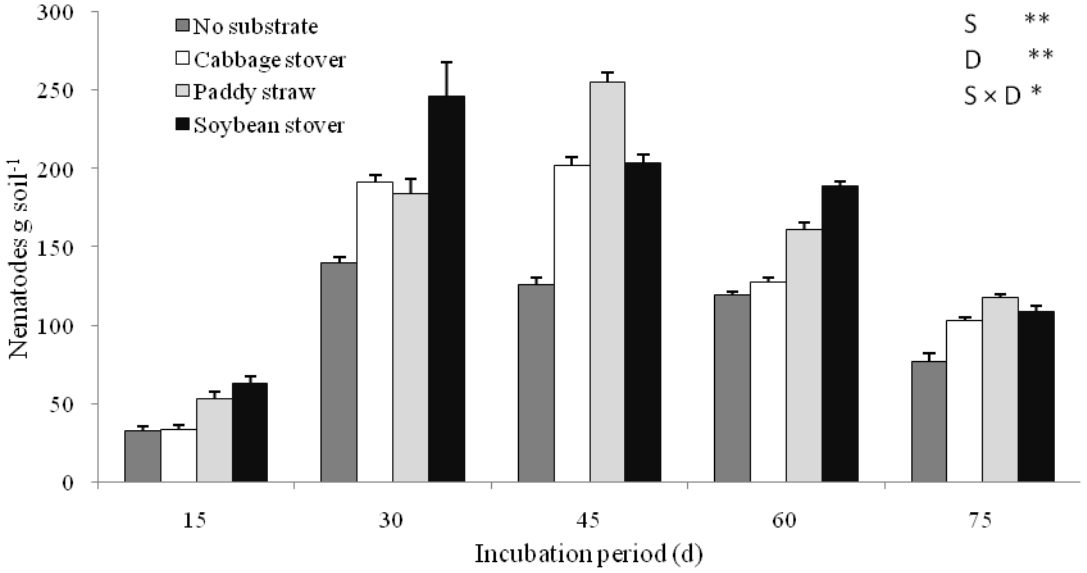
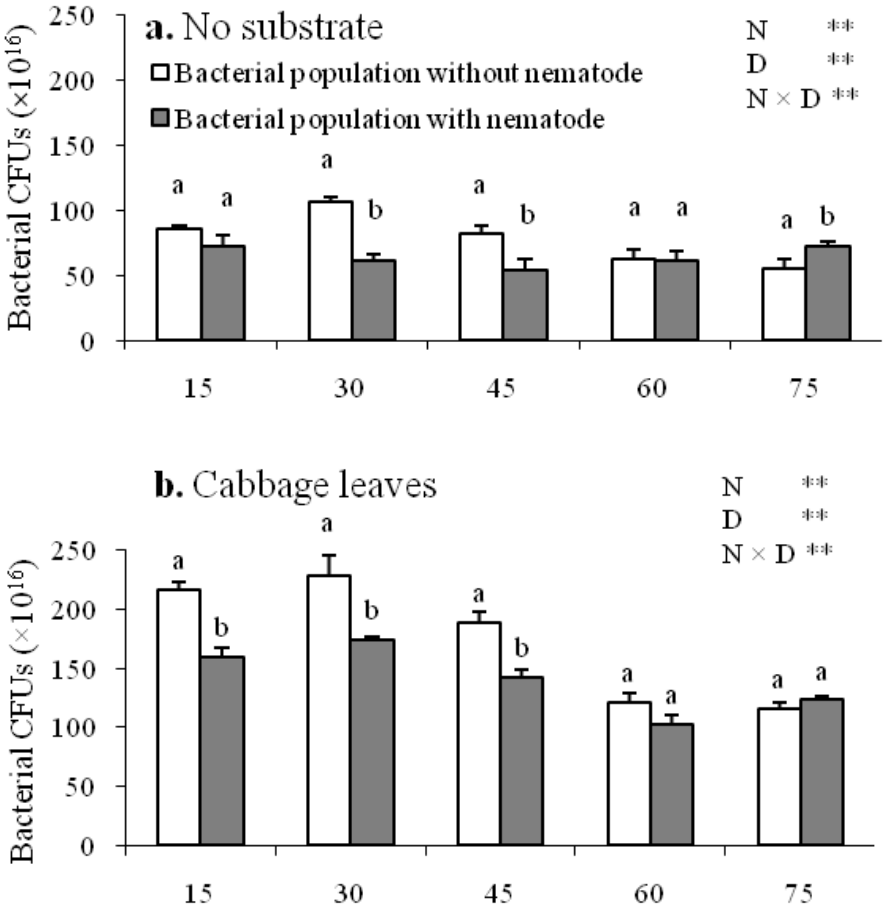


Figure. 1. *Population density of Cephalobus persegnis across different substrates over 75 days of incubation period during N and P mineralisation. Bars represent standard errors. Significance of the factors and their interactions (S = substrate, D = days, S x D = the interaction) are shown as * and ** which denote P < 0.05 and <0.001 respectively.*



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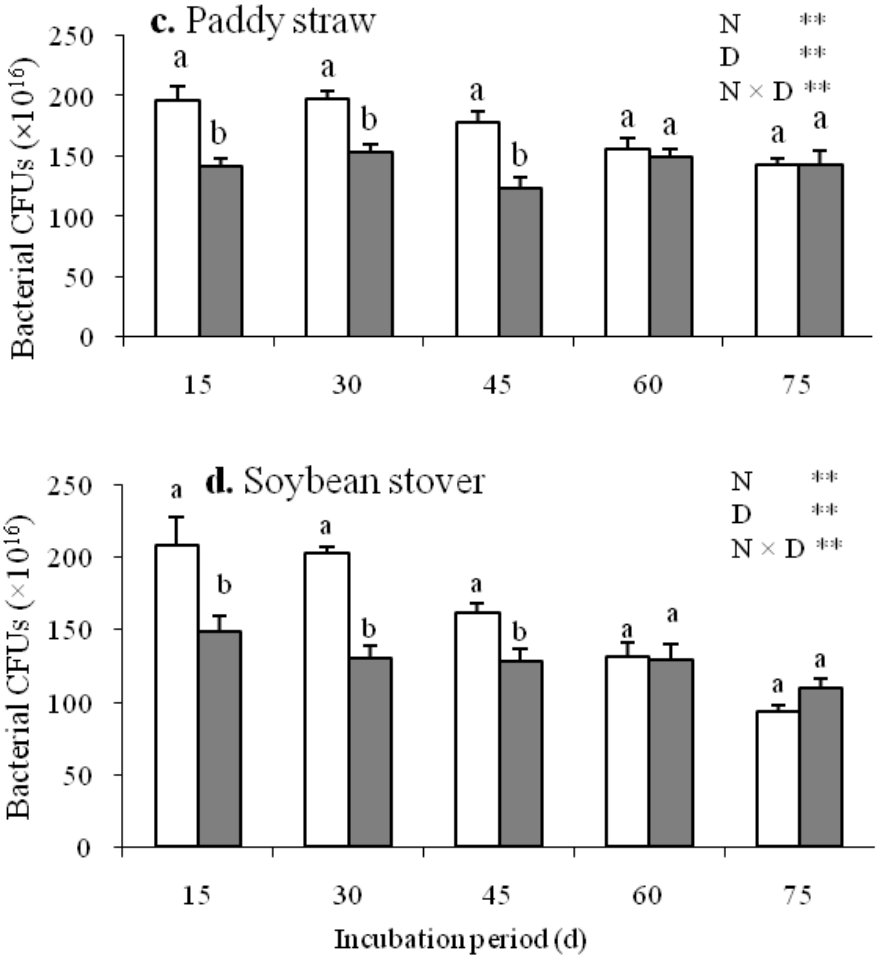

Figure. 2. *Bacterial population densities in presence and absence of Cephalobus persegnis across different substrates (Figure. 2a-d) over 75 days of incubation period. Bars represent standard errors. Different latter over pair of bars indicate a significant (P < 0.05) nematodes effect for that sampling period and substrate. Significance of the factors and their interactions (N = nematodes, D = days, N x D = the interaction) are shown for each plate as *, ** and ns, which denote P < 0.05, <0.001and non-significant respectively.*

The changes in nematode and bacterial densities were reflected in total microbial biomass carbon (MBC) on respective days of sampling (*Fig. 3*). The levels were significantly higher in presence of *C. persegnis,* than in its absence in all 3 substrates, on all days of observations, except day 15 in paddy straw treatments (*Fig. 3*). The progressive increase in MBC for 30-45 days of incubation, followed by a decline on days 60 and 75 were commensurate with the respective population densities of *C. persegnis* and bacteria (*Fig.1 and 2*). In the absence of organic substrate, this trend was not observed, rather a progressive decline was observed from 800 µg C g^{-1} dry soil to 400 µg C g^{-1} dry soil, from day 15 to day 75, indicating lowering microbial activity and mineralisation. The microbial biomass of carbon (MBC) constitutes labile carbon that declines faster and is restored faster than the non-labile carbon and is therefore a more sensitive indicator of carbon dynamics in agroecosystems (Blair et

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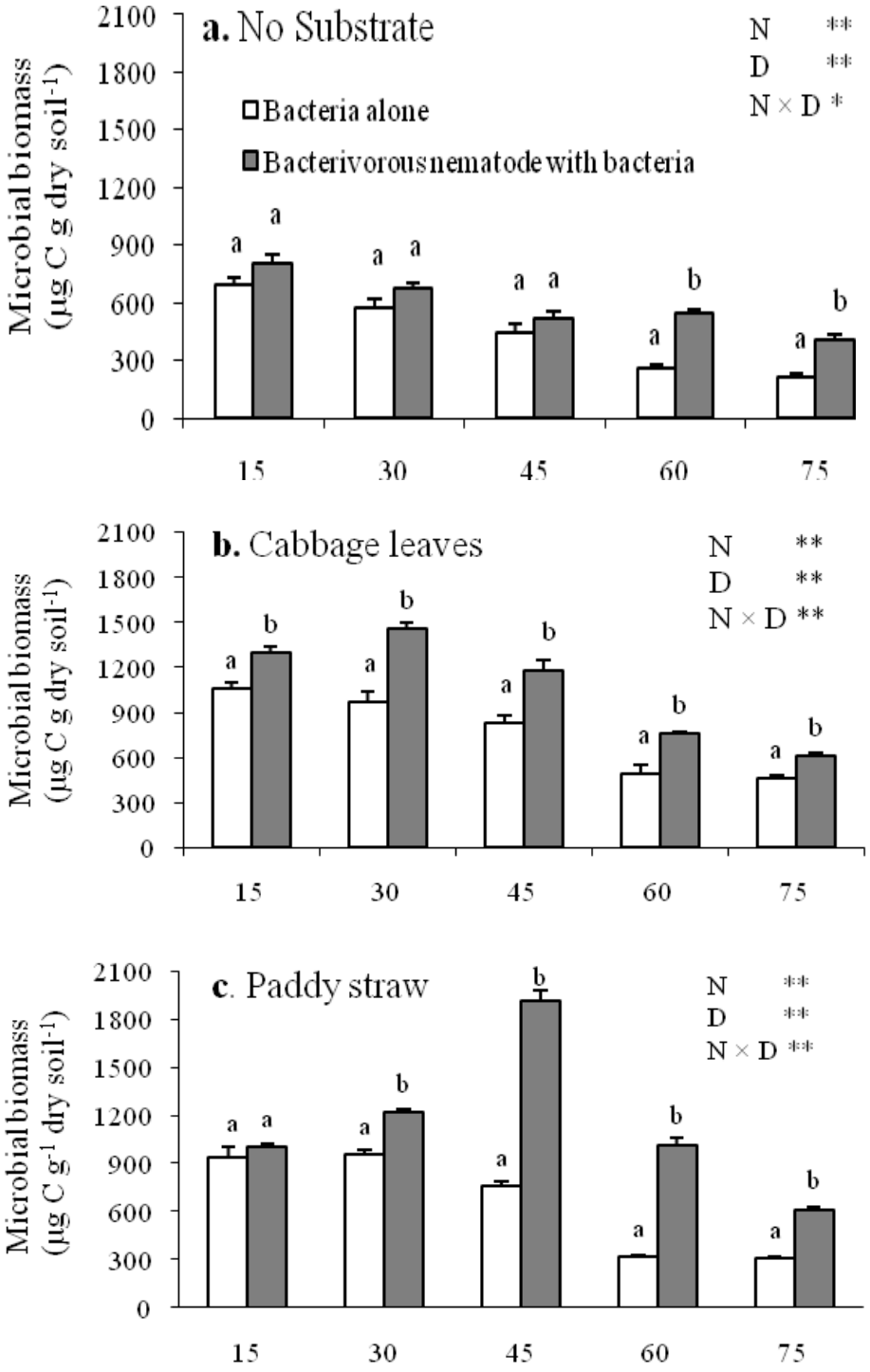
al., 1995). There are microbes which do not proliferate on the synthetic media, but account for the MBC and contribute towards the process of nutrient mineralization.



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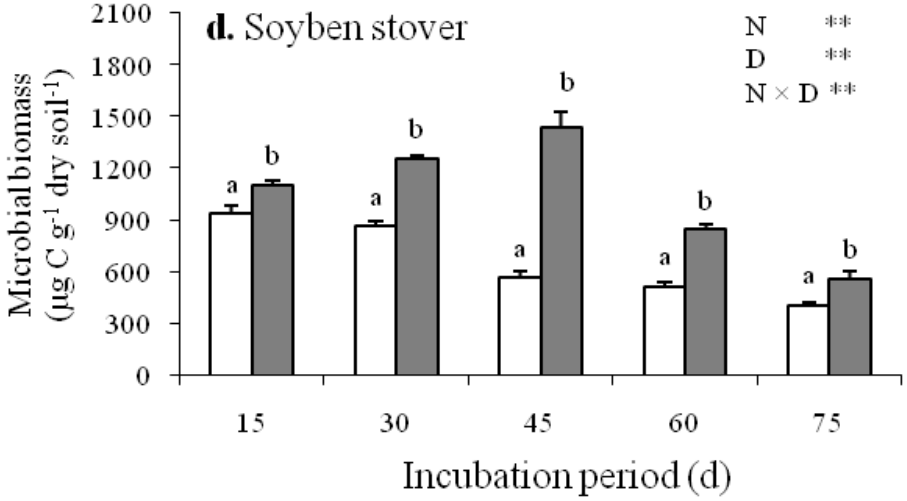
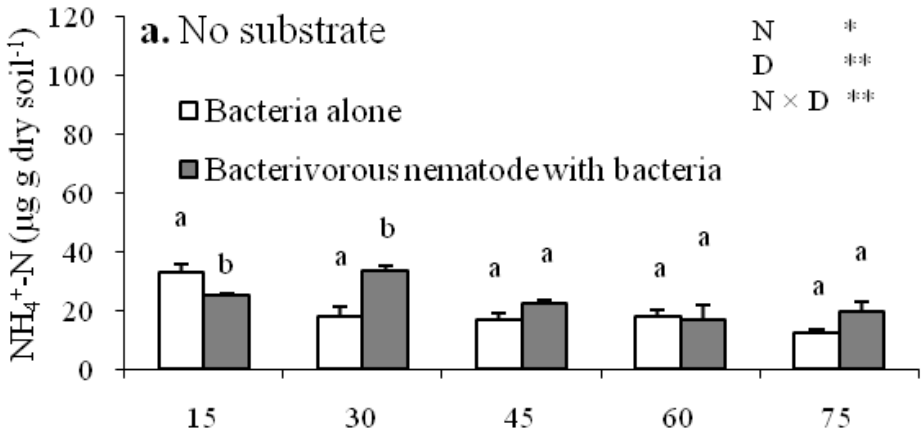


Figure. 3. *Influence of bacteria alone or Cephalobus persegnis plus bacteria on microbial biomass carbon across different substrates (Figure. 3a-d) over 75 days of incubation period. Bars represent standard errors. Different latter over pair of bars indicate a significant ($P < 0.05$) nematodes effect for that sampling period and substrate. See Figure 2 legend for the details on design and statistical results.*

The presence of *C. persegnis* resulted in significantly enhanced levels of NH_4^+–N in paddy straw treatments on days 15, 30 and 45 while in soybean stover treatment, these levels were significantly higher on all days of observation, except day 45 (*Fig. 4*). The trend that was noteworthy was the progressive increase in NH_4^+–N levels up to 45 days, followed by a decline on days 60 and 75 in the above two treatments, commensurate with the population density pattern of *C. persegnis* observed during that period. This supported the hypothesis that nematodes excrete inorganic nitrogen, mainly as NH4 (Ferris et al., 1998) and stimulate nitrogen mineralisation through their grazing activity. Increase in nitrogen mineralisation in the presence of bacterial-feeding nematodes has been reported in many studies (Ingham et al., 1985; Ferris et al., 1998; Lokupitiya et al., 2000; Kamra et al., 2003).



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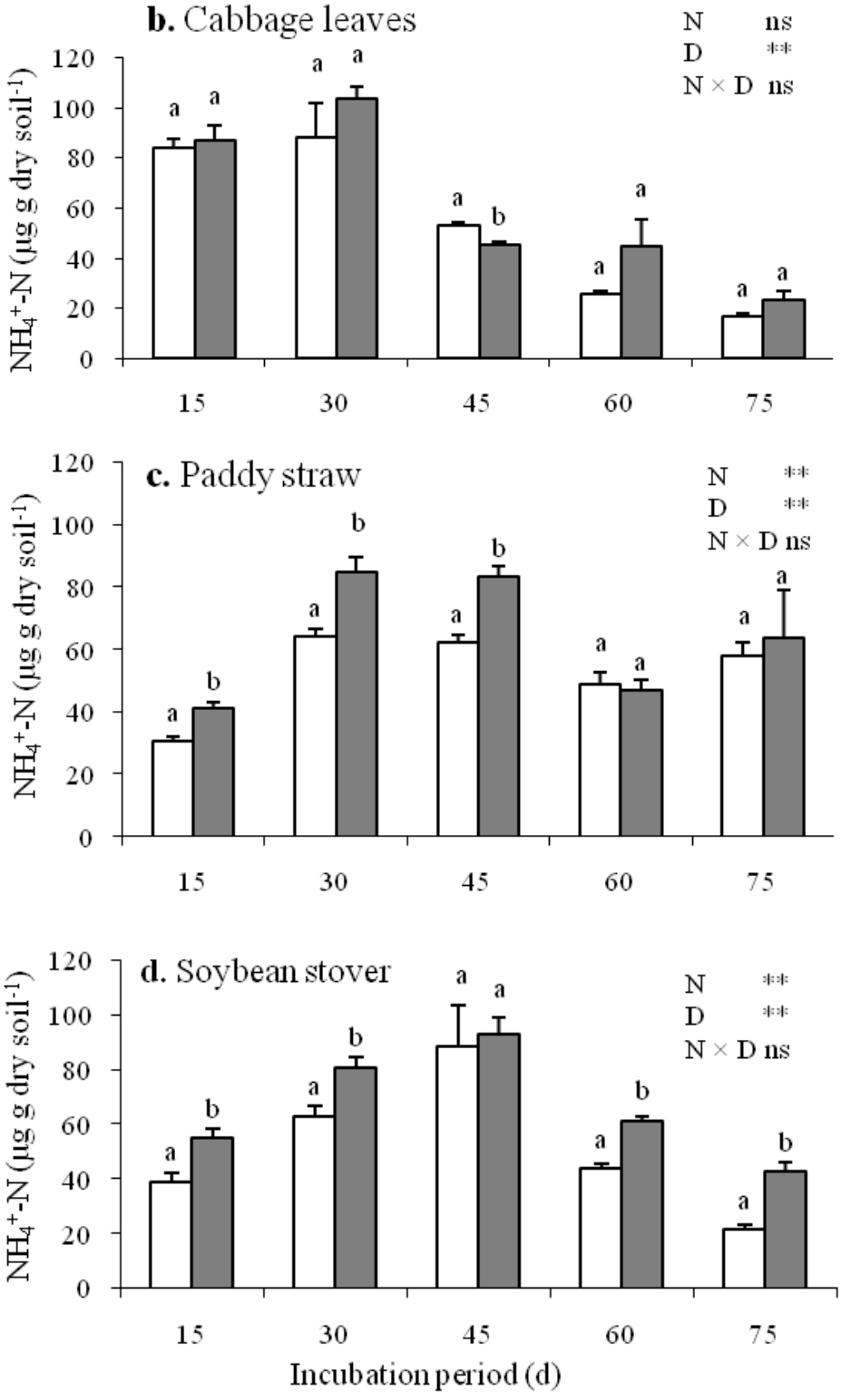

Figure. 4. *Influence of bacteria alone or Cephalobus persegnis plus bacteria on release of ammonical nitrogen (NH_4^+–N) across different substrates (Figure. 4a-d) over 75 days of incubation period. Bars represent standard errors. Different latter over pair of bars indicate a significant ($P < 0.05$) nematodes effect for that sampling period and substrate. See Figure 2 legend for the details on design and statistical results.*

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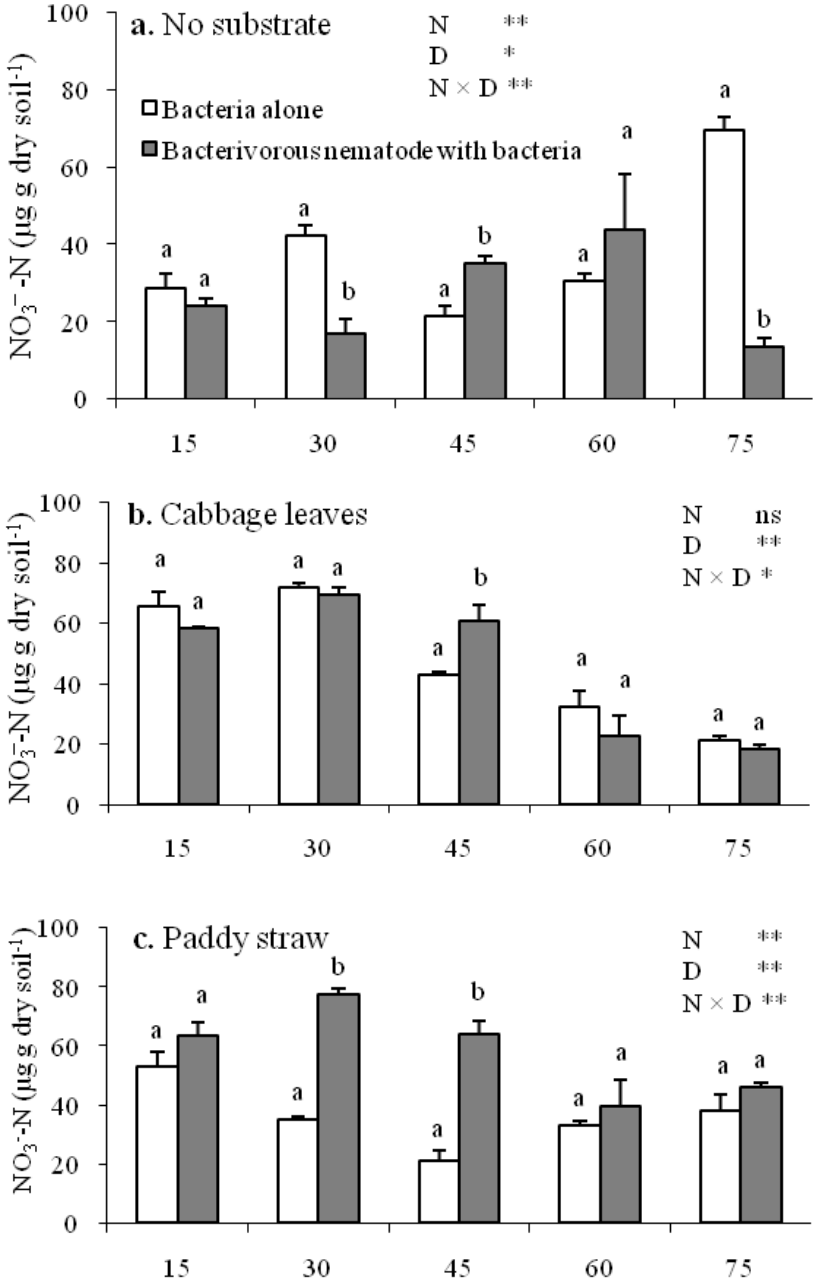
The NO_3^-–N levels showed an enhanced release in the presence of *C. persegnis* on days 30 and 45 in paddy straw treatments and day 45 in cabbage treatment (*Fig. 5*). Increase in nitrification in the presence of single species of bacterivorous nematode or the presence of nematode communities was also demonstrated by Bouwman et al., (1994) and Xiao et al., (2010). Gebremikael et al. (2014) also observed consistently higher concentration of NO_3^-–N in presence of nematodes than in their absence. However, release of NO_3^-–N in soyabean stover treatments is consistently less in presence of nematodes compared to their absence. In the absence of substrates, level NO_3^-–N is significantly lower on days 30 and 75. The inconsistent effects were possibly due to low levels of nitrifying bacteria and needs to be investigated.



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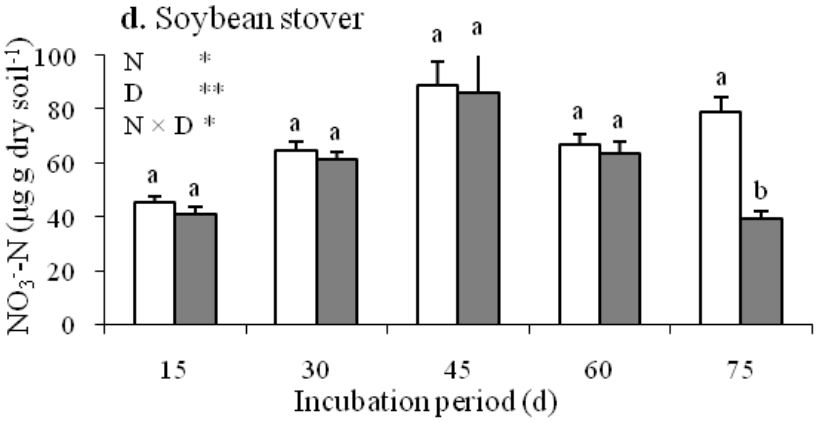
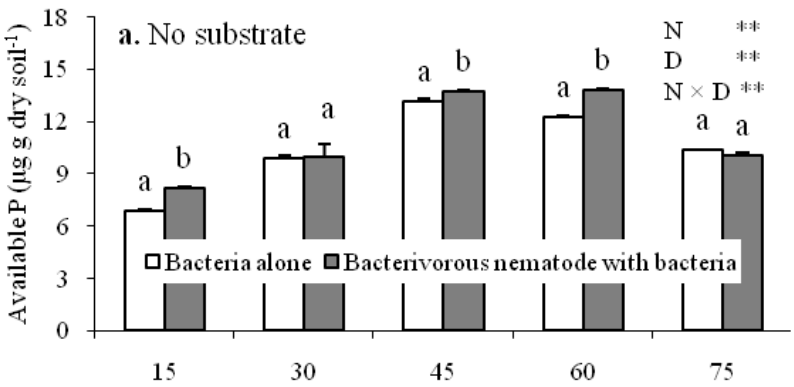


Figure. 5. *Influence of bacteria alone or Cephalobus persegnis plus bacteria on release of nitrate nitrogen (NO_3^-–N) across different substrates (Figure. 5a-d) over 75 days of incubation period. Bars represent standard errors. Different latter over pair of bars indicate a significant (P < 0.05) nematodes effect for that sampling period and substrate. See Figure 2 legend for the details on design and statistical results.*

The enhanced levels of P in presence of *C. persegnis* were observed on all days of sampling except day 45 in soybean stover, on days 45 and 60 in paddy straw treatment and days 15, 60 and 75 in cabbage treatment (*Fig. 6*). Therefore, a consistent relationship of enhanced P mineralisation in presence of *C. persegnis* could not be confirmed. Few studies have been made on the N and P content of nematodes. The P content is reported to be between 0.1--0.6 % biomass of nematodes (Dropkin and King, 1956; Hunt et al., 1987). The assimilation efficiencies are between 30--60%; thus, it is expected that nematodes would release P after feeding on bacteria. Enhanced levels of P have been reported in presence of bacterivorous nematode, *Mesodiplogaster* sp. by Coleman et al. (1977) and Anderson et al. (1981) in microcosms experiments. Coleman et al. (1977) found enhanced P mineralisation in microcosms with sterilised soil inoculated with bacteria alone or bacteria in combination with bacteriophagous amoebae or nematodes. In each case, mineralisation was greater in presence of the animal. However, Cole et al. (1978) and Woods et al. (1982) reported no enhanced P mineralisation in presence of bacterivorous nematodes.



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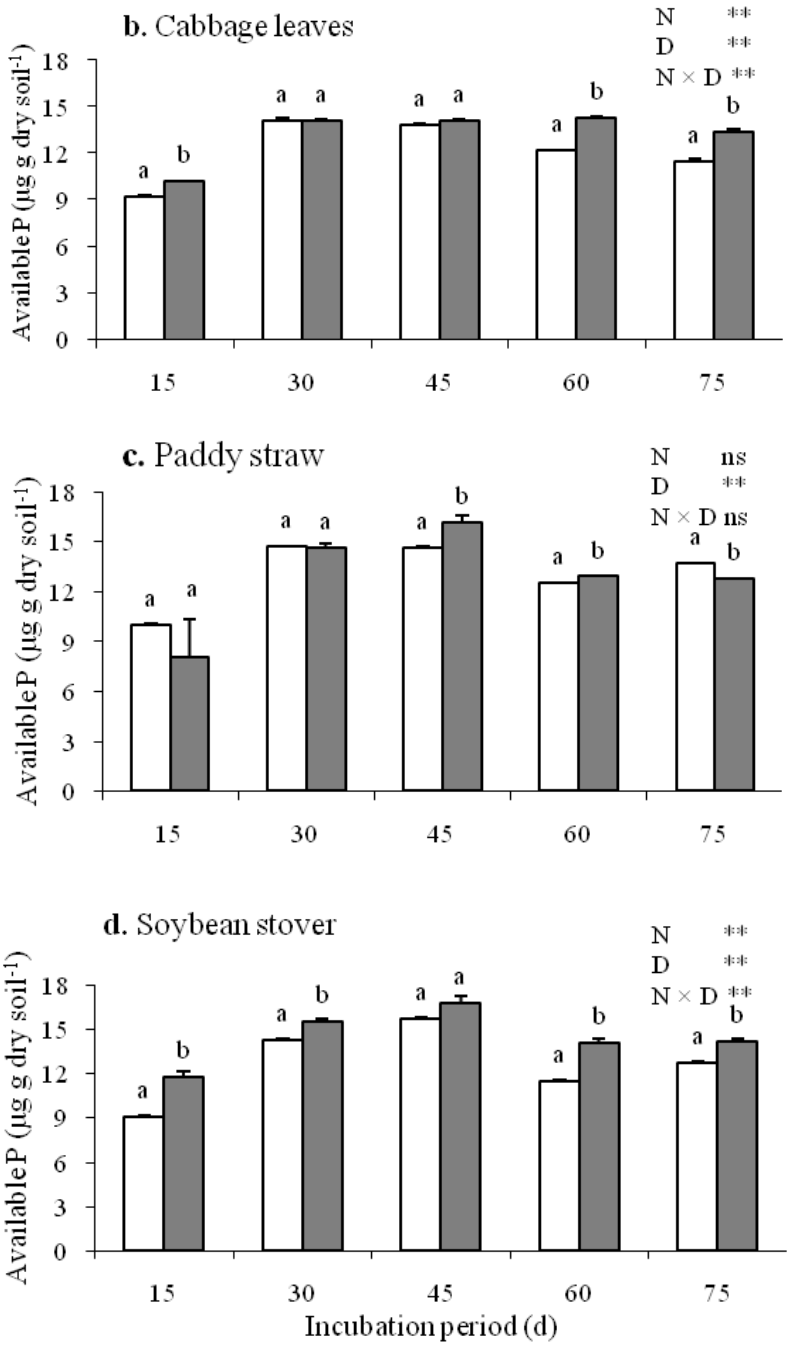

Figure. 6. *Influence of bacteria alone or Cephalobus persegnis plus bacteria on release of phosphorus across different substrates (Figure. 6a-d) over 75 days of incubation period. Bars represent standard errors. Different latter over pair of bars indicate a significant (P < 0.05) nematodes effect for that sampling period and substrate. See Figure 2 legend for the details on design and statistical results.*

Experiment 2

This experiment was conducted to determine the effect of bacterivorous nematode on rate of composting ie., if besides enhancing the rate of nutrient mineralisation,

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these nematodes could enhance the rate of composting, ie the time at which C:N ratio becomes constant (indicating no further decomposition) in the composting substrates.

The population densities of bacterivorous nematodes during aerobic composting showed similar trend in cabbage and soybean stover compost with respect to period of incubation. However, the nematodes were much higher on days 30, 45 and 60 in soybean stover compared to other substrates. In paddy straw composting, the densities of bacterivorous nematodes increased progressively up to 45 days followed by a progressive decline on days 60, 75 and 90. Similar trend was observed in the absence of organic substrate; although the density of bacterivorous nematodes was much lower (*Fig. 7*). As is evident from *Fig.8*, the decline in C:N ratios in various substrates was not affected significantly by presence of bacterivorous nematodes in cabbage or soybean composting on any day of sampling. It has been found that, in nutrient rich conditions (amended soil), the presence of nematodes may not significantly enhance organic matter decomposion and the subsequent nutrient mineralization (Ingham et al., 1985; Bjornlund et al., 2012; Gebremikael et al., 2015). Gebremikael et al (2015) found that there is no significant contribution of entire free living nematode community to C mineralisation either in native soil organic matter or added organic matter (grass covered amendments). However, this was attributed to lower nematode density in the experiment or significant decrease in nematode population density over the time. In paddy compost, the decline in C:N ratio due to presence of bacterivorous nematodes was found to be significant on days 75 and 90. This was supported by the relatively high number of bacterivorous nematodes (700 g^{-1} compost) maintained in paddy compost compared to a low number in cabbage (about 100g^{-1} compost) and soybean (250g^{-1} compost). There is a need to identify and quantify the microbial community present in each of these substrates to further understand and interpret the biotic interactions during composting.

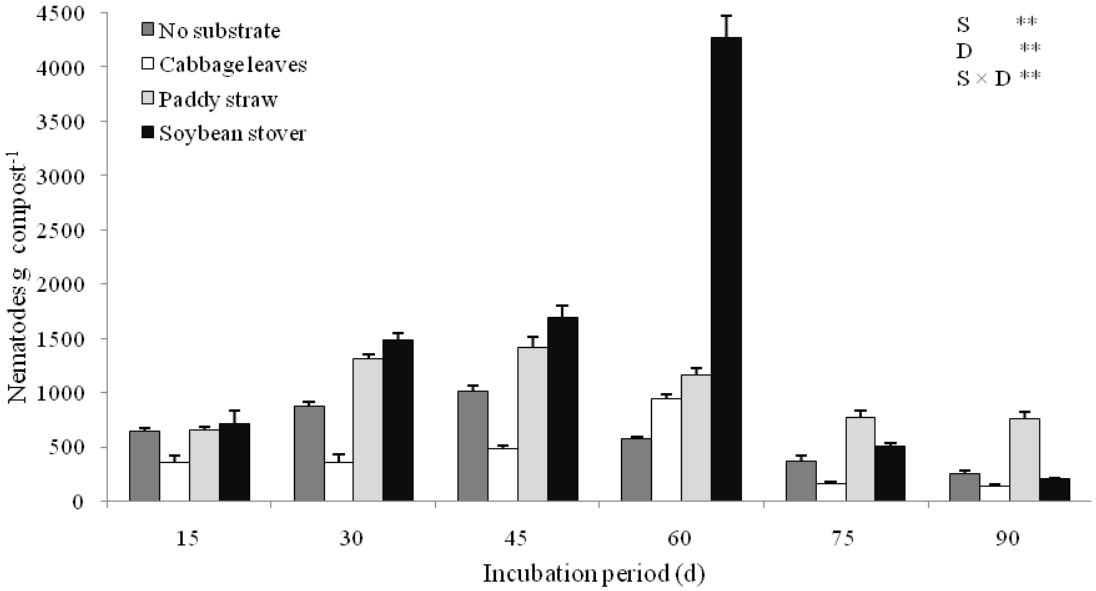

Figure. 7. *Population density of Cephalobus persegnis across different substrates over 90 days of incubation period during aerobic composting. Bars represent standard errors. Significance of the factors and their interactions (S = substrate, D = days, S x D = the interaction) are shown as ** which denote P < 0.01.*

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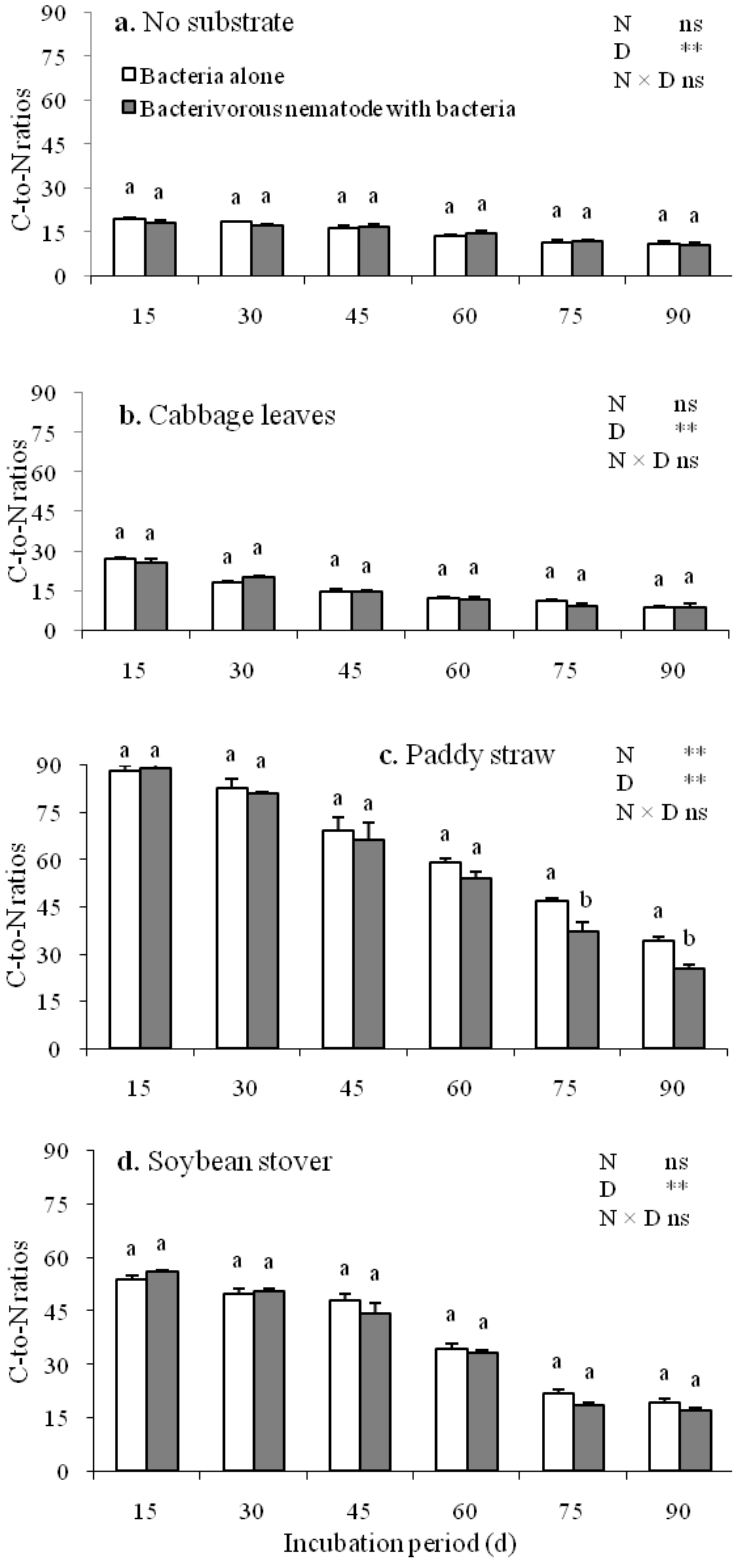

Figure 8. *Influence of bacteria alone or Cephalobus persegnis plus bacteria on C:N ratios across different substrates (Figure. 8a-d) over 90 days during aerobic composting. Bars represent standard errors. Different latter over pair of bars indicate a significant ($P < 0.05$) nematodes effect for that sampling period and substrate. See Figure 2 legend for the details on design and statistical results.*

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Bacterivorous nematodes can hasten the process of decomposition, as observed for a recalcitrant substrate like paddy straw. Compost can serve as an efficient delivery system for beneficial bacterivorous nematodes in soil as it can help in their establishment and also enhance the rate of mineralisation in soil, in presence of organic matter to achieve desirable C:N ratio (25-30:1). However, this hypothesis needs to be tested under field conditions.

Our results contribute to the evidence that the densities and activity of bacterivorous nematodes and bacteria present in the soil are influenced by the composition of the organic substrates. The presence of *C. persegnis,* significantly increased the microbial biomass carbon (MBC) and NH_4^+–N in a soil in presence of organic substrates like soybean stover or paddy straw. The nematode could also enhance the rate of decomposition of paddy straw during aerobic composting although the reduction in C:N ratios was significantly different from treatments without nematodes only on days 75 and 90 of incubation. However, in the present study contribution of single species of bacterivorous nematodes to nutrient mineralisation and organic matter decomposition was studied in the controlled environment but in realistic condition, decomposition process is regulated by several biotic and abiotic factors. Hence as suggested by Gebremikael et al (2015) studies are needed for realistic determination of contribution of entire free-living nematode communities including all feeding groups and indigenous microbial communities as occur in nature and management changes that are required for increasing the availability of nitrogen and other minerals especially in organic and low input farming systems.

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EFFECT OF RED DEER GRAZING ON ALPINE HAY MEADOWS: BIODIVERSITY AND MANAGEMENT IMPLICATIONS

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Abstract. Large mammalian herbivores are keystone species affecting the biodiversity and functioning of ecosystems, since herbivory directly influences plant species competitive ability. Thus, when managing habitats for conservation it is crucial to understand the selection patterns of large herbivores and their effect on plant diversity and assemblage. With this aim, we studied a pastoral system placed in central-eastern Alps, comparing late mowing with grazing by red deer.
We found that, with high stocking rates, red deer significantly affects the species and functional composition of alpine productive pastures, mostly by competitive exclusion of subordinate species due to the spread of poorly palatable, competitive, stress-tolerant tall grasses with strong avoidance strategies. We argued that grazing by red deer with high stocking rates is harmful when acts on formerly managed meadows, while it has a positive impact on plant diversity of long-term abandoned grasslands. Our results indicated that grazing by red deer with high stoking rates hinders the spread of successional/ruderal species, but allows the establishment of competitive dominant tall grasses. Our findings also confirmed the hypothesis that the impact of red deer on grassland biodiversity follows the intermediate disturbance hypothesis.
Keywords: *herbivory; grassland management; large herbivores; disturbance intensity; competitive exclusion*

Introduction

Large mammalian herbivores are keystone species, which affect the biodiversity and functioning of ecosystems (Rooney and Waller, 2003). Thus, when managing habitats for conservation it is crucial to understand the selection pattern of large herbivores and their effect on plant diversity and assemblage. One of the most widespread European wild large herbivores is red deer (*Cervus elaphus* L.). Red deer is an Intermediate Opportunistic Mixed feeding type (Hoffmann, 1989); it practices a marked degree of forage selectivity related to forage availability (Johnson et al., 2001) and quality (Wallis DeVries et al., 1999), and to the species composition of plant communities (Bellu et al., 2012). Red deer discriminates among pasture species, generally preferring legumes and forbs to grasses and avoiding fibers as much as possible (Kay, 1985). This ungulate shows remarkable anatomical modification of digestive apparatus linked to forage

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quality, such as the Surface Enlargement Factor and vascular development variation of rumen papillae (Hofmann, 1989), following the general trend of ruminants, in which modifications of rumen mucosa related to both pasture vegetative cycle and grazing on poorly palatable grasses occur (Scocco et al., 2013).

Herbivory directly affects the growth, reproduction, and survival of plants by consuming leaves, stems, flowers, and fruits, thus directly influencing plant species competitive ability (Lauenroth and Aguilera, 1998). Moreover, it was stated that differential defoliation leads to spatial variation in plant growth (Skarpe and Hester, 2008), nutrient cycling (Holdo et al., 2007) and, ultimately, vegetation structure (Wieren and Bakker, 2008). Therefore, grazing may lead to the variation in the abundance of plant species over longer periods by affecting competition processes (Augustine and Mc Naughton, 1998). Plants react against grazers by means of avoidance and tolerance strategies. Grazing avoidance involves mechanisms that reduce the probability and severity of grazing (mechanical and chemical defence or escape strategy), while tolerance consists in mechanisms that promote re-growth following defoliation (Briske, 1996). The effectiveness of these strategies depends on the level of primary production, the intensity of defoliation, and the foraging behaviour of the herbivores (Bullock, 1996). Defence mechanisms (besides resources acquisition and storage ability) of plants depend on their traits, which may be considered as biological characteristics of species responding to the dominant processes in an ecosystem (Lavorel et al., 1997). Trait assessment gives information about the mechanisms of processes like management treatments (Bullock et al., 2001), allowing to understand the changes acting in complex ecosystems and to predict vegetation modifications induced by different management types (Noble and Gitay, 1996).

Previous research on deer management largely focused on the effects of this ungulate on plant populations and habitat conditions and aimed to understand how to limit the impacts of these animals on ecosystem components and functions (Cote et al., 2004). In the last decades several studies were carried out worldwide about the impact of deer population on forest understory (e.g. Gill and Beardall, 2001), forest regeneration (e.g. Hegland et al., 2013), and dynamic processes of plant communities (e.g. Chollet et al., 2013). However, little is known about the impact of red deer on semi-natural herbaceous communities, especially as regards plant functional traits. Anyhow, this is a key issue, since the recent expansion of red deer resulted in an increased use of open areas with impacts, for instance, on forage production (Marchiori et al., 2012) and plant diversity (Schütz et al., 2003). Therefore, detailed knowledge about the effects of red deer grazing on biodiversity and trait-based species assemblage of pastures are needed to achieve a broader ecosystem perspective of grazing by wild herbivores (Mysterud, 2006). In this regard, detecting in which direction wild herbivores change the plant species assemblage is still a partially open question, since it depends on several factors such as herbivore species (Albon et al., 2007) and density (Putman, 2011; Ferretti et al., 2015), plant community type, productivity and composition (Bullock et al., 1996). Competition among sympatric wild herbivores (Lovari et al., 2014) and land use history (Gustavson et al., 2007) are key factors as well. Schütz et al. (2003) suggested that the red deer impact on grasslands follows the intermediate disturbance hypothesis (Grime, 2001). To test this statement, we compared the effects of late mowing and grazing by red deer. Late mowing is a non-selective type of disturbance (Kohler et al., 2005) that makes available to plants a long period to complete their reproductive cycle, leading to a complex trait-determined use of different temporal and spatial niches (Catorci et al.,

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2014b), thus acting like an intermediate disturbance intensity in productive environments. Instead, red deer browsing with higher density (e.g. more than 0.1 individuals/hectare – Putman et al., 2011; Ferretti et al., 2015; the average red deer density in the Alps is generally lower than 0.2 individuals/hectare - Marchiori et al., 2012), may be considered a high disturbance intensity, produced by an intermediate, opportunistic, mixed feeder, with a marked degree of forage selectivity (Hofmann, 1989), thus potentially leading to a high spatial heterogeneity of vegetation (Adler et al., 2001). Red deer selectivity changes at both large (plant community level) and small scale (patch and individual levels) due to differences in landscape and plant community mosaics (Hester et al., 1999; Cougenhour, 1991). Therefore, in order to assess the trait-related direction of changes in grassland composition caused by herbivory of red deer, we chose experimental controlled conditions, with captive red deer grazing exclusively on former hay meadows. In such experimental conditions we hypothesized that long-term grazing by reed deer with high stocking rates: i) decreases species richness and diversity; ii) alters the composition of pastures fostering the spread of poorly palatable species with strong competitive strategies, leading to the exclusion of the least competitive ones; iii) leads to a decrease of the pasture feed value through the reduction of the most palatable species (i.e. legumes and forbs).

Material and methods

Study area

The study was conducted in an old, traditional pastoral system of the Paneveggio Pale San Martino Natural Park, in the central-eastern Italian Alps. The study area (46°18’22’’N, 11°44’29’’E) is situated at 1500/1550 m a.s.l., on gentle south-southeast-facing slopes. The entire study site underwent the same management type until 1971 (mowing and fertilisation with mature manure after hay removal), when part of it was fenced and began to host a population of captive red deer.

In the fenced area (hereafter referred to as site A) annual cutting regime was imposed from late August to early September until 1990. In the fenced stand the manuring regime was continued during the 1980s. Starting from 1990 any type of management ceased. Red deer have not feed supply from May to late September, and are free to feed on fenced pastures (7 hectares). The stocking rate was very high, that is 0.4-0.5 individuals/hectare (see Schütz et al., 2003).

Differently, in the other part of the study area (6 hectares, hereafter referred to as site B), traditional mowing activities continued throughout the last forty years; the meadows are fertilized with manure annually and undergo hay cut in August (that is late mowing). Hay is stored and used during winter to feed deer.

Experimental design and data collection

We laid 43 plots of 50 x 50 cm in the grazed site and 34 in the mowed one. Lower left-hand corners of plots were placed at the nodes of a 50 x 50 m grid overlaid to the study area.

In each plot, we collected data on cover percentage of species, altitude (m a.s.l.), slope aspect (azimuth degrees) and slope angle (vertical degrees). Field relevés were carried out from late June to early July 2014. We selected a set of traits (life form, horizontal and vertical space occupation, type of vegetative propagation) focusing on

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plant competitive ability and resistance to disturbance (Díaz et al., 1999; Liira and Zobel, 2000; Cornelissen et al., 2003; Garnier et al., 2007). All traits were treated as categorical variables. A description of each trait, with the main associated plant functions, a list of the respective states and data sources is reported in the *Appendix 1*.

We used the Grime's strategies (Grime, 2001) to assess difference between treatments due to plant ecological adaptations and resource acquisition strategies. The CSR model postulates the existence of three primary plant strategies: competitors (C), stress-tolerators (S), and ruderals (R). In addition, the CSR model provides the existence of secondary strategies corresponding to the various trade-offs in adaptation to competition, stress and disturbance: competitive ruderals (CR), stress-tolerant ruderals (SR), stress-tolerant competitors (CS), and competitive stress-tolerant ruderals (CSR). Even though the ability of CSR theory to predict variation in species composition along environmental gradients, such as altitudinal and temperature ones, has been questioned (e.g. van der Werf et al., 1998), it has nonetheless been demonstrated to be a useful tool for assessing and quantifying variations along successional gradients (Caccianiga et al., 2006). Information about species life strategies (Grime, 1974, 2001) were gathered from Grime et al. (1988), BiolFlor database (Klotz et al., 2002), or checked by field observations, on the basis of the criteria and examples provided by Grime et al. (1988) and Grime (2001). Finally, since reed deer have a diet preference for forbs and legumes, we distinguished three functional groups: leguminous species, forbs, and graminoids.

In order to have an approximation of difference in soil nutrient content between sites, we used the nitrogen (N) Ellenberg's indicator values (Ellenberg 1974, 1996; Ellenberg et al., 1992) since Ellenberg's indicators have proven useful in analyzing the drivers of vegetation change (i.e. McCollin et al., 2000; Godefroid and Dana, 2007; Klaus et al., 2012). We drew N values from Pignatti (2005).

Species nomenclature followed Conti et al. (2005) and in some cases the International Plant Names Index (http://www.ipni.org).

Data analysis

To ascertain whether management type affects the species composition of grasslands, we performed canonical redundancy analysis (RDA) of the "plots x species cover (percentage)" matrix, constrained by land use (i.e. grazing and mowing conditions). Prior to RDA, cover data matrices were Hellinger-transformed to avoid considering double absence as a resemblance between sites (Legendre and Gallagher, 2001).

To assess the species diversity between the grazed and mowing conditions, we computed the Shannon-Wiener (H'), Gini-Simpson (D) and Shannon Evenness (E_H) indices.

To identify the indicator species of each management condition, we performed Indicator species analysis (ISA) on the matrix "relevés x species cover (percentage)", using management as grouping variable. ISA is a non-parametric method for identifying those items that show significantly preferential distribution (in terms of frequency and abundance). An Indicator Value (IV) is calculated by multiplying the relative abundance of each item in a particular group and the relative frequency of the item occurrence in the sample of that group (Dufrêne and Legendre, 1997). The number of randomized IVs higher than the observed ones is used to calculate the probability value (McCune and Grace, 2002). The statistical significance ($P < 0.05$) of the observed maximum IVs was tested using permutation tests with 4,999 iterations. We considered of interest only Indicator values higher than 20.

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We assumed that the indicator species set is a key tool to understand the changes in plant community composition, as the management modification mostly affects frequency and abundance of species identified as indicators species by ISA. Therefore, to verify whether functional composition of plant communities was related to changes in grassland management, the above-mentioned plant traits and Grime's strategies were attributed to the indicator species highlighted by ISA. To transform trait and Grime's strategies binary data (presence/absence) into quantitative data (i.e. aggregated cover values of each trait / strategy), we multiplied the "plots x indicator species cover" matrix by the "species x trait state / strategy (presence/absence)" matrix to provide a "plots x trait state / strategy of indicator species (cover percentage)" matrix, which formed the basis for the following analyses. We performed ISA on the matrix "relevés x trait state / strategy (aggregated cover values)", using land use as grouping variable.

We calculated cover-weighted mean values of nitrogen Ellenberg's indicator values for each plot and then performed descriptive statistics for sites A and B.

Statistical analyses were executed using R software (version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org), and vegan and labdsv R-packages (functions *rda* and *indval*, respectively) (Oksanen et al., 2014).

Results

Table 1 gives an overview of the dominant species (those with a mean cover value higher than 10% at least in one of the two sites) with percent frequency and mean cover percentage. The *Electronic Appendix 1.* shows the whole data set of relevés.

Table 1. *Frequency (%) and mean cover (%) of species recorded in 0.5 x 0.5 m plots in site A (grazed by red deer) and site B (mowed). Only species whose mean cover exceeds 10% at least in one of the two sites are indicated.*

Species	Site A		Site B	
	Frequency (%)	Mean cover (%)	Frequency (%)	Mean cover (%)
Bellardiochloa variegata (Lam.) Kerguélen	53.5	18.3	20.6	3.3
Brachypodium rupestre (Host) Roem. & Schult.	51.2	23.6	8.8	0.3
Deschampsia cespitosa (L.) P. Beauv.	53.5	17.6	11.8	2.0
Festuca microphylla (St.-Yves) Patzke	62.8	11.1	82.4	20.5
Festuca pratensis Huds.	0.0	0.0	26.5	11.3
Trifolium repens L.	83.7	14.4	91.2	13.2
Leontodon hispidus L.	0.0	0.0	85.3	14.8

RDA showed a separation between plots of mowed and grazed conditions (*Fig. 1*). The group of plots undergoing red deer grazing pressure segregated into two clouds: around *Deschampsia cespitosa* and *Bellardiochloa variegata* (1), and around *Brachypodium rupestre* (2). The topographic features shown in *Table 2* characterize these two clouds.

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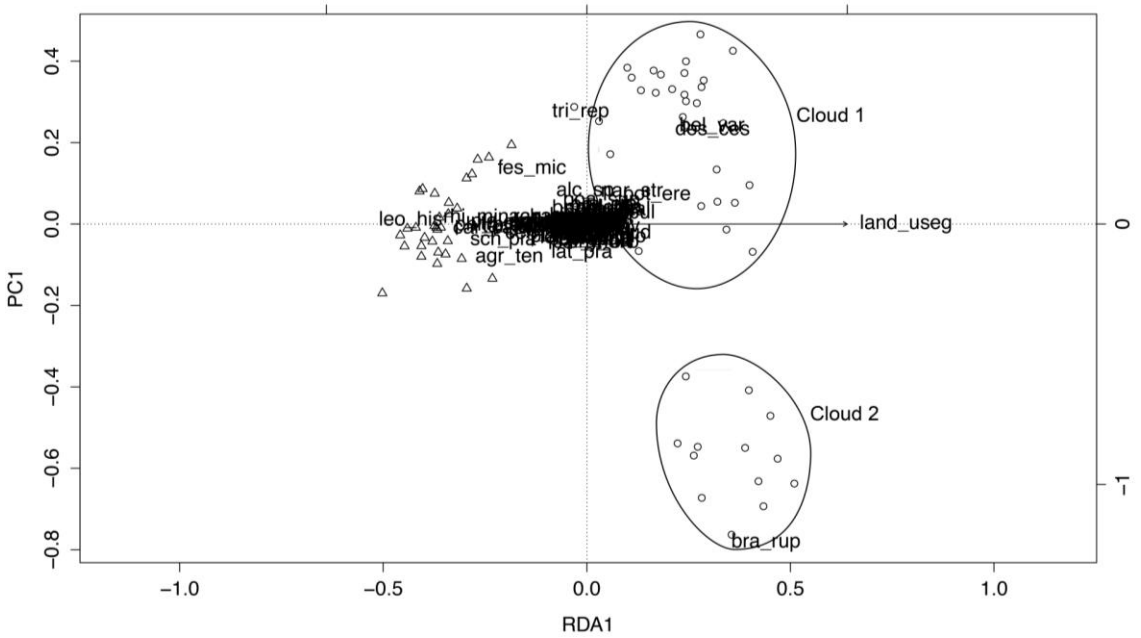

Figure 1. *Canonical redundancy analysis (RDA) ordination graph (scaling 2) of the "plots-by-species cover" matrix, constrained by land use (grazing by red deer / mowing). (Circles indicate plots laid in the site grazed by red deer, triangles refer to plots laid in the mowed site; land_useg: grazing; agr_ten: Agrostis tenuis; bel_var: Bellardiochloa variegata; bra_rup: Brachypodium rupestre; des_ces: Deschampsia cespitosa; fes_mic: Festuca microphylla; lat_pra: Lathyrus pratensis; leo_his: Leontodon hispidus; sch_pra: Schedonorus pratensis; tri_rep: Trifolium repens).*

Table 2. *Main topographic features of each group that segregates in the Redundancy analysis ordination graph in the site grazed by red deer.*

Topographic features	Group	Mean ± SD	Min.	Max.	1st quartile	Median	3rd quartile
Altitude (m a.s.l.)	1	1490.3 ± 4.1	1480.0	1494.0	1490.0	1491.0	1493.0
	2	1493.0 ± 5.7	1480.0	1501.0	1491.0	1493.0	1495.0
Aspect (azimuth degree)	1	122.1 ± 90.4	0.0	320.0	100.0	120.0	145.0
	2	137.5 ± 65.2	0.0	190.0	112.5	155.0	190.0
Slope (vertical degree)	1	6.7 ± 6.1	0.0	30.0	2.0	5.0	10.0
	2	12.3 ± 8.1	0.0	27.0	7.0	12.0	15.0

SD: standard deviation

Diversity and evenness indices values were greater under mowing (site B) than under grazing (site A) (*Table 3*).

Table 3. *Values of diversity indices calculated for each site (A: grazed by red deer, B: mowed).*

Site	Shannon (H')	Gini-Simpson (D)	Shannon Evenness (E_H)
A	1.79	0.76	0.43
B	2.35	0.86	0.50

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ISA for species (*Table 4*) highlighted two sets of indicator species (8 for site A and 22 for site B). Species with the highest IV ($P < 0.001$) were *Potentilla erecta* (0.60), *Brachypodium rupestre* (0.51), and *Deschampsia cespitosa* (0.48) for site A; *Rhinanthus minor* (0.94), *Leontodon hispidus* (0.85) and *Carex caryophyllea* (0.82) for site B.

Table 4. *List of indicator species of each site, as determined by Indicator species analysis. Only significant indicator values ($P < 0.05$) higher than 0.20 are shown (A: grazed by red deer, B: mowed).*

MaxSite	Species	IV	*P*
A	*Bellardiochloa variegata* (Lam.) Kerguélen subsp. *variegata*	0.454	0.001
	Brachypodium rupestre (Host) Roem. & Schult.	0.505	0.000
	Deschampsia cespitosa (L.) P. Beauv.	0.480	0.000
	Luzula sudetica (Willd.) Schult.	0.433	0.005
	Nardus stricta L.	0.209	0.008
	Potentilla erecta (L.) Räusch.	0.597	0.000
	Thymus pulegioides L. subsp. *pulegioides*	0.326	0.000
	Veronica officinalis L.	0.209	0.007
B	*Achillea millefolium* L.	0.579	0.002
	Agrostis capillaris L.	0.528	0.001
	Anthoxanthum odoratum L.	0.379	0.000
	Carex caryophyllea Latourr.	0.817	0.000
	Centaurea nigrescens (Willd.) subsp. *transalpina* (DC.) Nyman	0.379	0.000
	Colchicum autumnale L.	0.294	0.000
	Crepis aurea (L.) Cass.	0.324	0.000
	Festuca microphylla (St.-Yves) Patzke	0.533	0.010
	Schedonorus pratensis (Huds.) P.Beauv.	0.265	0.000
	Leontodon hispidus L.	0.853	0.000
	Leucanthemum vulgare Lam.	0.529	0.000
	Phleum pratense L.	0.618	0.000
	Plantago lanceolata L.	0.314	0.002
	Plantago media L.	0.524	0.000
	Prunella vulgaris L.	0.358	0.001
	Ranunculus acris L.	0.316	0.022
	Ranunculus montanus Willd. (group)	0.546	0.000
	Rhinanthus minor L.	0.941	0.000
	Taraxacum officinale Weber (group)	0.644	0.000
	Trifolium pratense L. subsp. *pratense*	0.570	0.000
	Veronica chamaedrys L. subsp. *chamaedrys*	0.397	0.001
	Vicia cracca L.	0.326	0.017

MaxSite: site with maximum indicator value; IV: indicator value; *P* proportion of randomized trials with an indicator value equal to or exceeding the observed indicator value

ISA for traits of indicator species (*Table 5*) highlighted that site A had a low number of indicator traits. Competitive stress-tolerant strategy had the highest IV (0.81; $P < 0.000$) for site A, while Competitors and Competitive Stress-tolerant Ruderals were the preferential strategies of site B. Moreover, ISA indicated rhizome and runner/runner-like rhizome as vegetative propagation type for site A, while absence of vegetative

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propagation (namely, only sexual reproduction), root, tuber and bulb splitters, besides roots with adventitious buds were identified as indicators for site B. In addition, ISA highlighted a high number of vertical space occupation types (sedge, rosette forbs, hemirosulate upright forbs, prostrate growth form), besides geophytes and therophytes for site B. Instead, chamaephytes, grasses, caespitose and reptant trait states emerged in site A. Finally, graminoids (IV = 0.64; $P < 0.001$) were identified as indicators for site A, leguminous plants and forbs for site B (0.66 and 0.84, respectively; $P < 0.001$).

Table 5. *List of indicator CSR strategies and trait states for indicator species of each site, as determined by Indicator species analysis. Only significant indicator values ($P < 0.05$) higher than 0.20 are shown (A: grazed by red deer, B: mowed).*

MaxSite	CSR strategy/Trait	Type of CSR strategy/Trait state	IV	*P*
A	CSR strategy	Competitive stress-tolerators	0.81	0.000
	Life form	Chamaephytes	0.33	0.000
	Vegetative propagation	Necessary	0.60	0.035
		Runner/runner-like rhizome	0.57	0.000
		Rhizome	0.55	0.000
	Horizontal space occupation	Caespitose	0.69	0.002
		Reptant	0.57	0.024
	Vertical space occupation	Grass	0.63	0.000
B	CSR strategy	Competitors	0.77	0.000
		Competitive Stress-tolerant Ruderals	0.60	0.004
	Life form	Geophytes	0.29	0.000
		Therophytes	0.93	0.000
	Vegetative propagation	Not necessary (only sexual reproduction)	0.92	0.000
		Root, tuber, bulb splitter	0.75	0.000
		Roots with adventitious buds	0.64	0.000
	Horizontal space occupation	Absent	0.72	0.000
		Pleiocorm	0.84	0.000
		Rosulate	0.94	0.000
	Vertical space occupation	Sedge	0.72	0.000
		Rosette forbs	0.97	0.000
		Hemirosulate upright forb	0.80	0.000
		Prostrate	0.28	0.020

MaxSite: cluster with maximum indicator value; IV: indicator value; *P* proportion of randomized trials with an indicator value equal to or exceeding the observed indicator value

Comparison of cover-weighted means of N Ellenberg's indicator values between the two sites indicated that soil of site A had a slight lower median value than in site B (3.8 vs. 4.5 - *Appendix 2*).

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Discussion

Consistently with several studies (e.g. Adler et al., 2001; Sebastiá et al., 2008; Fontana et al., 2014), we found (*Fig. 1*) that differences in long-term management lead to different species composition of plant communities. In particular, grazing by red deer promoted differences in species composition in comparison to mowing, leading to lower species diversity and evenness (*Table 3*). This is consistent with the hypothesized intermediate disturbance intensity produced by mowing (Catorci et al., 2014b), and, following the Grime's statements (2001), indicate that a high stoking rate of red deer should be considered as a heavy disturbance regime. Schütz et al. (2003) found that heavy grazing (about 0.2 individuals/hectare) by red deer led to a significant decrease in tall plant species compared to abandoned grasslands, while the richness and abundance of small size species and species with morphological protection from grazing or short life span increased. Instead, coherently with Augustine and McNaughton (1998) and Bergvall et al. (2006), we observed the opposite trend. In fact, we found a preferential distribution (namely, higher abundance and frequency) of some competitive stress-tolerant (indicator life strategy), coarse tall grasses (*Bellardiochloa variegata, Brachypodium rupestre*, and *Deschampsia cespitosa*) in the grazed site and of small (low stature) species in the mowed one (*Table 3*). These contrasting results suggest, consistently with other research (e.g. Gustavson et al., 2007), that the impact of grazing by red deer on pasture richness and composition depends, besides on red deer density, also on the former species composition of sites, and therefore on the land use history. We might argue that grazing by red deer with a high stocking rate has a negative impact on formerly managed meadows, while it is favourable for plant diversity on long-term abandoned grasslands. In fact, grazing by red deer with high stocking rate hinders the spread of successional/ruderal species lacking strong avoidance strategies (as indicated by Schütz et al., 2003), but fosters the establishment of competitive stress-tolerant, dominant tall grasses with strong avoidance strategies. Actually, the coarse tall grasses indicated by ISA for grazed site (*Table 4*) are poorly palatable species that are generally fostered by improper management due to under- or over-stocking conditions (Louault et al., 2002). Such type of dominant species invests a great amount of resources into root production and foraging rhizomes (indicator trait of the grazed site), overcoming the spatial competition for resources by strong clonal integration strategies (Hutchings and Wijesinghe, 1997). They minimize the loss of resources by the low palatability due to the silica-rich, tough and hairy leaves, the dead leaves protecting the new shoots, etc., and tend to dominate the plant community through specific traits such as tall canopies, extensive lateral spread, and litter deposition (Grime, 2001). Moreover, unpalatable plants typically grow and decompose slowly (Augustine and McNaughton, 1998). Finally, they influence the availability of resources, such as water and nutrient content of soil, and light radiation at the ground level (e.g. Vinton and Burke, 1995; Catorci et al., 2011a).

As a consequence of such strategies, several other species undergo competitive exclusion (Grime, 2001), as demonstrated in the study case by the higher number of indicator species in mowed site (*Table 3*), where several short graminoids (i.e. *Agrostis capillaris*, *Anthoxanthum odoratum*, *Carex caryophyllea*, *Festuca microphylla*, *Phleum pratense*, *Schedonorus pratensis*) besides forbs, rosette and prostrate plants (i.e. *Centaurea nigrescens* subsp. *transalpina*, *Crepis aurea*, *Leontodon hispidus*, *Taraxacum officinale*, *etc.*) were preferentially distributed (*Table 4*).

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These results are consistent with previous statements indicating that intermediate disturbance intensity plays a key role in limiting the competitive exclusion of subordinate species by the dominant ones (Grime, 2001). The aforementioned species are CSR or C strategists that were indeed identified by ISA for the mowed site (*Table 5*). Species with CSR strategy have intermediate characteristics between those of competitors, stress tolerators and ruderals and live in habitats in which coexistence of different strategies is possible because competition, stress and disturbance only occur during particular times in the year (Grime, 2001) or their combined effects are of moderate intensity (Grime, 1977), as under mowing management. This may explain the occurrence of competitors, mostly non-dominant graminoids, which can coexist with CSR strategists because competition for light is less intense.

The highest number of indicator traits was found under mowing management (*Table 5)*, while red deer grazing fostered plants with vegetative propagation based on lateral spread of runners and runner-like rhizomes, that are strategies allowing to face heavy disturbance intensity and flower consumption by herbivores (Catorci et al., 2012b). Moreover, such strategies are aimed at maximizing the species competitive ability when there is higher exploitation of soil resources in productive environments, allowing individuals to explore the neighbouring areas and find unexploited soil niches (Friedman and Alpert, 1991). Avoidance strategies like reptant horizontal space occupation and chamaephyte plant life form (Grime, 2001) were fostered as well. In addition, it emerged that grazing strongly reduces the feed value of pastures; actually legumes and forbs, preferentially selected by red deer (Radkowski and Barabasz-Krasny, 2007), were indicators of mowed condition. On the other hand, mowing allows the establishment of plants with leaves concentrated at the ground level (prostrate, rosulate), or just above it (hemirosulate), and of geophytes and therophytes, which benefit from canopy removal, thus avoiding competitive exclusion due to light depletion (Catorci et al., 2011b). Moreover, the vegetative reproduction types fostered by mowing (root, tuber and bulb splitters) allow a fast canopy pre-emption and the coexistence with competitive grasses by spatial and temporal niche differentiation in highly productive grassland communities (Catorci et al., 2012a). The observed small difference in nitrogen soil content does not seem to have significant implications from a floristic point of view. It was largely stated that higher the soil nitrogen values higher the spread of competitive tall grasses with a corresponding decrease of the species richness (Grime, 2001). However, we observed the opposite trend, likely indicating that within the observed small range of N indicator values, the control of dominant tall grasses by mowing has a major impact on species richness.

Features of established competitive tall grasses and management implications

Table 2 shows that the group of relevés with higher *Brachypodium rupestre* cover value (group 2 of *Fig. 1*) was characterized by the driest conditions, indeed it had higher average slope angles and more southerly aspects compared with those with higher cover of *Bellardiochloa variegata* and *Deschampsia cespitosa* (group 1 of *Fig. 1*). Therefore, we can infer that, in the study case, site conditions influence the type of invasive plants but not the spread of competitive species in former meadows undergoing red deer herbivory.

As regards *D. cespitosa*, its spread in disturbed habitats is largely due to seedling establishment; in closed, more stable communities, seedling establishment is a rare event and the species spreads by clonal growth (Davy, 1980). Thus, the high cover value of this species may be initially due to the trampling by red deer enhancing seed germination,

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subsequently reinforced by its clonal strategy and very low palatability (Davy, 1980). The competitive success of *B. rupestre* is related to its high tiller density and branching frequency (Pottier and Evette, 2010), as well as to its clonal growth and clonal integration strategy capabilities (de Kroon and Bobbink, 1997). These traits allow the spread of this species by the expansion of circular clonal tussocks, which often form (by coalescence of different clonal patches) nearly mono-dominant stands in the final phases of invasion (de Kroon and Bobbink, 1997). The spread of *B. rupestre* is induced by inadequate grazing pressure (Catorci et al., 2012c) and by high content of nitrogen (Willems et al., 1993). *B. variegata* is a poorly palatable species, which can grow on poor and acid soils (with a slow resource acquisition and storage strategy thanks to the presence of rhizomes) and tolerate the competition with tall grasses. Thus, its spread in the grazed site might be a side effect of the spread of *B. rupestre* and *D. cespitosa*.

In general, with reference to the management of coarse tall grasses, it was demonstrated that overstocking in fenced stands by domestic herbivores may be useful (Catorci et al., 2014a), especially if performed by equines (Catorci et al., 2012b) or cows (Schütz, 2003). Mowing has the same output (Bonanomi et al., 2009). However, mowing alone does not ensure the total control of dominant species endowed with competitive strategies (Catorci et al., 2011b), thus calling for management integration with different type of grazers (Gordon, 1988), such as late summer grazing by very little selective herbivores (i.e. equines).

Conclusion

We found that in conditions of high stocking rate, red deer significantly affects the species and functional composition of former hay meadows, leading to a trait-related change of species composition. We can also reinforce the hypothesis that the impact of red deer on grassland biodiversity follows the intermediate disturbance hypothesis. Indeed, as postulated by Grime (2001), starting from grassland systems experiencing intermediate, non selective disturbance regimes, red deer grazing reduces the species richness and diversity, mostly by competitive exclusion of subordinate species due to the spread of competitive, stress-tolerant tall grasses with strong avoidance strategies. Thus, we can argue that the substitution of mowing with red deer grazing on former hay meadows is not effective from a biodiversity conservation viewpoint, since palatable species (namely, leguminous plants) and those in need of seasonal canopy removal (e.g. prostrate plants with rosette leaf arrangement or plants with bulbs or tubers) were mostly lost in the long run.

Obviously, the results of the present study, obtained in a small area, are not directly generalizable to every case, but they can indicate the direction of changes of pasture composition from mowed meadows to abandoned lands under high red deer stocking rate, thus contributing to the overall understanding of the impacts due to reed deer population on herbaceous ecosystems. Ultimately, our study suggests that grazing by red deer could trigger the invasion of dominant tall grasses as well as the improper management of domestic herbivores. Moreover, we found some clues that exclusive red deer grazing may lead to the decrease of the pasture feed value, likely causing a decrease of habitat suitability for this ungulate, since animal welfare and reproductive performances are strongly dependent on feed amount and quality (see Catorci et al., 2014a; Lovari et al., 2014). In consideration of the above statements, we suggest that

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red deer impact on grassland composition should be considered in planning red deer population management.

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APPENDIX

Appendix 1. *Traits and trait states analysed in the present study, with a brief description, the main associated plant functions, and data sources.*

Trait	Trait state	Description	Main plant functions	Data source
Life form	Hemicryptophyte, Chamaephyte, Geophyte, Therophyte	Location of perennating organs as an adaptation used by plants to overcome the adverse seasons, according to Raunkiaer (1934) classification	Competitive ability Response to soil resources Response to disturbance	Pignatti (1982); checked by authors' observations
Vegetative propagation	Necessary/not necessary (only sexual reproduction); rhizome; runner/runner-like rhizome; root with adventitious buds; bulbils; root, tuber or bulb splitter	Occurrence and type of vegetative propagation, identified following Krumbiegel (2002), and Klimešová and de Bello (2009)	Competitive ability Response to soil resources Response to disturbance Reproduction	Klotz et al. (2002), Klimešová and Klimeš (2006), checked and supplemented by authors' observations
Horizontal space occupation	Caespitose (tuft forming); pleiocorm (system of compact, perennial shoots occurring at the proximal end of the persistent primary root); reptant (shoots and/or stems creeping on the ground); prostrate; rosulate (leaves basally arranged forming a rosette); absence of horizontal space occupation	Classification of horizontal growth form, according to the categories indicated in Krumbiegel (2002)	Competitive ability Response to soil resources Response to disturbance Defence from herbivory	Pignatti (1982), Klotz et al. (2002); checked and supplemented by authors' observations
Vertical space occupation	Leafy stem, narrow leaves (grass); no leafy stem, narrow basal leaves (sedge); upright forb with leaves arranged either scattered or tightly packed at the shoot, thus with short and long internodes (hemirosulate upright forb); upright forb with leaves equally spaced along the stem (erosulate upright forb); no leafy stem, broad basal leaves (rosette forb); leafy stem prostrate on the ground (prostrate forb)	Classification based on the width of leaves and on their position along the stem (Liira and Zobel, 2000; Krumbiegel, 2002 modified)	Competitive ability Response to soil resources Response to disturbance Defence from herbivory	Pignatti (1982), Klotz et al. (2002); checked and supplemented by authors' observations

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Appendix 2. *Descriptive statistics of cover-weighted mean nitrogen Ellenberg's indicator value in site A (grazed by red deer) and in site B (mowed).*

Statistics	**Site A**	**Site B**
Min	2.7	3.1
1st quartile	3.5	3.7
Median	3.8	4.5
3rd quartile	4.1	5.1
Max	6.2	7.1
Mean	3.8	4.5
Standard deviation	0.6	0.9

ELECTRONIC APPENDIX

Electronic Appendix 1: Species cover values (%) recorded in 50 x 50 cm plots laid in site A (grazed by red deer) and site B (mowed).

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SOIL ECOLOGICAL SAFETY EVALUATION FOR BIVALENT TRANSGENIC COTTON PLANTS: ROOT EXUDATES VERSUS SOIL ENZYME ACTIVITIES AND SOIL MICROBIAL DIVERSITY

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Abstract. Pot experiments were conducted to assess the possibly adverse effect of transgenic Bt- and CpTI-cotton (*Bacillus thuringiensis* and cowpea trypsin inhibitor cotton) on soil ecosystem. Soil enzymes activities and microbial molecular community and diversity based on 16s- and 18s-PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) profiles were determined by adding root exudates of Bt- and CpTI-cotton into soil. Results showed that the root exudates of transgenic cotton seedlings increased the soil catalase activity by 21.8% to 32.7%. The soil urease activity was decreased by 11.0% while the activity in the treatment of 1ml/350g soil was increased by 18.1% compared to control. The invertase activity in the treatment of 1 ml/350g soil was increased by 54.2%, however, the activity in the treatments of 2 and 4 ml/350 g soil was decreased by from 25% to 29.2%. The total 16sPCR-DGGE lane bands of bacterial community and diversity were almost not different. However, the effect pattern of root exudates of transgenic cotton on soil fungal community and diversity was differed from bacteria. The fungal community number and composition were changed. Though some dominant community disappeared and other newly dominant community appeared, the fungal stability index was increased. Results suggested there was little significantly adverse effect of Bt- and CpTI-cotton on soil ecosystem.
Keywords: *transgenic Bt+CpTI cotton, root exudates, soil enzymes,·soil microbial diversity,·biosafety assessment*

Abbreviations: Trangenic Bt+CpTI cotton-transgenic Bacillus thuringiensis + cowpea trypsin inhibitor cotton; CAT-catalase

Introduction

Genomic technologies have been used to improve cultivated crop species, which provides an opportunity to transfer new specific traits of interest into other valuable genotypes (GMO-genetically modified organism) within a short period of time, and greatly reduces costs by promoting crop yield and environmental risk by decreasing the use of chemical insecticides (Peferoen, 1997; Baute et al., 2002; Bourguet et al., 2002; Rahman et al., 2015). On a global basis in 2010, about 15.4 million farmers grew biotech crops on about 1 billion ha (James, 2010).

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Cotton (*Gossypium hirsutum* L.) is one of the most economically important crops in the world. The first commercially available transgenic cotton expressing an insecticidal protein (Cry1Ac from Bt) was produced in the United Sates in 1995 (Environmental Protection Agency, 1998). Subsequently the growing area of Bt cotton cultivars has steadily increased, especially in China and India (Wu et al., 2011; Raman et al., 2015; Zaman et al., 2015). Proteinase inhibitor (PI) gene-transformed cotton plants, especially the CpTI gene (cowpea trypsin inhibitor gene) found and derived from edible parts of cowpea, were also introduced for broader insect-resistance. Bt and CpTI, together or individually, defend transgenic cotton from attack and damage by specific pest insects.

As a novel technology, however, there have beenargues about the safety of transgenic crops that still continue today. Since the original production of transgenic plants, there has been worldwide debate about the safe use of these plants and their products. The potential risks and benefits of transgenic plants should be evaluated independently and objectively. Risk assessment of transgenic crops is a basic prerequisite for monitoring the possible risks that could arise upon the release and use of transgenic plants (Talas-Ogras, 2011). Recently regulations have been developed to address the risks of releasing transgenic plants into the natural environment. Clearly, future agricultural ecosystems, and ultimately also natural ecosystems, will be challenged by the large-scale introduction of transgenic plants, containing entirely novel genes and gene products in new combinations at high frequencies. All of these may have unknown impacts on their associated complex of non-target organisms, i.e. all organisms that are not targeted by the insecticidal proteins produced by the transgenic plants (Velkov et al., 2005).

Such effects have been investigated numerous times. Growing Bt rice cultivars effectively decreased *in situ* CH_4 emission fluxes and methanogenic archaeal and methanotrophic bacterial community abundance and diversity (Han et al., 2013). However, increasing the fructose content of transgenic cotton (Bt and chi) (Modirroosta et al., 2014) had no direct adverse impacts on honeybees feeding on transgenic cotton pollen (Liu et al., 2005a, 2005b). No Bt protein was found to be taken up from soil by non-Bt corn, carrot, radish, or turnip grown in soil in which Bt corn had been grown or into which biomass of Bt corn had been incorporated (Stotzky, 2004; Rahman et al., 2015; Zaman et al., 2015).No significant direct adverse effects of transgenic cotton pollen (Bt +CpTI) on the pollinating beetle *Haptoncus luteolus* (Chen et al., 2011) and on organisms (earthworms, nematodes, protozoa, bacteria, fungi) in soil or in vitro were found(Rahman et al., 2015; Zaman et al., 2015).

Interestingly, larvicidal proteins encoded by cry genes from *B. thuringiensis* are released in root exudates from transgenic *B. thuringiensis* corn, rice, and potato but not from *B. thuringiensis* canola, cotton, and tobacco (Saxena et al., 2004; Rahman et al, 2015). The observed pattern of arbuscular mycorrhizal fungal colonization of Bt transgenic cotton was virtually identical among both conventional and GM cultivars of cotton at each assessment, clearly indicating that colonization by AM fungi was not affected by the expressed transgenic traits (Knox et al., 2008). Morphologically, there are significant reductions in transgenic cotton plant height making them favorable for breeding. Yield was significantly increased for transgenic lines. Fiber quality of transgenic lines was not affected when compared with non transgenic lines but needs further studies to understand the complex molecular mechanisms for resistance management and biosafety studies to develop new Bt cotton varieties (Rashid et al., 2009; Rahman et al., 2015). Transgenic insect-resistant cotton expressing the Cry1Ac and/or CpTI protein has caused significant seasonal variation in the number of bacteria, fungi,

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azotobacter, denitrifying bacteria, and ammonia-oxidizing bacteria and in diversity indices of microorganisms, but no significant differences in microbial population sizes or diversity indices attributable to long-term cultivation of transgenic cotton (Li et al., 2011). Using transgenic plants expressing Cry1Ie might delay the development of Bt-resistant insects in the field while the former genes-transformed into cotton convey insect resistance to transgenic cotton (Zhang et al., 2013). Cultivating *Cry3Bb* protein Bt maize is unlikely to adversely affect soil ecology in the short term but it is still unknown in the long term cultivation (Devare et al., 2007).

No negative effect of different Bt rice varieties on the fitness of *Folsomia candida* through either diet or soil exposure in a laboratory test was obtained (Yuan et al., 2013). Transgenic cotton did not cause changes in populations of acarids and did not substantially reduce numbers of predators; its effects on aphids were inconsistent (Men et al., 2004). Molecular analysis of the bacterial community also showed no significant impact on the dominant members of the bacterial community and soil protease activity was not inhibited by the release of constitutively over-expressed protease inhibitor (Riglietti et al., 2008). No significant harmfu impact on CFUs of bacteria, actinomycetes and fungus between the Bt and non-Bt cotton rhizosphere during cropping season at one particular stage(Zwahlen et al., 2007; Zaman et al., 2015),even at far higher concentration of the Bt proteins (Rahman et al., 2015), no recombination event was detected between plant DNA and soil bacteria (Velasco et al., 2013), but other factors may be involved (Rui et al., 2005) and more studies are needed to assess the impact of the continuous release of *Cry1Ab* via root exudates and plant biomass on the soil ecosystem (Zwahlen et al., 2007).

Crop residues are the primary source of carbon in soil, and root exudates govern which organisms reside in the rhizosphere. Any change to the quality of crop residues and rhizosphere inputs could modify the dynamics of the composition and activity of organisms in soil. Insect-resistant Bt crops have the potential to change the microbial dynamics, biodiversity, and essential ecosystem functions in soil, because they usually produce insecticidal Cry proteins through all parts of the plant(Rahman et al., 2015; Zaman et al., 2015). It is crucial that risk assessment studies on the commercial use of Bt crops consider the impacts on organisms in soil. Generally, few or no toxic effects of Cry proteins on woodlice, collembolans, mites, earthworms, nematodes, protozoa, and the activity of various enzymes in soil have been addressed (Icoz and Stotzky, 2008; Rahman et al., 2015). Although some effects, ranging from no effect to minor and significant effects, of Bt plants on microbial communities in soil have been reported, they were mostly the result of differences in geography, temperature, plant variety, and soil type and, in general, were transient and not related to the presence of the Cry proteins (Icoz and Stotzky, 2008).

Considerable research has now been conducted on the effects of transgenic plants on soil microorganisms. Most studies to date suggest that Bt plants that have been released cause minor changes in microbial community structures that are often transient in duration. However, impacts of two-gene transgenic cotton (Bt- and CPTI- gene) on soil microbial community structure and functionhave received little attention.

Ecological stress or other environmental changes in soil ecosystem can be judged in advance through some sensitive/warning indicators, including biological and biochemical properties of the soil, e.g. microbial activity and the activities of soil enzymes (Nannipieri et al., 2003; Gomez et al., 2006). It is likely that those sensitive soil parameters may be affected by cultivation of Bt-CPTI-cotton. So, the objective of this study was to examine and assess the effect of Bt- and CpTI-cotton on microbial and

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biochemical indicators in soil of Bt+CpTI-cotton crops in a subtropical agro-ecosystem. The purpose of this study was to check unknown and possible changes due to the presence of root exudates of transgenic insect-resistant cotton, especially the responses of soil key enzyme activities and soil microbial community based on PCR-DGGE.

Materials and Methods

Transgenic and conventional cotton seed

Cotton seeds of bivalent (against Bt+CpTI) transgenic cotton, bred by Research Institute of Cotton, Chinese Academy of Agricultural Sciences, obtained from Jiangsu Academy of Agricultural Sciences, was used as transgenic cotton and cultivar Xinluhan 33, obtained from Jiangsu Academy of Agricultural Sciences, was used as the conventional cotton line. The genes Bt (*Cry1A*) and CpTI (Cowpea Trypsin Inhibitor), effective against cotton bollworm (*Helicoverpa armigera* Hubner), were contained in the transgenic cotton in the current study.

Collecting root exudates of transgenic cotton seedlings

The transgenic cotton and conventional parental cotton seeds were dipped in water for 48h and surface disinfected with 10% H_2O_2 for 20 minutes. Clean and rinse the seeds. The disinfected cotton seed was germinated in the incubator with 70-90% humidity and 25ºC. The seedlings with two leaves were transplanted into the soil in pot until the seedlings were grown up to the 5-6 leaf stage then the seedlings were dipped into 2000 ml of distilled water in a beaker for collection of root exudates. The root exudates were collected between 10:00 to 14:00 every day for 14 days. The distilled water containing root exudates of transgenic cotton seedlings were evaporated in a 45 ºC water bath until the liquid was concentrated to 2 ml (concentrated 1000 times) and subsequently stored at 4 ºC for further study.

Experimental design

The effect of transgenic cotton on soil enzyme activities and soil microbial diversity was assessed in a lab study. As control, the conventional parental cotton root exudates were also simultaneously assessed. Four treatments and three replicates were used in this single factor experiment. The concentrated root exudates (condensed 1000 times) of transgenic or conventional parental cotton xinluhan33 cotton seedlings were added into distilled water at four concentrations: 0 (CK), 1, 2, 4 ml (T1,T2,T3)of concentrated root exudates (transgenic cotton and non-transgenic cotton respectively) into 100 ml of distilled water. Each mixture of 100 ml of water and root exudates was blended into 350g of soil and mixed thoroughly and then this wet soil mixure was placed into a pot (14 cm x 8 cm). The pots were randomly distributed in a growth chamber with 10h of illumination, 25 ºC at daytime and 18 ºC at night time. After 10 days, sampling soil was analyzed for soil enzyme activities and microbial community and diversity.

Determination of catalase, phosphatase, urease and invertase activities in soil treated with root exudates

Antioxidant enzyme activity (CAT), redox related enzyme activities (phosphatase, urease) and hydrolic enzyme activity (invertase, sucrase, sugar conversion) in the soil

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were measured. All these enzymes were convinced to be associated with the functionality, productivity and buffer capacity in another new pot soil treated with the concentrated root exudates of transgenic and non-transgenic cotton.

Soil catalase activity assay

The activity of hydrogen peroxidase oxidoreductase (catalase) (EC 1.11.1.6) in soil was determined by back-titrating residual H_2O_2 with $KMnO_4$ (Johnson and Temple, 1964) with some modifications.

Place 2 g wind-dried soil into 100-ml beakers and add 40ml distilled water and 5 ml 0.3 % H_2O_2. Incubate the soil samples in the dark at 25 °C for 20 min at the back-forth vibrating incubator. Add 5 ml 6 mol L^{-1} sulfuric acid to stabilize the unbroken H_2O_2. Filter the suspension with slow-filter paper. Take 25 ml filtrate solution and titrate it with 0.3 mol L^{-1} $KMnO_4$ till light pink (record volume of $KMnO_4$ used as B). Meantime, titrate 25 ml 0.3% H_2O_2 with 0.3 mol L^{-1} $KMnO_4$ till light pink (record volume of $KMnO_4$ used as A). Here, (A-B)×actual concentration of $KMnO_4$ was the soil catalase activity. Soil catalase activity was calculated as milliliter of 0.3 mol L^{-1} $KMnO_4$ per gram of soil after 20 min.

Invertase activity assay

The invertase activity (substrate, 5 % sucrose) ,also sucrase (EC 3.2.1.26), was measured by reducing sugars release in the incubation period of 24h at 37 °C and colorimetrically (Frankenberger and Johanson, 1983) with some midifications.

Put 5 g wind-dried soil samples into 50 ml conic flask and add 15 ml 18% sugar solution, 5 ml pH5.5 phosphate buffer and 5 drops of toluene. Shake and mix the system. Place the mixture system into the incubator and incubate at 37 ºC for 24h. Filter the reaction system. Take 1 ml filtrate and place into 50 ml volumetric flask. Add 3 ml 3,5-dinitro salicylic acid. Heat the mixture for 5 min at the boiling water bath. Subsequently, move the mixture to running water to cold for 3 min. Add distilled water to dilute the yellowish mixture till 50 ml. Take 3 ml yellow solution to detect the colormetry at 508 nm wavelength. The sucrase activity was milligram of glucose produced from sucrose hydrolyzed by invertase after 24h per gram soil.ű

Soil urease assay

Soil urea amidohydrolase (urease) (EC 3.5.1.5), (substrate, 10% urea) were determined colorimetrically by the liberated NH_4^+ after 24h of incubation at 30 °C (Kandeler and Gerber, 1988) with modifications.

Place 5 g of wind-dried passed through 1mm into 100ml conic flask. Add 1 ml toluene into the flask and put it 15 min. Add 10 ml 10% urea solution and 20 ml citrate buffer (pH 6.7) and mix carefully, and the contents were allowed to stand for approximately 15 minutes until the toluene had completely penetrated the soil. A control, in which 10 ml distilled water was substituted for the urea, was run simultaneously for each soil sample. Incubate the mixture at 37 °C for 24h. Dilute the mixture with 38 °C hot water till 100 ml and shake carefully and filter the mixture into the conic flask. Take 3 ml filtrate and put it into 50 ml volumetric flask. Add 10ml distilled water and shake thoroughly. Add 4 ml sodium phenol and mix carefully, then add 3 ml sodium hypochlorite and shake thoroughly, put it 20 min. Dilute to 50 ml with distilled water. The blue mixture was

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determined at 578 nm wavelength within 1 h colormetrically. The soil urease activity was expressed as NH_3-N milligram per gram soil after 24h.

Phosphatase activity assay

Acid phosphomonoesterase (phosphatase) (EC 3.1.3.2) was determined colorimetrically by the liberated phenolphthalein after the substrate 1% sodium phenolphthalein phosphate, was incubated 1h at 30 °C following the modified method by Serrasolsas and Khanna (1995) with modifications.

Place 5 g wind-dried soil samples into 50 ml volumetric flask and add 1 ml toluene. Plug the mouth of the flask and shake gently for 15 min. Add 5 ml disodium phenol phosphate (6.75 g disoudium phenol phosphate dissolved in 1000 ml distilled water) and 5 ml responding buffer (pH5.0 acetate buffer for acidic phosphatase, pH 7.0 citrate buffer for nutral phosphatase, and pH 10.0 borate buffer for alkali phosphatase), disodium phenolphosphate replaced by 5 ml distilled water was used as control for each soil sample. Shake and mix carefully. Place into the incubator. Incubate at 37 °C for 24h.

Dilute the mixture inside the flask with 38 °C hot water till 50 ml (toluene floating up the scale-line) and filter. Take 1 ml filtrate and add into 100 ml volumetric flask. Add 5 ml pH 9.0 borate buffer and 3 ml 2.5 % potassium ferricyanide and 3 ml 0.5% 4-Aminoantipyrine. Shake and mix carefully. Add distilled water to volume. Optical density of the stable pink liquid after 30 min was measured at 570 nm. The phosphatase activity was calculated as phenol milligram per gram soil.

Extraction and purification of pot soil total DNA and DNA amplification with PCR

Each soil sample was homogenized before the total DNA was extracted from 1.0 g of the sample using a PowerSoil® DNA Isolation Kit based on the instruction manual provided by the manufacturer (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The DNA fragments were amplified with 16sDNA using GC-clamp primers (338F-GC: 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGG AGGCAGCAG-3', 518R: ATT ACC GCG GCT GG) for soil bacterium (Muyzer et al., 1993) and NS1-GC fung primers (White et al., 1990; May et al., 2001; Luo et al., 2009) for soil fungus (NS1: 5'-GTAGTCATATGCTTGTCTC-3', GC-fung: 5'-GC clamp-ATTCCCCGTTACCCGTTG-3' with GC clamps 5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCC CCGCCCC-3'). PCR amplification of a 16S rRNA gene fragment was performed with the primers 338F + GC and 518R to amplify the V3-V6 region. PCR amplification of an 18S rRNA gene fragment was performed with the primers GC -fung and NS1 to amplify the IST3 region. All bacterial and fungal PCRs were conducted in 25-µl and 50-µl volumes, respectively. The total soil genomic DNA extracted and purified was eluted with 100 µl of the DNA elution solution included in the kit.

The bacterial PCR system was composed of 1.0 µl of DNA template, 2 µl of forward primer, 2 µl of reverse primer, 12 µl of 2× PCR mix (*TaKaRa Taq polymerase* 1.25U/25 µl, TaKaRa, Dalian, China, dNTP Mixture 2×conc., 0.4 mM each, *Taq* Buffer 2×conc., 3 mM Mg^{2+}, dye Marker Tartrazine/Xylene Cyanol FF), and 10 µl of double-distilled water in a final volume of 25 µl. The bacterial PCR was conducted in a TP professional Standard Thermocycler (Biometra, Germany) and consisted of an initial denaturation at 94 ºC for 5 min, 35 cycles of denaturation at 94 ºC for 45 s, annealing at 55 ºC for 45 s, and extension at 72 ºC for 60 s, and a final extension at 72 ºC for 10 min.

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The fungal PCR system was composed of 2.0 μl of DNA template, 2 μl of forward primer, 2 μl of reverse primer, 25 μl of 2× PCR mix (*TaKaRa Taq polymerase* 1.25U/25 μl, TaKaRa, Dalian, China, dNTP Mixture 2×conc., 0.4 mM each, *Taq* Buffer 2×conc., 3 mM Mg^{2+}, dye Marker Tartrazine/Xylene Cyanol FF) and 19 μl of double-distilled water in a final volume of 50 μl. The fungal PCR was conducted in a TP professional Standard Thermocycler (Biometra, Germany) and consisted of an initial denaturation at 94 ºC for 4 min, 36 cycles of denaturation at 94 ºC for 60 s, annealing at 55 ºC for 45 s, an extension at 72 ºC for 45 s, and a final extension at 72 ºC for 10 min.

DGGE profiling and microbial community and diversity analysis of soil DNA

DGGE analysis was performed using the Dcode Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA) according to the instruction manual provided by the manufacturer (Quantity One software). Twenty-five milliliters of the PCR products obtained from the bacterial DNA and 50 ml of the PCR products obtained from the fungal DNA were loaded in each well, and electrophoresis was run at 60 ºC for 10 min at 200 V and for 16 h at 80 V in an 8 % acrylamide/bis-acrylamide (37.5:1) gel with a 40-60 % denaturant gradient for bacterial DNA and 25-40 % for fungal DNA, where 100 % denaturant contained 7 M urea and 40 % (v/v) formamide (deionized). The gels were silver stained (Radojkovic and Kusic, 2000) and were digitized and analyzed using the Quantity One 4.0 gel analysis software (BioRad). Because each migration position typically corresponds to a different sequence variant, each matrix obtained was considered a taxa presence-absence matrix for statistical analyses. The bands were numbered in order of appearance from the top to the bottom of the gels.

Statistical analysis of data

The data are presented as the means (±SE) from three replicates for each treatment. One-way analysis of variance (ANOVA) was performed using the SPSS Base Ver.11.5 statistical software (SPSS, IL, Chicago, USA). Least significant difference tests were used to test for significant differences in soil enzyme activities (soil CAT, urease, invertase and phospatase) exposed to different root exudates concentrations of transgenic cotton. The significance levels for all analyses were set at $p<0.05$.

The Shannon diversity index for microbial communities was calculated using the following equation: $H'=-\Sigma P_i \ln P_i$, where P_i is the relative abundance for the i^{th} band in the DGGE profiles. The Evenness index for microbial communities was calculated as $E=H'/\ln R$, where R (richness) represents the total bands in the DGGE profiles. The Stability index for microbial communities was calculated by $S=\Sigma(Pi/P_i max)/n$, where $P_i max$ is the maximal abundance in the i^{th} band in the DGGE profiles and n is the number of samples in the DGGE profiles.

Results

Effect of root exudates of transgenic cotton seedlings on soil catalase activity

The root exudates of transgenic or non-transgenic cotton seedlings increased the soil CAT activity first and decreased the acitivity of soil CAT when peaking at 2 ml/350 g soil. Compared with control (1.25 $KMnO_4$ ml g^{-1} soil), the soil CAT activity treated with root exudates of transgenic cotton seedlings increased by 21.8 % to 32.7 %,but there was no significant difference between transgenic and non-trangenic cotton (*Fig. 1*).

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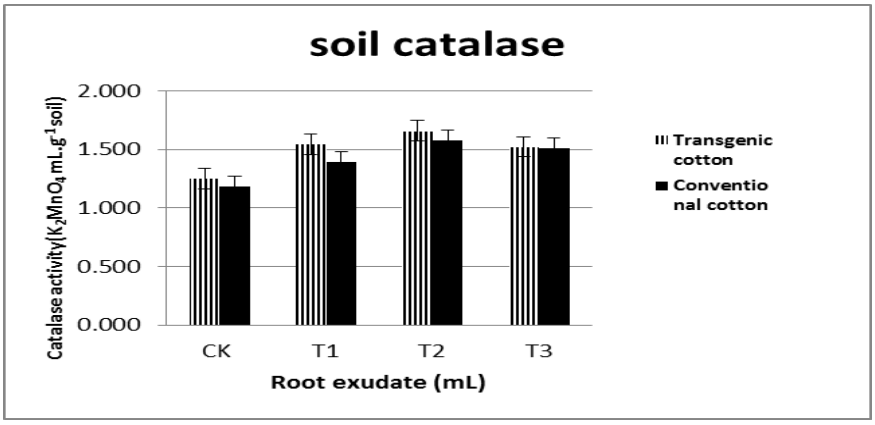

Figure 1. *Effect of root exudates from transgenic and nont-transgenic cotton seedlings (Xinluhan-33, containing Bt and CpTI genes) on the soil catalase(CAT) activity. The letters CK, T1, T2, and T3indicated the volume of the concentrated transgenic or non-transgenic cotton root exudates added into the pot soil. Bars are means + SE. Means with the same letters were not significantly different in LSD tests ($p<0.05$).*

Effect of root exudates of transgenic cotton seedlings on soil urease activity

The soil urease activity treated with root exudates of transgenic and non-transgenic cotton seedlings was firstly higher than control but then slightly higher and finally less than the control at the highest concentration (*Fig. 2*). In the highest concentration (4 ml/350 g soil), the soil urease activity treated with transgenic cotton was decreased by 11.0 % while the activity in the treatment of 1 ml/350 g soil was increased by 18.1% compared to control. No significant difference between transgenic and conventional cotton.

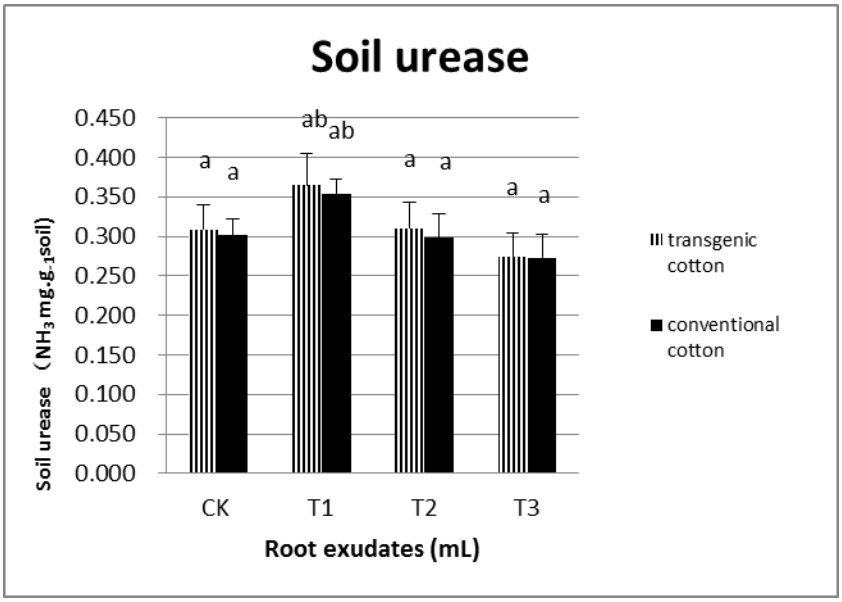

Figure 2. *Effect of root exudates from transgenic and nont-transgenic cotton seedlings (Xinluhan-33, containing Bt and CpTI genes) on the soil urease activities. The letters CK, T1, T2, and T3indicated the volume of the concentrated transgenic or non-transgenic cotton root exudates added into the pot soil. Values are means ±SE. Means with the same letters were not significantly different in LSD tests ($p<0.05$).*

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Effect of root exudates of transgenic cotton seedlings on soil invertase activity

Root exudates of transgenic or conventional cotton seedlings could significantly affect the invertase activity of soil. Except the soil invertase activity treated with 1 ml/350 g soil was abruptly ascended, the activity in the other treatments was all sharply descended (*Fig. 3*). The invertase activity in the treatment of 1 ml/350 g soil was increased by 54.2 %, however, the activity in the treatments of 2 and 4 ml/350 g soil was decreased by from 25 % to 29.2 % relative to control. No significant difference was found between treatment of transgenic and non-transgenic cotton.

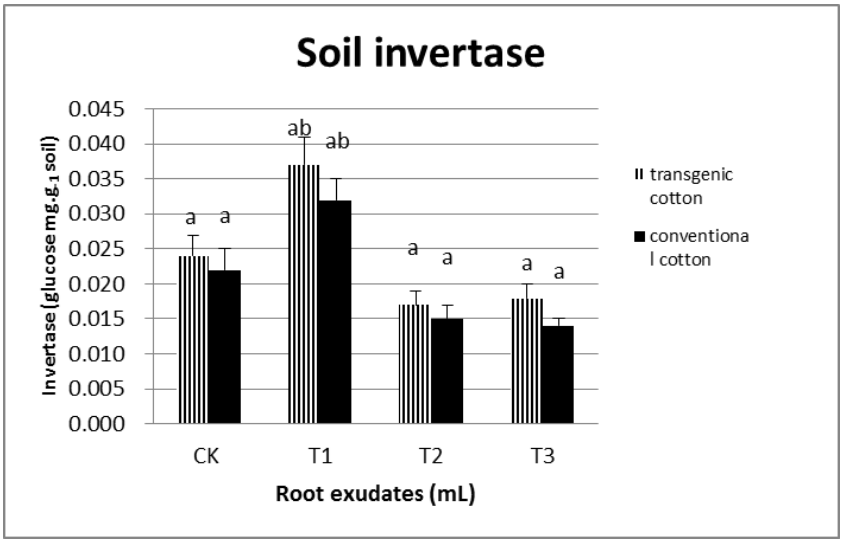

Figure 3. *Effect of root exudates from transgenic and nont-transgenic cotton seedlings (Xinluhan-33, containing Bt and CpTI genes) on the soil invertase activity. The letters CK, T1, T2, and T3 indicated the volume of the concentrated transgenic or non-transgenic cotton root exudates added into the pot soil. Values are means ±SE. Means with the same letters were not significantly different in LSD tests ($p<0.05$).*

Effect of root exudates of transgenic cotton seedlings on soil phosphatase activity

The soil phosphatase activity in all treatments of root exudates of transgenic or conventional cotton seedlings was higher than control (*Fig. 4*). But the increment of the soil phosphatase activity depended on the concentrations. The increase degree of the phosphatase activity was from 29.8 % to 63.1 % regarding to control. However, the activity was gradually declined with increasing concentration. Still, no significant variation between transgenic and non-transgenic cotton was obtained.

Effect of root exudates of transgenic cotton seedlings on soil bacterial and fungal community and diversity

The total 16sPCR-DGGE lane bands of bacterial community and diversity in soil treated with root exudates of transgenic or non-transgenic cotton seedlings were almost kept unvaried. The shannon index and even index were changed little. However, the stability index decreased markedly with increasing concentration, particularly in the highest concentration (4 ml/350 g soil). This indicated there is a little effect of root exudates of transgenic cotton on soil bacterial community while the bigger effect on soil

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bacterial community was obtained from the farther phylogenetic distance with control (*Fig. 5*). However, totally, the construction and composition of the soil bacterial community and diversity were not altered compared to control from the bacterial population stability. No obvious changes between transgenic and non-transgenic cotton were observed. This demonstrated that the key and dominant bacterial populations and community in the soil were not affected by the root exudates of transgenic cotton seedlings.

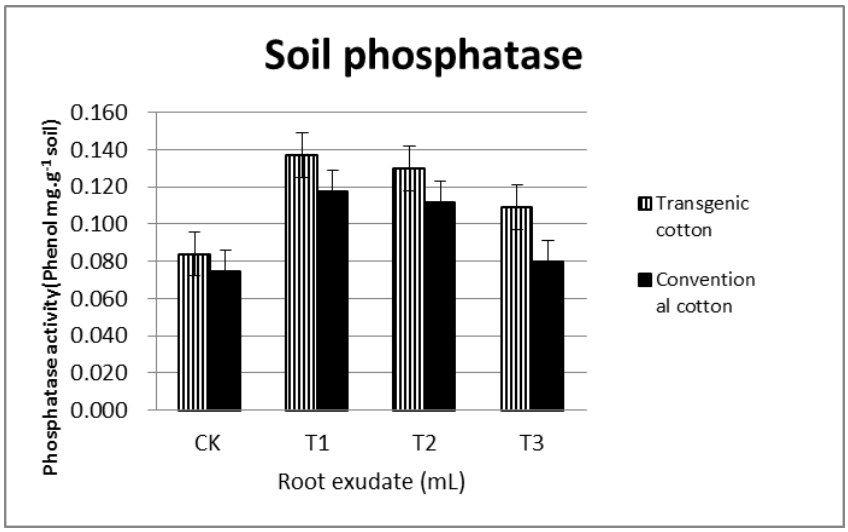

Figure 4. *Effect of root exudates from transgenic and nont-transgenic cotton seedlings (Xinluhan-33, containing Bt and CpTI genes) on the soil phosphatase activity. The letters CK, T1, T2, and T3 indicated the volume of the concentrated transgenic or non-transgenic cotton root exudates added into the pot soil. Values are means ±SE. Means with the same letters were not significantly different in LSD tests ($p<0.05$).*

However, the effect pattern of root exudates of transgenic cotton on soil fungal community and diversity was differed from bacteria. From total lane bands of 18 s PCR-DGGE profile, the fungal community was significantly decreased ranging from 37 bands to 18 (*Fig. 6,* fungi). *Fig. 6* showed the fungal community number and composition were changed. Some dominant community (bright lane bands) disappeared and other newly dominant community appeared. This clearly indicated root exudates of transgenic cotton could inhibit some fungus and stimulate some other fungus population. This was also evaluated from the evolution distance of phylogenetic tree of soil fungi based on 18s PCR-DGGE (*Fig .6*). Further analysis from diversity index, Shannon index for fungi was decreased depending on the concentration, while even index was little varied, showing evenly decrease of fungal species number by root exudates. However, the fungal stability index was increased with the increasing concentration of root exudates of transgenic cotton, indicating the fungal population and community in the soil was not affected by the much higher concentration of root exudates of transgenic cotton.

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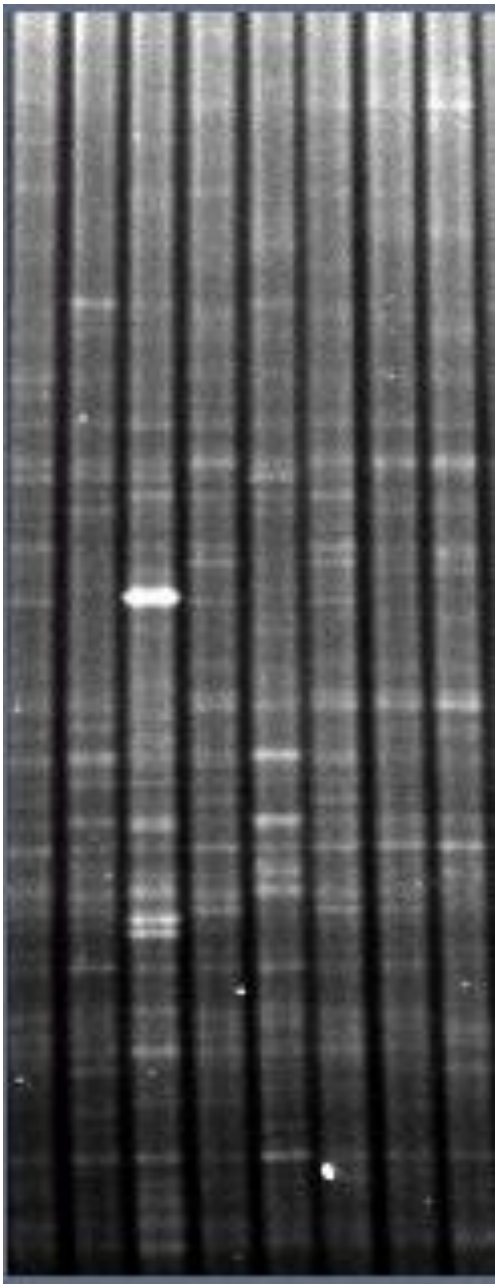

Figure 5. *Effect of root exudates from transgenic and nont-transgenic cotton seedlings (Xinluhan-33, containing Bt and CpTI genes) on the soil bactrial community and diversity based on 16s PCR-DGGE for bacteria. The letters CK, T1, T2, and T3 indicated the volume of the concentrated transgenic and non-transgenic cotton root exudates added into the pot soil. From left to right, lane 1, 2,3,4,5,6,7,8 responded to treatments of 0ml (control:no root exudates of transgenic cotton added), 0ml (control:no root exudates of non-transgenic cotton added), 1ml (root exudates of transgenic cotton added), 1ml (root exudates of non-transgenic cotton added), 2ml (root exudates of transgenic cotton added), 2ml (root exudates of non-transgenic cotton added), 4ml (root exudates of transgenic cotton added), 4ml (root exudates of non-transgenic cotton added).*

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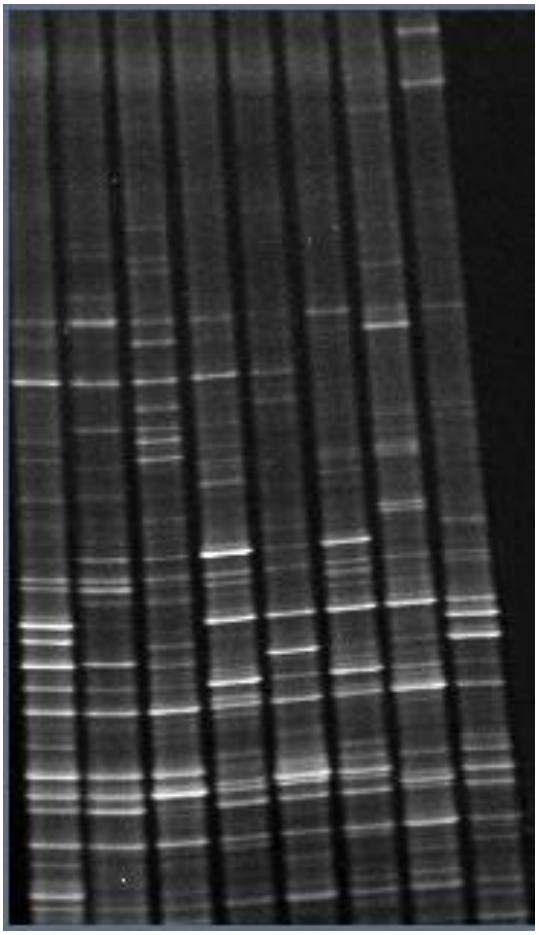

Figure 6. *Effect of root exudates from transgenic and nont-transgenic cotton seedlings (Xinluhan-33, containing Bt and CpTI genes) on the soil fungal community and diversity based on 18s PCR-DGGE for fungi. The letters CK, T1, T2, and T3 indicated the volume of the concentrated transgenic and non-transgenic cotton root exudates added into the pot soil. From left to right, lane 1, 2,3,4,5,6,7,8 responded to treatments of 0ml (control:no root exudates of transgenic cotton added), 0ml (control:no root exudates of non-transgenic cotton added), 1ml (root exudates of transgenic cotton added), 1ml (root exudates of non-transgenic cotton added), 2ml (root exudates of transgenic cotton added), 2ml (root exudates of non-transgenic cotton added), 4ml (root exudates of transgenic cotton added), 4ml (root exudates of non-transgenic cotton added).*

Discussion

Environmental problems related to plant genetic engineering may prohibit advancement of this technology and prevent realization of its full potential. One such common concern is the demonstrated escape of foreign genes through pollen dispersal from transgenic crop plants to their weedy relatives, creating super weeds or causing gene pollution among other crops or toxicity of transgenic pollen to non-target insects (Daniell, 1999). Another common issue is the effect of transgenic crops on their parental lines and other plants because plants interact with each other through root exudates in soil. Besides ecological effects on organisms in the aboveground compartment, effects on the below-ground

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compartment, in particular on soil and rhizosphere, have gained increasing attention. Soil has been recognized as a valuable resource for agriculture and therefore it has to be managed in a sustainable manner in order to maintain its quality (Widmer, 2007). The effect of transgenic Bt- and CpTI- cotton root exudates on the soil enzymes activities and soil microbial community and diversity was investigated in the current study.

Soil microbes and enzymes activities play a key role in the material decomposition, nutrients elements transformation, release and cycling, organic matter formation, soil fertility, promoting/suppressing plant growth, and various soil biological-physical-chemical processes (Zhang et al., 2014; Widmer, 2007; Critter et al., 2004; Hu et al., 2011). Soil enzymes can accelerate the above process, where the soil urease (urea amidohydrolase EC 3.5.1.5: catalyzing the hydrolysis of urea to CO_2 and NH_3) is very widely distributed in nature, hence, the efficient use of urea fertilizer in soil and the changes in urease activity can be used as an indirect indicator of the variation in the pool of potentially available N in a soil (Kulandaivelu et al., 2013; Rao and Ghai, 1985; Tabatabai, 1994; Bremner and Mulvaney, 1978). Activities of soil urease, acid phosphomonoesterase, invertase, and cellulase were stimulated by the addition of Bt cotton tissues (Sun et al., 2007). Catalase (H_2O_2 oxidoreductase, E.C. 1, 11, 1.6) is widely associated with the tissues of animals, higher plants, and aerobic micro-organisms. Activities of catalase and various other enzymes in soils have been correlated with such soil variables as particle size, carbon content, nitrogen content, numbers of micro-organisms, and fertility.

Catalase activity of a cultivated soil was correlated with bacterial and fungal counts, cation exchange capacity, dehydrogenase activity, and cotton yield (Rodriguez-Kababa and Truelove, 1982). Soil catalase activity is considered as a sensitive indicator of aerobic microorganisms' activity and is related to both the number of the aerobic microorganism and soil fertility (Shiyin et al., 2004).Our results of several soil enzymes activities were also in agreement with this increase trend in the experimental concentrations much higher than possible actual concentrations of root exudates or residues of transgenic Bt and CpTI cotton, as the concentrations were condensed 1000-time in this study. However, the CAT activity was not always increased. Actually, the root exudates of transgenic cotton seedlings increased the soil CAT activity first and decreased the acitivity of soil CAT when peaking at 2 ml/350 g soil (*Fig. 1*). Our results showed the soil urease activity treated with root exudates of transgenic cotton seedlings was firstly higher than control but then slightly higher and finally less than the control at the highest concentration (*Fig. 2*).

Phosphatases have been extensively studied in soil, because they catalyse the hydrolysis of ester–phosphate bonds, leading to the release of phosphate (P), which can be taken up by plants or microorganisms . It has been shown that the activities of phosphatases (like those of many hydrolases) depend on several factors such as soil properties, soil organism interactions, plant cover, leachate inputs and the presence of inhibitors and activators (Speir and Ross, 1978; Nannipieri et al., 2010). Acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) are phosphomonoesterases which are believed to play a significant role in soil P cycling, where these enzyme activities have been suggested as potential components of groups of indices to assess soil quality (Staddon et al., 1998). In the current study, the soil phosphatase activity in all treatments of root exudates of transgenic cotton seedlings was higher than control (*Fig. 4*). The increase degree of the phosphatase activity was from 29.8 % to 63.1 % regarding to control. However, the activity was gradually declined with increasing concentration. This was conformed by Shen et al. (2006) there were few

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significant differences in enzyme activities between Bt and non-Bt cottons at any of the growth stages and after harvest (Shen et al., 2006)

The soil invertase drives C cycling by catalyzing the hydrolysis of sucrose – thus, testing the activity of soil invertase may be useful for evaluating soil capability of decomposing complex organic compounds into subunits that can be assimilated by microorganisms or plants, in other words, such enzymatic indices would integrate other chemical, physical, and biological characteristics and could monitor the effects of agricultural management on soil's long-term productivity (Hu et al., 2011; Mikhailouskaya and Bogdevitch, 2009). In the present investigation, root exudates of transgenic cotton seedlings could significantly affect the invertase activity of soil. Except the soil invertase activity treated with 1 ml/350 g soil was abruptly ascended, the activity in the other treatments was all sharply descended (*Fig. 3*). The invertase activity in the treatment of 1 ml/350 g soil was increased by 54.2 %, however, the activity in the treatments of 2 and 4 ml/350 g soil was decreased by from 25 % to 29.2 % relative to control. However, there was no significant difference among them. There were few significant differences in enzyme activities between Bt and non-Bt cottons at any of the growth stages and after harvest; amendment with cotton biomass to soil enhanced soil enzyme activities, but there were no significant difference between Bt and non-Bt cotton; the richness of the microbial communities in rhizosphere soil did not differ between Bt and the non-Bt cotton; the functional diversity of microbial communities were not different in rhizosphere soils between Bt and non-Bt cotton indicating no adverse effects of Bt cotton on the soil ecosystem (Shen et al., 2006). This was comply with our results.

Although there is large-scale adoption of Bt cotton by the farmers as immediate financial gain, there is concern that Bt crops release Bt toxins into the soil environment decreasing soil chemical and biological activities (Singh et al., 2013).Significantly higher dehydrogenase enzyme activity and $KMnO_4$-N content of soil were observed in Bt cotton with cover crop of peanut over pure Bt cotton followed by pure peanut at all the crop growth stages (Singh et al., 2013).

Higher microbial population in soil was maintained by pure peanut over intercropped Bt cotton. By growing cover crop of peanut between Bt cotton rows, bacteria, fungi, and actinomycetes population increased by 60 %, 14 %, and 10 %, respectively, over Bt cotton alone. Bt cotton fertilized by combined application of urea and farm yard manure maintained higher microbial population over urea alone. Significant positive correlations were observed for microbial population of soil of Bt cotton, which indicates no harmful effects of Bt cotton on soil biological parameters and associated cover crop (Singh et al., 2013). This was also supported by our results. The total 16s PCR-DGGE lane bands of bacterial community and diversity in soil treated with root exudates of transgenic cotton seedlings were almost little different, indicating almost no variation of bacterial community and diversity. Further, the shannon index and even index were changed little (*Fig. 5*), while the bigger effect on soil bacterial community was obtained from the farther phylogenetic distance with control (*Fig. 5*). However, totally, the construction and composition of the soil bacterial community and diversity were not altered compared to control from the bacterial population stability. This demonstrated that the key and dominant bacterial populations and community in the soil were not affected by the root exudates of transgenic cotton seedlings.

However, the effect pattern of root exudates of transgenic cotton on soil fungal community and diversity was differed from bacteria. From total lane bands of 18s PCR-DGGE profile, the fungal community was significantly decreased ranging from 37

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bands to 18 (*Fig. 6,* fungi), where the fungal community number and composition were altered. Some dominant community (bright lane bands) disappeared and other newly dominant community appeared. This clearly suggested root exudates of transgenic cotton could inhibit some fungus and stimulate some other fungus population. This was also further evaluated from the evolution distance of phylogenetic tree of soil fungi based on 18s PCR-DGGE (*Fig .6*, fungi). Fungal diversity index, Shannon index for fungi in soil was decreased depending on the concentration of transgenic cotton, while even index was little varied, showing evenly decrease of fungal species number by root exudates (fungi). However, the fungal stability index was increased with the increasing concentration of root exudates of transgenic cotton, indicating the fungal population and community in the soil was not affected by the much higher concentration of root exudates of transgenic cotton (fungi). All these demonstrated that there was no significant adverse effect of transgenic Bt and CpTI-cotton on soil biology and microbes.

However, the concentration of Bt and CpTI cotton plant in the field soil was not reached so much high, maybe 1/10-1/100 as much as this study. Hence, the change of both soil enzymes activity and soil microbial community and composition in the current work will not be likely to happen. Of course, the investigation for a long term, 3-5 year, would need to further verify.

Conclusions

There was little but not significant adverse impact of transgenic Bt+CpTI cotton root exudates on soil microorgnisms and soil enzymes activity based conventional and molecular investigation, indicating posing little risks of Bt and CpTI-cotton to soil ecosystem. Though some changes of soil fungal community, biodiversity and composition were found, the so high concentrations of root exudates of transgenic Bt+CpTI cotton (condensed 1000 times) would not be possibly occurred in the actual field soil.

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SEMI-CONTINUOUS ANAEROBIC CO-DIGESTION OF COW MANURE AND BANANA WASTE: EFFECTS OF MIXTURE RATIO

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Abstract. Anaerobic co-digestion of cow manure (CM) and banana waste (BW) was investigated. The experiments have been carried out and were tested for different mixture ratios of CM:BW in terms of volatile solids : 100:0, 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100 with the aim to maximize methane yield and methane production from the anaerobic co-digestion of cow manure and banana waste. The results obtained show that a maximum methane yield of 229 mL_{STP}/g VS added was obtained at a (CM): (BW) ratio of was 40:60 with a biodegradability of 86% for an organic loading rate in the range of 1.49-3.57 kg waste mixture/(m^3d). The pH and alkalinity for all experiments were controlled and kept within an optimum interval of 7.1-7.6 for pH and 2-4 g $CaCO_3$/L for alkalinity. It was shown that banana waste and cow manure could easily be degraded by anaerobic co-digestion under mesophilic conditions, resulting in methane yields sufficient for commercial production of methane.
Keywords: *anaerobic co-digestion, mixture ratio, cow manure, banana waste, mesophilic*

Introduction

Anaerobic digestion is a sustainable green technology that has been extensively adapted for the treatment of various types of biological waste materials. The main product of this process is biogas, a renewable energy, formed mainly of methane and carbon dioxide. Co-digestion is the simultaneous anaerobic digestion of two or more organic wastes in one digester, it is used to increase methane production from low-yielding or difficult to digest materials. It has been shown that it enhances the biogas production, especially when treating certain residues such as cattle manure (Marañón et al., 2012). In the agricultural sector, anaerobic digestion has been established as a valuable resource for the production of biogas as a renewable source of energy; hence animal manures have become a requested and important raw material (Mata-Alvarez et al., 2010; Mata-Alvarez et al., 2011). The methane potential of manure comes from the digestion of the organic components in the faeces and in the straw used as bedding material, which is mainly: carbohydrates, proteins and lipids (Moller et al., 2004).

Cow manure, which contains water, nutrient and minerals as well as important microbes, is an excellent substrate for the production of biogas when co-digested with other kinds of waste materials such as organic industrial waste, household waste and sewage sludge. As such, cow manure serves as an excellent "carrier" substrate during the mixed digestion of wastes and allows anaerobic digestion of concentrated industrial waste, which would be difficult to treat separately (Angelidaki and Ellegaard, 2003). Its

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high water content acts as solvent for dry waste materials, its high buffering capacity regulates the optimum pH level in the reactor, and its high concentration of nutrients, is required for the optimal growth of bacteria (Angelidaki and Ellegaard, 2003). However, due to its low contents of carbon sources; anaerobic digestion of manure is often associated with poor methane yields (Mata-Alvarez et al., 2010; Mata-Alvarez et al., 2011). Thus, Co-digestion of manure with other substrates has been applied as a cost-effective alternative to improve the process efficiency, and consequently resulting in economically feasible methane yields of biogas plants (Astals et al., 2013; Frigon et al., 2012; Banks et al., 2011). The advantages of co-digesting of animal manure together with other kinds of waste materials have been reported in different research studies. In that way, Angelidaki and Ellegaard (2003) reported the increase in biogas yield due to co-digestion of cow manure together with waste materials in anaerobic digestion process.

Banana residues are among the best anaerobic digestion feedstosk, due to its high organic composition. In agricultural sector, banana is the world's fourth most produced food commodity, after rice, wheat and apple. Bananas are grown in more than hundred countries, mostly in the developing world where they represent an important staple food (Khan et al., 2009). The cultivation, harvesting and processing of banana generates huge amounts of waste, mainly from leaves, stems and peels and to some extent the degraded banana fruit itself (Khan et al., 2009). Indiscriminate disposal of these wastes exhibits an environmental hazard due to the release of foul gases such as hydrogen sulphate and ammonia during its decomposition. The disposed waste materials serve also as a potential hatchery for insects and pests (Khan et al., 2009). Fruit-processing wastes, especially banana waste is highly biodegradable because of their rich organic matter and high moisture content. It has been found earlier that biowaste residues with a moisture contents above 50% are more suitable for bio-conversion processes rather than thermal-conversion processes (Bardiya et al., 1999). The banana waste is a concentrated source of putrid organic waste, ideal for anaerobic digestion to produce energy while fermentation products can serve as fertilizer with high nutritional value, as well as a valuable energy source in form of biogas (El-Mashad and Zhang, 2010).

The goals of the present research work are to study the effect of mixture ratio at volatile solids basis on a semi-continuous anaerobic co-digestion of cow manure with banana waste (whole fruit), as well as investigating the methane yield, organic loading rate which can be treated, and process stability during the digestion. Seven experiments at laboratory scale were carried out under mesophilic condition using continuously stirred tank reactors. This study can provide a profitable solution for increasing methane potential production by introducing banana waste as co-substrate with cow manure, especially for rural areas and developing countries in which there is a high production of these types of waste.

Materials and methods

Experimental Setup

The reactors used in the laboratory for anaerobic digestion were continuous stirred-tank reactors (CSTR), with a volume of one litre (*Figure 1*). These reactors have four orifices, the first to insert the substrate, the second one for the ventilation of biogas, the third for inert gas injection (nitrogen) to maintain anaerobic conditions, and the last one to remove effluents. The content of the reactors was mechanically stirred and

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mesophilic conditions were maintained using thermostatic jacket containing water heated to 37°C. All reactors were worked in semi-continuous mode. The volume of methane produced during the process was measured using 1.5-L Boyle-Mariotte reservoirs connected to each reactor. To remove the CO_2 produced during the process, tightly closed bubblers containing a NaOH solution (6 N) were connected between the two elements. The volume of methane displaced an equal measurable volume of water from the reservoirs. This volume was corrected in order to remove the effect of water steam pressure and the measured methane was then expressed at standard temperature and pressure conditions (STP: 0ºC and 1 atm) (Belhadj et al., 2014).

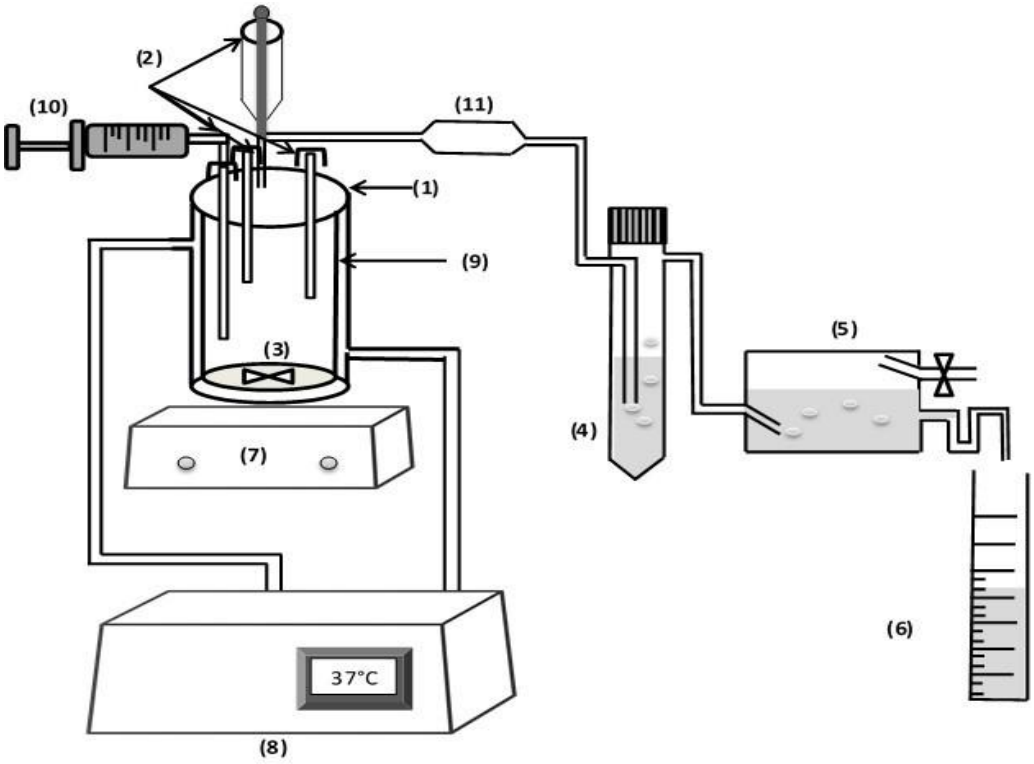

Figure 1. *Schematic diagram of CSTR reactor system used. 1-CSTR digester; 2-Inlet/outlet sample; 3-Magnetic stir bar; 4-Tightly closed bubblers containing a NaOH solution (6 N); 5-Reservoir containing water; 6-Graduated cylinder; 7-Magnetic stirrer; 8-Thermostatic bath; 9-Thermostatic jacket; 10-Syringe; 11-Biogas sampling tube.*

Table 1. *Experimental design*

Digesters	**CM :BW (based on VS)**
R1	100 :0
R2	80 :20
R3	60 :40
R4	50 :50
R5	40 :60
R6	20 :80
R7	0 :100

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Substrate

The substrates used for the anaerobic co-digestion were cow manure and banana waste. Cow manure was collected from Tiflet farm (West of Morocco) and stored in the laboratory at 4°C until usage. Banana waste was collected from a local supermarket. It was mixed, ground and subsequently frozen to -4°C in plastic containers for subsequent storage. The main characterisation of both cow manure and banana waste are shown in *Table 2*.

Table 2. *Substrates and mixtures ratios main characterization.*

		pH	Alkalinity (g $CaCO_3$/L)	Total Solids (g/kg)	Volatile Solids (g/kg)	VS/TS(%)
CM :BW	100 :0	7.40±0.10	1.80±0.05	169±2.56	142.12±1.45	84±4
	80:20	7.4±0,1	3.10±0.50	168.38±0.50	140.30±1.87	83±1
	60:40	7.1±0,1	2.60±0.15	161.85±2.43	136.5±1	85±1
	50:50	6.8±0,1	2.10±0.3	155.25±0.07	134.75±0.6	87±1
	40:60	6.4±0,1	1.40±0.25	145.50±0.9	135.6±1	94±2
	20:80	5.8±0,2	0.65±0.01	143.04±0.6	131.1±1.18	91±1
	0 :100	5±0.10	0.90±0.01	146.36±0.86	136.57±1.31	93±03
Inoculum		6.7±0.2	ND	29.10±0.30	20.50±0.20	70±2

Result = mean ± standard deviation (SD)
ND: not determined

The reactors were initially loaded with 7 g VS/L of digested sludge from a municipal wastewater treatment plant (MWTP) as inoculum.

The pH value is a measure of the acidity or alkalinity of the liquid content of the reactor. Most methanogenic microorganisms have an optimum pH level for growth between 7 and 8, while the acid-forming bacteria often have a lower optimum pH level for optimal growth (Raposo et al., 2011; Angelidaki and Sandres, 2004). If the pH value of the waste to be tested is outside the optimal range, and if there is insufficient buffer capacity, the anaerobic process will be inhibited. During all experiments, the pH was controlled before and after each load.

Experimental procedure

In order to bio-stimulate the biomass prior to the experiments, reactors were first fed with a synthetic solution composed of glucose, sodium acetate and lactic acid. During this initial period, the organic load added to the reactors was gradually increased from 0.5 to 1.0 g VS/L over a 15-day period. For acclimatization of the biomass resources, the reactors were fed with loads of 0.5g VS/L in which the percentage of mixtures in the feeding process was increased from 25% to 100% after several loads. Subsequently, during each set of experiments with pure mixture, the organic load added to the reactors

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was gradually increased. In all cases three replicates of each load were fed to the digesters in order to ensure reproducibility and the volume of methane was measured as a function of time and samples were taken and analysed before and after feeding.

Chemical analysis

Analysis of the total solids (TS), the minerals solids, the volatile solids (VS), the pH, and the bicarbonate alkalinity, were performed according to the Standard Methods for the examination of Water and Wastewater (APHA, 1989). For the determination of total solids, the samples were dried at 105°C for 24 h, and total solid contents were calculated from the differences between weights before and after drying. The dried matters were heated at 550°C for 2 h, and organic matter contents were calculated from the losses on ignition. Alkalinity was determined by a titration method at pH 4.5.

Results and discussion

Stability parameters of the process

The pH evolution

Figure 2 shows the evolution of pH value during the period of anaerobic digestion, the pH value remained stable throughout the digestion process and there was no need to add alkalinity to the digester. To avoid an underestimation of the methane potential, most anaerobic digsetion experiments are carried out at pH values ranging from 7.0 to 7.8. If the pH value needs to be adjusted, diluted solutions of NaOH, lime, or an acid solution such as HCl, could be used (Li et al., 2011). In our experiments we usually use the normality of 0.1mol/L for both solutions.

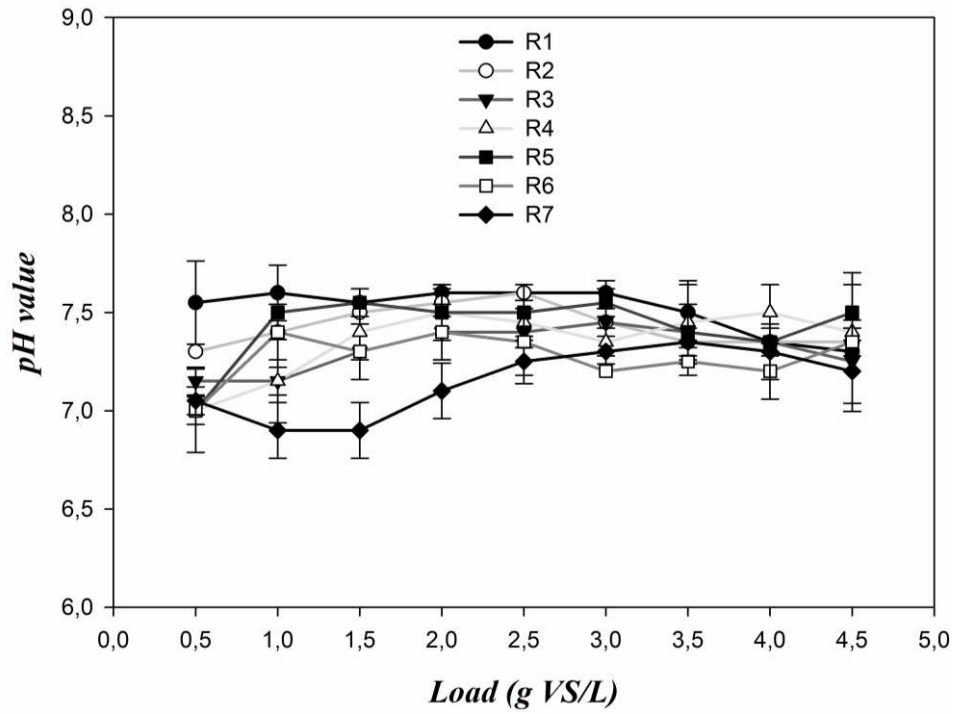

Figure 1. *pH evolution in the substrate treatment phase of all anaerobic co-digestion experiments.*

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Alkalinity assessment

All experiments have shown an acceptable and stable range between 2 and 4 g $CaCO_3$/L of alkalinity during the process. The evolution of alkalinity throughout the different experiments is shown in *Figure 3*. (Nizami et al., 2009) reported that a concentration of 2–5 g $CaCO_3$/L provides a sufficiently high buffering capacity allowing a large increase in volatile fatty acids (VFA) at a minimum decrease of the pH value. It is also known as buffering capacity, due to the presence of various compounds mainly bicarbonate, carbonate and hydroxides. (Raposo et al., 2011).

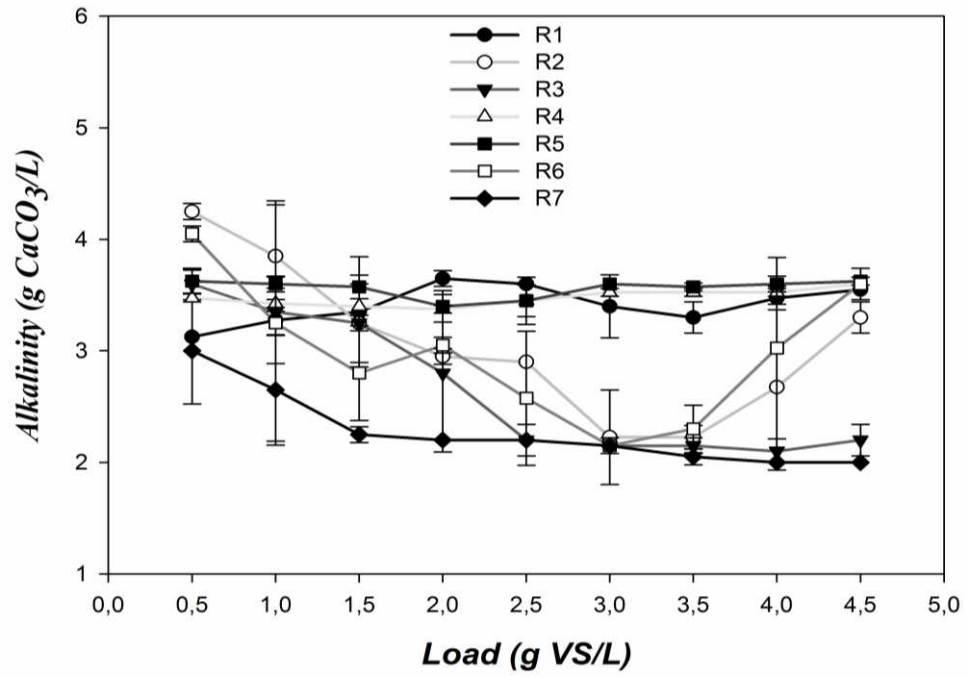

Figure 2. *Alkalinity evolution in the substrate treatment phase of all anaerobic co-digestion experiments.*

Biodegradability and methane yield

As shown in the *Figure 4*, the anaerobic digestion of cow manure gave a low biodegradability (50%), and the increasing of the ratio of banana waste in the mixture improved the treatment efficacy, while the biodegradability of banana waste used as single substrate in anaerobic digestion was high (94%).

The low biodegradability obtained by anaerobic digestion of cow manure can be justified by the fact that VS in cow manure is not highly degradable compared to that of other animal manures due to the high digestion efficiency of the rumen system in cows, along with their fibrous diet (Ann-Wilkie, 2005).

In addition, it can be observed from *Table 3* that the content of lignin of CM is high 24.4 (%TS) compared with that of BW (8.30 %TS), and as known, the content of lignin of a substrate affects negatively the biodegradability. In that way, the banana biowaste input contributes mainly to increase considerably the biodegradability to 94% (*Figure 4*), which is the positive result of co-digestion also seen in other studies.

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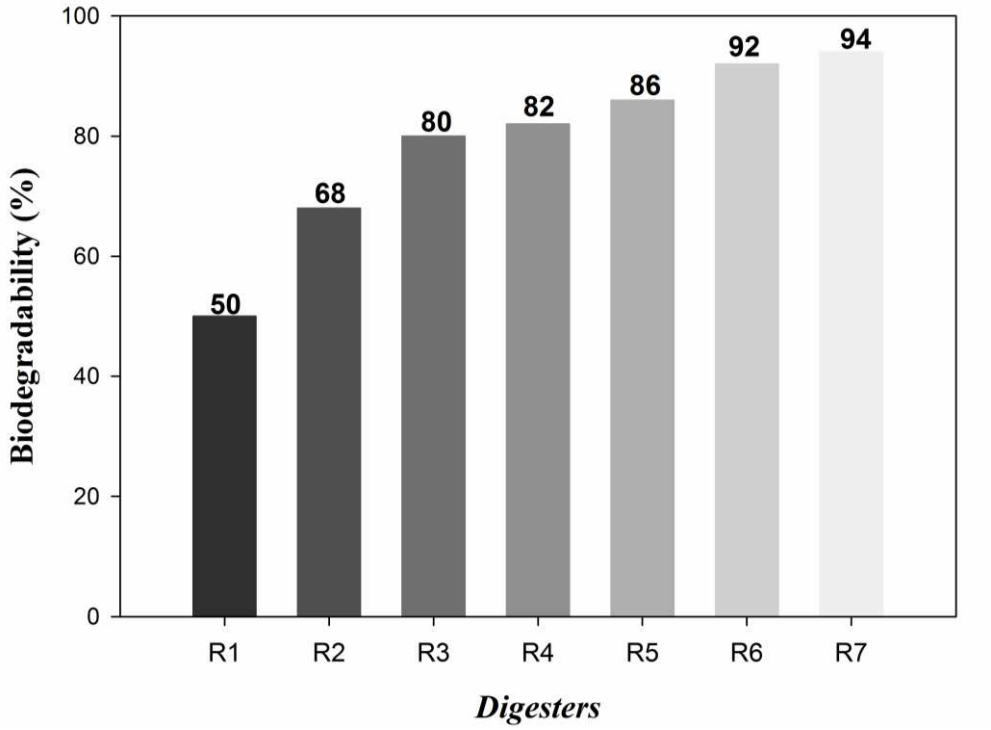

Figure 3. *Biodegradability of all anaerobic co-digestion experiments.*

Table 3. *The full set of characterisation data of cow manure and banana waste (Gustavsson et al., 2014).*

Nutrient and sugar rich feedstock	Total Solids[a]	Ash content (%TS)	Glucan (%TS)	Xylan (%TS)	Arabinan (%TS)	Klason Lignin (%TS)
Cow manure[b]	93.88	20.02	27.61	18.06	2.51	24.14
Banana waste[c]	95.40	6.19	77.50	3.00	3.00	8.30

[a]This is the dry matter of the dried and milled sample.
[b] Nutrient rich feedstock
[c]sugarrich feedstock

For methane yield, as shown in *Figure 5*, by fitting pairs of values of the maximum experimental volume of methane produced in each load (mL_{STP}/L)-VS to a straight line, the methane yield coefficient coincides with the slope of the regression line. *Table 4* shows the methane yield coefficient of all experiments. The methane yield obtained from mono-digestion of CM was 107 mL_{STP}/g VS was slightly lower than reported by Moller et al. (Moller et al., 2004) who obtained a methane yield of 148 mL_{STP}/g VS. Concerning the methane yield obtained by mono-digestion of BW, the value obtained was 316 mL_{STP}/g VS. This value is in line with that obtained by the study of Gunaseelan, (Gunaseelan, 2007), who tested the mesophilic anaerobic digestion of three varieties of banana peel, the methane yield obtained was between 314mL/g VS and 321 mL/g VS. In addition the rich composition in glucose (glucan) of banana waste 77.5% TS (*Table 3*) contribute positively to increase methane production.

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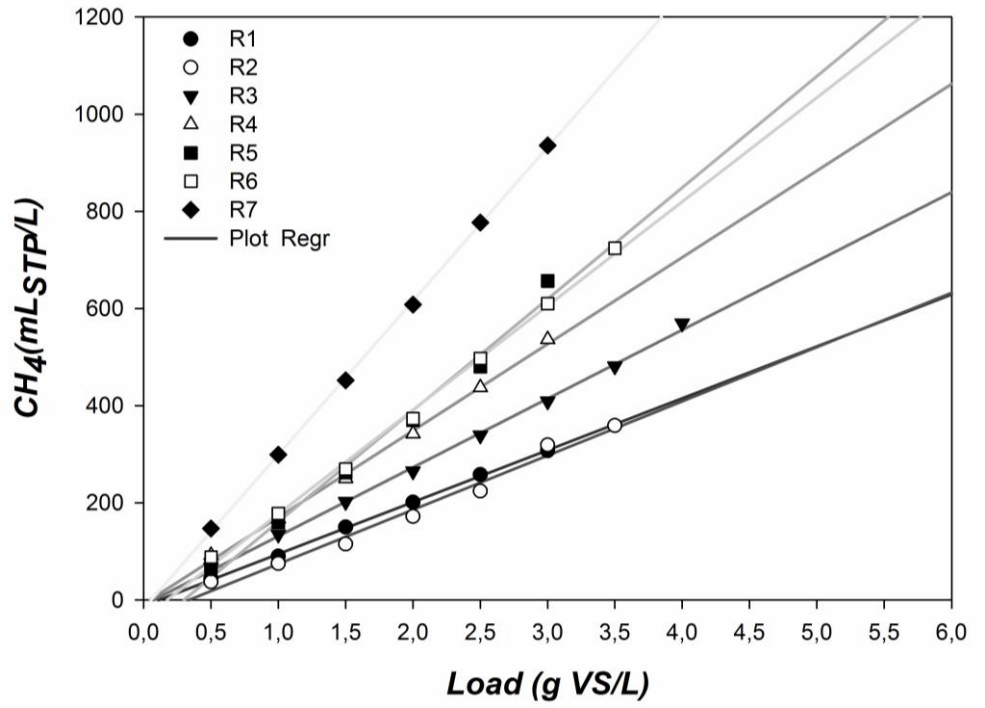

Figure 4. *Variation of the experimental maximum methane volume produced with the VS added to obtain the methane yield coefficient of the process of different digesters.*

For anaerobic co-digestion experiments, the results indicate that an increase in banana waste also increases the methane yield (*Figure 5*). An optimum was reached with a BW concentration of 60 %. There was no further improving when increasing the BW concentration to 80 %, the maximum methane yield of 229 mL_{STP} CH4/g VS was obtained by digester R5 where the CM/BW ratio is 40/60. Thus, our study shows the added value of banana waste as co-substrate with cow manure, and the increasing the proportion of BW from 20% to 60% improve the methane yield from 112 to 229 mL_{STP} CH4/g VS; the results obtained are in line with other findings e.g. Fang et al. (Fang and Angelidaki, 2011) who studied the thermophilc anaerobic digestion of by-products from sugar production with cow manure. They found a methane potential of 240 mL/g VS in CM/Sugar beet by-products of 50:50 (wet basis). It should be noted that despite the high percentage of biodegradability which reached 92% in digester R6, we obtained a methane yield lower than that observed in digester R6 which have a biodegradability of 86%. In fact, Triolo et al. (Triolo et al., 2011) reported that methane yield cannot be directly related to biodegradability, since methane yield reflects the destruction of organic materials, and the methane potential of each organic component in the volatile solids (VS) varies widely.

Kinetics of methane production

Kinetic studies of anaerobic digestion process are useful to predict the performance of digesters and design appropriate digesters and are also helpful in understanding inhibitory mechanisms of biodegradation (Rao and Singh, 2004). A first order kinetic model was used to characterize each set of experiments kinetically as was described by Borja et al. (1995). This kinetic model fit the experimental methane production volumes

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against the time for low substrate concentrations. The kinetic characterization allows comparing the proposed anaerobic co-digestion with other ones (Belhadj et al., 2014).

$$G = G_m \times \left(1 - e^{-K \times X \times t}\right) \quad \text{(Eq. 1)}$$

(Eq. 1) allows relating the accumulated volume of methane (G, mL) with time (t) once the concentration of sludge (X) and the kinetic constant (K) are known. Moreover, the previous equation can be reordered in the form shown in (Eq.2), as microorganism concentration is considered to be constant (Serrano et al., 2014).

$$G = G_m \times \left(1 - e^{-K' \times t}\right) \quad \text{(Eq. 2)}$$

The K' and G_m values for each load were calculated numerically from the experimental data obtained by nonlinear regression using Sigma-Plot (version 12.5).To evaluate the variations in experimental data, the theoretical values of maximum methane production (G_m) for all experiments were calculated using (Eq. 2) and plotted against their corresponding experimental values. *Figure 6* shows an example of the theoretical values of maximum methane production (G_m) adjusted to their corresponding experimental values (G_T) of digester R5.

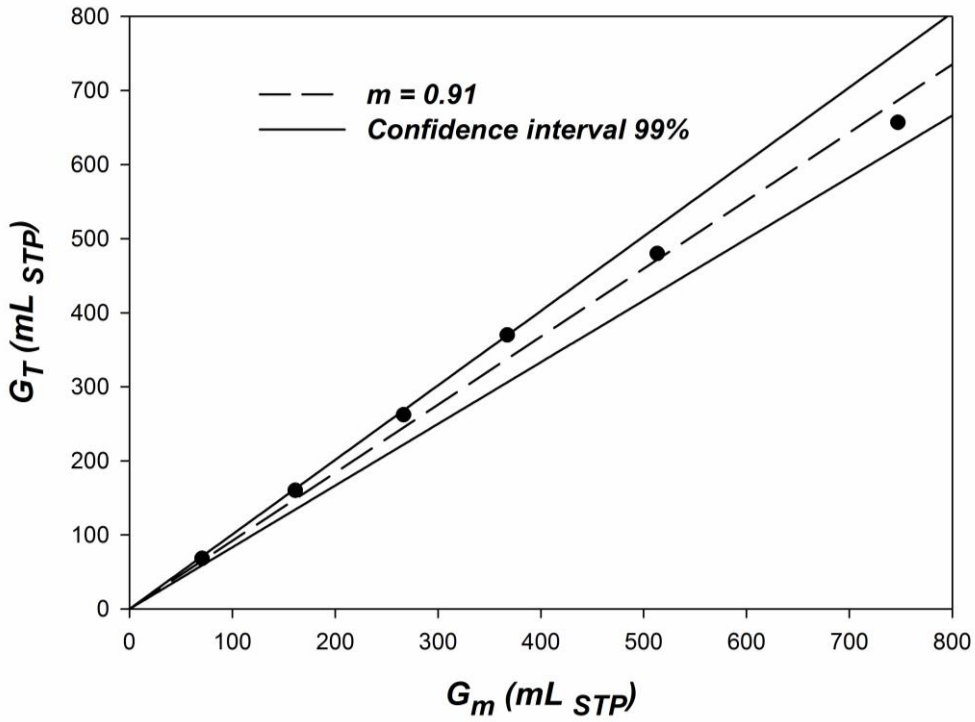

Figure 5. *Comparison between the experimental maximum methane production values (GT) and the theoretical values (Gm) of digester R5 predicted by (Eq. 2).*

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Moreover, the theoretical methane yield of all experiments was calculated by fitting pairs of values of the maximum theoretical volume of methane predicted in each load (mL_{STP}/L)-VS to a straight line, the theoretical methane yield coefficient coincides with the slope of the regression line (*Table 4*). The predicted methane potential (Yt) increased with increased portion of BW except in the case of digester R6 in which the experimental as well as theoretical methane potential decreased when the BW was increased to 80. It should be noted that the theoretical methane yield (Yt) was higher than the experimental methane yield (Ye) in all co-digestion experiment, except in digester R6 and R7. The data of the experimental methane yield (Ye) was found to fit well (R2 values between 0.9823 and 0.9996). At the same time, digester R7 got the maximal theoretical methane yield of 315 mL_{STP}/gVS.

Table 4. *Difference between experimental and theoretical methane yield, and the correspond OLR of each digester.*

Digesters	R1	R2	R3	R4	R5	R6	R7
Ye_{CH4}(mL_{STP}/gVS)	107	112	141	178	229	214	316
r^2	0.9991	0.9823	0.9981	0.9975	0.9867	0.9967	0.9996
Yt_{CH4}(mL_{STP}/gVS)	134	138	160	187	259	193	315
OLR (kg VS/(m^3.d))	0.17-1.1	0.48-2.53	0.53-2.74	1.17-2.87	1.49-3.57	0.66-1.74	0.58-2.8

Organic loading rate

Organic Loading Rate (OLR) is defined as the rate of organic matter to be introduced to a digester. OLR is an important control parameter in anaerobic digestion; overloading could lead system to fail, due to the accumulation of inhibiting substances. Therefore the organic loading rate (OLR) relates the amount of waste added to the reactors with the reactor volume and time. The operational conditions used in this study allow the added substrate to be biomethanised as much as possible.

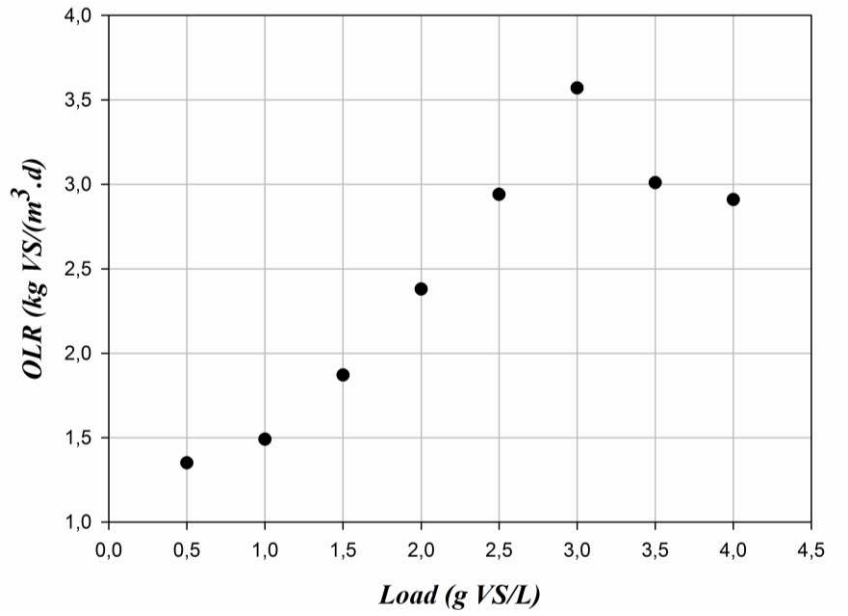

Figure 6. *Variation of organic loading rate against the load added of digester R5.*

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Figure 7 shows the variation of the OLR (kg VS/ (m^3.d)) with the added load to the digester R5. OLR was calculated considering the substrate concentration added to the reactors (kg VS/m^3) and the time (day) required to reach 95% of the total methane production for each load (Serrano et al., 2013). Digester R5 gave a maximum amount of organic matter which could be treated; its OLR values increased from 1.49 to 3.57 kg VS/(m^3.d). Although the methanogenic potential obtained in the case of mono-digestion of banana waste is high to that obtained from the co-digestion, but it should be noted according to the *Table 4* that the OLR which can be treated in the case of the co-digestion experiments (digester R5) is much more important.

Conclusion

A mesophilic anaerobic co-digestion of Cow Manure (CM) and Banana Waste (BW), was evaluated using a 1-liter Labe-scale CSTR anaerobic digesters. Seven CM/BW mixtures ratios were tested. Mono-digestion of cow manure and banana waste gave methane yield of 107 and 316 mL_{STP}/g VS respectively. For co-digestion experiments, a maximum methane yield of 229 mL_{STP}/g VS was obtained when mixing 40% CM with 60% BW at a final biodegradability of 86%.The addition of banana waste as co-substrate in the mixture improved methane yield as well as OLR which was reached a value of 3.57 kg VS/(m^3.d).

The results show a high biogas potential and high suitability of banana waste as a feedstock for economically viable waste-treatment technology like anaerobic digestion for the purpose of energy generation in the form of methane. Hence, anaerobic co-digestion of cow manure mixed with banana waste could generate significant amounts of energy supply in form of biogas that could be used to cover essential needs.

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REMOVAL RATE OF HERBICIDE ACLONIFEN WITH ISOLATED BACTERIA AND FUNGI

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Abstract. In this research the microbial biodegradation of aclonifen was investigated using liquid and soil experiments with identified cultures and mixed consortia. Isolated fungi and bacteria consortia showed the highest degradation at 93% of the Chemical Oxygen Demand (COD) parameter over five days. Bacteria mix and fungi mix performed 90% and 91% degradation in five days, as COD, while 71% and 91% were active ingredients. For Total Organic Carbon (TOC) experimental results, bacteria mix, fungi mix, and bacteria and fungi mix, showed 86%, 88% and 88% respectively. Soil studies with mixed cultures of bacteria and fungi performed the most efficient degradation, at 97% after five weeks. The degradation of aclonifen by 2 ml mixed cultures showed about 63% of degradation in five weeks and 5 ml of mixed cultures showed about 90% in six weeks.
Keywords: *microbial biodegradation, aclonifen, mixed consortia, chemical oxygen demand, total organic carbon*

Introduction

One of the main factors of environmental pollution is the excessive use of chemicals and pesticides, used on a global scale, to increase production and for the protection of crops. In addition to the main problems with soil, ground water, and surface water pollution, there are also many risks to human health from these chemicals, such as direct poisoning and their residues contaminating drinking water (Nemeth et al., 2002). As a consequence, these environmental problems, particularly regarding the presence and accumulation of pesticides in surface and ground water as well as soil, are becoming increasingly importance with every passing day (Carrizosa et al., 2001).

Studies relating to soil degredation focus on degrading residual pesticides and their components. Degradation amounts of pesticides are becoming critical and their rate of spread is an important environmental risk.

Meanwhile, microbial degradation is not only an important mechanism for controlling pesticides in soils it is also an environmentally friendly method. Temperature and humidity are the main parameters for controlling microbial degradation of pesticides in soils. Pesticide degradation in dry soils is slower than in wet soils (Masutti, 2003).

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Aclonifen is a diphenylether herbicide in which one ring is unsubstituted and the other, NH_2^-, Cl_2^-, NO_2^-, is substituted. This substitution, especially NH_2^-, is a particular feature in the diphenylether herbicidal family. In contrast with other herbicides of the same family (acifluorfen, oxyfluorfen, bifenox), this compound is not only acting through a phytotoxic protoporphyrin IX accumulation but also through an inhibition of carotenoid biosynthesis (Kilinc et al., 2001).

Nitrodiphenyl ether herbicides are potent herbicides. Some metabolites and parent compounds are considered as possible mutagens and endocrine disruptors. Both properties pose serious health and environmental risks (Keum et al., 2001).

Several diphenyl ether degrading strains have been reported, such as *Coriolus versicolor* (Hiratsuka et al., 2001), *Azotobacter chroococcum* (Chakraborty et al., 2002) and *Sphingomonas wittichii* RW1 (Keum et al., 2001). Moreover, as one of the same kind of herbicides, only a few reports on fomesafen degrading strains are available with only two such strains, namely, *Aspergillus niger* S7, having been reported (Li et al., 2009).

The metabolism pathways of the microbial degradation of several diphenyl ethers have been studied. It is reported that the main degradation pathway of oxyfluorfen by *A. chroococcum* was the reduction of nitro group to amino compound, further acetylation of amino derivative, O-dealkylation and dechlorination (Chakraborty et al., 2002). The experiments were performed to study the degradation of chlornitrofen (Kamei and Kondo, 2006), and diphenyl ethers (Federici et al., 2011), using *Phlebia brevispora* and *Lentinus tigrinus*, respectively.

In this study, the microbial degradation of aclonifen was studied using bacteria and fungi isolated from an agricultural area previously unexposed to aclonifen. Soil samples, from a sunflower field with a known history of extensive pesticide usage, located in Kirklareli City, Turkey, were also collected as a source of pesticide degrading microbes. In the experiments, aclonifen degradation was investigated using five species of bacteria and six species of fungus that were isolated from the soil samples, using different media plates.

Biological degradation is the most frequently used method for the remediation of pesticides in soil and water. The results of experiments have shown that levels of biodegradation depend on the removal of pesticide residuals. Biodegradation/bioremediation is a low cost and theoretically alternative process that does not result in toxic final products (Massiha et al., 2011).

Materials and Methods

Chemicals and reagents

An agricultural products shop supplied the aclonifen herbicide under the trade name Chekic 600. This herbicide contains 600 gr L^{-1} of aclonifen active ingredient. Aclonifen standard was supplied as a yellow powder with 99.9% purity from Dr. Ehrenstorfer GmbH Co. All media for the isolation and enrichment of bacteria and fungi were obtained from Sigma Aldrich. Acetone and hexane were obtained from Merck Company. All the chemicals used were of HPLC grade. Analytical standards for calibration were in the range of 0.1-100 mg L^{-1}. Methanol was chosen as the diluting solvent.

Instruments

The quantification of aclonifen was performed by LC-MS-MS (Dionex Ultimate 3000) equipped with a C18 Thermo Accucore column 100mmx2.1mm 2.6 mic. Owen

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temperature of the column was 400°C and Auto Sampler temperature was 50°C . Retention time was 9.0 min. Mass (Thermo Access Max) HESI ion source was 3500 volt and Ion Transfer Tube temperature was 270°C . Sheath and Aux gas were 50 Arb and 15 Arb, and the quantification limit was 15 ppb. Collisure gas pressure was 1.5 mTorr. LOD and LOQ values were 4 ppt and 15 ppt. Ion transmissions were; for primary ion 265.1, second ions 182.1 and 247.9. The quantification (method detection) limit was 0.1-1 mg kg^{-1}. The average value of determination coefficient (R^2) of the calibration curve was 0.999.

All samples were spiked with surrogate and internal standards in order to determine the recovery rate, with tetrachloro-m-xylene (TCMX) used as the surrogate standard. The surrogate standard was spiked to the sample prior to extraction. Quintozene was used as the internal standard, and was spiked just before capping the chromatography vials. Average recovery rate was 86 percent. The limit of detection (LOD) values was calculated for each congener as average blank concentrations plus three times the standard deviations. Any sample concentrations falling below the LOD value were ignored. Blank samples were corrected for each set of analysis, and all results were blank corrected.

Soil sample collection

The experiments were conducted on soil samples obtained from agricultural areas in Turgutbey Village, Kirklareli City. The majority of the farms selected in the area have been cultivating sunflowers and wheat for several years. Soil with no background of aclonifen concentration was collected from a field. All the samples were collected from five selected points (*Figure 1*), from the uppermost 0–20 cm of soil following the standard procedure and stored in glass vessels at 4°C temperature in a thermos (Carter and Gregorich, 2006). The soil samples were analyzed at the Trakya Agricultural Research Institute and the results of the analyses are shown in *Table 1*.

Figure 1. *Turgutbey Village and soil sampling area*

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Table 1. *Analyses results of the soil sample used for isolation of cultures*

PARAMETER	VALUE
Depth, cm	0-20
pH	6.5
Organic carbon, %	2.1
Clay, %	62
Sand, %	32
Silt, %	5
Moisture Content, %	21

Culture media preparation

The plate count, dextrose casein peptone, potato dextrose, dichloran rose bengal chlorinated, sabouraud dextrose as media agar and malt extract, sabouraud dextrose, were prepared as media broth, and used according to manufacturer's instructions (Sigma Aldrich-USA). After cooling, diluted agricultural soil (containing no trace of aclonifen) in a sterile isotonic solution (0.08% NaCl) was added into petri dishes. The medium pH was adjusted to 6.5.

Isolation and enrichmet of bacteria and fungi

For the isolation of bacteria and fungi, soil samples taken from a depth of 0 to 20 cm before herbicide application were placed in sterile glass jars (Zelles et al., 1991). Approximately 10 g of soil sample was diluted to 10^{-4} in 0.8% sodium chlorate isotonic water. 0.1 ml of this diluted sample was taken and sown into plate count agar, dextrose casein peptone agar, potato dextrose agar, dichloran rose bengal chlorinated agar, malt extract agar, sabouraud dextrose agar and yeast extract agar which was prepared by being sterilized in an autoclave at 121°C for 15 min at 1 atm pressure in a sterile cabin. After preparation, the petri dishes were put in a 20°C incubator; the growing phase for the bacteria was about three days, and ten days for the fungi. Growing bacteria in petri dishes were marked as B1-B5 and fungi as F1-F6. These bacteria were then added to sabouraud dextrose broth and fungi were added into malt extract and incubated at 20°C for enrichment.

Identification of fungi and bacteria species

Molecular characterization procedures were applied to Fungi, isolating the Genomic DNA from Yeast; and to bacteria, isolating the Genomic DNA from gram positive and Gram Negative Bacteria (Beutler et al., 1990).

Fungi studies

The fungi marked on the petri dishes were sown in PDA (Peptone Dextrose Agar) petri dishes by streak plates to ensure the reproduction from sport fungi. The fungi that were grown at room temperature and from single colony isolation were transferred to other PDA petri dishes and were kept at room temperature until they reached the appropriate size for DNA isolation. The growing fungi were scratched using a sterile blade and crushed with liquid nitrogen in sterile conditions, after which, DNA was isolated from the fungal hyphae.

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An ordinary taq polymerase was conducted for PCR (Polymerase Chain Reaction) using many combinations of ITS (Internal Transcribed Spacer) region primers, which are often used in the definition of DNA. The PCR conditions are given below.

Final concentrations (total 25 µL reaction volumes): 1X Taq polymerase buffer / 1.5 µM MgCl2 / 0.4 µM forward primer / 0.4 µM reverse primer / 0.5 µM dNTP / 1 U(unit) Taq polymerase (F1, F4, and F6) or 1.25 U Taq polymerase (F2, F3, and F5) and 200ng DNA.

Heat cycle conditions: 1 cycle: 94°C -3 min / 35 cycles: 94°C - 15 s, 55°C - 30 s, 72°C - 30 s / 1 cycle: 72°C 1 - 5 min.

In the PCR, the expected length of the bands was obtained only for F1 (*Penicillium thrichoderma*), F4 (*Metacordyceps chlamydosporia*) and F6 (*Alternaria alternata*). For the other fungi; F2 (*Penicillium simplicissimum*), F3 (*Penicillium Talaromyces*) and F5 (*Stachybotrys chartarum*) One-Taq polymerase was used. The three primers designed by Avcioglu (personal communication, 2014) gave two results. These tapes, which were cut from the agarose gel and cleaned (in the case of multiple bands) or as a single band, PCR reaction were sent directly for sequence analysis. A Thermo-Scientific Gene JET Gel Extraction Kit was used in the cleaning of the bands cut from the agarose gel. In cases of a sequence reaction on the bands (cut from the agarose gel) not performing well, re-amplification was made (by One Taq polymerase).

Bacteria studies

Phire Hot Start II DNA Polymerase was used for PCR, because it allows making no DNA isolation. Then, longer PCR bands of various lengths (1000–3000 bp) were obtained through bacterial 16S ribosomal general primers. The pipette instructions and cycling protocols are given below

For final concentrations: (total 20µL reaction volume): 1X Phire Animal Tissue PCR Buffer (includes dNTPs and $MgCl_2$) / 0.5µM forward primer / 0.5µM reverse primer / Phire Hot Start II DNA polimeraz and H2O.

Heat cycle conditions: 1 cycle: 98°C – 5 min / 40 cycles: 98°C – 5 s, 72°C – 20 s / 1 cycle 72°C – 4 min/4°C-∞

Bacteria isolated and denoted from B1 to B5 were identified using 16sRNA Universal Primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′; Escherichia coli positions 8–27) 16S rRNA universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′; Escherichia coli positions 8–27) (Edwards et al., 1989). 1492R 5' TACGGYTACCTTGTTACGACTT 3' positions 1492–1512) (Weisberg et al., 1991; Park et al., 1995).

Microbial biodegradation studies

In order to assess the degradation ratio of the aclonifen from the fungi and bacteria, 5 species of each bacterial cultures and their mixtures, 6 species of each fungi cultures and their mixtures, and the mixtures of 5 bacteria and 6 fungi cultures were grown with shaker controlled at 20°C through the broth enrichment techniques. In this enriched media, Chemical Oxygen Demand (COD) measurement was carried out in 24-hour intervals (according to the Standard Methods 5220C closed reflux titrimetric method, SMC, 2009b) and decreasing of the substrate followed. After the end of the seventh day (at the end of the log phase on the growth curve) 1 ml of culture was taken at sterile

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conditions from these culture media, added into liquid media with isotonic pesticide solution, and bioremediation studies were started.

Studies in liquid media

In the liquid medium study, in 98 ml of 0.8% isotonic sodium chloride solution, 1 ml of the Chekic 600 (includes 0.6 gr aclonifen active ingredient) and 1 ml of growing cultures from the broth media (approximately 10^9 unit/1ml) were added. The aclonifen was prepared from Chekic 600 (trade name of the herbicide) in the same concentration as used in the field (200 ml/1000 m^2). The growing media used in the experiments were the previously isolated and the enriched bacteria and fungi mixtures, with 1 ml of the solutions obtained from mixtures of all kinds or from the separately enriched solution (only fungus or bacterium) used in the experiments.

In this phase, these enriched solutions were shaken continuously at Gallenkamp orbital incubator at 20°C. Solution samples were monitored at 24-hour intervals on the basis of the COD, the active ingredient, and TOC parameter. For COD studies, standard method 5220 C (SMC, 2009b) and for TOC studies, standard method 5310 A (SMC, 2009a) were used. For determination of the active ingredient; firstly EPA 3510 C Seperatory Funnel Liquid-Liquid extraction method (EPA, 1996) was used, followed by the EPA 1614 method (EPA, 2007a).

Studies in soil media

According to the results of bioremediation studies in liquid media, the best removal efficiency was observed in a mixture of bacteria and fungi cultures; therefore soil media studies were also conducted in these mixed culture media. For this study, sterilized glass jars measuring 10 x 10 x 10 cm were filled with soil samples obtained before herbicide application. These soil samples were blended 3-4 times a day and kept in a drying oven at 105°C for four days. At the end of day four, soils were cooled at room temperature and made ready for experimental studies. The amount of soil in each jar was about 700 g. To provide suitable soil humidity, a total of 350 ml microbial culture in various volumes (2, 5 and 10 ml) and aclonifen containing culture media were added as defined in Dileep (2008). Four different solutions, including a replicate sample, were used to add to soil samples in jars. 2400 µg of aclonifen was added into three of these solutions of 350 ml. In this experimental setting, the humidity of the soil was maintained at about 50-60% with stabilized tap water for 12 weeks. Each week (every seven days), 20 g soil was taken from each soil medium, 10 g of which was used for analytical studies and 10 g of which was used for determining humidity.

Over 12 weeks, 10-gram samples were mixed with anhydrous sodium sulphate to form a free-flowing powder. The samples were extracted with solvent once using ultrasonic extraction (EPA, 2007b), and a portion of the extract was collected for cleanup and was analyzed. In order to determine the percent dry weight, a separate portion of the sample was weighed out at the same time as the portion used for analytical determination. Immediately after weighing the sample aliquot to be extracted, 10 g aliquot of the sample was measured out into a tared crucible. This aliquot was dried overnight at 105°C and allowed to cool in a desiccator before weighing, although this oven-dried aliquot was not used for the extraction. The percent dry weight was calculated as follows: approximately 10 g of the sample was

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weighed into a 200 ml beaker, and 1.0 ml of matrix spiking and surrogate spiking solutions were added to each sample. The sample was scanned ultrasonically twice for 30 min with 50 ml of the extraction solvent mixture (50% acetone and 50% Hexane for LC-MS-MS analyses). The extract decant was filtered through Whatman No.41 filter paper using a Buchner funnel. In agricultural soils there are many organic substances that can interfere with the target analyte, so clean-up procedures are essential prior to chromatographic measurement. In order to eliminate possible interfering organics such as PAHs, and PCBs, alumina-silicic acid column was prepared. These two chemicals were baked at 450°C for six hours and then cooled down to room temperature in a desiccator. Separation column was formed by 3 g silicic acid (3% water), 2 g neutral alumina (6% water), and 2 g Na_2SO_4 (Jantunen, et al., 2000). Next, column was pre-washed with 20 mL of Dichloromethane and 20 mL of PE, respectively. Sample was evaporated to 1-2 ml and then poured to column. Finally, 20 ml dichloromethane was added to elute the pesticides (Cindoruk, 2011). The aliquot of the sample was placed into a concentrator tube in a warm bath and evaporated to 1 ml volume using a gentle stream of clean, dry nitrogen, after which it was analyzed. The internal wall of the concentrator tube was rinsed several times with solvent during concentration. Then, the extract was analyzed for the target analytes using the EPA 1614 method (EPA, 2007a) over eight weeks.

Results

Results of identified bacteria and fungi

The results of identification of fungi and bacteria isolated from sunflower growing field are given for fungi species in *Table 2*, and for bacteria species in *Table 3*.

Table 2. *First and second primers, sequences and references used to identify fungi (Erguven, 2015)*

Fungi Code and Approximate species identity	**First Primer 5'-3' sequence and reference**	**Second Primer 5'-3'sequance and reference**
Penicillium thrichoderma	ITS* GCATCGATGAAGAACGCAGC (White et al., 1990)	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar, 2014)
Penicillium simplicissimum	ITS GAAGGTGAAGTCGTAACAAGG (Cooke et al., 2000)	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar, 2014)
Penicillium talaromyces	ITS TCCTCCGCTTATTGATATGC (White et al., 1990)	ITS GAAGGTGAAGTCGTAACAAGG (Cooke et al., 2000)
Metacordyceps chlamydosporia	ITS GAGACCGCCACTGTATTTCG (Avcioglu-Dundar, 2014)	ITS GCATCGATGAAGAACGCAGC (White *et al.,* 1990)
Stachybotrys chartarum	ITS TCCGTAGGTGAACCTGCGG (White et al., 1990)	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar, 2014)
Alternaria alternata	ITS GCATCGATGAAGAACGCAGC (White et al., 1990)	ITS ATCCCTACCTGATCCGAGGTC (Avcioglu-Dundar, 2014)

*ITS:Internal Transcribed Spacer

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Table 3. *Identified bacterial codes and their species*

Accession Number	Bacterial Code and Approximate Species Identity	Identity	Reference
KF831394.1	*Bacillus simplex*	99%	(Heyrman et al., 2005)
HE646789.1	*Bacillus muralis*	99%	(Li et al., 2014)
KF555623.1	*Micrococcus luteus*	99%	(Bahig et al., 2008)
KC634108.1	*Micrococcus yunnannesis*	99%	(Chitra et al., 2014)
HG530135.1	*Clostridium tetani*	99%	(Ortega et al., 2012)

Results of removal rate of aclonifen in liquid media

COD and active agent results of aclonifen with identified fungi and bacteria are given in *Table 4*. The removal rate of aclonifen with fungi at the end of day five is given in *Figure 2*, for bacteria are given in *Figure 3*. The removal rate of the active agent for the bacteria mixture, fungi mixture and mixture of bacteria + fungi are given in *Table 5*. Removal rates of COD for mixture cultures are given in *Figure 4* and TOC removal values are given in *Figure 5*.

Table 4. *Removal rate of aclonifen with bacteria and fungi species*

Fungi	Day	COD Aclonifen (mg/l)	COD Aclonifen (%)	Aclonifen (mg/ml)	Aclonifen (%)	Bacteria	COD Aclonifen (mg/l)	COD Aclonifen (%)	Aclonifen (mg/ml)	Aclonifen (%)
Penicillium taloremyces	0	15600	53	2,45	49	Bacillus simplex	15600	91	1,68	72
	1	14720					13600			
	2	13920					6800			
	3	12480					3360			
	4	8250					1680			
	5	7330					1360			
Penicillium thrichoderma	0	15600	77	1,49	69	*Bacillus muralis*	15600	78	2,28	62
	1	13920					14720			
	2	7330					13600			
	3	6880					7700			
	4	5160					4620			
	5	3590					3430			
Metacordceps chlamydosporia	0	15600	91	1,39	71	*Micrococcus luteus*	15600	70	2,7	55
	1	14720					14720			
	2	13920					13600			
	3	8600					7700			
	4	3480					4680			
	5	1040					4680			
Penicillium simplicissimum	0	15600	68	1,97	59	*Micrococcus yuannesis*	15600	93	1,62	73
	1	14720					7360			
	2	13920					5460			
	3	8600					3600			
	4	5440					1700			
	5	4990					1090			
Stachybotrys chartarum	0	15600	82	1,25	74	*Clostridium tetani*	15600	80	2,22	63
	1	14720					14040			
	2	12480					7700			
	3	4680					6160			
	4	4620					4620			
	5	2800					3120			
Alternaria alternata	0	15600	69	1,92	60					
	1	14720								
	2	13920								
	3	10290								
	4	6880								
	5	4840								

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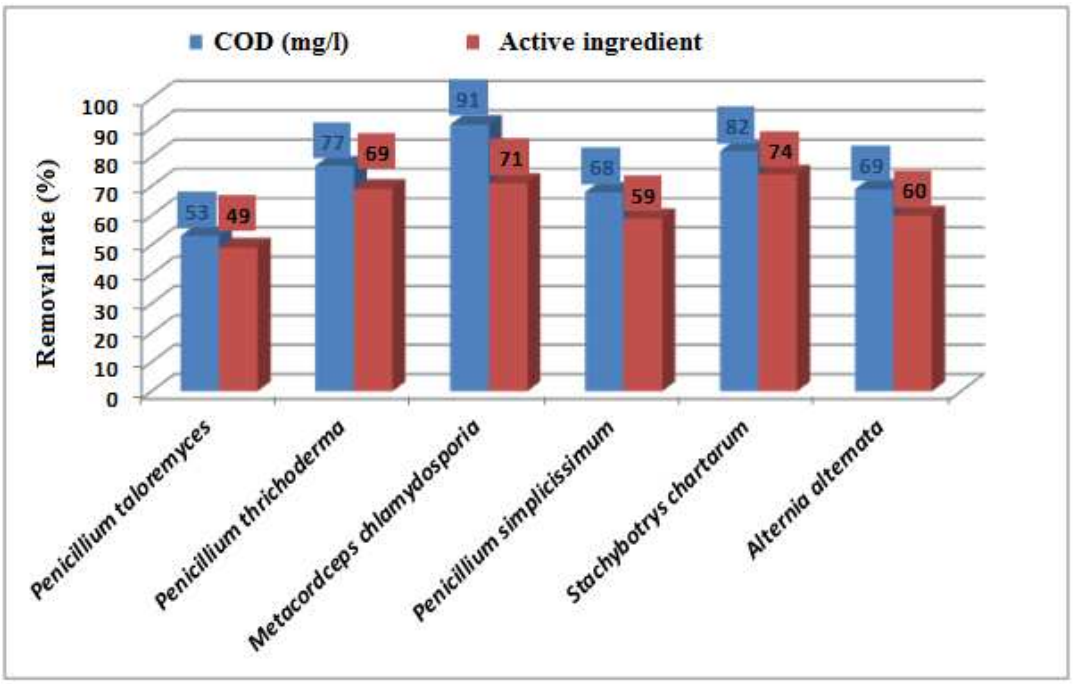

Figure 2. *Removal rate of aclonifen with all isolated fungi*

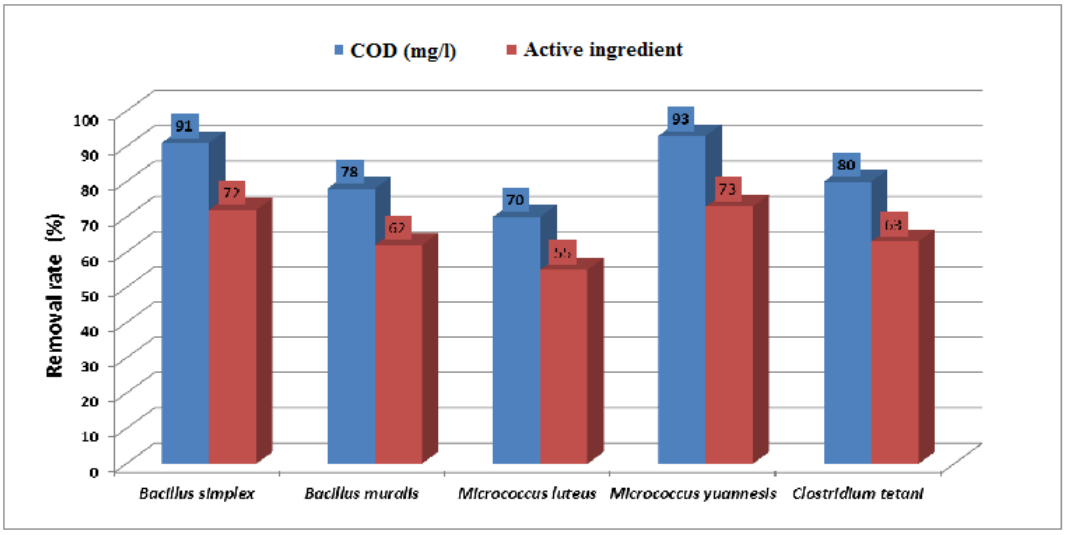

Figure 3. *Removal rate of aclonifen with all isolated bacteria*

Table 5. *Removal rate of aclonifen with mixed cultures*

Sample Type	Day	Aclonifen* mg/ml	Aclonifen Removal Efficiency (%)
Mixture of bacteria	1	5,46	9
	2	5,16	14
	3	5,04	16
	4	3,00	50
	5	1,74	71

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Mixture of fungi	1	5,58	7
	2	5,04	16
	3	4,14	31
	4	2,28	62
	5	0,54	91
Mixture of bacteria + fungi	1	4,5	25
	2	3,48	42
	3	2,64	56
	4	1,44	76
	5	0,06	99

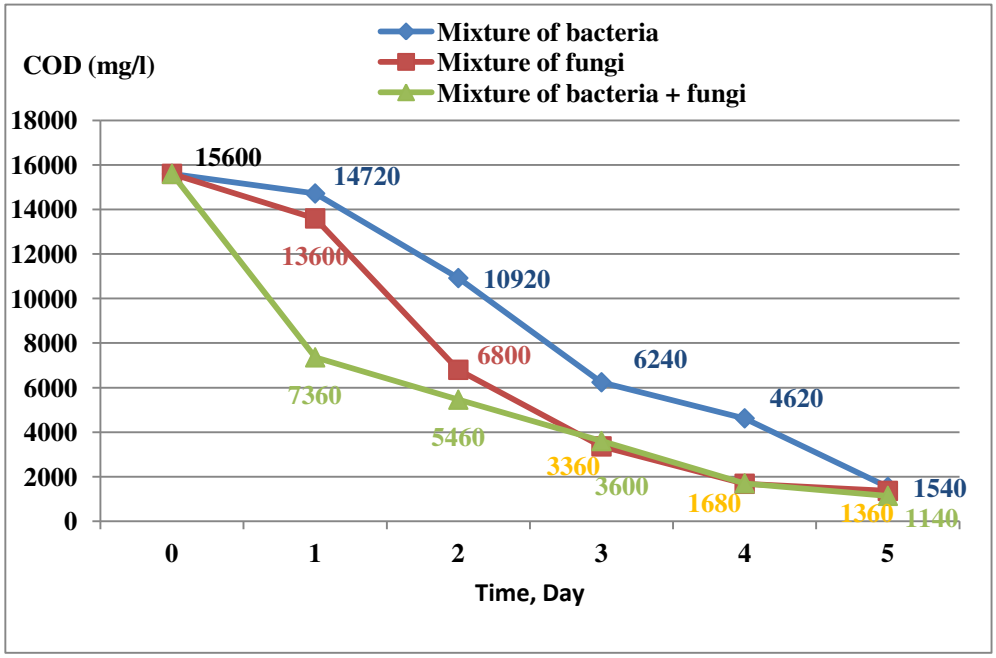

Figure 4. *Removal rate of aclonifen as COD with mixed cultures*

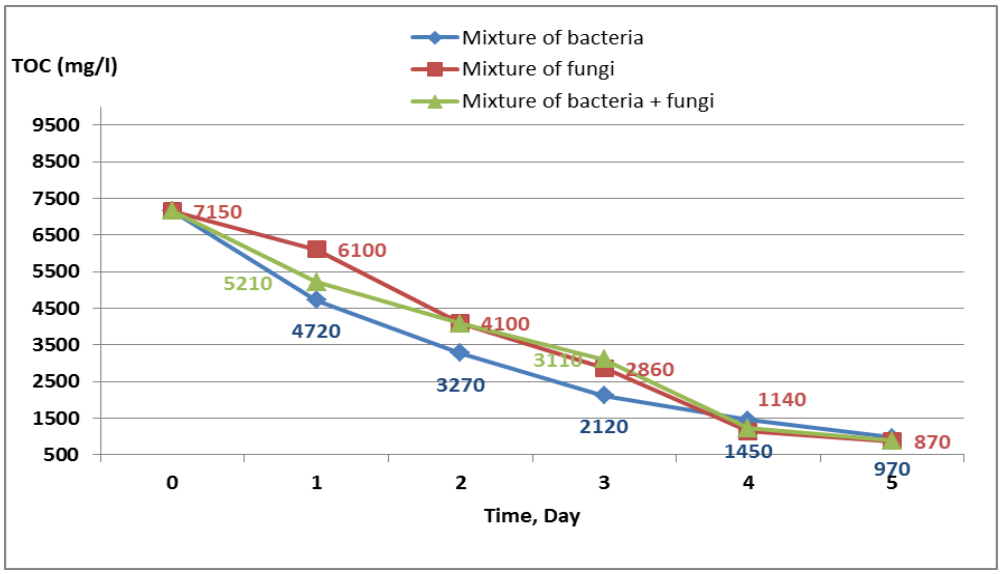

Figure 5. *Removal rate of aclonifen as TOC with mixed cultures*

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Results of removal rate of aclonifen in soil media

Bioremediation results obtained from mixed cultures in soil with aclonifen are given in *Table 6*, and removal rates are given in *Figure 6*.

Table 6. *Removal rate of aclonifen in soil media with mixed cultures*

Week	Mixture of bacteria + fungi				Mixture of bacteria + fungi			
	Blank	2ml	5 ml	10 ml	Blank	2ml	5 ml	10 ml
	Removal rate (%)				Concantration (ng/g)			
1	0,9	9,2	19,0	52,0	3399,1	3114,4	2778,3	1646,4
2	5,8	7,1	18,8	63,7	3231,1	3186,5	2785,2	1245,1
3	10,7	19,2	28,4	67,0	3063,0	2771,4	2455,9	1131,9
4	18,0	35,8	45,8	72,5	2812,6	2202,1	1859,1	943,3
5	25,8	48,7	55,5	82,9	2545,1	1759,6	1526,4	586,5
6	31,1	61,8	62,1	96,7	2363,3	1310,3	1300,0	113,2
7	37,4	62,5	68,7	96,9	2147,2	1286,3	1073,6	106,3
8	42,3	60,6	70,5	97,2	1979,1	1351,4	1011,9	96,0
9	49,3	61,1	71,1	99,4	1739,0	1334,3	991,3	20,6
10	53,4	64,9	69,5		1598,4	1203,9		
11	59,1				1402,9			
12	69,1				1059,9			

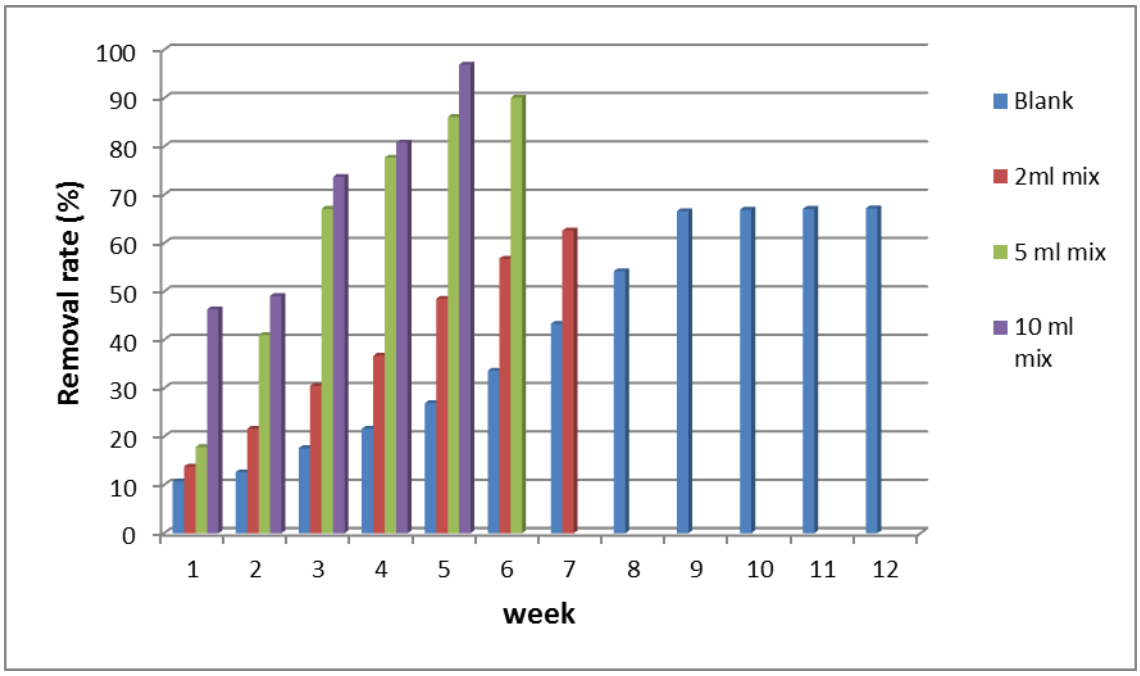

Figure 6. *Removal rate of aclonifen with mixture cultures*

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In the studies conducted on aclonifen-added experimental media, the best removal rate of the active agent within five days was achieved with *Stachybotrys chartarum* at a rate of 74%. Within this time period, the lowest removal efficiency was observed with *Penicillium talaromyces* at a rate of 49%. As for the results of the bioremediation of this herbicide by fungi, 6.00 mg/l of active agent decreased to 1.25mg/l with *Stachybotrys chartarum*. This value is 2.45 mg/l for *Penicillium talaromyces*. COD removal increased between 91% and 53% for the same herbicide. According to these results, *Metacordyceps chlamydosporia* has the best removal performance. 15600mg/l of COD decreased to 1040 mg/l after the end of day five. The lowest removal performance was seen with *Penicillum talaromyces* was 15600mg/l to 7330 mg/l as COD. Results for the other four species occur between these values.

In the studies conducted in liquid media administered with bacteria and the addition of aclonifen, the best removal rate based on the active agent within five days was achieved with *Micrococcus yuannesis*, at a rate of 73%. Within this time period, the lowest removal was with *Micrococcus luteus* at 55%. Regarding the active agent concentration, if we consider the bioremediation of the same herbicide by bacteria, 6.00 mg/l of active agent decreased to 1.62 mg/l with *Micrococcus yuannesis* by the end of day five, and to 2,70 mg/l with *Micrococcus luteus*. COD removal rate was observed to be between 93% and 70% with same herbicide. According to these results, *Micrococcus yuannesis* has the best removal performance. The COD, which was calculated as 15600mg/l decreased to 1090 mg/l at the end of day five. The poorest removal performance was observed with *Micrococcus luteus*, decreasing the COD from 15,600mg/l to 4,680 mg/l. Results for the other four species varied between these values.

In the studies conducted in liquid media administered with bacteria, fungi, and bacteria + fungi mixtures the best removal rate of aclonifen based on the active agent within five days was achieved with the bacteria + fungi mixture at a rate of 99%. Within this time period, the lowest removal rate was observed with the mixture of bacteria at a rate of 71%. Regarding the active agent concentration, if we consider the bioremediation of the same herbicide by bacteria, fungi, and bacteria+ fungi, the active agent of nearly 6.00 mg/l was decreased to 0.06 mg/l with the mixture of bacteria + fungi at the end of day five, to 1.74 mg/l with the mixture of bacteria, and to 0.54 mg/l with the mixture of fungi (*Table 5*). The COD removal was observed between 93% and 90% with the same herbicide. According to these results, the mixture of bacteria + fungi had the best removal performance. The COD calculated 15,600mg/l decreased to 1,140 g/l at the end of day five. The poorest removal performance was observed with the mixture of bacteria, decreasing the COD of 15,600mg/l to 1,540 mg/l. Other removal efficiencies varied between these values. In the study, the removal efficiency of TOC was observed to be between 86% and 88%. According to these results, the best removal was achieved with the fungi mixture and the mixture of bacteria + fungi. The TOC, which was calculated to be 7,150 mg/l, decreased to 870 mg/l at the end of day five with the mixture of fungi, and to 880 mg/l with the mixture of bacteria + fungi. The lowest removal efficiency was observed with the mixture of bacteria, decreasing the TOC to 970 mg/l at the end of day five.

According to the bioremediation studies on soil media with aclonifen, removal rate with a mixture of 10 ml culture solution was 99%, which is higher than the values obtained with the mixture of 2 ml and 5 ml culture solutions. The next best removal efficiency was observed with 5 ml culture solutions with a rate of 70% at the end of

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week nine, however, the 2 ml. solution reached 65% at the end of week 10. When the results of the study were evaluated based on the active agent, nearly 3,420 ng/g of aclonifen, which was added to the soil, was observed to decrease to 21 ng/g at the end of week nine, in 10 ml solution mixture, while the amount decreased to 1,204 ng/g at the end of week 10 in the 2 ml mixture solution, and to 991 ng/g at the end of week nine in the 5 ml mixture. According to these results, it was understood that increasing the amount of mixed cultures in soil shortens the removal time of aclonifen and increases the rate of removal.

Discussion

Herbicides and surfactants differ in chemical composition and react differently when incorporated into the soil system due to differences in chemical properties and interactions with soil components and environmental factors (Smith et al., 1982; Ray et al., 1985). Microbial community structure, often used as an indicator in monitoring soil quality, is affected by various environmental and growth factors, such as moisture, temperature, nutrient availability, and management practices (Petersen et al., 2002).

In aerobic laboratory soil degradation studies, the overall geometric mean DT50 value of aclonifen at 20°C is 62.3. Degradation of aclonifen in soil under anaerobic conditions was investigated in one of the studies presented in the dossier but it was not considered reliable (EFSA, 2008). Aclonifen may be considered moderate to highly persistent (DT50 = 32.2 – 134 d) in soil, under aerobic conditions, at 20°C. Based on the findings from the screening test on ready biodegradability, water/sediment simulation test and soil aclonifen appears to be susceptible to primary degradation (a degradation of >70% within 28 days) and not ultimate mineralization (Humburg, 1989).

Conclusion

The route of degradation in soil under dark aerobic conditions was investigated in two studies in a total of five soils. In our study, it was observed in the weekly active ingredient soil studies that the removal efficiencies were higher when the concentrations of mixed microorganism culture were increased. The values were 63% for 2 ml culture in 7 weeks, 90% for 5 ml culture in 6 weeks, and increased to 97% for the 10 ml culture in 5 weeks.

This study showed that biodegradation of aclonifen was found sufficiently high, especially for the mixed cultures. Isolated fungi and bacteria consortia showed the highest degradation of 93% as COD parameter in 5 days. Bacteria mix and fungi mix performed 90% and 91% degradation in 5 days as COD while 71% and 91% as active ingredient. Soil studies with mixed cultures of bacteria and fungi performed the most efficient degradation as 97% after 5 weeks. The degradation of aclonifen by 2 ml mixed cultures showed about 63% of degradation in 7 weeks and 5 ml of mixed cultures showed about 90% in 6 weeks. As a conclusion it can be said that the results of the experiments showed important implication potential in the development of in-field treatment systems for pesticide-contaminated soils.

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EFFECT OF THINNING ON EVAPORATION OF SCOTS PINE FOREST

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Abstract: This paper presents the results of a study carried out on a Scots pine stand, aged over 70 years, growing on ICP Forests land. The stand underwent late thinning, this being the last operation performed prior to complete felling, which will take place when the trees reach an age of approximately 100 years. The thinning, which reduced the basal area by 20%, caused a decrease in LAI by approximately 30% on average and an increase in the gap fraction by almost 50%. The thinning of the tree canopy increased by more than 40% the quantity of light reaching the lower levels of the tree stand and the soil. Comparison of the results for actual evapotranspiration and tree transpiration with potential evapotranspiration (T ETP^{-1}, EVT ETP^{-1}) revealed an 8% increase in tree transpiration, while the actual evapotranspiration of the ecosystem increased by 14% after thinning. This increase was partly the result of the higher tree transpiration, but another factor may have been the increased evapotranspiration of forest floor vegetation and the soil, resulting from the greater quantity of light reaching the forest floor. The ratio of the pines' transpiration to the actual evapotranspiration of the ecosystem (T EVT^{-1}) was practically unchanged, from 0.60 before thinning to 0.61 after. Modelling was also carried out (based on a Plant Water model) for average meteorological conditions to determine the effect of thinning on the rate at which the trees depleted supplies of soil water. This effect was greatest in September, when plant available water was present for 9 days longer than prior to thinning. In the months with the highest levels of transpiration (from May to August) the period in which plant available water was present lengthened by 2–3 days.
Keywords: *actual evapotranspiration, ICP Forests, Pinus sylvestris, Poland, transpiration*

Introduction

Thinning is an important forest management operation carried out on a very large total area every year. In Poland it is performed annually on several hundred thousand hectares of forest (for example, in 2013 the State Forests National Forest Holding carried out thinning on a total of 440 000 ha; Anonim, 2013).

Thinning is carried out to improve the conditions of tree growth by reducing competition between trees for access to nutrients, water and light, and by increasing the space occupied by individual trees (Martin-Benito et al., 2010). Reducing the number of trees also affects the trees' photosynthetic activity (Gershenson et al., 2009; Högberg, 2010), root activity, and inputs of labile organic carbon (Kuzyakov, 2002; Zhu and Cheng, 2011). This leads to higher incremental growth and improved growth efficiency (Valinger et al., 2000; Pukkala et al., 2002; Mäkinen and Isomäki, 2004). Apart from its effect on tree growth, thinning also has a strong impact on the forest ecosystem. It increases the upper soil temperature and accelerates nitrogen mineralization (Thibodeau et al., 2000), increases soil moisture (Davidson et al., 2006), and makes trees more resistant to insect attack (Coyea and Margolis, 1994).

It is of interest to consider the impact of thinning on soil water conditions, particularly when forest management operations are carried out in changing climatic conditions, particularly in case of soil drought.

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The aim of this work was to determine how the thinning of an older stand of Scots pine *(Pinus sylvestris* L.*)* affected factors related to the water cycle in the ecosystem. An analysis was made of tree transpiration, the actual evapotranspiration of the ecosystem, leaf area index (LAI), light conditions, and soil water. It was also determined how thinning affects the time of soil water depletion.

Materials and methods

Description of the study area

The study was carried out in an area managed as part of the ICP Forests programme (DeVries et al., 2003), situated approximately 25 km south of central Warsaw, in the region overseen by the Chojnów Forest Administration. The area has poor, sandy soil of type Dystric Arenosol (IUSS Working Group WRB, 2006), with *Querco-roboris Pinetum typicum* (W.Mat. 1981) J.Mat. 1988 vegetation structure. The tree stand consists of 75-year-old Scots pine (as at 2013) with some birches and young oaks. The study area covered 0.42 ha, and prior to thinning contained a total of 415 trees: 358 pines, 48 oaks and 9 birches. In thinning carried out in the winter of 2012–2013, a total of 122 trees were removed (112 pines, 6 oaks and 4 birches), and the basal area was reduced from 15.2 m^2 (36.2 m^2 ha^{-1}) to 12.1 m^2 (28.8 m^2 ha^{-1}), representing a 20% decrease. The trees removed were chiefly those which were the most weakly developed or were entirely shaded by the canopy.

The average annual temperature in 30 last years was 8.5 ºC and average precipitation achieved the about 560 mm according to IMGW data for the nearest meteorological station Warszawa-Okęcie, distant about 20 km from the research plot.

Water circulation conditions in the area are studied in accordance with the ICP Forests methodology (Raspe et al., 2010). Measurements are made of soil moisture at depths of 10 cm, 25 cm, 50 cm and 85 cm in three soil profiles, and of throughfall. The soil moisture and throughfall are measured in 1 hour time interval. Soil retention properties were determined by plotting a pF curve (*Table 1*), and the leaf area index was calculated using hemispherical photographs. Adjacent to the area is a weather station which measures meteorological parameters in an open space in 10 minutes time interval (radiation, precipitation, air temperature on 2.0 m, 0.5 m, 0.05 m, humidity on 2.0 m and 0.5 m, wind speed and direction).

Table 1. *Volumetric water content [cm^3cm^{-3}] in characteristic points of pF-curve [cm] of Dystric Arenosol on research plot*

Depth [cm]	pF								
	0	1	1.7	2	2.5	3	3.4	3.7	4.2
0-15	0.457	0.418	0.282	0.225	0.112	0.071	0.059	0.058	0.045
15-30	0.450	0.428	0.284	0.232	0.089	0.055	0.045	0.041	0.034

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Evaporation

Evaporation was analysed in the periods from 1 May to 30 September in 2012 (before thinning) and in 2013 (after thinning) for days with complete measurement data on which soil water availability was unlimited (pF between 2.0 and 3.7) and there was no rainfall. Analysed pF was chosen in the 2.0-3.7 range to avoid the influence of the water seeping through the soil (pF < 2.0) and to exclude unavailable water to plants (pF > 3.7). Evaporation was determined by the following methods:

Actual evapotranspiration (EVT)

Based on measurements of soil moisture (θ_{TDR}) in 3 profiles (on 4 depths: 10 cm, 25 cm, 50 cm, 85 cm) the actual soil water supply (SWS) was determined. Daily water supply changes (ΔSWS) subject to the conditions: pF 2.0 < θ_{TDR} < pF 3.7: precipitation = 0; ΔSWS < 0 determine the value of actual daily evapotranspiration.

Transpiration by pines

The transpiration of individual pines (T) was determined based on sap flow (F) measurement using a Thermal Dissipation Probe (Dynamax Inc.). Measurements were performed on three pines (*Table 2*), using 2 sensors per tree. Based on the measured rate of water flow in the tree stem (V), the computed white cross-sectional area (A_{sw}) and the area of the projection of the crowns of individual trees (A_c), the tree transpiration (T) was calculated:

$$F = V \cdot A_{sw} \ [dm^3 s^{-1}] \quad \text{(Eq. 1)}$$

$$T = F \cdot A_c^{-1} \ [mm] \quad \text{(Eq. 2)}$$

Table 2. *Characteristics of examined Scots pines*

	DBH (cm)	Height (m)	Crown Position Class
Pine no 1	22.5	19.0	Co-dominant
Pine no 2	25.3	20.5	Dominant
Pine no 3	24.3	20.5	Dominant

Potential evapotranspiration (ETP)

Calculation of the potential evapotranspiration makes it possible to determine how the meteorological conditions affect the process of evaporation. Potential evapotranspiration was computed by means of the Penman–Monteith formula (Allen et al., 1998).

Effect of drought – modelling

A criterion for determining the effect of thinning on the occurrence of soil drought was taken to be the time in which the trees deplete the supply of soil water in the useful

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retention range (from pF 2.0 to pF 3.7). In the case of Scots pine, the mass of the small roots responsible for water uptake is mainly located in the upper soil layer (Janssens et al., 2002). Changes in water supply were therefore analysed down to a soil depth of 0.25 m. The computed soil water supply in that layer was 43.4 mm. The trees' uptake of water was calculated using the Plant Water model, one of the group of Soil-Plant-Atmosphere-Continuum models (Kowalik and Turner, 1983; Kowalik and Eckersten, 1984). A detailed description of the model is contained in Kowalik et al. (1987). In the Plant Water model, a plant is regarded as a water reservoir through which water flow takes place as a result of the loss of easily exchangeable water in the transpiration process (T, g m^{-2} s^{-1}) and the inflow of water taken up by the roots (U, g m^{-2} s^{-1}). The plant's water content (V) is given by the formula:

$$V = V_i + \int_0^{\tau} (U - T)d\tau \ [g\ m^{-3}] \qquad \text{(Eq. 3)}$$

where V_i is the plant's initial water content, and $d\tau$ is the time interval after which the changes are considered.

The uptake of water through the root system (U) is calculated from the flow-producing gradient of potentials of leaf water (ψ_l, MPa) and of soil water (ψ_s, MPa), taking account of the resistances encountered by the flowing water, using the formula:

$$U = \frac{\psi_s - \psi_l}{r_r + r_p} \ [g\ m^{-2}\ s^{-1}] \qquad \text{(Eq. 4)}$$

where r_r is the resistance to the flow of water between the soil and root (MPa m^2 s g^{-1}), and r_p is the resistance of the plant's vascular bundles (MPa m^2 s g^{-1}).

Modelling was carried out for the months from April to September, applying the average meteorological parameters recorded at the weather station in those months (*Table 3*).

Table 3. *Average meteorological conditions for the days subject to modelling*

	Mai	June	July	August	September
Air temperature [°C]	13.2	16.7	18.0	17.2	12.0
Humidity [%]	74.5	77.9	74.7	76.3	83.5
Radiation [W m^{-2}]	124.2	143.0	148.6	120.2	65.8

LAI, gap fraction, light conditions

The leaf area index of the tree stand, the gap fraction, and the solar radiation below canopy were determined from hemispherical photographs, which were analysed using Hemiview software. The photographs were taken before and after thinning, during the period of maximum leaf area, at the same 10 points. During the

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study period no other foliage - reducing factors (insects, pathogen, drought, pollution, etc.) were identified.

Statistics

The distribution of EVT, ETP and T are non-normal. The significance of the differences in EVT, T, EVT ETP^{-1}, T ETP^{-1} and T EVT^{-1} before and after thinning was tested using Mann-Whitney test.

Results and discussion

Thinning leads to a reduction in the number of trees and the density of the canopy. Parameters providing a good description of a tree canopy are the leaf area index (LAI) and the gap fraction. The LAI is defined as the surface area of green leaves and needles per unit horizontal area of ground (Watson, 1947). This parameter concerns the main surfaces on which carbon dioxide and water are exchanged between the upper layer of the crown and the atmosphere. It is also a key variable for the functioning of the ecosystem. Many processes taking place in forests depend on the LAI, including rain and light interception (Gash, 1979), tree stand productivity (Davi et al., 2006), transpiration (Granier et al., 2000), and soil respiration (Davidson et al., 2002). Spatial differences in LAI are correlated with evapotranspiration and soil moisture (Grier and Running, 1977; Long and Smith, 1990; Burton et al., 1991; Jose and Gillespie, 1997). Thinning which reduced the basal area by 20% caused a decrease in LAI by approximately 30% on average, and an increase in the gap fraction by almost 50% (*Table 4*). The thinning of the canopy brought about an increase by more than 35% in the quantity of light reaching the lower layers of the trees and the soil.

Table 4. *Leaf Area Index (LAI). gap fraction (%) and radiation below canopy (R_{bc}, MJ m^{-2} $year^{-1}$) before and after thinning*

No. hemispheric image	Before thinning			After thinning			Percentage of change [%]		
	Gap fraction	LAI	R_{bc}	Gap fraction	LAI	R_{bc}	Gap fraction	LAI	R_{bc}
1	6.6	2.60	396	23.7	1.45	1493	259.5	-44.2	277.0
2	7.6	2.60	458	23.6	1.51	1360	210.9	-42.1	196.7
3	15.6	1.95	1042	17.9	1.49	1051	14.8	-23.4	0.9
4	19.8	1.68	1382	20.4	1.43	1228	3.0	-15.0	-11.1
5	17.9	1.92	1202	21.9	1.47	1246	22.1	-23.4	3.7
6	11.9	2.26	829	19.4	1.73	1204	62.1	-23.3	45.3
7	10.0	2.43	714	17.4	1.71	1160	73.0	-29.7	62.5
8	16.9	2.04	1228	18.1	1.37	1055	7.0	-32.5	-14.0
9	14.6	2.19	918	22.0	1.32	1270	51.3	-39.6	38.3
10	17.5	1.99	1170	21.3	1.46	1221	21.8	-26.7	4.4
Mean	13.9	2.17	934	20.6	1.49	1229	48.5	-31.0	31.6

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The persistence of the effects of canopy density reduction depends on the rate of crown growth. The growth of crowns to fill available space has been observed in the case of pine as well as other trees (Baldwin et al., 2000; Lockow, 2003). However the incremental growth of the crowns is smaller in older trees than in younger ones. A study by Juodvalkis et al. (2005) showed that a very significant rise in the increment of crown volume can be achieved through thinning in the case of young trees – for example, those aged 10–20 years in the case of pines. For older trees, crown growth will fall to below 10%. It can therefore be expected that the increase in throughfall will persist for longer in the case of older trees, but with younger ones, because of the higher rate of crown growth, the effect will persist for a significantly shorter time.

Many researchers have reported that larger quantities of precipitation reaching the soil may be one of the chief factors behind the increase in the available soil water supply following thinning (Zahner and Whitmore, 1960; Cregg et al., 1990; Stogsdill et al., 1992; Breda et al., 1995; Baumler and Zech, 1997;). They also identify reduced evapotranspiration or transpiration as a second factor. The effect of thinning on transpiration has been studied by many authors, but there are discrepancies in the results reported. Breda et al. (1995) found a relationship between reduced tree transpiration and reduced basal area in stands of *Quercus petraea* (Matt.) Liebl. A similar result was obtained by Morikawa et al. (1986) in a stand of *Chamaecyparis obtusa* Endl. However, in a study of transpiration in *Pseudotsuga menziesii* (Mirb.) Franco, conducted by Black et al. (1980), thinning was found to have little or no effect on tree transpiration. A similar result was obtained for a stand of *Pinus taeda* L. (Stogsdill et al., 1992), and in a study of *Pinus sylvestris* L. Vesala et al. (2005) also found that thinning did not affect the total tree transpiration.

The present study of changes in transpiration in pines and in the evapotranspiration of the pine ecosystem indicates that these processes intensify following thinning (*Fig.1*). Transpiration in the pines showed a statistically significant increase ($p = 0.0012$) of 41%, while actual evapotranspiration increased by 42%, which was again statistically significant ($p = 0.00005$). The ratio of the pines' transpiration to the evapotranspiration of the ecosystem ($T\ EVT^{-1}$) was practically the same before and after thinning, amounting to 0.58 before thinning and 0.60 after (difference not statistically significant, $p = 0.4547$). The change in conditions following thinning had an equal effect on the transpiration of individual trees and on the evapotranspiration of the whole ecosystem (*Table 5*). This finding also indicates the relatively low water requirements of the Scots pine. It is possible that the fact that the pines do not use up all of the water available at a given time is one of the main factors that have enabled that species to take hold in a wide range of forest habitats, from extremely dry to marshy.

Table 5. *Median values of potential evapotranspiration (ETP), actual evapotranspiration (EVT), transpiration of trees (T), and relations between ETP, EVT and T*

	Parameter					
	ETP [mm]	EVT [mm]	T [mm]	$EVT\ ETP^{-1}$	$T\ ETP^{-1}$	$T\ EVT^{-1}$
Before thinning	3.36	1.74	1.06	0.48	0.32	0.58
After thinning	3.48	2.48	1.50	0.65	0.39	0.60

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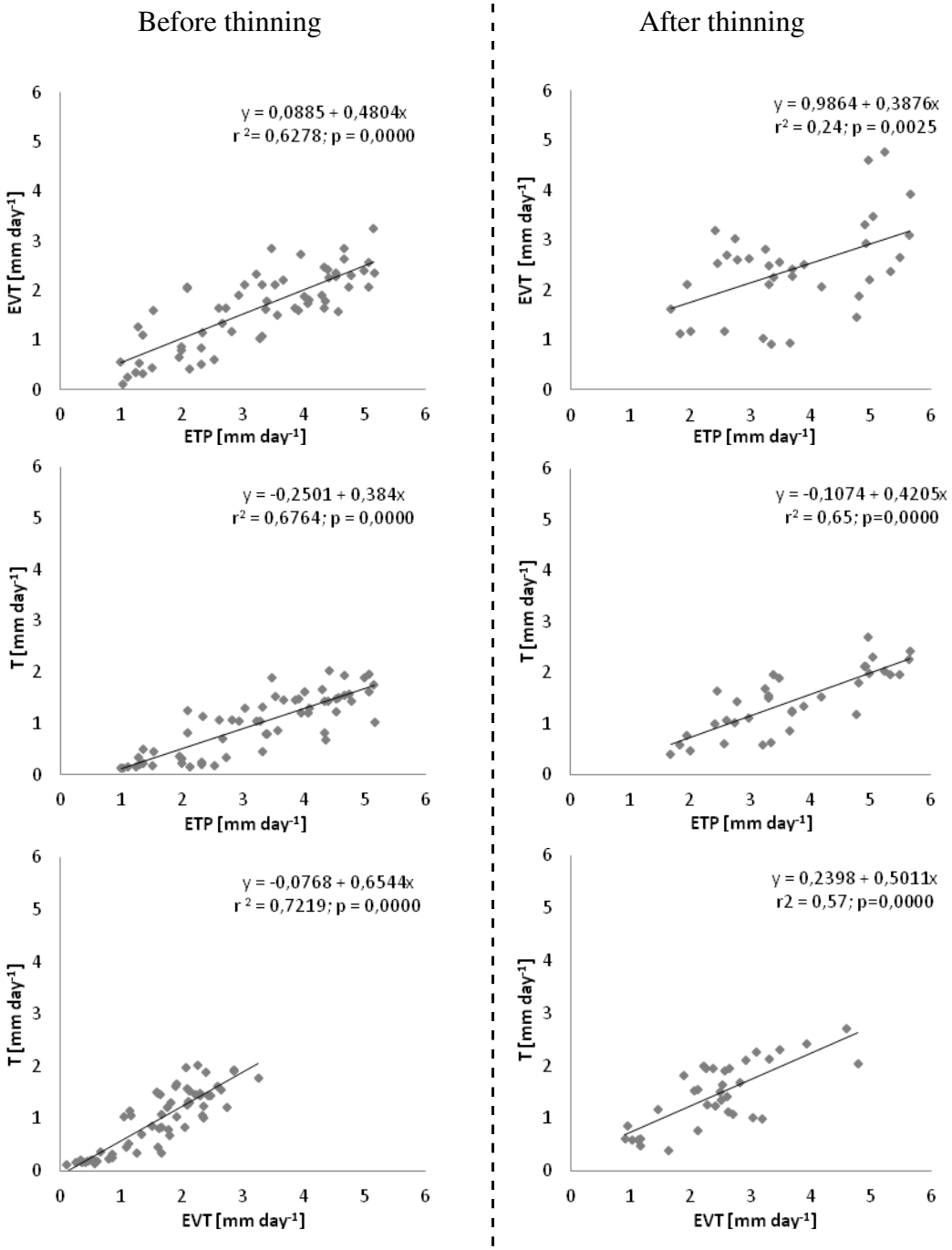

Figure 1. *Relationships between transpiration. actual evapotranspiration. potential evapotranspiration before and after the forest thinning*

Comparison of the results with potential evapotranspiration (difference not statistically significant, p = 0.1199), which provides an analysis for similar meteorological conditions determining the evaporation process, shows that tree transpiration increased by 22% (the ratio T ETP^{-1} was 0.32 before and 0.39 after thinning; difference statistically significant, p = 0.0003). The reason for this may be the reduction in the density of the crowns, which entails not only an increase in the growth

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space for individual trees, but possibly also the creation of improved conditions for transpiration. After thinning, a greater quantity of light reaches the crowns (Whitehead et al., 1984), and the creation of space between the crowns favours better exchange of water vapour and its rising above the crowns. Ensuring increased accessibility of light to the tree crowns is one of the reasons for which thinning is carried out; the growth of trees is thus accelerated by creating better conditions for photosynthesis. At the same time, better conditions are created for tree transpiration.

Evapotranspiration of the ecosystem showed a 35% increase following thinning (the ratio EVT ETP^{-1} was 0.48 before and 0.65 after thinning (difference statistically significant, $p = 0.0005$). In this case, the increase resulted partly from the higher tree transpiration, but another contributing factor may have been increased evapotranspiration of forest floor vegetation and the soil, as a result of the greater quantity of light reaching the forest floor. An increase in ecosystem evapotranspiration following a significant decrease in LAI was also observed by Knoche (2005).

An increased quantity of light penetrating through the tree canopy, along with increased soil moisture, will stimulate the development of lower vegetation layers. Lüttschwager et al. (1999) note the great importance of the ground layers of vegetation for evapotranspiration in pine stands, as do Müller et al. (1998). They showed that forest ground cover dominated by such species as *Calamagrostis epigejos* (L.) Roth and *Deschampsia flexuosa* (L.) Trin can, in favourable conditions, transpire up to almost 50% of the total water uptake of the forest ecosystem. A characteristic feature of these ground layer species is their high light requirement, hence a large reduction in the tree canopy may have a favourable effect on the occurrence and development of those species following thinning, which will lead to an increase in the total evapotranspiration of the forest ecosystem.

An important challenge faced by foresters in central Europe is the need to adapt tree stands to the effects of climate changes. It is projected that such changes will lead to warmer weather and lower rainfall during the vegetation period in central Europe (Degirmendžić et al., 2004; Briffa et al., 2009; Dubrovsky et al., 2009;). As a result, insufficiency of water in the environment will become more common, particularly in habitats with weak sandy soils with a deep groundwater level. Thinning, i.e. reducing the density of tree stands, may be a way of adapting the forest to the changing climate (Misson et al., 2003; Martin-Benito et al., 2010). Whitehead et al. (1984) believe that thinning will reduce the susceptibility of tree stands to drought, by reducing interception and increasing the soil water supply. Similar conclusions have been reported by Aussenac and Granier (1988) and Gracia et al. (1999). Thinning has been found to have a positive impact on drought stress in a study of 32-year-old black pines in Spain (Martin-Benito et al., 2010) and in 22-year-old spruce in the Belgian Ardennes (Misson et al., 2003).

The modelling performed here for average meteorological conditions, neglecting the effect of thinning on microclimate, showed that the number of days on which the pines deplete the soil water supply to a state in which no water is available to plants is greatest at the start and at the end of the vegetation season. There is similar variation in the number of additional days of water availability following thinning. The effect of the reduction in tree density was greatest in September, when available water was present for 9 days longer than was the case prior to thinning. In the months with the highest levels of transpiration (May to August) the time for which available water was present increased by 2 or 3 days (*Fig. 2*)

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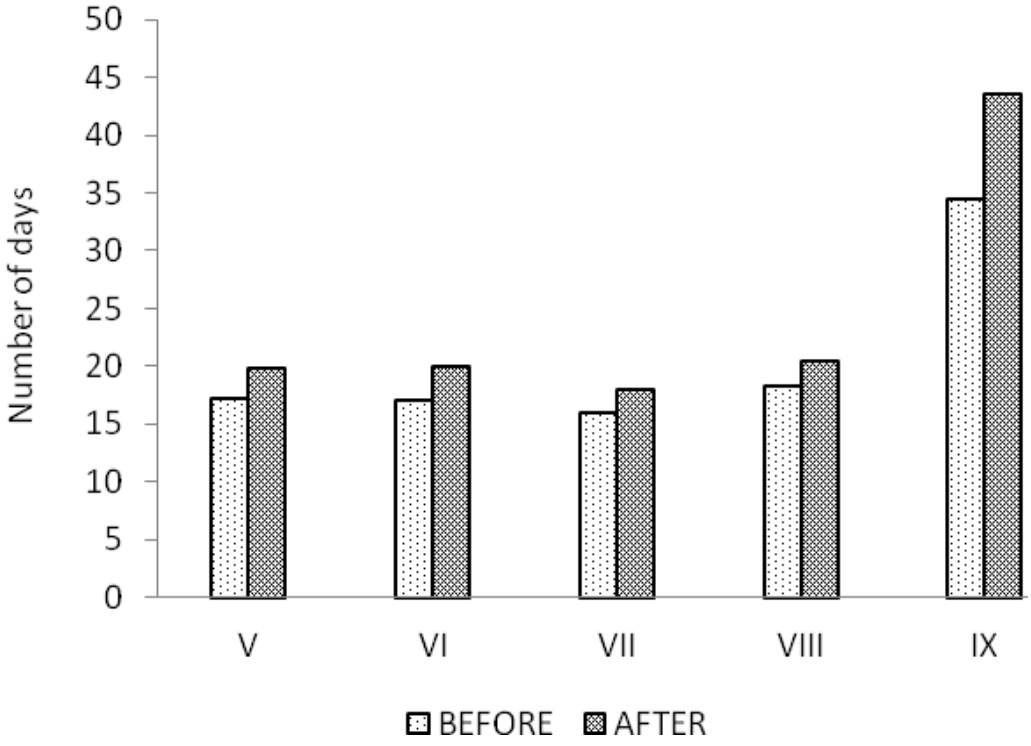

Figure 2. *Number of days on which the pine stand depleted the supply of soil water within the useful retention range (from pF = 2.0 to pF = 3.7) before and after thinning from May (V) to September (IX)*

The relatively small change in the length of the period in which plant available water was present may be of great importance for the tree stand's survival in rainless periods. In particular, Lagergren and Lindroth (2002) showed that a decrease in available soil water content (SWC) greatly reduces transpiration in pines, and hence even a small increase in SWC will have a strong impact on a stand's transpiration ability.

However, the modelling of meteorological conditions does not take account of micrometeorological changes occurring in the tree stand after thinning. These changes will lead to an increase in tree transpiration and the evapotranspiration of the ecosystem, as described above. Therefore, in order to draw accurate conclusions, it is necessary to carry out a study within the tree stand itself, although this may be difficult in view of the very high variability of the meteorological conditions found within the stand.

Conclusions

Reducing the number of trees will lead to a decrease in transpiration, and consequently a decrease in the evapotranspiration of a forest ecosystem. However, this study has shown that thinning in fact causes an increase both in the transpiration of individual trees and in the actual evapotranspiration of the ecosystem. Although thinning reduces the total transpiring leaf area, the reduction in the density of the canopy creates better conditions for intensity of transpiration, by increasing the quantity of solar radiation reaching the tree crowns, and also facilitates the rising of water vapour above the crowns. The actual evapotranspiration of the ecosystem is increased partly

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due to the greater tree transpiration, but another factor may be the higher transpiration of forest floor vegetation and soil evaporation, caused by the greater quantity of light penetrating the tree canopy. The contribution of the transpiration of the Scots pine to the evapotranspiration of the ecosystem was at a similar level of around 0.60 both before and after thinning.

A model-based study of the lengthening of the time in which soil water was available to plants showed that thinning produced a positive effect. It should be borne in mind, however, that the study was based on data from a weather station situated outside the forest. Thinning strongly modifies the micrometeorological conditions existing within a tree stand. Hence modelling based on data from standard weather stations may carry a certain degree of error.

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IDENTIFICATION OF SYNTOPIC ANURAN SPECIES IN EARLY TADPOLE STAGES: CORRESPONDENCE BETWEEN MORPHOMETRIC AND GENETIC DATA

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Abstract. Many European frogs and toads are relatively secretive species and except during breeding season, adults can rarely be seen during time-restricted fieldwork. In contrast, their tadpoles are easy to record and could be very useful in a brief biodiversity assessment. It is important to perform quick and accurate taxonomic identification of tadpoles, yet genetic methods are costly and cannot be routinely applied. We tested suitability of morphometric analysis for taxonomical distinction among tadpoles of early breeding local anuran species. Tadpole samples were collected simultaneously at three different locations in Republic of Serbia (South-eastern Europe) in habitats known to be breeding sites shared by brown frogs and the common toad. DNA barcoding verified *Rana dalmatina*, *R. temporaria* and *Bufo bufo* species, each collected in different location. The results of linear morphometric analyses suggested that relative head length and head width could be good discriminative characteristics for tadpoles of these two *Rana* species and those of *B. bufo*. To distinguish between tadpoles of two analyzed brown frog species, relative tail length could be used. For further development of the identification procedures for tadpoles of particular species, it is essential to involve geometric morphometrics and to analyze different larval developmental stages.
Keywords: *brown frogs, common toad, early breeders, taxonomic identification*

Introduction

Analyses of tadpole morphology have been shown as very applicable in anuran taxonomy and phylogeny (Duelmann and Trueb, 1994; Sidorovska et al., 2002; Grosjean, 2005; Vejarano et al., 2006), but nowadays they become interesting also for conservation studies in a broadest sence (Buskirk, 2009; Severtsova et al., 2012; Pujol-Buxo, 2013; Schulze et al., 2015). Some anuran genera include morphologically similar and partly syntopic taxa and their species can be recognized mainly on the basis of genetic differences and differences in advertising calls (Larson and Chippindale, 1993). Moreover, many European frogs and toads are secretive crepuscular or nocturnal species so adults, except during the breeding season, can rarely be seen during short visits to the place (Arnold and Ovenden, 2002). In contrast to adult individuals, their tadpoles are easily detectable through the whole aquatic life stage (McDiarmid, 1994). In such cases, confident taxonomic identification of tadpoles is sometimes the only way

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to do quick and complete assessment of anuran fauna in the area of interest (Gascon, 1991). Although topics relating to ecological studies and environmental impact assessments are demanding, genetic methods still cannot be routinely applied due to restricted funds, which is a common problem in such studies.

Morphometric analysis of tadpoles has been widely used in interspecific comparisons, but often based on the ratios of total length or snout-vent length, where intra-specific and intra-populational variability should be taken into consideration (Sidorovska et al., 2002; Grosjean, 2005; Arendt, 2010). Regarding the tadpole's morphology, there are two phases with remarkable changes: before stage 25 and after stage 42 (McDiarmid and Altig, 1999). Many authors described various tadpole larval phases in their work (details in Lima and Pederassi, 2012) but most of these studies focus on the stages between 37 and 39 (Lima and Pederassi, 2012).

The aim of this paper was to evaluate the use of tadpole morphometric analysis for taxonomic distinction among locally-occurring syntopic, early-breeding anuran species. Unlike other studies, our work was focused on early development stages (hatchlings, stages 23 to 25, according to Gosner, 1960), as at this phase distinction among those species is sometimes difficult in the field (Arnold and Ovenden, 2002). The development of procedures for confident taxonomic identification of anuran tadpoles, including hatchlings, could help in more effective faunistic and ecological surveys of amphibians. This is particularly important in areas where the narrow zones of sympatry and occurrence of syntopy among various anuran species are recorded, such as in Republic of Serbia in South-eastern Europe (Dufresnes et al., 2013; Vukov et al., 2013).

Material and Methods

The brown frogs of genus *Rana* (family Ranidae) are among the earliest-breeding European anurans; the adults are mostly terrestrial, with an aquatic larval stage. Some European brown frog species spawn in fast highland streams (e.g. *R. graeca* in the Balkans; Arnold and Ovenden, 2002) but most breed in various types of stagnant or moderately fast-running waters, occurring in lowland habitats and at altitudes up to 2745 m a.s.l. in the Alps (Veith et al., 2003). It has been noted that Eurasian brown frogs are sometimes difficult to classify (Che et al., 2007) which is an issue particularly in the areas of syntopy.

Three species of brown frog, namely, *Rana dalmatina*, *R. temporaria* and *R. graeca* are common in South-east Europe (Sillero et al., 2014) with *R. dalmatina* and *R. temporaria* being widely distributed throughout Europe (Gasc et al., 1997; Arnold and Ovenden, 2002; Sillero et al., 2014). As noted in Hartel (2005), syntopic habitats of these two species are rare and interspecific competition may contribute to their niche separation (Riis, 1988). A study along the Târnava Valleys in Romania showed that the domination of *R. dalmatina* over *R. temporaria* was a common phenomenon in the lower to middle parts of the Valley, while in the upper section (>600 m elevation) *R. temporaria* began to dominate (Hartel, 2005). On the contrary, the long-term studies in Western Europe (Gollmann et al., 2002) showed an inverse relationship i.e. the domination of *R. temporaria* upon *R. dalmatina* (Hettyey and Pearman, 2003; Hartel, 2005). *R. graeca* is a brown frog species, endemic in the Balkan Peninsula, where it inhabits river gorges and canyons at altitudes from 300 m to 1000 m (Asimakopoulos, 1997).

We collected samples of tadpoles in early spring 2013 in three locations in Serbia, where the occurrence of brown frog species was common knowledge (Crnobrnja, 1982;

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Arnold and Ovenden, 2002; Tomašević et al., 2008). In Serbia, the agile frog (*R. dalmatina*) has a widespread distribution (Vukov et al., 2013). In contrast, the grass frog (*R. temporaria*) has a rather scattered distribution and for this reason is considered to be a species of conservation concern (Crnobrnja-Isailović and Paunović, 2015; Vukov et al., 2015). Both agile and grass frogs start breeding activities shortly after the snow has melted and their mating season ends quickly (Hartel, 2005; Iosob and Prisecaru, 2014; Crnobrnja-Isailović et al., 2015). *R. graeca* mostly breeds in cool and fast-running watercourses in hilly and mountainous areas (Arnold and Ovenden, 2002) and its occurrence in certain locations in Serbia overlaps with either *R. dalmatina* or *R. temporaria* (Vukov et al., 2013).

The common toad (*Bufo bufo*) is the only other anuran species in the area that breeds almost as early in the spring as brown frogs (Arnold and Ovenden, 2002). Both the eggs masses and the tadpoles of these two genera, *Bufo* and *Rana,* are easily distinguished (Arnold and Ovenden, 2002). However, it could not work on very early developmental stage: shortly after hatching, the tadpoles of brown frogs and the common toad could have similar appearance and cannot be taxonomicaly identified *in situ* by simple visual inspection.

Description of sampling locations

Geographic position of sampling locations is presented in *Table 1* and *Fig. 1*.

Figure 1. *Geographic position of sampling locations.*

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Table 1. *Geografic coordinates of the sampling locations.*

Location	Longitude	Latitude	Altitude (*m*)
Zuce reservoir	44º 40' 55.9"	20º 33' 7.4"	240
Bigar Hill	44º 13' 26"	21º 52' 20"	720
Trešnjica River gorge	44º 07' 18.46"	19º 29' 57.3"	200

Each sampling location is distinctive regarding habitat characteristics and anthropogenic pressure. Lake Zuce, an artificial water reservoir, is situated in an agricultural area at the foot of Mountain Avala near the capital of Republic of Serbia, Belgrade, so the area is under considerable anthropogenic pressure. Geologically, Mt. Avala consists of limestone, marl, sandstone and serpentine rock. It is a conical hill, mostly covered by forest vegetation, both native and planted, trees including durmast oak (*Quercus petraea*), Turkish oak (*Quercus cerris*), hornbeam (*Carpinus betulus*), beech (*Fagus sylvatica*), linden (*Tilia europaea*), black pine (*Pinus nigra*) and black locust (*Robinia pseudoacacia*). Meadow vegetation is also present, but less extensive. The lake itself is surrounded by remnants of the deciduous forest (Tomašević et al., 2008). The only brown frog species recorded there is *R. dalmatina* (Crnobrnja-Isailović et al., 2012).

In comparison with the Zuce Reservoir, the other two sampling sites are under lower anthropogenic influence. The hilly-mountainous stream of Bigar is located on Bigar hill, part of the Homolje mountain range in eastern Serbia. The hill is composed of limestone and its vegetation consists of beech forest communties (*Acero Carpinetum betuli* [maple and hornbean]), *Fagetum montanum* (mountain beech) and *Fagetum montanum subas, Corydalo Fagetum* (community of beech with Holewort and Spring fumewort) and *Acero Fagetum* (beech with maple). In early spring, these communities are dominated by annual species, mostly ground flora, such as *Corydalis solida, Corydalis cava, Dentaria bulbifera* etc. Also, the area is covered in grassland, in the form of mowed meadows. The Bigar stream is one of the biggest permanent streams in this area. The stream bed is up to 1m in width and 30cm deep on average, and it joins with the Valja Saka stream to form the Jagnjilo River, which continues to flow to the north. Like all watercourses in this part of Serbia, Bigar stream belongs to the Danube Basin (Paunović et al., 2014). Two brown frog species occur in the area – *R. dalmatina* and *R. temporaria* (Crnobrnja, 1982).

Trešnjica River (2-3m in width and up to 50cm deep) is located in western Serbia. This clean mountain river emerges below Povlen Mountain in western Serbia, and after 23 km it flows into the Drina River, also belonging to the Danube catchment. Immediately before joining the Drina, Trešnjica flows through a several-kilometre long limestone gorge, in places characteristic of a canyon valley. The vegetation mainly comprises Oriental hornbeam (*Carpinus orientalis*), Turkish oak (*Quercus cerris*), Italian oak (*Quercus frainetto*), black pine (*Pinus nigra*), prickly juniper (*Juniperus oxycedrus*), and several other thermophilous species. Trešnjica River gorge is one of Serbia's nature reserves (Amidzić et al., 2007). The only known brown frog species spawning in this river is *R. graeca* (Arnold and Ovenden, 2002).

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Sampling procedure

On every locality, ten individuals were gathered from the same aggregation of tadpoles by a standard deep or hand net, and they were preserved in 70% ethanol. In laboratory, tissue samples for genetic analyses (e.g. tip of the tail) were taken after measurement procedure and deposited in 95% ethanol. The collected tadpoles were in early development stages – hatchlings (stages 23 to 25, according to Gosner, 1960). Their body colour in all samples was black, while only specimens from Bigar Hill had visible external gills. Samples were deposited at the Department of Hydro-ecology and Water Protection, Institute for Biological Research "Siniša Stanković", University of Belgrade. Collection permit was issued by Ministry of Energetics, Development and Nature Protection of Republic of Serbia, No. 353-01-54/2013-08.

DNA extraction, amplification and sequencing

We have randomly chosen three of ten tadpoles from each locality from the same dense aggregation of tadpoles for genetic identification. Total DNA was extracted from 5 mm of tadpole's tail muscle, using the *AccuPrep* Genomic DNA Extraction kit, according to the manufacturer's instruction (Bioneer Corporation, Daejeon, R. Korea). The tissue was incubated in a tissue lysis buffer at 50°C, for one hour. After precipitation and washing, DNA was eluted using a 75microL elution buffer. Depending on the species, an (approximately) 380 bp fragment of mitochondrial *16S rRNA* gene was amplified by PCR with termalprofile and primers (16Sar: 5'-CG CCTGTTTATCAAAAACAT-3' 16Sbr: 5'-CCGG TCTGAACTCAGATCACGT-3') described in Veith et al. (2003). Special care was taken to ensure sterile conditions, and for each PCR run negative control (with water instead of a template) was used as a contamination check. Amplicons were sequenced in both directions using a BigDye® Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA) and sequences were base called and assembled with ABI software: Sequencing Analysis 5.1 and SeqScape software, v 2.5. The obtained sequences were deposited in GenBank database (http://www.ncbi.nlm.nih.gov/genbank) under accession numbers KR136355 - KR136364 (Appendix 1).

Additional sequences for analysis were downloaded from GenBank. All sequences were aligned by ClustalW (Thompson et al., 1994) and visually inspected in Bioedit 7.2.5 (Hall, 1999). A best-fit substitution model in aligned sequences was examined by JModelTest v.2.1.4. (Darriba et al., 2012) while phylogenetic analysis was conducted using MEGA 6.0 (Molecular Evolutionary Genetics Analysis software; Tamura et al., 2013). Tadpole species were identified using a DNA barcoding approach, based on sequenced *16S rRNA* gene part.

The Maximum Likelihood (ML) method, integrated in MEGA 6.0 software, was used to build a phylogenetic tree. Bootstrap analysis was done to determinate the strength of support for a clade on the phylogenetic tree.

The *Bombina variegata* species' sequence was used to root the tree.

Morphometric analysis

The tadpoles were placed in a Petri dish with constantly refreshed water, in order to avoid desiccation. The subjects' dorsal, lateral and ventral sides were photographed using a binocular magnifier Carl Zeiss, Stemi 2000-C with magnification 6.5 and a

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digital camera AxioCamERc 5s, Zeiss. ZEN 2011 software and ImageJ (Abramoff, 2004) were used for all measurements.

Morphometric characteristics used in subsequent analyses were chosen in accordance with available literature (Van Buskirk and Relyea, 1998; Sidorovska et al., 2002; Vences et al., 2002; Dey and Gupita, 2002; Grosjean, 2005; Vejarano et al., 2006; Altig, 2007; Arendt, 2010; Di Cerbo and Biancardi, 2010; Severtsova et al., 2012; Johansson and Richter-Boix, 2013). These were: ed – eye distance, hh – head height, e, – eye diameter, tl – tail length, cc – central tail muscle, th – tail height, m – mouth length, hwv – head width, hlv – head length and bl – body length. Measurements were standardized by body length (bl) to obtain standardized values for further analyses (ED – eye distance, HH – head height, E – eye diameter, TL – tail length, CC – central tail muscle, TH – tail height, M – mouth length, HWV – head width, HLV – head length).

The normality test (Shapiro-Wilk's test) was used to determine if the data set had normal distribution. Descriptive statistics were used to calculate various measurements, such as mean, standard deviation, minimum and maximum, range, median etc. of the obtained data. Levene's test of equality of variances between variables was used to assess homogenity of data (Levene, 1960). Test of equality of covariances (Box's test) (Box, 1949) was used to assess homogenity of covariance matrices. One-way ANOVA with contrasts was used to analyze the significance of differences between group means for tested variables. The linear regression was applied for modeling the relationships between tested variables. Pearson product-moment correlations were used to check linear correlation between analyzed variables. Uncorrelated variables were excluded from further multivariate analysis (Canonical Discriminant Analysis; CDA). The CDA was used to determine which variables discriminate analyzed data of these naturally co-occurring groups, and to visualize its relationships (Quinn and Keough, 2002; Young and Young, 1998; Simonović, 2004; Ivanović and Kalezić, 2009; Hair et al., 2010).

Performed statistical analysis were done by using the software package Statistica 7.0 (StatSoft, 2004).

Results

Phylogenetic analysis

The final dataset for phylogenetic analyses included 27 sequences from GenBank and 10 sequences obtained during our analysis (*Appendix 1*). As there is a lack of *R. graeca* sequences in GenBank, especially in the case of *16S rRNA* gene, we sequenced the tissue sample (a fingertip) collected earlier from one *R. graeca* adult from Serbia (Access no. KR136364). After careful examination, obtained sequences of *16S rRNA* gene, 421 - 537 bp long, were cut to 348 bp reliable sequence and used in phylogenetic analysis. The performed model test determined Tamura Nei model (1993) with invariant sites (TN93+I) as the most suitable. The maximum likelihood (ML) method produced a tree (*Fig. 2*) with good support for the analyzed species.

Two main clades were distinguished on obtained phylogenetic tree corresponding to analyzed *Rana* and *Bufo* gene sequences. The *Rana* clade included two subclades - one containing *R. temporaria* and the other with *R. dalmatina* and *R. graeca*. Within *R. dalmatina,* samples from Serbia formed a separate cluster, with good support from other analyzed conspecific sequences from Moldova, Spain, Germany and France. Within *R. temporaria* subclade, two main lineages were observed. Samples from Serbia were clustered with those from Spain, Czech Republic, Sweden, Russia and Ukraine. The

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other lineage contained conspecific sequences, mostly from the Western Europe. The *Bufo bufo* clade was well defined in the obtained tree (consider that *B. bufo* PT sequence information taken from the GenBank is in fact *Bufo spinosus* PT, due to taxonomic revision of *Bufo bufo* species group after the paper by Recuero et al., 2012). Besides samples from Greece, samples from Serbia also formed a separate cluster. We obtained uncosistent results regarding sample from Trešnjica river: DNA analysis showed that those tadpoles belong to *B. bufo* rather than to *R. graeca* what was expected based on geographical position and type of the breeding site.

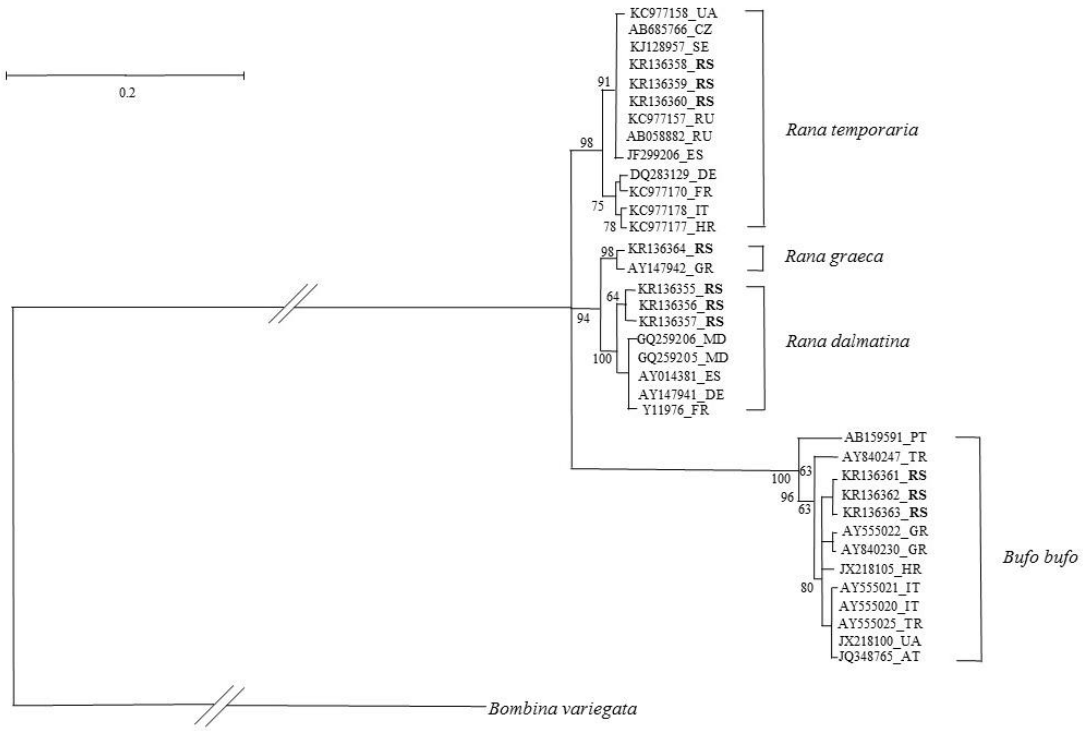

Figure 2. *Phylogenetic analysis of 16S rRNA gene fragment by Maximum Likelihood method based on the Tamura-Nei model. Bootstrap value (<60%) was showed and confirmed the clade at the end of a branch.*

Morphometric analysis

The value ranges of selected raw measurements are presented in *Table 2*.

Table 2. *Value ranges of selected raw body measurements; n –sample size. Abbreviations of the variables are explained in Material and Methods. All the measurements were in mm.*

Species (n)	bl	hl	hw	tl	th
R. dalmatina (10)	10.08 – 11.27	3.47 – 3.90	1.60 – 2.05	5.92 – 7.29	1.64 – 2.46
R. temporaria (10)	8.90 – 10.68	2.75 – 4.20	1.32 – 1.84	4.91 – 6.27	1.38 – 1.94
B. bufo (10)	10.12 – 11.20	4.04 – 4.70	2.15 – 2.64	5.61 – 6.44	1.77 – 2.17

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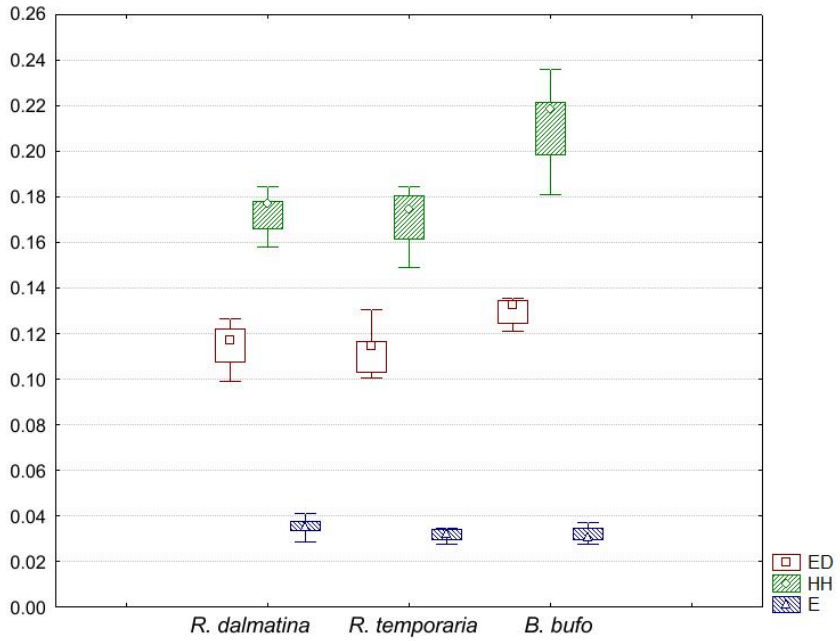
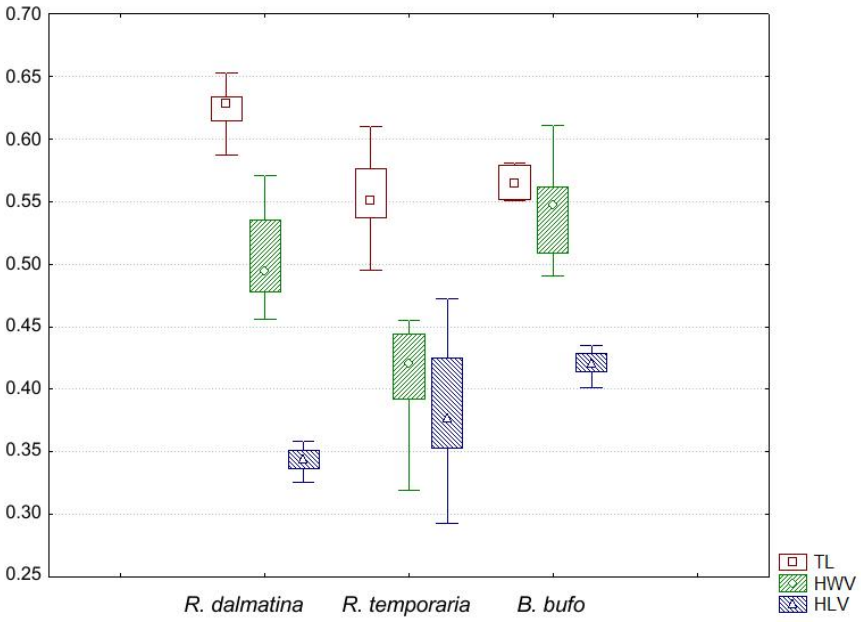
The measurements were further standardized by body length (bl) to obtain values for subsequent analyses (ED, HH, E, TL, CC, TH, M, HWV and HLV). Since the sample size was less than 50, Shapiro-Wilk's test was used to check the normality of data. In the tested data set, the obtained p-value was greater than the chosen alpha level (α=0.05), confirming that the analyzed data had a normal distribution.

The results of descriptive statistics (*Fig. 3.*) highlighted the similarity of ED and HH values among the analyzed *Rana* species, compared to *B. bufo,* suggesting that these two parameters could distinguish the two genera at early tadpole stages.

a)



b)



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c)

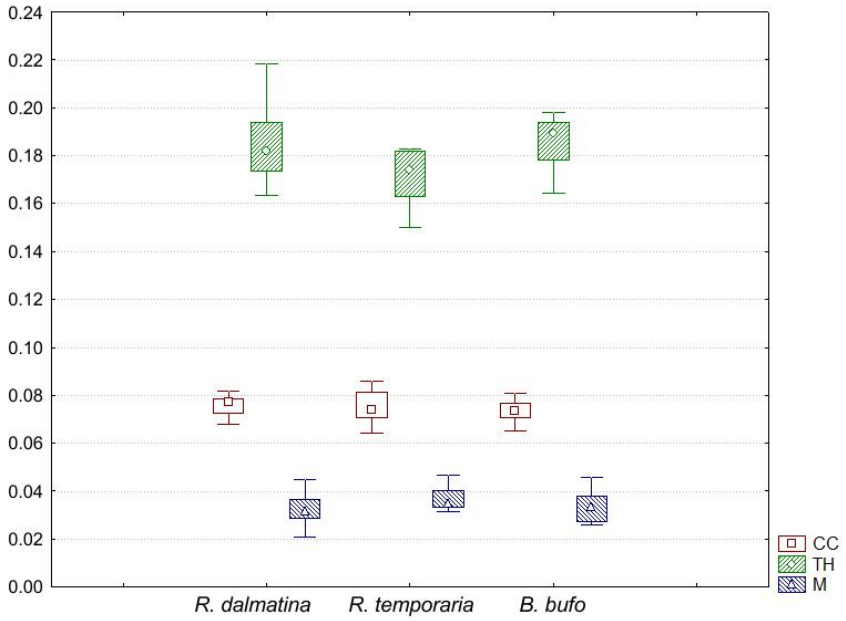

Figure 3. *Descriptive statistics of the standardized measurements in tadpoles of analyzed anuran species: a) variable ED, HH and E; b) variable TL, HWV and HVL; c) variable CC, TH and M. Abbreviations of the variables are explained in Material and Methods.*

Moreover, TL, HLV and especially HWV differed in all three analyzed data sets. Thus, these parameters could be used in species recognition. It was notable that E, TH, CC and M values were relatively similar in all three data sets.

Performed tests of homogeneity (Levene's test and Box M test) showed homogeneity (equality of variances/covariances) for all tested variables with the exception of variable HLV.

To test statistical significance of differences between means of used variables (for analyzed species), one-way ANOVA with contrasts was applied. As it could be seen from *Table 3*, variables which differ significantly ($p<0.05$) among examined species, were ED, HH, TL, HWV and HLV.

Table 3. *Results of one –way ANOVA with contrasts (significant differences ($p<0.05$) are in bold); Estimate –Estimated mean value; Std. Err –Standard Error; t – t value; p – p value; Cnf. Lmt (-95%), Cnf. Lmt (+95%) – Confidence Intervals for Mean.*

	Estimate	Std. Err	t	p	Cnf. Lmt (-95%)	Cnf. Lmt (+95%)
ED	-0.032690	0.006351	-5.14736	**0.000021**	-0.045721	-0.019659
HH	-0.085195	0.011152	-7.63957	**0.000000**	-0.108077	-0.062314
E	0.003240	0.003502	0.925069	0.363125	-0.003946	0.010425
TL	0.047562	0.017595	2.703096	**0.011736**	0.011459	0.083665
CC	0.005678	0.004581	1.239504	0.225825	-0.003721	0.015078
TH	-0.016355	0.010192	-1.60466	0.120203	-0.037267	0.004558
M	0.002175	0.005979	0.363826	0.718821	-0.010092	0.014443
HWV	-0.156075	0.037526	-4.15910	**0.000290**	-0.233073	-0.079078
HLV	-0.106239	0.025620	-4.14680	**0.000300**	-0.158806	-0.053672

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Prior to applying multivariate analysis, Pearson's product-moment correlation was run to eliminate uncorrelated parameters. Eye diameter (E), central tail muscle (CC) and mouth length (M) showed no correlation ($p<0.05$) and were thus excluded from further analysis (*Table 4*).

Table 4. *Pearson correlations among tested variables (marked correlations that are significant at $p < 0.05$ are in bold). Abbreviations of the variables are explained in Material and Methods.*

	ED	**HH**	**E**	**TL**	**CC**	**TH**	**M**	**HWV**	**HLV**
ED	1.000	**0.450**	-0.047	-0.314	-0.244	0.065	0.135	0.320	**0.413**
HH	**0.450**	1.000	0.004	-0.225	-0.114	**0.365**	0.091	**0.576**	**0.453**
E	-0.047	0.004	1.000	0.121	0.139	-0.028	-0.344	-0.110	0.074
TL	-0.314	-0.225	0.121	1.000	0.229	0.300	-0.217	0.211	**-0.384**
CC	-0.244	-0.114	0.139	0.229	1.000	0.113	0.142	0.014	-0.005
TH	0.065	**0.365**	-0.028	0.300	0.113	1.000	0.087	**0.521**	-0.137
M	0.135	0.091	-0.344	-0.217	0.142	0.087	1.000	-0.077	0.088
HWV	0.320	**0.576**	-0.110	0.211	0.014	**0.521**	-0.077	1.000	-0.226
HLV	**0.413**	**0.453**	0.074	**-0.384**	-0.005	-0.137	0.088	-0.226	1.000

Canonical Discriminant Analysis (CDA) revealed that HWV and HLV are the most informative characters for taxonomical distinction between tadpoles of brown frogs and those of the common toad, while TL was considered the most informative for distinguishing between tadpoles of two brown frog species.

Table 5. *The roots of the discriminant analysis (CDA), their discriminatory power and loads (the most discriminative variables are in bold).*

	Root 1	**Root 2**
ED	-0.31469	0.480344
HH	-0.27051	-0.075231
TL	-0.19050	**1.049517**
TH	-0.11888	0.113444
HWV	**-0.66539**	0.068916
HLV	**-0.83712**	-0.426270
Eigenval	12.58955	3.311605
Cum.Prop	0.79174	1.000000

The complex relationship among selected morphometric parameters in analyzed tadpole samples was presented in two-dimensional space of first and second discriminant axes (*Fig. 4.*).

The first axis (root) distinguished *B. bufo* (to the left of the graph) from genus *Rana* (to the right of the graph). Distinction of the analyzed *Rana* species occurred along the second root.

Linear regression analysis was used for a detailed investigation of the relationship between the most significant variables, by canonical discriminant analyses (HLV, HWV

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and TL). As values of the coefficient of determination (r^2) were low (< 0.3), only the TL/HWV relationship was presented on the graph (*Fig. 5*).

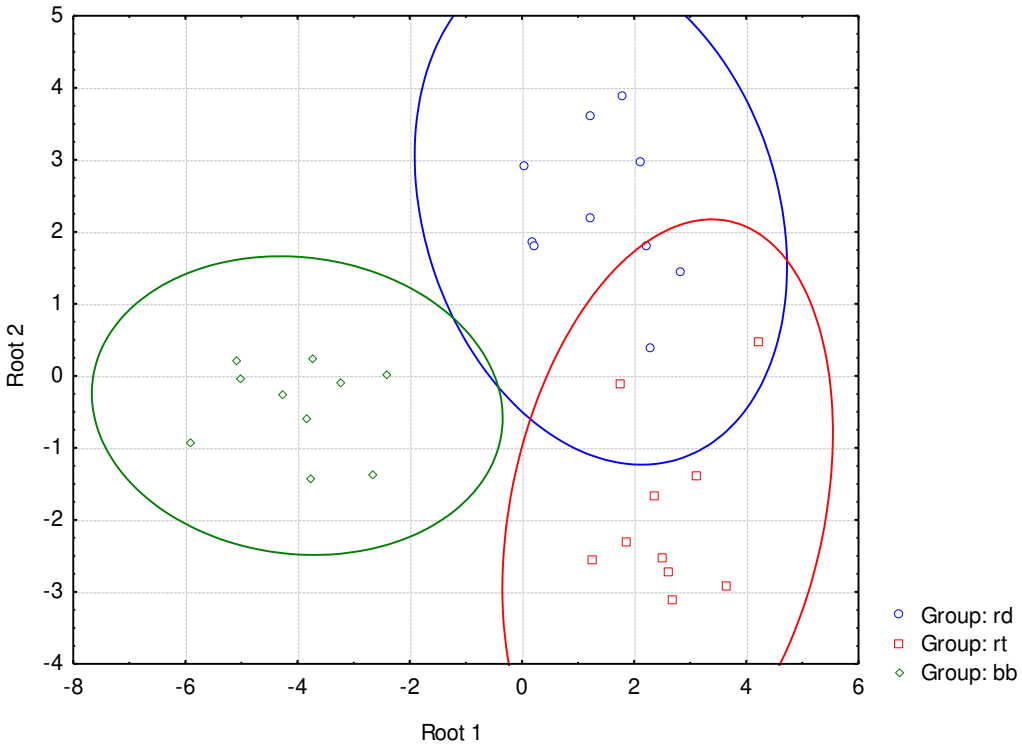

Figure 4. *Canonical Discriminant Analysis: rd – R. dalmatina, rt – R. temporaria, bb – B. bufo.*

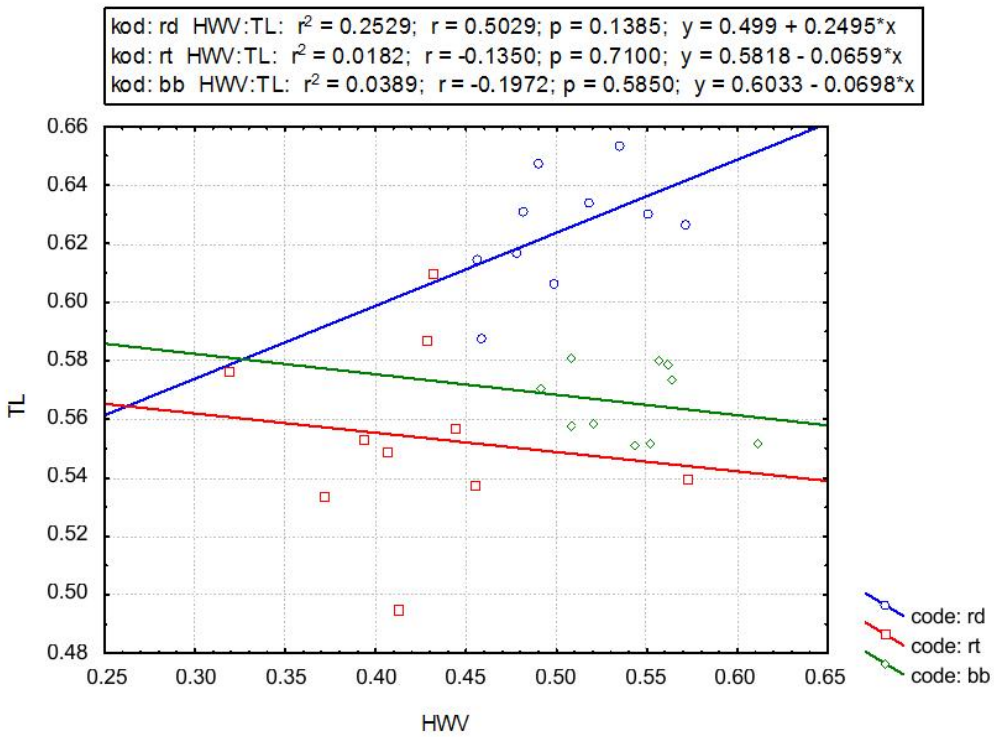

Figure 5. *Regression analysis of head width (HWV) on tail length (TL); rd – R. dalmatina, rt – R. temporaria, bb – B. bufo.*

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It was notable that *R. dalmatina* showed a positive correlation, in contrast to the negative one occurring in *R. temporaria* and *B. bufo*. The best approximation with a linear regression line was observed in *R. dalmatina* (r^2=0.25), while the weakest one was detected in *R. temporaria* (coefficient of determination was 0.01).

Discussion

Gene sequence, based on *16S rRNA* gene, confirmed the morphological identification of the analyzed species. The obtained phylogenetic tree showed that the *R. temporaria* clade was the most heterogeneous, as mentioned previously in the work of Reh and Seitz (1990) and Vences et al. (2013). Further, analyzed sequences from Serbia were shown to be closer to the North-Eastern group (samples form Czech Republic, Russia, Ukraine and Sweden) than to specimens from the South and Western group (samples from Croatia and Italy). A similar relation was detected in another typical boreal species e.g. *Zootoca vivipara* (ex *Lacerta vivipara*), where Surget-Groba et al. (2001) showed a closer similarity between Bulgarian populations and East European ones than with adjacent parts of the Balkan Peninsula and Western Europe (Crnobrnja-Isailović, 2007). Although the common frogs' postglacial re-colonization of Europe was not as straightforward as had been previously assumed, with numerous small, cryptic refugia (Teacher et al., 2009), our results could denote Serbia as one of these refugia (or re-colonization hotspots) in the case of North-East Europe.

R. dalmatina samples from Serbia were well separated from others collected in Spain, Moldova, France and Germany. In the *B. bufo* clade, samples from Serbia were placed together with those from Greece, Italy, Austria and Croatia, but somewhat apart from the sample from Portugal, being in line with the results of Recuero et al. (2012) who revealed that common toads from the Iberian Peninsula belong to *B. spinosus,* while Appenine, Central European and Balkan ones represent *B. bufo.*

Descriptive statistics and one-way ANOVA showed fewer differences in samples of *R. dalmatina* and *R. temporaria* tadpoles in comparison with *B. bufo*. Canonical Discriminant Analysis showed that the most useful measures for distinguishing tadpoles of the three analyzed species were body size standardized tail height (TL), head width (HWV) and head length (HLV). Relative head size is a determinative characteristic for distinguishing between *Rana sp.* and *B. bufo*, while the relative tail length is the most informative for distinguishing between the two *Rana* species. Additionally, in all cases linear regression analysis of the most informative variables (HLV, HWV and TL) showed a separation of *R. dalmatina* from the other two analyzed species, while the coefficient of determination (r^2) was low in all cases.

Furthermore, we learned from this study that *B. bufo* and *R. graeca* tadpoles could be visually misidentified at very early developmental stages if they occur syntopically in highland running waters in the early spring (a common spawning type for the Greek frog but not so usual for the Common toad). Our further analysis will include comparison of the same morphometric measures used in this study, taken simultaneously from tadpoles of all three brown frog species and the common toad, all raised in the laboratory from fertilized eggs collected in the natural habitat in order to follow, compare and detect significant allometric changes occuring within and among three species. Additionally, our future plans include also a comparative body shape analysis on samples of three species of brown frogs and the common toad, done at several stages of tadpole development by using geometric morphometrics.

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APPENDIX

Appendix 1. *The list of sequences that were used in the analyses (GenBank accession numbers and countries of origin).*

Species	Country	Access no.
Rana dalmatina	Serbia	KR136355
Rana dalmatina	Serbia	KR136356
Rana dalmatina	Serbia	KR136357
Rana dalmatina	Germany	AY147941
Rana dalmatina	France	Y11976
Rana dalmatina	Spain	AY014381
Rana dalmatina	Moldova	GQ259206
Rana dalmatina	Moldova	GQ259205
Rana graeca	Serbia	KR136364
Rana graeca	Greece	AY147942
Rana temporaria	Serbia	KR136358
Rana temporaria	Serbia	KR136359
Rana temporaria	Serbia	KR136360
Rana temporaria	Spain	JF299206
Rana temporaria	Germany	DQ283129
Rana temporaria	France	KC977170
Rana temporaria	Russia	AB058882
Rana temporaria	Russia	KC977157
Rana temporaria	Sweden	KJ128957
Rana temporaria	Ukraine	KC977158
Rana temporaria	Czech Republic	AB685766
Rana temporaria	Italy	KC977178
Rana temporaria	Croatia	KC977177
Bufo bufo	Serbia	KR136361
Bufo bufo	Serbia	KR136362
Bufo bufo	Serbia	KR136363
Bufo bufo	Portugal	AB159591
Bufo bufo	Italy	AY555021
Bufo bufo	Italy	AY555020
Bufo bufo	Turkey	AY840247
Bufo bufo	Turkey	AY555025
Bufo bufo	Greece	AY555022
Bufo bufo	Greece	AY840230
Bufo bufo	Croatia	JX218105
Bufo bufo	Ukraine	JX218100
Bufo bufo	Austria	JQ348765
Bombina variegata	HE794027	

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INFLUENCE OF INITIAL CELL CONCENTRATIONS ON THE GROWTH RATE AND BIOMASS PRODUCTIVITY OF MICROALGAE IN DOMESTIC WASTEWATER

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Abstract. The aim of this study was to compare the specific growth rate and biomass productivity of microalgae in domestic wastewater according to the initial cell concentration. The initial microalgae cell concentrations tested started from 10^3 cell/mL, 10^4 cell/mL, 10^5 cell/mL, 10^6 cell/mL, and 10^7 cell/mL under outdoor condition. The result revealed that the highest biomass productivity occurred at 10^6 cell/mL concentration with a value of 1.24×10^4 cell/mL/day, 0.26 day^{-1} of specific growth rate, and a doubling time of 2.63 days. Meanwhile, the lowest biomass productivity occurred at 10^3 cell/mL concentration with the lowest specific growth rate of 0.1 day^{-1} and the longest doubling time, which reached up to 7.14 day. As a result, the initial cell concentration of microalgae did influence the algal biomass productivity and growth rate differently. Thus, the maximum growth rate and biomass productivity were obtained at 10^6 cell/mL concentration which is recommended to be used in biotechnology industries and any wastewater treatment.
Keywords: *microalgae, growth rate, biomass productivity, wastewater, and cell concentration*

Introduction

Currently, growing microalgae in wastewater has become a topic of interest among researchers worldwide for its potential in phycoremediation, biofuel and hydrocarbon production, greenhouse gases mitigation and other factors (Gani, et al., 2015a; Gani, et al., 2015b; Onalo et al., 2014; Komolafe, et al., 2014; Rasoul-Amini et al., 2014; Lim and Vadivelu, 2014; Prakash et al., 2014; Shin et al., 2015; Abdelaziz et al., 2014; Mehrabadi et al., 2014; Slade and Bauen, 2013; Guo et al., 2013; Kothari et al., 2012; Park et al., 2011). The success of culturing mostly depends on the availability of nutrients in wastewater, culturing techniques, and environmental factors such as light, temperature, salinity, pH, and photoperiod (Komolafe, et al., 2014; Cai et al., 2013; Udom et al., 2013; Zhu et al., 2013; Sacristán de Alva et al., 2013; Kothari et al., 2013; Mata et al., 2012; Asulabh et al., 2012; Kirrolia et al., 2012; Qin and Li, 2006). The uniqueness of microalgae is that they like ordinary crops. In this case, microalgae absorb CO_2 in the atmosphere while producing O_2 by assimilating nutrients in wastewater (Muñoz and Guieysse, 2006; Mata et al., 2010; Rawat et al., 2011; Brennan

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and Owende, 2010; Abdel-Raouf et al., 2012; Sivakumar and Rajendran, 2013; Slade and Bauen, 2013; Ramachandra et al., 2013; Gani, et al., 2015c). For example, nutrients taken up by microalgae in wastewater such as nitrogen, phosphorus, and carbon are able to reduce eutrophication in aquatic environments (Karthikeyan et al., 2012; Choul-gyun 2002; Chu et al., 2014; Aslan and Kapdan, 2006; Rasoul-Amini et al., 2014; Boonchai et al., 2012; Kim et al., 2013; Valley et al., 2012; Can et al., 2013).

As discussed by Oswald (1957) and De la Noüe et al. (1992), there are many species of algae in nature, some of which has already been grown by other researchers using wastewater as the alternative to synthetic media, such as *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp., and others. For example, Mata et al. (2012) cultivated *Scenedesmus obliquus* in synthetic brewery wastewater to analyse the potential of biomass production. They found that the maximum dry biomass was 0.9g per litre of culture. In the same study, *Scenedesmus obliquus* successfully reduced the value of COD and TN up to 57.5% and 20.8%, respectively. Other than that, Zhu et al. (2013) has successfully grown freshwater microalgae (*Chlorella zofingiensis*) in piggery wastewater to characterize the algal growth and nutrients removal. Their study used a column photobioreactor with varying nutrient concentration and found that biomass productivity was different subject to several concentrations. Also, Kothari et al. (2013) used dairy industry wastewater for the cultivation of *Chlamydomonas polypyrenoideum* on biodiesel production. Results indicated that dairy wastewater was a great medium for algae growth when 75% concentration was applied. Meanwhile, culturing *Botryococcus braunii* in urban wastewater was studied by Can et al. (2013). The author found that the best growth using different loading concentrations also occurred in 75% urban wastewater, while without dilution (100%) the urban wastewater was better in terms of lipid production.

Although many researchers have investigated microalgae *Botrycococcus* sp., a few of them focused on synthetic medium (Eroglu et al., 2011; Molnár et al., 2012; Wang et al., 2014; Dayananda et al., 2005; Cheng et al., 2013; Ashokkumar and Rengasamy, 2012; Suzuki et al., 2013) compared to domestic wastewater as the medium (Can et al., 2013; Órpez et al., 2009). So it is necessary to perform deeper research on *Botryococcus* sp. to be grown in domestic wastewater for the potential of biomass production. The aim of this paper is to compare the specific growth rate and biomass productivity of *Botryococcus* sp. in domestic wastewater according to the initial cell concentration, while the raw characteristics of domestic wastewater are also determined.

Materials and Methods

Preparation of microalgae

Microalgae used in this experiment were collected and isolated from a tropical rainforest in the Southern region of Peninsular Malaysia (between N 02° 30.711" E 103° 20.984" and N 02° 30.740" E 103° 20.996"), namely *Botryococcus* sp. Initial stock cultures of *Botryococcus* sp. were maintained in modified Bold's Basal medium (Bischoff and Bold, 1963) containing the following chemicals: NaNO3, CaCl2.2H2O, MgSO4.7H2O, K2HPO4, KH2PO4, NaCl, EDTA, KOH, FeSO4.7H2O, H2SO4 and micronutrients (ZnSO4.7H2O, MnCl2.4H2O, MoO3, CuSO4.5H2O and Co(NO3)2.6H2O). The culture was inoculated in outdoor condition for 14 days. Prior to inoculation, microalgae cultures were harvested using a centrifuge at low speed (3500rpm) for ten minutes and washed three

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times with sterilized distilled water. This was followed by cell observation and cell concentration count using Neubauer haemocytometer.

Sampling and characterization of wastewater

The wastewater used in this study was effluent wastewater obtained from a domestic wastewater treatment plant located in Batu Pahat, Johor, Malaysia. The samples were collected in the morning at around 7:00am to 9:00am using acid washed sample bottles at the site and immediately transferred to the laboratory and preserved at temperatures below 4°C in a refrigerator. Then, wastewater quality parameters were immediately characterized once the samples reached the laboratory to avoid changes due to chemical and biological reactions. Chemical oxygen demand (COD), biochemical oxygen demand (BOD), total phosphorus (TP), dissolved oxygen (DO), and pH analysis were measured according to the standard methods (APHA, 2012). While total nitrogen (TN), total organic carbon (TOC), total carbon (TC), and inorganic carbon (IC) were obtained using TOC Analyzer (Brand: TOC-VCSH, Japan, Shimadzu). Before the inoculation process, the wastewater sample was filtered using a nylon membrane filter (Whatman) with a 0.45µm pore size to remove other microorganisms and suspended solids.

Experimental setup

A total of 15 Erlenmeyer flasks (500mL) were filled with 200 mL wastewater and were used in this experiment as the domestic wastewater. The wastewater experiment flasks (triplicate) were inoculated with microalgae starting with an initial cell concentration of 10^3 cells/mL based on the standard methods (APHA, 2012) and increased up to 10^7 cell/mL (10^3 cell/mL, 10^4 cell/mL, 10^5 cell/mL, 10^6 cell/mL, and 10^7 cell/mL) (Kothari et al., 2013). The flasks were covered with sterile cotton plugs and kept under outdoor natural condition during the experimental period. All samples were shaken from time to time to ensure that the *Botryococcus* sp. was uniformly homogenized in the wastewater.

Determination of microalgae growth

The samples were taken daily from the culture for cell growth counting in wastewater started on day 3 using Haemocytometer (improved Neubauer chamber) according to Andersen's (2005) technique. The growth of *Botryococcus* sp. was determined according to the specific growth rate (µ/day), division per day (Dd), doubling time (td), and biomass productivity (cell/mL/day) using equations 1, 2, 3, and 4, respectively (Zhu et al., 2013; Komolafe et al., 2014; Asmare et al., 2013; Issarapayup et al., 2009; Wang et al., 2010; Andersen 2005). Nf and Ni were defined as the cell concentration (cell.mL^{-1}) at time Tf and Ti, respectively. The graph over time was required to plot the growth of batch culture to predict the exponential stage of the culture. At least three-time points were considered to satisfy or confirm the exponential stage (Andersen, 2005).

$$\text{Specific growth rate } (\mu/\text{day}) = \frac{\ln(Nf/Ni)}{Tf-Ti} \quad \text{(Eq. 1)}$$

$$\text{Division per day (Dd)} = \frac{\mu/\text{day}}{\ln 2} \quad \text{(Eq. 2)}$$

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$$\text{Doubling time (td)} = \frac{1}{Dd} \quad \text{(Eq. 3)}$$

$$\text{Biomass productivity} = \frac{Nf - Ni}{Tf - Ti} \quad \text{(Eq. 4)}$$

Statistical analysis

All experiments were conducted in triplicates for each culture. Data analysis of average, mean differences, standard deviation, and the graph for each experiment were done using Microsoft Office Excel Professional Plus 2010.

Results and Discussion

Wastewater characterization

Wastewater characterization is compulsory and important for determining the nutrient supplements required for microalgae growth during the cultivation process. *Table 1* shows the physical and chemical parameters of domestic wastewater compared to the effluent standard which has been used in the formation of culture media in microalgae growth experiments. Concentrations of COD and BOD were 76.1 mg/L and 44 mg/L, respectively; this concentration was different from that used in other research papers. For example, Mostafa et al. (2012) used untreated domestic wastewater containing 50 mg/L COD and 15 mg/L of BOD to cultivate cyanobacteria and *Chlorella vulgaris*. Zhang et al. (2013) cultivated mixotrophic microalgae strains in domestic wastewater containing 142 mg/L of COD. However, the concentration of both COD and BOD in this study remains still under the limit of Standard B but slightly over the limit of Standard A. The wastewater used also contained 3.27 mg/L of TP, which was below both standard limits, while TN was 15.79 mg/L. Both parameters were compared to a study conducted by Zhang et al. (2013), who found that TP and TN were 1.59 mg/L and 27.7 mg/L, respectively. Other parameters were also determined such as TSS and TOC in which the values were 2250 mg/L and 21.06 mg/L, respectively. TSS concentration indicates that the wastewater was incomparable to the effluent standard limit, which was more than 100 mg/L of Standard B. Other than that, the pH value showed acceptable concentration compared to the effluent standard and suitable enough for microalgae cultivation (Creswell, 2010).

A few researchers (Zhang et al., 2013; Teles et al., 2013; Can et al., 2013; Ji et al., 2013) have shown the potential of microalgae in domestic wastewater treatment to biotransform pollutants into valuable biomass before the discharge of the cleaned water to the environment. As previously discussed in the introduction, the growth efficiency of microalgae in wastewater mostly depends on different variable factors such as the availability of nutrients and the influence of environmental factors. Thereby, this study may allow the use of domestic wastewater for the development of culture method in biomass production and for further purposes of phycoremediation study coupled with hydrocarbon production.

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Table 1. *Characteristics of the raw domestic wastewater used as the growth media*

Physiochemical parameters	**Concentration ([a]mg/L)**	**Effluent standard, mg/L (Environmental Quality Act, 1974)**	
		Standard A	**Standard B**
Chemical oxygen demand (COD)	76.10	50	100
Biochemical oxygen demand (BOD)	44.00	20	50
Total phosphorus (TP)	3.27	5	10
Total nitrogen (TN)	15.79	-	-
Total suspended solid (TSS)	2250	50	100
Total dissolved solid (TDS)	4900	-	-
Total carbon (TC)	21.06	-	-
Total organic carbon (TOC)	2.19	-	-
Inorganic carbon (IC)	18.86	-	-
Dissolved oxygen (DO)	14.76	-	-
pH	6.93	6.0 – 9.0	5.5 – 9.0

[a]All parameters unit in mg/L except for pH
*All experiments conducted in triplicate (*n*=3)

Growth of Botryococcus sp. and biomass productivity

In general, for most experiments, the growth curve which showed existing lag phase and the exponential phase was then followed by gradually increasing the biomass concentration over time except for 10^7 cell/mL concentration. There were no growth activities in this cell concentration due to the overpopulation. However, in other cell concentration experiments, a stationary and declining phases were also observed. All of the above explanations could be referred further in *Figure 1* below. Similar growth curve has been reported by Can et al. (2013); Cabanelas et al. (2013); Teles et al. (2013) and Chaput et al. (2012), who used domestic wastewater to grow microalgae but with different nutrients and pollutant load concentrations.

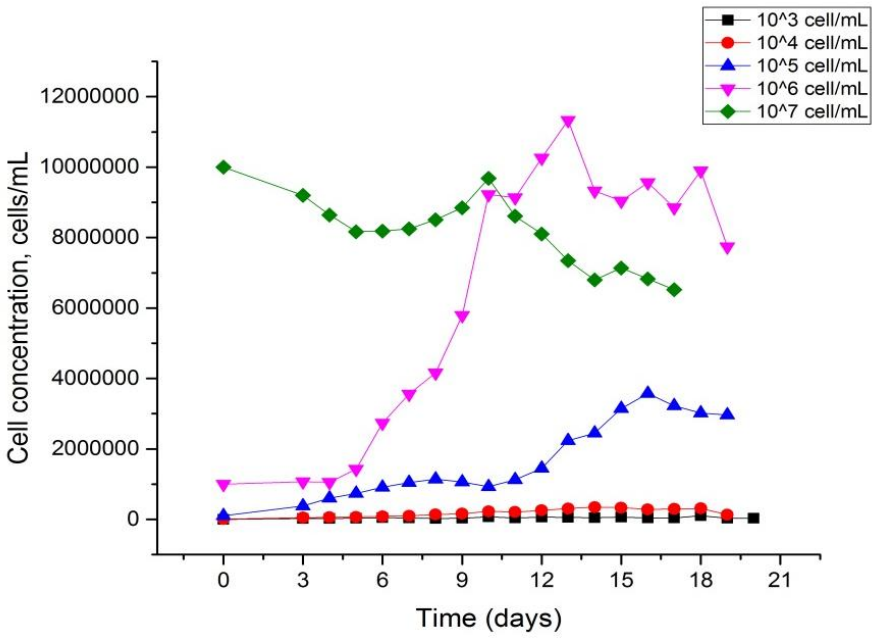

Figure 1. *Growth of Botryococcus sp. in different cells concentration*

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Obviously, *Figure 1* shows that the growth in 10^6 cell/mL concentration was enhanced dramatically compared to other cell concentrations such as 10^3 cell/mL and 10^4 cell/mL while 10^5 cell/mL seemed to show us a better increment but still lower than 10^6 cell/mL. This means that the maximum peak growth for both cell concentration, 10^5 cell/mL and 10^6 cell/mL, occurred on day 16 at 3.57×10^6 cell/mL and day 13 at 1.1×10^7 cell/mL, respectively.

The exponential phase of *Botryococcus* sp. growth could be predicted by drawing a straight line in *Figure 1*, in which the points touched the straight line at least thrice according to Andersen's (2005) technique for each cell concentration except 10^7 cell/mL. Based on the 10^6 cell/mL concentration curve, the exponential phase occurred starting from day 5 to day 13 while for 10^5 cell/mL concentration the exponential phase started from day 11 to day 16. Also, according to the data in *Figure 1*, it was apparent that 10^3 cell/mL and 10^4 cell/mL concentration showed much less growth, with a maximum cell density achieved of only up to 1×10^5 cell/mL and 3.5×10^5 cell/mL, respectively. *Table 2* below thoroughly describes their biomass productivity.

Table 2. *Computation of specific growth rate (μ/day), division per day (Dd), doubling time (td) and biomass productivity of Botryococcus sp. grown on domestic wastewater*

Cell concentration (Cell/mL)	Specific growth rate (μ/day)	Division per day (Dd)	Doubling Time, td (day)	Biomass productivity, Cell/mL/day (10^4)
1×10^3	0.10	0.14	7.14	0.51
1×10^4	0.18	0.26	3.85	3.48
1×10^5	0.23	0.33	3.03	48.98
1×10^6	0.26	0.38	2.63	123.77
1×10^7	0	0	0	0

*All experiments conducted in triplicate (n=3)

After identifying the exponential phase, the specific growth rate (μ/day), doubling time (day), and biomass productivity (cell/mL/day) were determined scientifically based on the formula given in the methodology section in this paper. *Table 2* shows that the highest specific growth rate was at 10^6 cell/mL concentration with 0.26 day^{-1} compared to other concentrations. So, this result is quite similar to the results obtained by Teles et al., (2013) with specific growth rate value up to 0.23 day^{-1} when cultivating *Chlorella vulgaris* in domestic wastewater. Then, the lowest specific growth rate occurred at 10^3 cell/mL concentration with 0.1 day^{-1} as expected. Biomass productivity results are also provided in *Table 2* and have been plotted in a graph over cell concentration compared to specific growth rate in *Figure 2*.

From the data illustrated in *Figure 2*, obviously in various cell concentrations, biomass productivity, and the growth rate of *Botryococcus* sp. were very different. According to *Figure 2*, it occurred on 10^6 cell/mL (x-axis) concentration with the maximum biomass productivity and specific growth rate were 1.24×10^6 cell/mL/day and 0.26 day^{-1}, respectively; this finding is different from other previous research paper. For example, Shin et al. (2015) used *Scenedesmus bijuga* for growth on diluted food wastewater and was able to achieve biomass productivity between 39.4 mg/L/day to 50.75 mg/L/day in terms of dried weight. This difference is likely due to the strength of nutrients concentration available in the wastewater used. It may also be affected by environment factors, particularly weather conditions.

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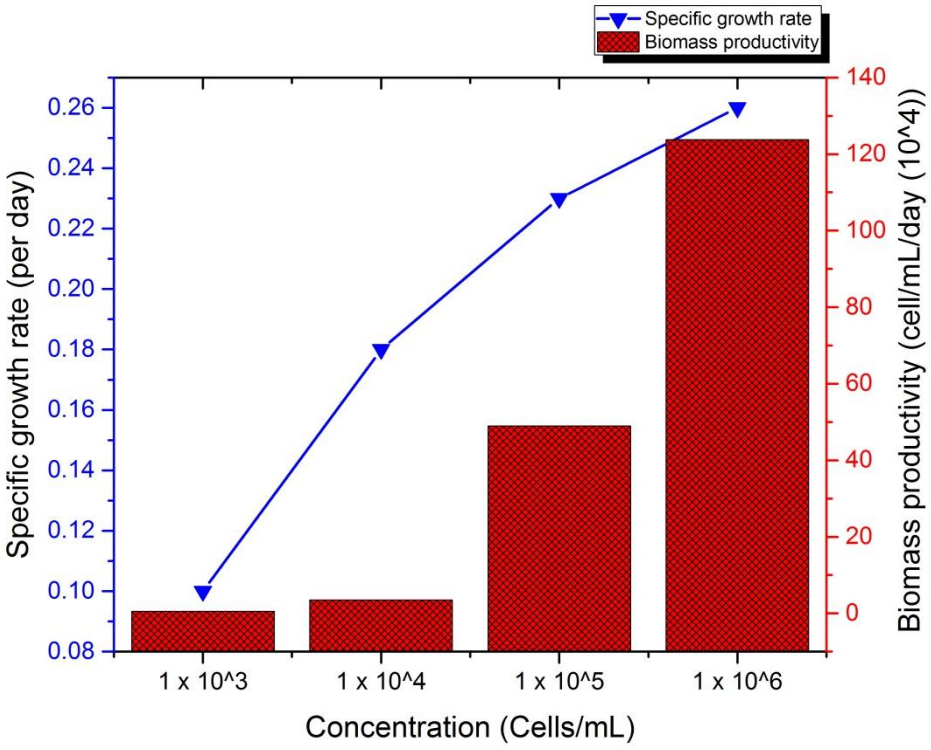

Figure 2. *Specific growth rate (μ/day) and biomass productivity (cell/mL/day) of Botryococcus sp. in different cell concentration (cell/mL) on domestic wastewater*

Conclusion

Recently, interest in cultivating microalgae as a source of biomass has grown. Growing microalgae requires a large quantity of water and nutrients. Amazingly, the use of domestic wastewater to cultivate microalgae is good from a sustainability perspective. However, the success of culturing microalgae in domestic wastewater is highly dependent on cell concentration at the initial stage of cultivation. This study has demonstrated how different cell concentration of microalgae makes it possible to produce microalgae biomass with high levels of productivity. Further research in this field regarding the role of microalgae *Botryococcus* sp. would be helpful for phycoremediation and hydrocarbon production.

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BIOTRANSFORMATION OF C-5 AND C-6 SUGARS OF CELLULOSIC COTTON WASTES TO BIOETHANOL THROUGH PHYSICAL AND ENZYMATIC MODES

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Abstract. The present research focuses on the feasibility of utilizing cotton waste for energy resurgence after physical and biological pretreatment followed by simultaneous saccharification and fermentation processes (SSF). The cotton waste was pretreated with microwave irradiation (MW) and subsequently subjected to enzymatic hydrolysis for the release of sugars utilizing the cellulase enzyme producer *Trichoderma reesei* (MTCC 164). Based on Box-Behnken design, pH, temperature and hydrolysis time period were selected as the most significant variables for the production of cellulase. The simultaneous fermentation and enzymatic saccharification (SSF) of pretreated substrate with immobilized *Saccharomyces cerevisiae* (MTCC 172) yielded 2.65%, 1.48% and 1.32% of ethanol with hard waste, carding waste and cotton seed waste, respectively. The findings suggest that these cotton wastes can be used effectively for the ethanol production.
Keywords: *cotton waste, SSF, cellulase production, pre-treatment, Box-Behnken design, ethanol*

Introduction

Globally, ethanol has become an immediate viable alternative for the rapidly exhausting fossil fuel deposits, and increasing concerns over the everlasting environmental pollution (Galbe and Zacchi, 2002). An extensive range of substrates can be used as feedstock for ethanol production, comprising fermentable sugars in the form of lignocellulosic wastes through series of steps involved in the reduction of complex polysaccharides. Furthermore, cellulosic biomasses are the most abundant in the world and thus, are now being considered as one of the best substitute raw materials for the production of ethanol (Jorgensen et al., 2007). India has large number of cotton fabrics manufacturing industries in the world. The difficulty of disposing the final scrape from these industries has now assumed serious dimensions, as it has no salability and poses pollution hazards (Raj et al., 2009). Bioconversion of these cotton wastes into valuable product such as ethanol could be considered as suitable solution.

The basic idea in pretreatment technology of cotton waste in the bioconversion process is to alter or remove lignin and hemicelluloses, increasing the surface area and decreasing the crystallinity of cellulose (Zhu et al., 2009). Degradation or loss of carbohydrate and formation of inhibitory by-products should also be considered during pretreatment (Taherzadeh et al., 2007). Microwave irradiation has high heating efficiency, and thus it is able to degrade cellulose and hemicelluloses as well as increase in enzymatic susceptibility (Zhu, 2005).Microwave heats the target object directly by applying an electromagnetic field to dielectric molecules as compared to conduction/convection heating which is based on intramolecular heat transfer

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(Newnham et al., 1991). Several researchers have used microwave pretreatment as a potential method for pretreatment of various lignocellulosic materials (Alvira et al., 2010; Shi et al., 2011; Jackowiak et al., 2011) and to damage the recalcitrant lignin (Hu and Wen, 2008). Further fermentation of the hydrolysate will help to yield ethanol using fermenting microorganisms.

SSF has gained a lot of concern, as it is both logistically and cost-effectively favorable in terms of higher ethanol yield (Tomas-Pejo et al., 2009).The processes usually employed in the fermentation of lignocellulosic hydrolysate are Simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). Conventionally or traditionally the SHF process has been employed but SSF is superior for ethanol production since it can improve ethanol yields by removing end product inhibition and eliminate the need for separate reactors. It is also cost effective but difference in optimum temperature conditions of enzyme for hydrolysis and fermentation poses a few limitations. The higher ethanol yield coefficient from SSF would be partially due to more conversion of xylose to xylitol under the SSF conditions (Sarkar et al., 2012). Hydrolysis is usually the rate limiting step in SSF (Philippids and Smith, 1995). In SSF, cellulases and xylanases convert the carbohydrate polymers to fermentable simple sugars. These enzymes are extremely susceptible to feedback inhibition by the products such as glucose, xylose, cellobiose, and other oligosaccharides. The advantage of SSF is that, a saccharification and ethanol fermentation is concurrently carried out in a single vessel (Hasunuma and Kondo, 2012). To achieve a high ethanol concentration from cellulosic materials in a SSF system, a high substrate loading is required, which results in high insoluble solids content in the system. A major concern in the SSF process is fast liquefaction of cellulose to overcome the mixing problem at high loadings of cellulosic materials for microbial simultaneous fermentation of releasing glucose. Therefore, microwave pretreated biomass subjected to enzymatic saccharification in a high substrate concentration; SSF enhances the yield of ethanol production. Against this background, the present study was carried out to analyze the use of cellulase producing *T. reesei* (MTCC 172) and *S. cerevisiae* (MTCC 164) in the conversion of cotton waste to bioethanol.

Materials and methods

Fermentable substrates and cultures

Cotton wastes were collected from textile mills in and around Coimbatore, Tamilnadu, India. The wastes were mechanically processed to reduce the length of the fibres and also to eliminate the debris. The fibers were boiled in water at 100°C for 30 min. Subsequently, the fibers were rinsed with deionized water and air-dried. The cultures, *T. reesei* (MTCC 164) and *S. cerevisiae* (MTCC 164) were obtained from Microbial Test Culture Centre (MTCC), Chandigarh, India. The cultures were revived in the Czapek-Dox broth for further analysis.

Microwave pretreatment of cotton waste and compositional analysis

Exactly 5 g of each type of cotton waste (Cotton seed waste-CSW, Carding waste-CW and Hard waste-HW) in 500 ml of distilled water was placed at the center of the rotating platform in the microwave oven for 5, 10, 15, 20, 25 and 30 min in a general

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purpose laboratory microwave oven (Godrej appliances, model: GMX 20GA1 MIZ) at 230 V and ~ 50 Hz (Keshwani et al., 2007). After treatment, the cotton wastes were filtered and shade dried The moisture, cellulose and hemi-cellulose contents of the cotton wastes were determined as per to ASTM test methods (1995). DNS method of Miller (1959) was used to estimate reducing sugars.

Optimization and purification of cellulase enzyme by T. reesei

The experimental design and statistical analysis were performed employing RSM Box-Behnken experimental design using Design-Expert software (Trial Version 7.1.5, Stat-Ease, Minneapolis, 2008) for optimizing the pH, temperature and incubation time for the production of cellulase enzyme. The design matrix with 17 experimental runs in two blocks and triplicates for the midpoint was used for the experiment. The culture was grown for 72 h at 30°C in Czapek- Dox broth and was used for further analyses. The partial purification of the culture medium was performed using dialysis. The enzyme produced under optimal condition was dialysed using dialysis membrane. The dialysed cellulase enzyme was further checked for their activity using spectrophotometric assay method.

Immobilization of S. cerevisiae

Exactly 50 ml of sodium alginate solution (7.2% in distilled H_2 O) was mixed with equal volume of distilled H_2O containing 0.1 ml of overnight yeast extract broth culture of *S. cerevisiae* (MTCC 172) and was gradually dropped into 0.1 M $CaCl_2$ solution for the formation of sodium alginate beads. The beads were stored at 4°C after washing with distilled H_2 O. The viability of the immobilized *S. cerevisiae* cells was verified as per standard procedure (Duarte et al., 2013).

Simultaneous saccharification and fermentation (SSF)

To 100 ml of fermentation broth [yeast peptone dextrose broth (YPDB)], 2.5 g of pretreated cotton waste, 1 ml of dialyzed cellulase enzyme and 0.1 g of *S. cerevisiae* sodium alginate beads were added with fermentation broth and incubated for 7 days at 37°C in shaker. After incubation, the ethanol production was estimated by Williams and Darwin dichromate method (Williams and Darwin, 1950).

Results and Discussion

Microwave pretreatment of cotton wastes

The microwave irradiation for 5 mins proved to be efficient in releasing free glucose from cotton wastes (*Table 1*). From the results, it is evident that 5 min of MW irradiation released 140 mg/ml, 165 mg/ml and 180 mg/ml of glucose from CSW, CW and HW, respectively. Zhu et al. (2009) reported that microwave irradiation at a power of up to 700 W at various exposure times resulted in weight loss due to the degradation of cellulose, hemicelluloses and lignin. Microwave pretreatment increasing the yield of total available reducing sugars has been reported by (Azuma et al., 1984); (Kitchaiya et al., 2003); (Ooshima et al., 1984). Lin et al. (2012) stated that microwave irradiation of cotton waste is an alternative approach to heating.

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Table 1. *Glucose concentration of microwave pretreated cotton waste*

Time of exposure (min)	Glucose Concentration (mg/ml)		
	CSW	CW	HW
0	70	80	100
5	145	175	185
10	140	165	180
15	135	125	130
20	130	130	160
25	100	110	115
30	100	100	120

Compositional analysis of cotton waste

The compositional analysis showed that the cellulose and hemicelluloses content (%) decreased after five minutes of microwave irradiation of the cotton waste (*Table 2*). The results prove that the pretreatment aids in the conversion of the complex polysaccharides into simple sugars so as to aid in the subsequent fermentation process. It was also noted that, there was 7%, 5% and 5.6% decrease in the moisture content for CSW, CW and HW wastes, respectively after the microwave treatment. The carding waste and hard waste recorded maximum reduction of cellulose (15%) and hemicelluloses (10%) after microwave irradiation. Exactly, 13.84 % and 10.40% increase of free sugar in hard waste and carding waste respectively, was noted. There was 6.1 % and 6.9 % decrease acid residues in cotton seed waste and hard waste respectively after pre-treatment. In a similar study, Venkatramanan et al. (2014) stated that the moisture content and acid insoluble residue was 12.63% and 17.84%, respectively, in untreated cotton waste.

Table 2. *Compositional contents of cotton waste*

Treatments	Cotton Type	Compositional analysis				
		Moisture content %	Cellulose %	Hemicelluloses %	Free sugars mg/ml	Acid residue %
Before pretreatment	CSW	15.30	57	10	20.21	18.70
	CW	14.60	59	11	24.40	15.40
	HW	8.80	68	16	41.32	7.20
After pretreatment	CSW	8.30	42	04	30.61	11.20
	CW	9.60	51	07	33.17	9.30
	HW	3.20	45	06	55.16	0.32

Analysis of response surface, enzyme production and hydrolysis

The results showed that the independent factors viz., temperature and incubation time had significant effect on the enzyme production. The quadratic term of the three different factors namely, temperature, pH & incubation time also had a significant effect (*Table 3*). The regression model for the enzyme activity noted to be significant ($p < 0.01$) with satisfactory value of 0.8849 as co-efficient of determination (R^2). The result

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indicates a good agreement between experimental and predicted values and implies the statistical model is reliable for enzyme production. Response surface plots (*Fig. 1-2*) depict that the hydrolysis time and temperature significantly affect the enzyme production. The two dimensional contour plot with respect to pH and incubation time showed sloping nature on the plot, suggesting that temperature and hydrolysis time was interdependent. This proves that incubation time for enzyme production is dependent on the temperature. However, the shapers of the response surface curves indicate no positive interaction between temperature & incubation time and pH & incubation time. The correlation between predicted and measured values validates the model and existence of an optimal point. "Prob > F" value less than 0.0500 indicate model terms are significant. The analysis of variance showed that this regression model is significant ($p < 0.01$) with F value of 14.66. There was only a 0.01 % chance that a "Model F-Value" was insignificant which largely could occur due to noise. Values of "Prob > F" less than 0.0500 indicated that the model terms were significant. In this case A, B, A2 and C2 were significant model terms. Values greater than 0.1000 indicated that the model terms were not significant. The lack of fit value of 0.067 is not significant hence the model was perfectly fit (*Table 4 and 5*). "Adeq Precision" i.e., signal to noise ratio was 13.85 which indicated an adequate signal. Hence, this model could be used to navigate the design space. The maximum cellulase activity of 2.76 IU/ml was recorded at pH 4, temperature 25 °C at 78 h. Our results are in accordance with Das et al. (2015) who stated that, optimization of cellulase enzyme production by Box- Behnken design showed that all the three factors plays viz., pH, temperature and incubation time play an important role in the cellulase enzyme activity.

Table 3. *Box- Behnken Experimental design with different variables*

Runs	Variables			
	Temperature C (A)	**pH (B)**	**Incubation time (h) (C)**	**Enzyme activity IU/ml**
1	40	6.5	78	0.80
2	55	6.5	144	0.10
3	40	9	12	0.11
4	25	6.5	12	0.30
5	40	6.5	78	0.51
6	55	9	78	0.27
7	40	4	12	2.55
8	25	9	78	2.76
9	55	4	78	2.64
10	40	4	144	2.27
11	40	6.5	78	0.67
12	25	6.5	144	1.78
13	40	6.5	78	0.66
14	55	6.5	12	0.39
15	40	6.5	78	0.75
16	40	9	144	0.20
17	25	4	78	2.65

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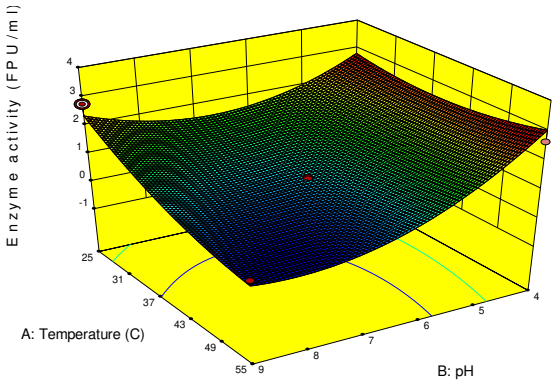

Figure 1. *Effect of incubation time on enzyme production using T. reesei*

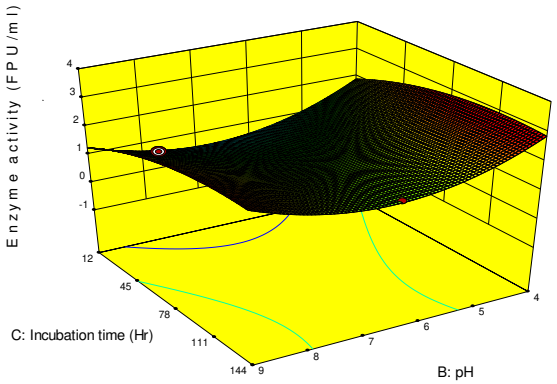

Figure 2. *Effect of temperature on enzyme production using T.reesei*

Table 4. *ANOVA of surface quadratic model*

ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]						
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	16.11	9	1.79	14.66	0.0009	Significant
A	1.85	1	1.85	15.13	0.0060	
B	6.15	1	6.15	50.40	0.0002	
C	0.12	1	0.12	1.01	0.3481	
AB	1.53	1	1.53	12.57	0.0094	
AC	0.78	1	0.78	6.38	0.0395	
BC	0.033	1	0.033	0.27	0.6208	
A^2	0.60	1	0.60	4.94	0.0617	
B^2	4.38	1	4.38	35.84	0.0005	
C^2	0.72	1	0.72	5.91	0.0454	
Residual	0.85	7	0.12			
Lack of Fit	0.80	3	0.27	21.31	0.0064	
Pure Error	0.050	4	0.013			
Cor Total	16.97	16				

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Table 5. *ANOVA of the first –order regression model*

Lack of Fit Tests					
Source	**Sum of Squares**	**Df**	**Mean Square**	**F Value**	**p-value Prob > F**
Linear	8.79	9	0.98	77.65	0.0004
2FI	6.45	6	1.07	85.40	0.0004
Quadratic	0.80	3	0.27	21.31	0.0064
Cubic	0.000	0	0.013		
Pure Error	0.050	4			

The results of cellulase hydrolysis of the MW irradiated cotton waste at different exposure times showed that maximum of 665 mg / ml (M2), 670 mg / ml (M1) and 910 mg / ml (M2) of reducing sugars were released from CS, CW and HW, respectively (*Table 6*). The data proves that the enzymatic treatment enhances the release of sugars from the MW irradiated cotton wastes. From the *Table 6* it is evident the HW yielded more reducing sugars after enzymatic treatment compared to CS and CW. Hence the HW irradiated with MW for 10 minutes was selected for further analysis. Enzymatic hydrolysis is cost effective because it produces better yield than acid hydrolysis and also the cost of enzyme is subsequently reduced by using advanced technologies in enzyme industries (Pan et al., 2005).

Table 6. *Biological treatment of pretreated cotton waste.*

MW irradiated cotton waste	**Release of reducing sugars (mg/ml) after cellulase treatment**						
	M0	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**
CS	100	650	665	660	620	545	540
CW	100	670	665	660	650	600	540
HW	210	850	910	890	710	750	730

M0- Control, M1- Exposure time 5 min, M2- Exposure time 10 min, M3- Exposure time 15 min, Exposure time 20 min, M5- Exposure time 25 min, M6- Exposure time 30 min.

Simultaneous Saccharification and Fermentation (SSF)

The SSF resulted in maximum ethanol production of 2.65% with MW irradiated and enzymatically treated HW. All the untreated cotton wastes yielded poor percentage (0.02 – 0.11 %) of ethanol. The increase in the ethanol yield with cotton wastes was in the order of as MW irradiated>enzymatically treated>combinatorial MW and enzymatic treatment (*Table 7*).

In this process, the glucose produced by the hydrolyzing enzyme is consumed instantly by the fermenting microorganism present in the culture. Since the inhibition effects of cellobiose can be minimized by keeping low concentration of these sugars in the media. SSF gives higher reported ethanol yields from cellulose than SHF and requires low amount of enzyme (Eklund and Zacchi, 1995). Hahn-Hagerdal et al. (2006)

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has stated that *S. cerevisiae* is the most commonly and conventionally used microorganism for fermenting ethanol from sugar based residues at industrial scales and accordingly the same culture is used in the present study. The fermentation process correlated with the report of Tengborg et al. (2000) which states that, the optimum conditions for fermentation using *S. cerevisiae* at 38 °C comprises the optimal conditions for hydrolysis. Unfortunately, the ethanologenic species, *S. cerevisiae* cannot ferment the C5 sugars into ethanol efficiently. If only hexose sugars from cellulosic biomass are fermented, (with pentose sugars left behind) feedstock consumption for bioethanol production will be significantly high, and in the interim the unfermented pentoses will remain with the distillage. On the other hand, it will increase energy consumption in the treatment of the distillage (Zhao et al., 2012). Hence research in this area is highly imperative, since efficient conversion of both C-5 and C-6 sugars is necessary to maximize ethanol yield under controlled costs.

Table 7. *Percentage ethanol production of pretreated cotton waste (SSF)*

Samples	**Percentage Ethanol Yield (%)**			
	Control	**Microwave**	**Biological**	**Microwave + Biological**
Cotton seed waste	0.02	0.10	0.25	1.32
Carding waste	0.10	0.24	0.42	1.48
Hard waste	0.11	1.35	1.55	2.65

Conclusion

The universal production of biofuels has increased radically in the past few years, primarily due to increase in oil prices, national security concerns, environmental considerations and the efforts to revitalize rural communities. India is one of the countries having a huge number of textile industries. The objective of physical pretreatment is to reduce the fibre size and crystallinity, increasing the surface area, reducing the degree of polymerization and shearing the biomass. Choice of pretreatment processes of lignocellulosic biomass is an extremely important step in the synthesis of biofuels. The current study focussed on microwave pretreatment so as to avoid degradation of hemicelluloses and to improve the pentose yield. Among various methods available for the lignocellulose hydrolysis, the current study employed the cellulase hydrolysis and hence an imprerical polynomial model for the production of cellulase enzyme by *T. reesei* was designed using the Box-Behnken design. The simultaneous saccharification and fermentation yields 2.65% of ethanol from MW irradiated cotton waste. This study thus establishes that the cotton waste from the textile mills can be converted into valuable product for a variety of industrial application.

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ECOLOGICAL ROLES OF COMMERCIAL MANGROVE PLANTATION FORESTS FOR BENTHIC MACROINVERTEBRATE COMMUNITIES IN THAILAND

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Abstract. Mangrove plantation for renewable energy production has an important economic value for Thai inhabitants. Planted mangroves may also serve as valuable areas for biodiversity. We aimed to investigate benthic community structure and estuarine environment in mangrove plantation sites (young, intermediate, mature sites and a semi natural mangrove forest) in Samut Songkram province. The mangrove plantation is a monoculture of *Rhizophora apiculata* compared with a semi natural mangrove forest where 5 species of trees were recorded. Values of mangrove height and diameter at breast height (DBH) increased with the age of the mangrove plantation forests. Overall, the parameters reflected good water quality conditions (temperature 30.75-32 ^{0}C; pH 6.69-7.73; salinity 16.33-21.66 ppt; DO 3.45-4.57 mg/l). Nutrient concentrations and BOD were rather low indicating less impact from nutrient sources. In term of biodiversity, phytoplankton and zooplankton appeared to be greater in younger mangrove plantation sites. Intertidal benthic macroinvertebrate communities tended to increase with the age of the plantation site and invertebrate assemblages have developed through time. Hence, it could be stated that mangrove plantation used as charcoal production not only generates income for the local people but also plays an important role in estuarine ecosystems as the crucial habitat for benthic macroinvertebrate species.
Keywords: *mangrove, biodiversity, water quality, Thailand*

Introduction

The expansion of extensive shrimp farming along coastal areas of Thailand has substantially reduced the extent of mangrove forests. For example, it was reported that mangrove losses attributable to shrimp aquaculture expansion are estimated to be up to 32% of the total mangrove area destroyed in 1993 (Dierberg and Kiattisimkul, 1996). Losses of mangrove forests have had a huge impact on a broad range of coastal ecosystems and aquatic resources that have declined (Primavera, 1993). In fact, mangrove forests play a key role in ecosystems as well as providing goods and services to humans (Kaplowitz, 2001; Vo et al., 2012). Mangroves provide food sources for marine animals and act as shelter and a nursery ground for fish larvae and other important economic aquatic species such as sea bass, grouper, crab and shrimp (Ronnback, 1999; Kathiresan, 2010). Mangrove trees also help nourish soil organic matter, recycle nutrients and protect shoreline from erosion and tsunamis (Wafar et al., 1997; Osti et al., 2008; Kathiresan, 2012). In terms of the economy, mangroves can be used as a renewable resource for charcoal production that generates income for local inhabitants (Bandaranayake, 1998; Kridiborworn et al., 2012).

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During the past decades, shrimp farming in Thailand has been experiencing outbreaks of viral diseases such as white spot syndrome and reduced shrimp production due to degradation of the environment (Flegel, 1997; Chayaburakul et al., 2004). Disease damage has been extensive, covering large areas of culture and many shrimp farms were abandoned (Dierberg and Kiattisimkul, 1996). As a result, farmers have realized the ecological importance of mangrove forests and have rehabilitated the abandoned areas. Commercial mangrove plantation for charcoal production is also an alternative for local people instead of continuing shrimp culture. It was reported that after the failure of shrimp farming, some locals turned to mangrove plantation as a source of income (Kridiborworn et al., 2012).

In Thailand, local communities have commercially used plantation mangrove forests for sustainable wood extraction for charcoal production for more than 50 years such as in Yeesarn village of Samut Songkhram province (Tontaweewong, 2000). Mangrove plantation is considered sustainable and does not result in a reduction in the area of mangrove forest since the planted mangrove areas that are cut for charcoal production equal the areas where new mangrove forests are planted (Kridiborworn et al., 2012). Mangrove plantation for charcoal production not only provides local venture benefits but also supports and brings back biodiversity. It usually takes approximately 10-15 years before the planted forests reach the mature stage and can be used for charcoal production (Kridiborworn et al., 2012). During this period, planted mangrove forests can provide habitat for aquatic flora and fauna that help to maintain the overall biodiversity of the area despite the fact that the species richness and abundances of coastal fauna may vary in comparison with undisturbed mangrove forest areas (Macintosh et al., 2012; Andradi-Brown et al., 2013).

Therefore, this research aimed to compare the mangrove community and structure of planted and natural mangrove forests. We also determined and compared properties of water and biological resources between the mangrove planted area over time (different years of plantation) and natural mangrove areas at the village of Yeesarn, Samut Songkhram province. In particular, we studied benthic macroinvertebrate communities that may differ among mangrove sites. The results of this study showed the importance of planted mangrove forests as habitats for biodiversity apart from being renewable sources of alternative energy. It also gave us insight into the environmental conditions and variation of biodiversity in the mangrove forests planted in different years.

Materials and Methods

Study site and charcoal production in Yeesarn

Yeesarn village (13°18'14.3"N 99°54'07.2"E) is located in the coastal zone of Amphawa district, Samut Songkhram province and is known as a source of good quality charcoal production (*Fig. 1*). In fact, Yeesarn village is the first community in Thailand that planted and used mangrove forests for energy production (Chaiyasarn, 2007). *Rhizopihora apiculata* is the main mangrove species used for making charcoal in this area. The estimated total plantation area of *R. apiculata* was 2,000 ha and almost 40% (300 out of 716) of households in Yeesarn were involved in mangrove plantations for charcoal production purpose in a 2010 survey (Kridiborworn et al., 2012). In 1 ha of mangrove plantation, there are around 6,875 trees planted. The rotation period is between 10-15 years with an average wood diameter of 5-8 cm (Chaiyasarn, 2007; Kridiborworn et al., 2012).

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Figure 1. *Yeesarn village located in Amphawa district, Samut Songkhram province. Source: www.google.co.th/maps*

Mangrove community structure and diversity

This study compared the mangrove structure, water quality and biodiversity among 3 mangrove plantation sites used for charcoal production and a semi natural forest area with similar environmental conditions. Sampling took place in October 2014 in 3 ages of planted mangrove forests (young aged 0-5 years, intermediate aged 5-10 years and mature aged 10-15 years) and also took place in the semi natural mangrove forest. The semi natural mangrove forest actually used to be a plantation of *Rhizopihora apiculata* and was left unmanaged. Subsequently the local owner donated the land for conservation purposes. Each site covered an area of around 1.6 ha and in total, there were 4 sites studied. The assessment of each mangrove community structure was conducted randomly using a 3×3 m^2 rope plot at each mangrove site. All vegetation present in each quadrat was identified to the species level and the number of individuals was counted. In each plot, a client manometer (clinometer) was used to measure the height of 5 random trees (m) and in addition, the diameter (cm) (DBH or diameter at breast height) was also recorded using a tape measure.

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Water sampling

Water quality was determined in a narrow, tidal, man-made creek dredged through each site (6 replicates). These creeks are made and used specifically for transporting cut wood after harvest. We measured water depth (m), transparency (using a Secchi disc; m), water temperature (°C) and salinity (refractometer: ppt) on site. A portable multi-meter (Consort 933) was also used to study pH, conductivity (mS/cm), total dissolved solid (TDS; mg/l) and dissolved oxygen (DO; mg/l). In addition, 2 bottles of water samples (2 l) were collected at about 30 cm below water surface and transferred to a plastic bottle. One bottle of water was used for analysis of total suspended solid (TSS; mg/l), biochemical oxygen demand (BOD; mg/l) and chlorophyll a (mg/l). Another bottle of water sample was preserved by adding a few drops of concentrated sulfuric acid until the pH was lower than 2 and kept in a plastic container (temperature below 4 °C) for further analysis (total nitrogen (TN; mg/l), total phosphorus (TP; mg/l), based on standard methods at the laboratory of the Department of Environmental Technology and Management, Kasetsart University and the Central Lab Company.

Biodiversity sampling

We studied the species composition and abundances of phytoplankton and zooplankton by pouring 5 l of water through a plankton net with mesh sizes of 20 and 64 μm, respectively. Samples were then transferred to a plastic bottle and preserved with 70% ethanol. Identification and the number of phytoplankton and zooplankton were studied under a light microscope at the Department of Fishery Biology, Faculty of Fisheries, Kasetsart University. Six replicates were studied.

We used 2 methods to study macroinvertebrate communities in mangrove forests. The first method was the collection of benthic macroinvertebrates using a 1×1 m^2 quadrat placed randomly in each site (with 6 replicates). Any macroinvertebrate fauna that were present on the ground within a quadrat were collected by hand. The specimens were then put in a plastic bag and preserved in 70% ethanol. The top layer of soil (10 cm) in a quadrat was also removed using a shovel and transferred to a plastic bag to investigate sedimentary invertebrate fauna. Sediments were taken to the laboratory, washed and sieved through a 0.5 mm sieve. Specimens of animals were separated and kept in a plastic bottle containing 70% ethanol for subsequent identification and counting. The second method was the collection of macroinvertebrate animals present onsite by hand by 2 people for 30 min as one sample (adapted from Macintosh et al., 2002). We randomly collected as many invertebrates as possible throughout each site. Specimens were kept in a plastic bag and preserved with 70% ethanol and identified in the laboratory up to the species level.

Data analysis

The data were averaged and presented as mean±standard deviation. An F-test at the 95% confidence level was used to differentiate mangrove forest structure and water quality among studied sites (young, intermediate, mature and semi natural sites) using the SPSS 2013 software. We also used Primer (Plymouth routines in multivariate ecological research) 6 for MDS analysis (multidimensional scaling) to study the similarity or dissimilarity of physicochemical variables among sites. Cluster analysis was also applied to determine patterns in species composition and assemblages among sites. Data were transformed by square root and standardized.

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Results

The results revealed that mangrove plantation was a monoculture and the only species in the mangrove plantation used for charcoal production was *Rhizophora apiculata (Table 1).* In contrast, 5 species were found on the semi natural site consisting of *R. apiculata, Xylocarpus granatum*, *Nypa fruticans*, *Cycas circinalis* and *Derris trifoliate*. The heights of the trees differed significantly among sites. Trees aged 0-5 years were shortest (5.12±0.49 m) compared to trees aged 10-15 years that were highest (12.27±0.81 m). The DBH ranged between 3.53 and 6.43 cm and also differed significantly between the younger mangrove site and the other sites. The DBH values increased with the age of the trees. The number of trees on each site varied from 11,111 to 27,778 trees per ha. The density decreased as the age of the trees increased.

Table 1. *Detailed investigation of mangrove plantation at Yeesarn village, Samut Songkhram (n=15)*

Parameter	Mangrove site			
	Young 0-5 years	**Intermediate 5-10 years**	**Mature 10-15 years**	**Semi natural**
Species	1	1	1	5
Height (m)	5.12±0.49^{a}	8.75±0.53^{b}	12.27±0.81^{c}	11.01±3.32^{c}
DBH (cm)	3.53±0.48^{a}	4.87±1.02^{b}	6.04±1.58^{b}	6.43±2.35^{b}
Density (trees/ha)	27,778±4,006^{a}	14,444±4,006^{b}	11,111±3,333^{b}	17,778±3,334^{b}

Remark: *Mean values followed by different letters are significantly different ($p < 0.05$).*

All studied creeks were relatively shallow *(Table 2).* Water quality analysis revealed that most values of physicochemical characteristics were comparable among sites and remained in good conditions. Temperature values were in the range of natural conditions and the pH values were neutral at all sites. Dissolved oxygen values varied between 3.45 and 4.57 mg/l. Conductivity values corresponded well with the total dissolved solids and salinity indicating saline conditions in the studied areas. At all sites, nutrient concentrations were low. Total nitrogen concentrations were similar among sites but total phosphorus concentrations were detected only at the mature mangrove area and the semi natural site. Concentrations of chlorophyll a significantly differed among sites. The highest value of chlorophyll a was detected at the mature mangrove aged 10-15 years. BOD concentrations were somewhat low at all sites indicating low contamination of organic matter contents from human activities.

Table 2. *Physicochemical characteristics of water at mangrove plantation sites in Yeesarn village (n=6)*

Parameter	Mangrove site			
	Young 0-5 years	**Intermediate 5-10 years**	**Mature 10-15 years**	**Semi natural**
Water depth (m)	1.4±0.1^{a}	1.3±0.6^{b}	0.7±0.05^{b}	1.0±0.1^{c}
pH	7.07±0.06^{a}	7.15±0.02^{a,b}	7.33±0.12^{b}	6.96±0.05^{c}
Temperature (c)	31.25±0.17^{a}	30.75±0.37^{a}	32.0±1.47^{a}	30.83±0.29^{a}
Transparency (cm)	17.0±1.7^{a}	19.7±1.9^{a,b}	24.3±5.4^{b}	19.5±1.6^{a,b}
DO (mg/l)	4.57±0.54^{a}	4.06±0.37^{a,b}	3.63±1.02^{a,b}	3.45±0.61^{b}
TDS (mg/l)	7.48±1.09^{a}	6.98±0.20^{a,b}	5.71±1.25^{b}	8.10±0.61^{a,b}

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TSS (mg/l)	158.16±19.42[a]	99.64±11.56[b]	83.61±23.31[b]	89.69±27.53[b]
Conductivity (mS/cm)	13.65±1.58[a,b]	12.47±0.35[a]	9.76±2.72[a]	14.37±0.37[b]
Salinity (ppt)	21.7±2.7[a]	18.5±0.5[a]	16.3±0.5[b]	20.0±0.0[c]
TN (mg/l)	3.53±3.51[a]	2.76±1.36[a]	2.41±0.66[a]	1.83±0.48[a]
TP (mg/l)	nd	nd	0.17±0.40[a]	0.17±0.40[a]
BOD (mg/l)	3.49±0.87[a]	1.86±0.47[b]	2.92±0.80[a,b]	2.34±0.45[b]
Chlorophyll a (mg/l)	9.53±2.29[a]	5.67±0.85[b]	22.73±5.70[c]	6.69±1.36[a,b]

Remark: *Mean values followed by different letters are significantly different ($p < 0.05$). nd is non-detectable.*

The results of MDS analysis clearly distinguished similarities and dissimilarities in the general water quality among sites *(Fig. 2).* The water quality in the young and mature mangrove plantation sites was rather dissimilar as indicated by the sampling points that were far apart. On the other hand, the MDS analysis showed the similarity in the environmental variables between the intermediate mangrove plantation site and the semi natural mangrove forest.

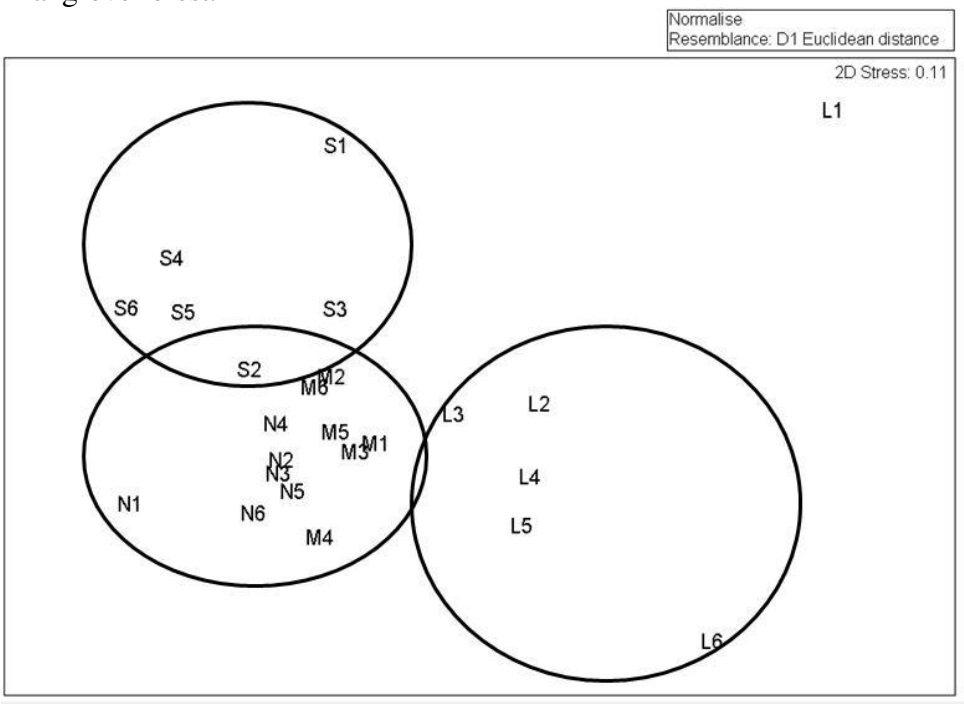

Figure 2. *Multidimensional scaling analysis of environmental variables among studied sites (S = young, M = intermediate, L = mature and N = semi natural sites), the number represents sampling points (Stress = 0.11 indicates fair goodness of fit).*

The results of the biological studies showed that the species and densities of phytoplankton tended to be higher in the creek in the younger mangrove plantation sites compared with the older sites *(Table 3).* The most dominant species of phytoplankton present were *Navicula* sp., *Gyrosigma* sp. and *Nitzschia* sp. Similarly, the numbers of zooplankton were highest in the creek of the young mangrove site aged 0-5 years and were lower at the other older sites. However, the numbers of zooplankton species appeared to be similar among sites. *Tintinnopsis gracilis*, *T. tubulosa* and copepod nauplius were the main species recorded.

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The overall species of benthic macroinvertebrate animals (both collected by hand and by quadrat) increased with the age of the mangrove sites *(Table 3).* Common macroinvertebrates (snails) collected by hand belonged to the Family Elloblidae that were present at all sites. The main species were *Melampus* sp., *Cassidula aurisfelis* and *Laemodonta punctigera*. The abundances of macroinvertebrates collected by hand were relatively comparable among sites. However, the abundance of macroinvertebrates collected using a quadrat appeared to be greater in the older mangrove sites and lower in the younger mangrove areas. The main sedimentary species were in the Families Capitellidae and Spionidae. Other abundant animals collected using a quadrat included the Family Elloblidae (*Melampus* sp.) similar to those collected by hand.

Table 3. *Species and abundances of biological resources in forest plantation sites of Yeesarn village, Samut Songkhram province (n=6)*

Biological resource	**Mangrove site**			
	Young 0-5 years	**Intermediate 5-10 years**	**Mature 10-15 years**	**Semi natural**
Phytoplankton				
Species	22	11	15	8
Density (cell/l)	1,737	584	584	218
Zooplankton				
Species	16	15	16	8
Density (ind./l)	328	218	195	81
Benthic fauna (by hand)				
Species	8	9	13	12
Density (ind./30 min of collection)	159	160	157	138
Benthic fauna (by quadrat)				
Species	8	11	14	11
Density (ind./m^2)	20	33	49	43

Cluster analysis shows distinct groups of benthic macroinvertebrates among sites *(Fig. 3).* The analysis revealed similar patterns of benthic communities collected by hand and by quadrat. Considering hand collection, there was a high level of similarity (80%) of benthic macroinvertebrate communities between mature mangrove (L) and semi natural mangrove (N) sites. There was also similarity (76.5%) in the species composition of benthic macroinvertebrates between the young (S) and intermediate (M) mangrove plantation sites. Two groups of benthic macroinvertebrates collected by quadrat were also detected at the high level of similarity (76%) between the mature mangrove (L) and the semi natural mangrove (N) sites and there was 66% similarity (76.5%) in the benthic macroinvertebrate composition of the young (S) and intermediate (M) mangrove plantation sites.

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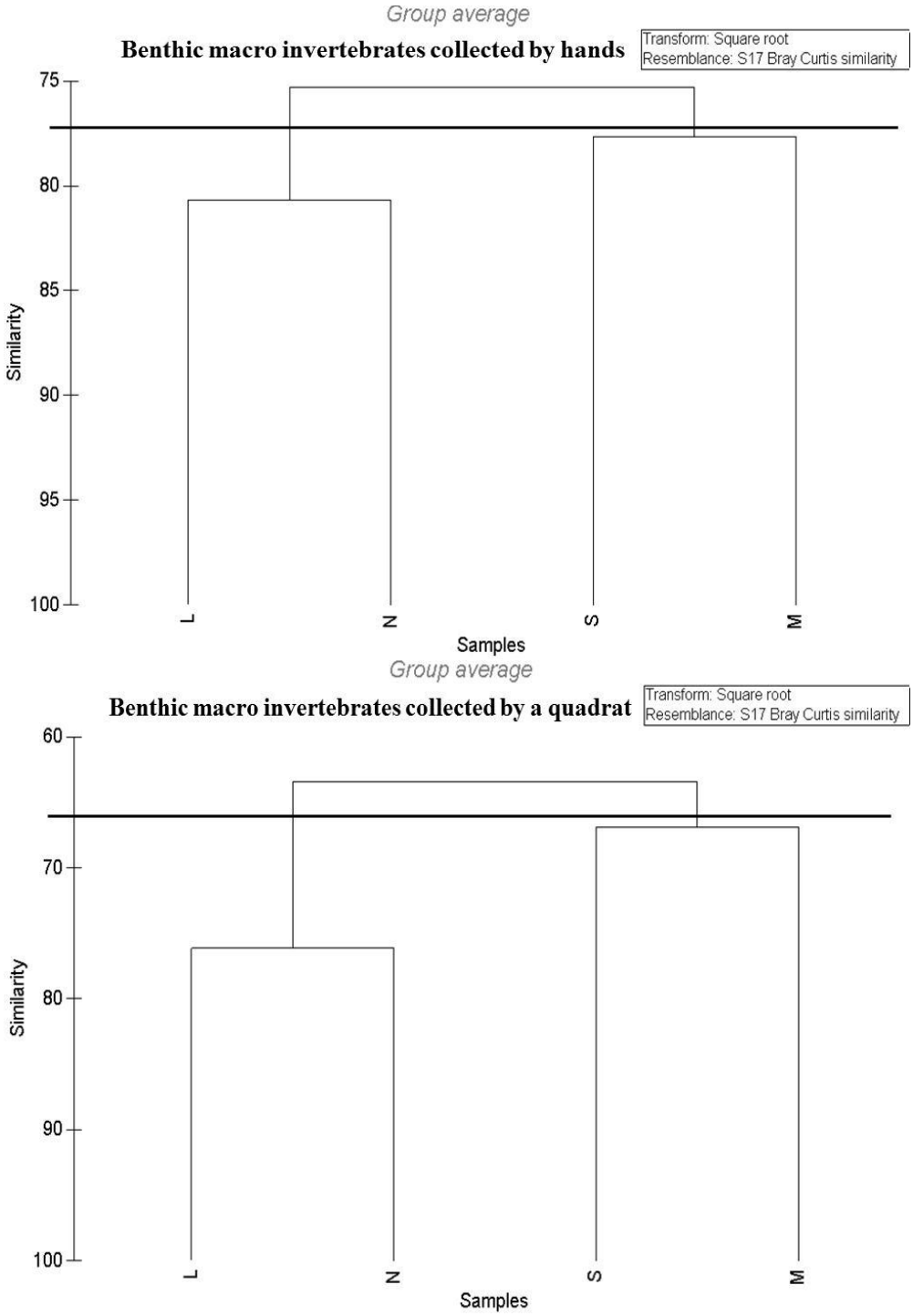

Figure 3. *Dendrogram of cluster analysis showing 2 groups of similarity of benthic macroinvertebrates among mangrove sites (S = young, M = intermediate, L = mature and N = semi natural sites).*

Discussion

Regarding mangrove plantation structure, our results were rather consistent with other studies (Hassan, 2006; Kridiborworn et al., 2012). The recent study conducted in 2012 reported that *Rhizophora apiculata* was the main mangrove species planted for

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charcoal production in Yeesarn village. An average density of mangrove in plantation sites recorded in 2012 was around 22,089 trees/ha similar to the density of the mangrove plantation at age 0-5 years in our study (27,778 trees/ha). The average height (10-11 m) and DBH (3-4 cm) of mangrove trees at the harvest age (9-12 years) from the previous study (Krindiborworn et al., 2012) were also comparable with the current study. *Rhizophora apiculata* appears to be a suitable species for charcoal production in Yeesarn village because it grows well in the estuarine and intertidal environment as well as having high quality in terms of the carbon content and calorific value (Krindiborworn et al., 2012).

Water quality in the creeks of all mangrove sites was good and suitable for aquatic organisms. The results of the present study were also similar to other studies in the region (Gandaseca et al., 2011; Jitthaisong et al., 2012). The water was salty as it is a specific characteristic of an estuarine environment. Phosphorus concentrations were rather low at most sampling stations and the nitrogen concentrations were comparable among sites. BOD concentrations were also low. Mangrove plantations are usually located far from local community settlements or are around the edge of the land (transition zone) and therefore are less impacted by household waste and pollution. The present study is in contrast with Tripathy et al. (2005) who found high concentrations of nutrients in an Indian mangrove ecosystem due to adjacent sources of nutrient. In addition, in the current study, the water quality appeared not to be affected much by mangrove sites since during data collection, as all mangrove sites were not inundated due to a low tide period.

The results of the biodiversity studies at the mangrove sites revealed interesting findings. The phytoplankton population and density appeared to be higher at the younger mangrove plantation site compared with the mature-aged sites. It was likely due to shading created by the canopy at the older-aged mangrove sites which reduced the light availability in the creeks and thus suppressed the growth of phytoplankton. Communities of zooplankton were also high and seemed to correspond well with the phytoplankton population.

The community of invertebrate animals changed with the age of the mangrove forest stand similar to Ashton et al. (2003). In particular, species of benthic macroinvertebrate fauna (collected by hand) tended to increase more at the mature mangrove sites than at the younger plantation sites. The present study of common macroinvertebrates recorded was also consistent with Macintosh et al. (2012) who reported that snails of the families Neritidae and Ellobiidae were the most abundant and common molluscs in mangrove forests. The communities of benthic and sedimentary invertebrates (collected by quadrat) were present in high numbers in the mature plantation mangrove forest and semi natural areas. This could be explained by the fact that the mature mangrove plantation forests provided rich and crucial food sources for benthic macroinvertebrate communities. Long colonization and assemblages of benthic mancro invertebrates in the mature mangrove plantations could also account for the greater numbers of animal species that we recorded. In the young plantation forests, it may take several more years for macroinvertebrates to colonize and establish the population. From observation, the plantation trees at the younger sites may also create a drier soil environment due to light penetration through the open canopy, thus being less suitable for some animals such as molluscs and annelids compared to the wetter condition in the older-aged plantation forests with tall trees having a large closed canopy. The low frequency of inundated environmental conditions may also be responsible for the presence or absence of some

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mangrove fauna (Ashton et al., 2003). Several studies have indicated that different groups of benthic animals prefer different environments; for example, fiddler crabs prefer semi-open mangrove habitat with some light penetration while sesarmid crabs prefer a more closed canopy created by mature forest stands (Macintosh et al., 1984; 2012, Ashton et al., 2003).

Conclusions

In conclusion, this study showed differences in the community structure of intertidal benthic macroinvertebrates between the younger mangrove plantation sites and the mature old-aged mangrove forests. It could also be stated that benthic macroinvertebrate communities and populations in mangrove plantation areas have developed through time and the older the mangrove forests, the higher biodiversity and abundances of the benthic fauna. Accordingly, our findings support the fact that apart from having high economic value for charcoal production, mangrove plantation forests also play an important role in estuarine ecosystems since mangrove plantation forests, especially mature forest stands, provide suitable habitats and food sources for many species, especially molluscs and crustaceans.

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APPENDIX

Table A1. *A list of invertebrate species collected by using a quadrat*

Phylum Annelida
Class Polychaeta
Family Capitellidae
Mediomastus sp.
Family Spionidae
Prionospio sp.
Oligochaeta
Phylum Sipuncula
Sipunculus sp.
Phylum Arthropoda
Class Crustacea
Family Amphipoda
Gammaridea
Family Alpheidae
Alpheus euphrosyne
Family Grapsidae
Episesarma mederi
Sesarma (Chiromantes) *eumolpe*
Sesarma mederi
Family Ocypodidae
Macrophthalmus teschi
Uca forcipata
Phylum Mollusca
Family Gastropoda
Assiminea sp.
Family Elloblidae
Melampus siamesis
Melampus sp.
Cassidula aurisfelis
Laemodonta punctigera
Family Neritidae
Neritina violacea
Family Mytllidae
Modiolus philippinarum
Family Potamiddae
Cerithium sp.
Family Dounacidae
Donax sp.

Table A2. *A list of invertebrate species collected by hands*

Phylum Annelida
Class Polychaeta
Family Capitellidae
Mediomastus sp.
Family Spionidae
Prionospio sp.
Oligochaeta
Phylum Sipuncula
Sipunculus sp.
Phylum Arthropoda
Class Crustacea
Family Amphipoda
Gammaridea
Family Alpheidae
Alpheus euphrosyne
Family Grapsidae
Episesarma mederi
Sesarma (Chiromantes) *eumolpe*
Sesarma mederi
Family Ocypodidae
Macrophthalmus teschi
Uca forcipata
Phylum Mollusca
Family Gastropoda
Assiminea sp.
Family Elloblidae
Melampus siamesis
Melampus sp.
Cassidula aurisfelis
Laemodonta punctigera
Family Neritidae
Neritina violacea
Family Mytllidae
Modiolus philippinarum
Family Potamiddae
Cerithium sp.
Family Dounacidae
Donax sp.

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MAPPING OF HEAVY METAL CONTAMINATION CHARACTERISTICS USING CIAPRG METHODOLOGY

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Abstract. The aim of this study is to produce a digital contamination map which is interactively rectified with a database, projected and scaled. For this reason, a detailed investigation was conducted for an understanding of contamination dispersal characteristics in terms of the contribution of heavy metal concentrations, such as mg/kg of Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn in a city center. CIAPRG Methodology (Contribution Independent of Amount through Positional Recoding and Grading) has been developed and used for the first time in this study by the authors in order to constitute a map of total heavy metal contamination distribution characteristics. Heavy metals from fifteen soil sample points, which were situated at different locations in the study area, were collected for chemical analysis. Data obtained by the chemical analysis was inserted into CIAPRG methodology. The majority of total heavy metal contribution is shown to be based on Cu, Mn and Ni. The effects of the contribution to pollution values which were recoded and graded, can be shown in relation to buildings, independent of the quantity of pollutant within the context of CIAPRG methodology. The resulting map designated that the mid and south-western parts of the study area were more intensive than the other parts in terms of heavy metal contamination. The graded contaminator values for soil and roadside dust were together compared with graded values for soil graded contaminator values, and roadside dust graded contaminator values, separately, using independent sample t tests with the results being interpreted.
Keywords: *CIAPRG methodology, heavy metal contamination distribution, roadside dust, soil*

Introduction

Considering accelerated industrialization and urbanization, almost half of the world's population now lives in urban areas. Their dense activities result in an increasing amount of contaminants discharged into the urban environment. A variety of environmental problems have emerged, of which heavy metal pollution in urban soil is a major issue (Madrid et al., 2002; Tanner et al., 2008; Lu et al., 2010; Apeagyei et al., 2011; Xia et al., 2011). As typical contaminants in an urban environment, heavy metals are useful indicators of environmental pollution. For example, Pb, Cu and Zn could be an indicator of traffic pollution. Most heavy metals, such as Pb, Cr and Cd, will continue to accumulate in an urban environment due to their non-biodegradability and their lasting nature. As a result, they are often termed 'chemical time bombs'. In urban areas, these 'bombs' have become a potential threat to human health and safety and have severely disturbed the natural geochemical cycle of the ecosystem. Metals have a

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direct influence on public health as they can easily enter the human body through dust ingestion, dermal contact or breathing (Lee et al., 2006; Kadi, 2009; Chen et al., 2010; Bućko et al., 2011).

The components of soils in a city center are affected significantly by human activity, even though soils are composed of mineral constituents, organic matter, living organisms and air and water that are of vital importance to human health. Vehicle exhaust particles, caused by traffic, contribute directly to heavy metal pollution (Lee et al., 2006; Shi et al., 2008; Christoforidis and Stamatis, 2009; Wei and Yang, 2010; Duoon and Lee, 2011).

In order to explain pollution characteristics precisely, a model should be constructed with all parameters included in the model. CIAPRG methodology has been developed and used in this study for the first time. The methodology in question identifies a related characteristics assessment concerning different pollutant sources.

Materials and Methods

The study area

The workspace covers an area of 2.8 km East-West and 1.3 km North-South (3.64 km^2) and is 750-800 m above sea level. It is located on alluvial ground covering most of a housing area *(Figure 1).*

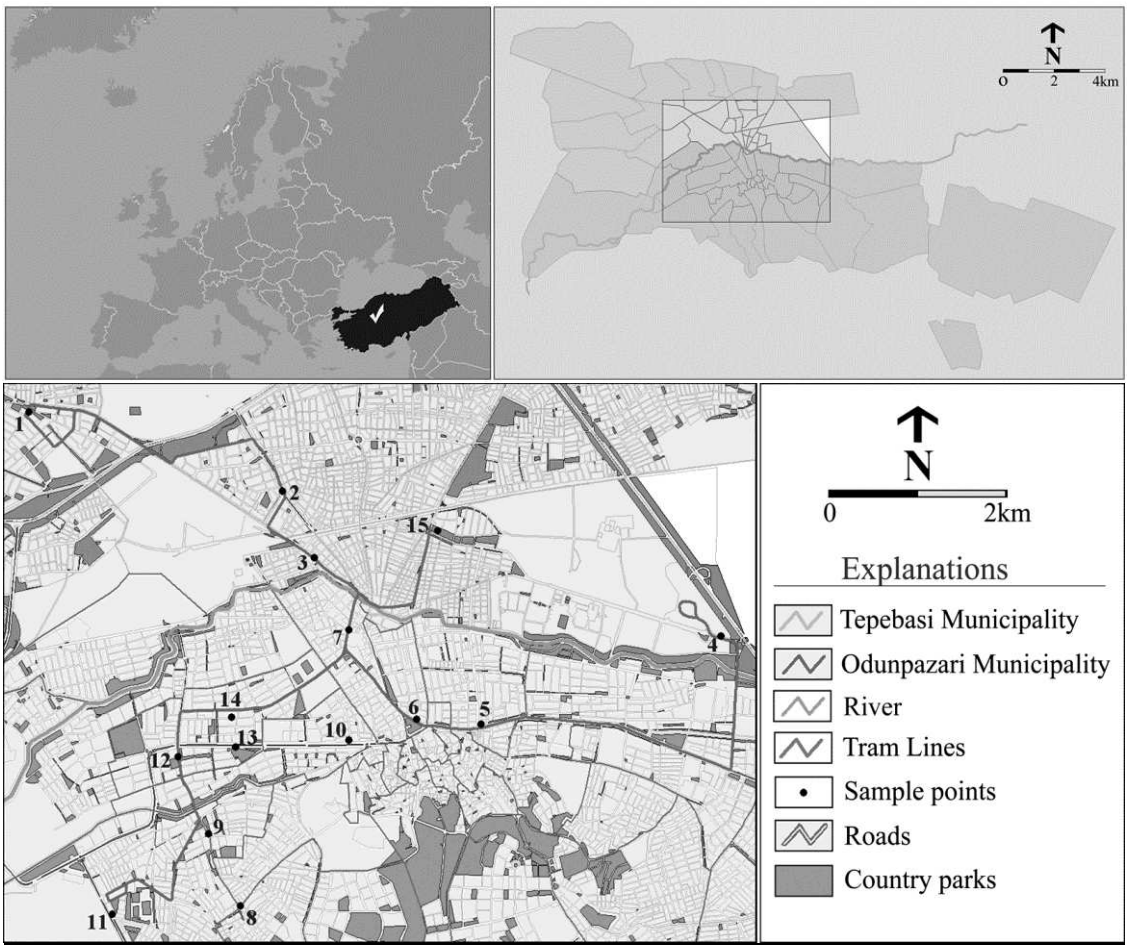

Figure 1. *Location map of the study area and sampling locations*

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This area contains geological units such as new alluvium, old alluvium and rock (Nefeslioglu et al., 2003). The new alluvium ground possesses a high earthquake risk due to its liquefaction potential (Ayday et al., 2001). These liquescent areas are concentrated on low speed grounds (Tun et al., 2004).

This new alluvium which fills Eskişehir lowland consists of loose sediment with no cementation between the particles. Generally, below this level, there is silt clay in which the silt percentage is higher. Certain areas below this level consist of a thick clay layer. At the lower layers, there is a sand level and below this there is silty sand. At the lower layers the sand percentage increases and gravelly sand is evident. The workspace comprises old alluvium in the north-west, rock in the south-east and new alluvium in between (Tun et al., 2004).

Extensive CIAPRG methodology

CIAPRG (Contribution Independent of Amount through P**o**sitional Recoding and Grading) methodology is a new method first used in this paper.

The aim of the method is to define the effects of the components of pollution, to model the locations of these components and to map them, taking into account the structural relationships between the components.

During the application part, firstly, the components of the pollution distribution to be mapped are defined. Each of these components causes pollution in the study area. These pollution values have a distribution and, by modeling, they are taken as a mean value of each layer. It is important to be cautious during the map construction stage. The processes of coordination and measurement should be planned, taking into account the aims of the study. The definitions of projection should be particularly defined considering the geographical properties and the location of the study area. Each variable which contributes to the pollution distribution and each component which constructs that contributor should be structured at a different layer where the locational relationships will be modeled. It is important that each of the layers be mapped separately in order to avoid confusion when denoting the map, examining one component distribution and evaluating the additive contribution of each contributor. The layers which have common components are rectified, projected, and structurally analyzed by location and modeled. When vector based mapping, in particular, is used, then the resolution is high.

The data base related to the numerical map, using strata contribution to the pollution, is constructed cautiously. The data base is a numerical platform used in modeling, analyzing the contribution of each component. The data base works interactively with the graphical unit. Each analysis result constructs a separate stratum on the graphical data. Similarly, the results of the analyses and modeling on the graphical data constructs a data set for new analyses.

Each component which contributes to the pollution in the area takes a score as the pollution contributor. At this stage, the vector based pollution distribution map layer is transformed by the grid based map layer. The extent of contribution to the pollution is defined by the relative contributions of other components. The contribution of each component depends on its own characteristics. These individual characteristic scores are written at the grid related coordinate and are correlated with their own characteristic information. Hence, it can be included in the calculations using the grid value. The contribution to the pollution of each component in the same coordinate is different to that of the other components. The important point here is that each grid value represents

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the degree of contribution to the pollution in the related coordinate. After this, the noted score at each grid is added to the other grid values at the related coordinate. Therefore, the recorded score at the related coordinate and grid will give a changeable result with the correlation of the extent of contribution to the resulting value which denotes the total result.

In the last stage, the amount of contribution to the pollution was recoded due to its contribution to the pollution and its own characteristic. The resulting map is a grid-based, measured, coordinated, projection-defined and data base interactive numerical map. On that map, for each of the coordinates, a grid based examination is possible. The contribution to pollution can be examined for every separate coordinate, and therefore, the contribution of the component itself can be obtained as a ratio or the contribution of all the components can be obtained as a total. In this way, the researcher can interpret the results in terms of the different contributions of the components.

Sampling

In this study, the workspace of the sampling area focused on Eskişehir city center. The heavy metal concentrations were used as input data for pollution maps to study the distribution of heavy metals in this urban area. A systematic sampling strategy was adopted to prove a sampling strategy over the entire workspace.

Gridding procedures were applied to the study area for homogeneous evaluation of the geographical data. Gridding was performed, based on a grid size of $5x5m^2$, using all of the input points available within a variable search radius. In this method, the closest point to the center of the grid unit is estimated. Heavy metals, which are highly enriched in soil and roadside dust were identified and compared.
The coordinates of the sample locations were recorded by GPS and the sampling grids are shown in *Figure 1*. An assessment of the overall contamination of the soil is based on the degree of contamination.

Analytical methods

Fifteen roadside dust and fifteen soil sites were selected in Eskişehir, including locations with a high density of buildings, main roads and tram lines *(Figure 1).* At each sampling site, roadside dust and soil samples of ~1 kg were collected by sweeping, using a polyethylene brush and tray, during the dry season in June 2009. Sampling was performed on the first 10 cm below the surface of the soil along the roadside. All of these samples were initially air-dried in a laboratory, and then sieved through a 0.5 mm polyethylene sieve before being dried for 3 hours at 105^{o} C (to a constant mass). A small portion, typically 500 mg of dried and sieved samples, was transferred to a 10 ml Teflon PFA (perfluoroalkoxy) vial. An aliquot of ultrapure HNO_3 (10mL) was added and the cap of the vial was closed tightly. The acidified sample was kept at room temperature for 2-3 hours and then the bomb was heated at 175 ºC for 10 minutes. After cooling, the digests were passed through Whatman 41 filter paper (Whatman Company, UK). The digestion tubes were rinsed four times, passing through the filter, and the filters were made up to 100 mL volume using ultrapure water. Heavy metal concentrations were determined using soil and roadside dust samples of 500 mg which were microwave digested (Cem Mars 5) (USEPA 2007). Finally, the content of heavy metals in the soil and roadside dust was determined by Inductively Coupled Plasma-Optic Emission Spectrometry (ICP-OES) (Varian 720 ES).

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Software support

In this study, two types of software were used for analyzing, modeling and developing maps. The software used for digitizing, mapping, spatial analysis and vector-based analyses was GeoMedia Professional, and for recode procedures, overlay analysis and other raster and grid-based analyses and modeling GeoMedia Grid Analysis was applied.

Spatial Analysis with CIAPRG Methodology for Contribution to Map Layers

Each of the heavy metal pollutants examined respectively was based on values which were obtained by soil and roadside dust analyses. Subsequently, digital dispersal maps were produced of the city center for each of the pollutants using this analyzed data. Each of contributors to heavy metal pollution exhibited different qualities. Therefore, initially, the concentration values of contributors were normalized in accordance with Equation 1.

Normalised Concentration was expressed by the following equations:

$$NC_i = \frac{x_i - \mu}{\sigma} \quad \text{(Eq. 1)}$$

and μ is given by :

$$\mu = \frac{1}{n}\sum_{i=1}^{n} x_i \quad \text{(Eq. 2)}$$

where $\boldsymbol{x}$ is a raw score to be standardized, $\boldsymbol{\mu}$ is the mean of the value of contributor and $\boldsymbol{\sigma}$ is the standard deviation of the value of the contributor.

Secondly, recoded maps were generated for every contributor in terms of characteristic pollution factors; average, % contribution, normalized concentration and exceeded risky factor. At this level of the study, equation 3 was used for every distribution map in order to grade the contribution degree to heavy metal pollution.

Contamination of heavy metal contributers (CHC) was expressed by the following equations, where NC is Normalised Concentration, C is Contribution, Av is Average Value and CF is Coefficient Factor.

$$CHC_i = \sum_{i=1}^{8} NC_i \cdot C_i \bullet Av_i \cdot CF_i \quad \text{(Eq. 3)}$$

Finally, superimposed maps, used as traffic density volume maps and recoded contributor maps according to distributions of heavy metals with characteristic pollution factors, were also generated, classified and recoded. Equation 4 was also used for every distribution map, in order to generate and to grade total contamination of heavy metal contributors according to contribute to pollution. The final heavy metal pollution

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distribution map, generated using CIAPRG Methodology, included all heavy metal pollutant contributions.

The total contamination of the heavy metal contributers (TCHC) is expressed by the following equations:

$$TCHC = \sum_{i=1}^{8} \prod_{j=1}^{4} X_{ij} \quad \text{(Eq. 4)}$$

Related parameters of heavy metals for i, 1,2,3,4,5,6,7 and 8 refer to Cr, Ni, Fe, Pb, Mn, Cd, Cu and Zn respectively. Related parameter of contaminations for j, 1,2,3,4, refer to the relevant values of Normalized Concentraion (NC), Contribution of Contributor (C), and Coefficient Factor (CF) respectively, with TCHC as the Total Contamination of Heavy Metal Contributors. Equations 1-4 are considered by authors in terms of the CIAPRG Methodology in this study. The superimpose process in terms of contribution of heavy metal contamination as an example for only one grid, located at the same coordinate in each contribution map, is shown in *Figure 2*. This process was applied to each grid cell, located at the same coordinate for every contribution distribution map.

The final total contaminations of heavy metal contributers, generated using contributions thanks to scaled, rectified and projected heavy metal contributors, could be correctly superimposed with rectified high resolution satellite imagery.

Results and Discussions

When heavy metal pollution was evaluated for both the soil and roadside dust samples, Ni concentrations were found to be significantly above limit values at almost all sample points (75 mg/kg) (Malkoc et al., 2010). When pollution was graded, Ni was seen as most abundant for soil at 41.52%, and for roadside dust 36.76%. Cr was found as the second most abundant for soil at 18.57% and for roadside dust at 20.95%. Although Fe was found as the third most abundant for soil at 16.93%, it was found to be the least abundant for roadside dust at 0.20%. It cannot be concluded that Fe is an abundant parameter, because the color of Eskisehir soil is brown, taking that color from Fe. A low ratio of Fe in roadside dust shows the probable Fe pollution. The reference values for Fe and Mn do not exist in soil pollution regulations, hence Environmental Protection Agency (EPA) standards (USEPA, 2004) were taken into account as reference values. For the soil samples, the contaminators can be arranged in order as follows: Ni>Cr>Fe>Cd>Cu>Mn>Zn>Pb. For the dust samples, they can be arranged in order as follows: Ni>Cr>Cu>Cd>Zn>Mn>Pb>Fe. Neglecting Fe at all sample points, the Pb ratio would be the lowest for both soil at 1.96% and for dust at 3.85%. This does not exceed the limit values at any of the sample points (300 mg/kg). When Cd, Cu, Mn and Zn value orders were compared for soil and dust, the degree of the contaminators were seen to vary, but the limit values were not exceeded. The ratios of contaminators for the soil samples were, Cd 7.78%, Cu 4.92, Mn 4.21% and Zn 4.12, while the ratios of contaminators for the roadside dust samples were, Cu 13.79%, Cd 10.56 %, Zn 9.38% and Mn 4.51% *(Table 1).*

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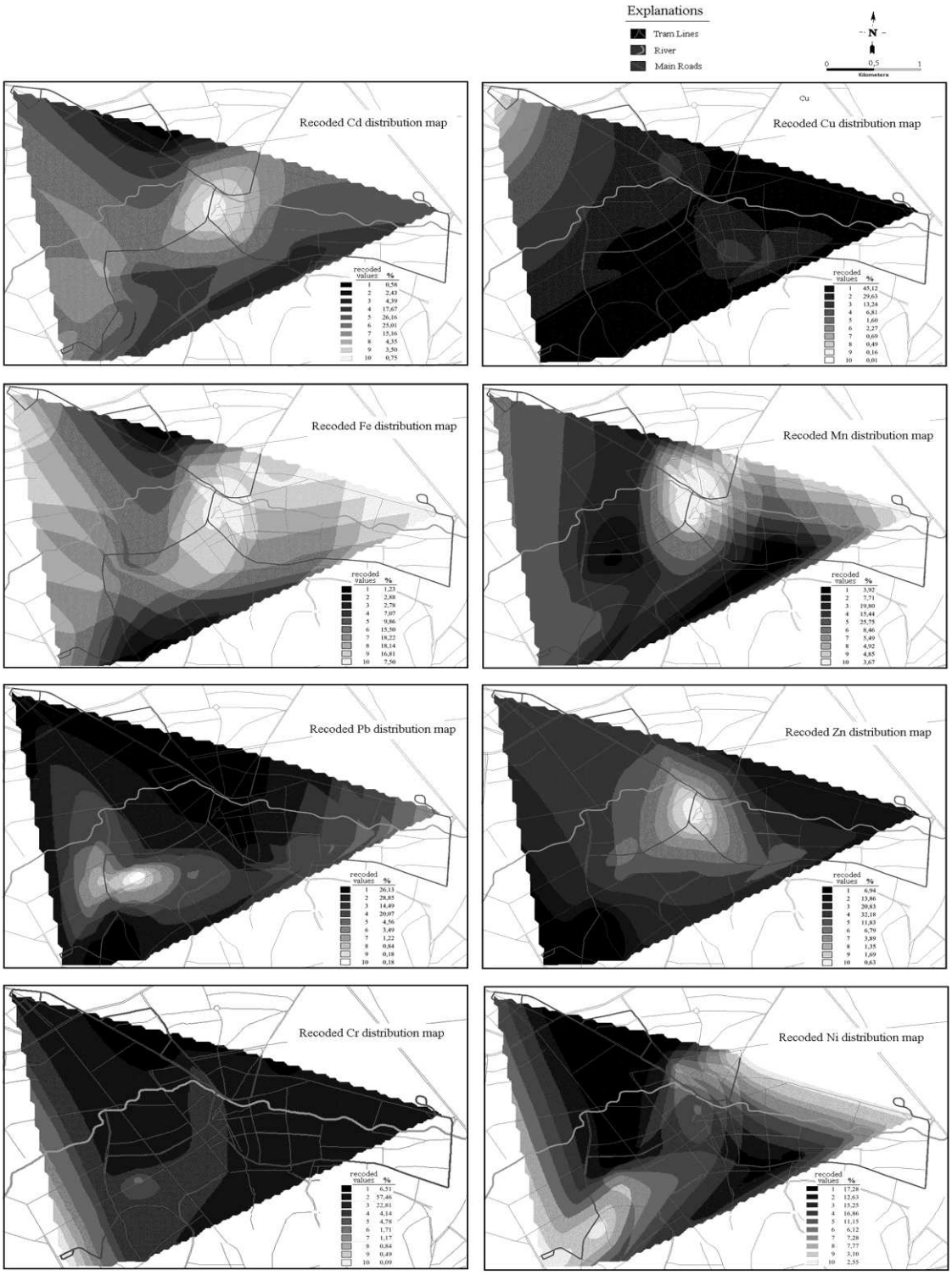

Figure 2. *Graded heavy metal pollution distribution maps for soil and road dust*

The most important result of this study is that none of parameter values exceeded the corresponding limit values for dust samples at three types of sample points; for only tram, only traffic and for both traffic and tram sample points. However, for the soil samples, those values were below the limits only at the sample points at both the traffic

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and tram lines. According to the results, vehicles and passing trams are not the only sources of pollution at the sample points in question. Also traffic density, vehicle duration time at traffic lights (different at almost all the sample points), volume of traffic, types of vehicles, road construction work, meteorological conditions, and wind direction may affect results.

Table 1. *Contamination degrees for metals in soil and road dust*

	Soil			Road Dust		
Metal	Range (mg/kg)	Average (mg/kg)	Contribution (%)	Range (mg/kg)	Average (mg/kg)	Contribution (%)
Cd	0.59-3.39	1.23	7.78	0.60-1.99	1.08	10.56
Cr	7.37-231.97	97.85	18.57	34.98-132.73	71.42	20.95
Cu	9.22-112.29	36.31	4.92	16.25-274.80	65.84	13.79
Fe	8809.17-39877.56	20517	16.93	8636.57-24865.85	153.14	0.2
Mn	243.65-571.37	399.24	4.21	191.76-502.19	276.81	4.51
Ni	8.84-299.47	164.11	41.52	61.2-182.32	94	36.76
Pb	6.9-97.39	30.92	1.96	11.13-113.61	39.43	3.85
Zn	33.50-156.88	65.18	4.12	28.79-250.65	95.96	9.38
Degree of contamination	9119.25-41350.32		100	8981.29-26324.15		100

The southern and western parts of Eskişehir city center were found to be more polluted than the other parts of the study area in terms of Ni and Cr concentration distributions, as shown in *Figures 3* and *Figure 4*. Since we could not find any value which exceeded the critical limit for Cd, Cu, Fe, Mn and Zn, it was not necessary to calculate a Coefficient Factor for these heavy metals. In this process, the first original values of the contributors were used. These were then recalculated in terms of their contribution to total heavy metal pollution as layers, and then these values, graded according to their contribution to total pollution, were calculated as a percentage of the total area. These values, which were calculated, recoded and graded for each map, were generated for characterization of heavy metal pollution *(Figure 5).*

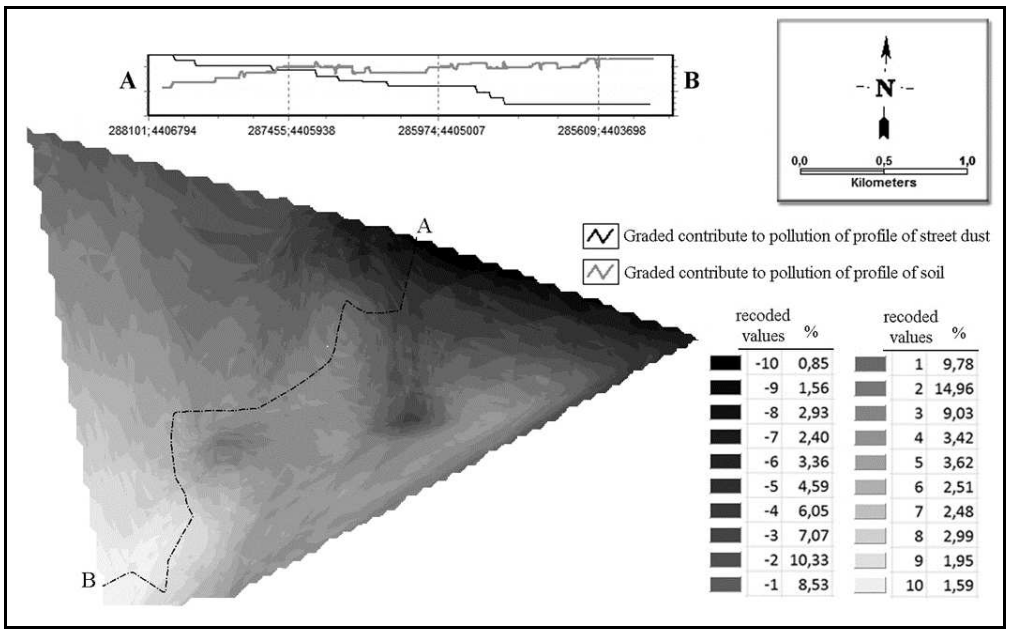

Figure 3. *Contamination profiles of recoded values from soil and road dust*

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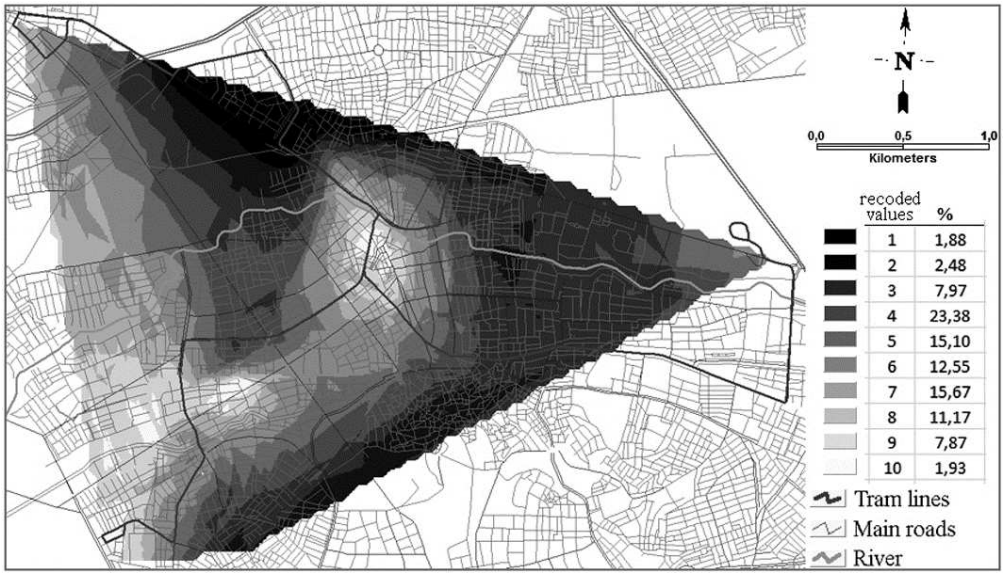

Figure 4. *Digital map of the city center superimposed with graded pollution map*

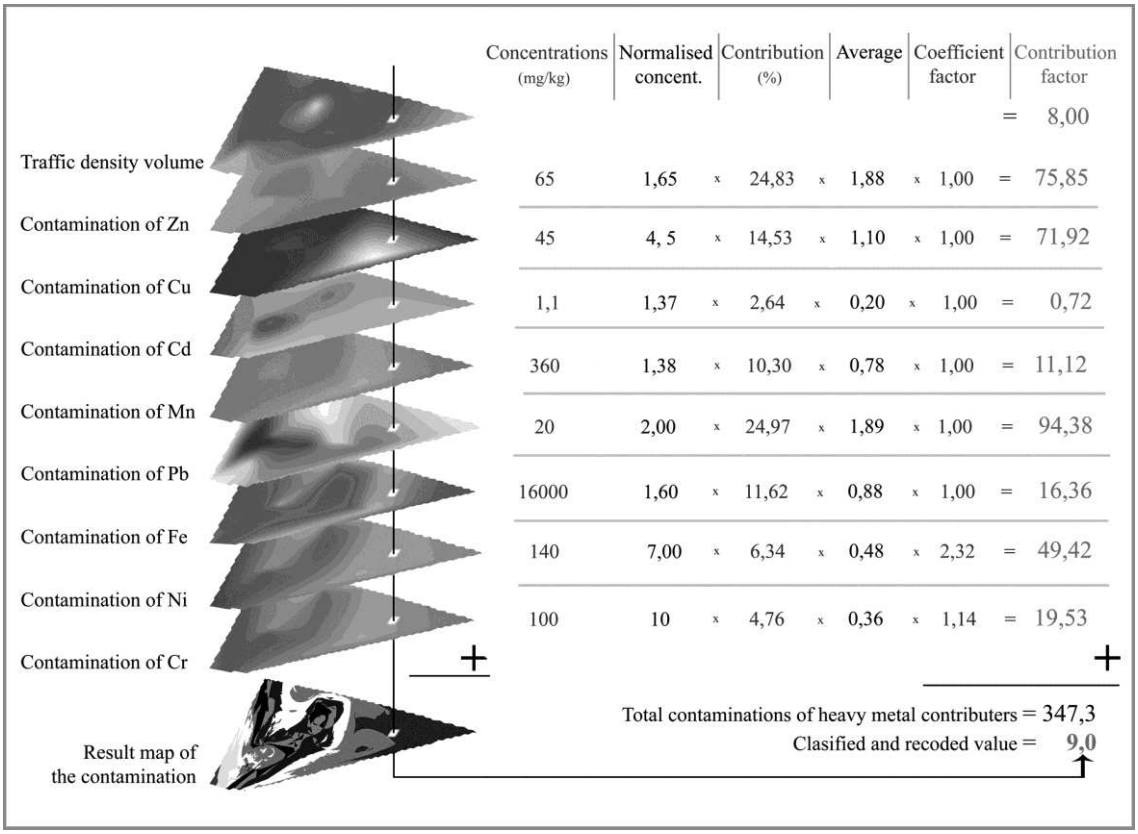

Figure 5. *Example showing the superimposed process, in terms of contribution of heavy metal contamination, for one grid*

Pollution distributions were mapped for soil and roadside dust separately, and then the percentage of pollution degrees were calculated on the total studied area *(Figure 2).*

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At this part of the study, the heavy metal pollution maps, generated for soil and roadside dust separately, were not used to determine a contribution to total pollution, because any contribution to pollution was caused not only by soil, but also roadside dust, at the same coordinates of the studied area and at the same points as grids on the digital map.

In order to determine total pollution, contribution values caused by both of the sources were collected at the same pool, and these were then recoded and graded in terms of a percentage contribution to total heavy metal pollution, instead of each source being evaluated individually *(Table 2).* Each contribution grade value of heavy metal pollution is shown in *Table 2*. Grey colored cells and bold written values refer to contributions of heavy metal pollution caused by roadside dust, and values written in ordinary font refer to contributions of heavy metal pollution caused by soil. For a grading contribution degree to total pollution for each contributor, all values of soil and roadside dust were graded and sorted at the same scale. Therefore, all of the graded values were mixed in terms of contribution to pollution *(Table 2).*

All of the pollutant contribution values were graded from 1 to 10. Cu, Mn, Ni and Pb elements in the soil and Cd, Cr and Fe elements in the roadside dust were investigated by analyses to identify their contribution to the heavy metal pollution.

Related Zn values are approximately at the same percentage for the soil and the roadside dust *(Figure 6).*

	Soil %	Roadside dust	equal %
Cd	**18,1**	**73,51**	**8,39**
Cr	**21,47**	**58,22**	**20,31**
Cu	65,75	22,67	11,58
Fe	**38,26**	**48,42**	**13,32**
Mn	75,1	19,2	5,7
Ni	65,43	23,05	11,52
Pb	68,46	10,81	20,73
Zn	**41,3**	**41,35**	**17,35**

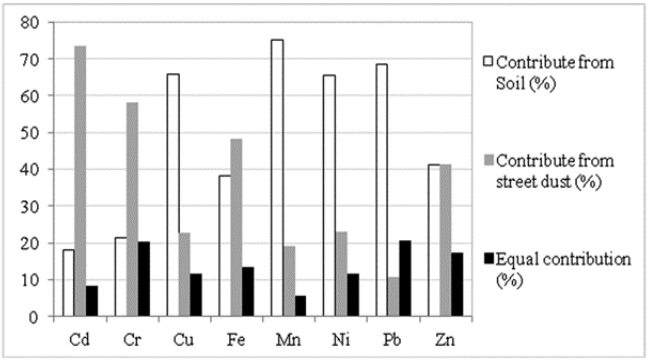

Figure 6. *Percentage values for each pollutant contributing to pollution on entire study area*

An AB-profile was obtained for only one tram line, in terms of explaining this difference visually. In the AB-profile, the bold grey solid line refers to the graded contribution to pollution values for soil, and the thin black solid line refers to the graded contribution to pollution values for roadside dust. A differentiation map was obtained by subtracting the graded values of contribution to pollution caused by roadside dust from the graded values of contribution to pollution caused by soil. Negative results mean that the contributions to pollution from roadside dust are more than that of soil. Because more pollution is caused by roadside dust at around point A, the black thin solid line located at the top of the AB-profile approached point A. Due to the xy=287455.4405938 coordinate being located at the other side of the area, a bold grey line was drawn at the top of the AB-profile, close to point B. Heavy metal pollution in the soil increased in this part of the studied area *(Figure 3).*

In this part of the study, the graded data values for soil and roadside dust together were compared with the graded values for soil using an independent sample t test. As a result of analyses, the difference between the soil and roadside dust values and the soil values for Cd (p=0.488), Mn (p=0.903), Pb (0.948) were not found to be statistically significant.

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However, the differences in question were found to be statistically significant for Cr (p=0.016), Cu (p=0.000), Fe (p=0.020), Ni (p=0.013) and Zn (p=0.000).

Table 2. *Mixed and sorted pollution contribution values caused by soil and road dust*

Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn
1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1
2	1	1	1	2	1	1	1
2	1	1	1	2	1	1	1
3	1	1	1	2	1	1	1
3	1	1	1	2	1	1	1
3	1	1	1	3	1	2	1
3	1	1	1	4	1	2	1
3	1	1	1	4	1	2	1
3	1	1	1	4	1	2	1
4	1	1	2	5	1	2	1
4	1	1	2	5	1	3	1
4	1	1	2	5	1	3	1
4	1	1	2	5	1	3	1
4	1	1	2	6	1	3	1
5	1	1	2	6	1	3	1
5	1	1	2	6	1	3	1
5	1	2	2	6	1	4	1
6	1	2	2	6	1	4	1
6	1	2	3	6	1	4	1
6	1	2	3	7	1	5	1
6	1	2	4	7	1	5	1
6	2	2	4	7	1	5	1
7	2	2	4	7	1	5	1
7	2	2	5	7	1	5	1
8	2	2	5	8	1	5	1
8	2	2	6	8	2	5	2
8	3	2	6	8	3	6	2
8	3	2	6	8	4	6	2
8	4	2	7	8	4	6	2
9	4	3	7	8	5	6	2
9	5	3	7	9	6	6	2
9	5	3	7	9	6	6	2
9	5	3	7	9	7	7	2
9	6	3	7	10	9	7	2
10	7	3	8	10	10	7	2
10	8	3	8		10	7	2
10	9	3	8			8	2
	10	4	8			8	2
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The soil and roadside dust contamination data set was also compared with the roadside dust contamination data set for the same heavy metals using their graded values. As a result of independent sample t testing, the difference between the graded values for soil and roadside dust and the roadside dust data for Cd (p=0.712), Mn (p=0.735) and Pb (p=0.199) were not found to be statistically significant. However, the difference between the graded values for soil and roadside dust contamination and roadside dust contamination for Cr (p=0.005), Cu (p=0.011), Fe (p=0.019), Ni (p=0.024) and Zn (p=0.007) were found to be statistically significant.

Since the graded contaminator values together for roadside dust and soil were found to be greater than those for soil and roadside dust separately, these were taken into account in the digital mapping.

A digital map of the city center was superimposed with a distribution map, generated in terms of total pollution contributions from heavy metals in soil and roadside dust scaled on buildings *(Figure 4).*

In this way, the reformed superimposed map provided better visualization of the contributions of the heavy metal pollutants in the soil. This map also was used to study the relationship between metal enrichment in soils, roadside dust and related traffic volumes. Detailed heavy metal pollution distribution in soil of an urban area can be shown, based on buildings, in a similar manner.

The obtained final total pollution distribution map is scaled, rectified and projected; it was therefore, correctly and easily superimposed using high resolution satellite imagery. Hence, heavy metal pollution distribution was determined due to the characteristics of the contributors included and the effects of the embedded pollutants, based on buildings *(Figure 7).*

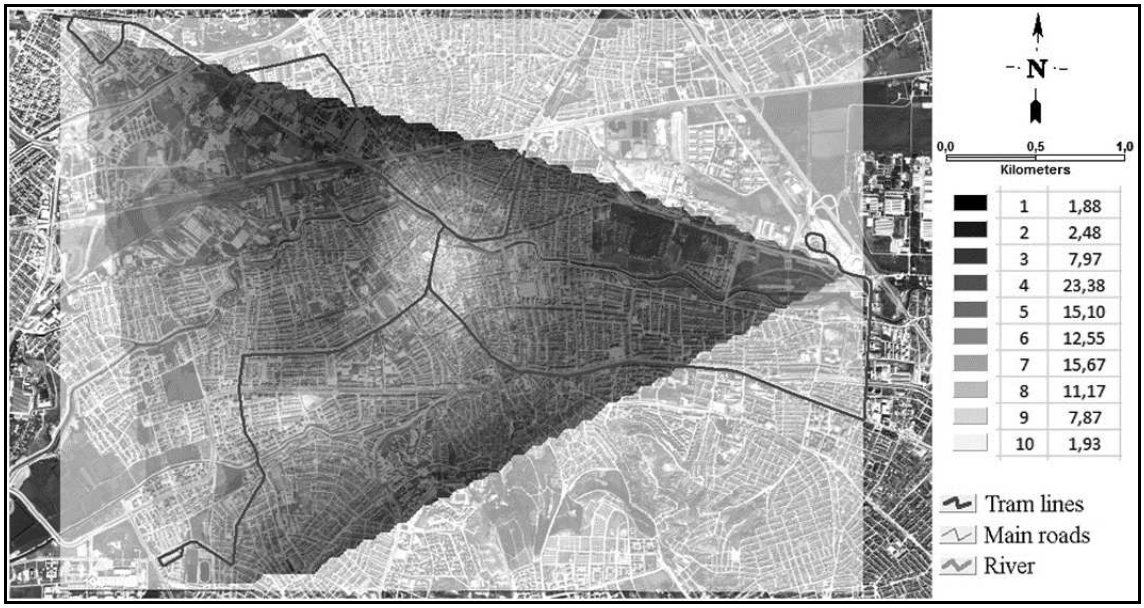

Figure 7. *Superimposed map with satellite image and graded map of heavy metal pollutants*

The final map, including the contribution to total heavy metal contamination of Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn, illustrates that the mid and south western parts of the study area were more contaminated in terms of heavy metal contamination. According

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to statistical results, using values obtained from soil and roadside dust together give us a more accurate result than using them separately.

CIAPRG methodology is recommended for use in similar studies concerning any distribution characteristic assessment as an alternative solution for locational modeling with an interactive data base. CIAPRG methodology, suggested by the authors for this study, produced more efficient results than other conventional methods. In addition to this, CIAPRG methodology provides an assessment based on each rectified source, supported by the data base. Local authorities should be more cautious about buildings at the 8 to 10 graded scores and take necessary precautions in order to reduce risk *(Figure 7).*

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ASSESSING THE IMPACT OF LAND USE AND LAND COVER ON WATER QUALITY IN THE WATERSHED OF A RESERVOIR

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Abstract. Natural forest of Río Tercero watershed (Argentina) was replaced by urban expansion and agricultural activities causing negative impacts in water quality of Río Tercero reservoir. This paper classifies land use and land cover (LULC) in the watershed, trying to find a relationship with nutrient loads of the reservoir's tributaries. Each tributary was analysed during 2006 bimonthly for physico-chemical variables. LULC was determined using a Landsat 5 TM image. Statistical analyses were carried out to identify relationships between water quality and LULC. Results suggest that urban and agricultural activities are the primary driving forces behind the variations in nutrient loads measured in tributaries. Sub-watershed most affected by human activities displayed the highest values of nutrient loads. If agricultural and urban activities continue to increase, water quality at watershed scale will decline. It is recommended the development of a global approach management plan at a watershed scale.
Keywords: *basin, land use change, nutrient loads, water quality, water resources*

Introduction

Lakes and reservoirs are important freshwater sources used to satisfy environmental and human needs. Unfortunately, in recent years there is a rapid declining availability of usable freshwater in terms of water quality and quantity due to unsustainable land use practices and urban land development.

Studies demonstrate that surface water quality is controlled by both the natural processes (i.e. precipitation inputs, erosion, and weathering) and the anthropogenic activities via point sources, such as industrial effluents and wastewater treatment facilities, and diffuse, such as runoffs from farming land and urban area (Ahearn et al., 2005; Li et al., 2009). On this sense, Seeboonruang (2012) and Su et al. (2015) found that different land use types and spatial patterns greatly affect water quality. According to Lee and Bastemeijer (1991), many problems of water pollution are caused by changes in land use patterns on watershed areas as population pressure and economic activity increase. Li et al. (2008) found that there is a strong relationship between declining water quality and increasing agricultural and urban land development at watershed scale. Ngoye and Machiwa (2004) concluded that areas close to human and agricultural activities significantly contributed to higher concentrations of nutrients concentration than natural areas. Tong and Chen (2002), studying the contribution of pollutants from different types of land use, found that agricultural land use produced the highest and bare land use the least amount of contaminants. Awotwi et al. (2015) found that human

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activity is one of the major driving forces leading to changes in land cover characteristics and subsequently hydrologic processes. Wang (2001) found that industrial land and agricultural land decrease water environment quality and forest land and grassland have a negative influence on water pollutant concentration. Water quality in rivers is generally linked with land use in the watershed that can affect the amount and quality of runoff during and following rainfall. This is support by Richards and Host (1994) who suggest that forestry, agriculture, industrialization and urbanization modify watershed cover characteristics that influence runoff quality. Therefore, there is an increasing need to monitor and assess agricultural activities and urban expansion precisely at watershed scale (Howell et al., 2012; Ouedraogo et al., 2010).

Satellite remote sensing offers an alternative option for analysing land use and land cover (LULC) at a regional scale because it facilitates observations across larger regions and at higher frequencies than ground-based observations (Schulz et al., 2010; Shen et al., 2013). Among different sensors that have been used with this aim, the Landsat series of satellites provides the longest continuous record of satellite-based observations, being the primary source of medium spatial resolution Earth observations used in decision-making and an invaluable resource for monitoring global change (Hadjimitsis et al., 2010; Loveland and Dwyer, 2012). This series of satellite missions has collected imagery of the Earth's surface since 1972, providing an unparalleled record of the status and dynamics of Earth (Cohen and Goward, 2004). The Landsat 5 TM is equipped with a multi-spectral scanning equipment, which operates on seven spectral bands located between the visible and infrared regions of the spectrum. The spatial resolution is 30 m for the visible through middle infrared channels and 120 m for thermal infrared band, allowing the detection of small scale spatial variability across a reservoir surface (Xu et al., 2013). This sensor present a revisit time of 16 days and a radiometric resolution of 256 digital numbers (DN) (Oguro et al., 2003; Loveland and Dwyer, 2012).

In the central region of Argentina, considerable changes in LULC have taken place over the past four decades mostly due to population increase, the spread of settlement and the increasing use of land resources for agriculture and economic development. Thus, the main objective of this study was to classify LULC in the watershed of Río Tercero reservoir (Argentina), trying to find a relationship with nutrient loads supplied of its tributaries. This study, which is the first study that explores the current extend of land cover types in Central Argentina, tries to demonstrate the useful of LULC detection by remote sensing in those regions where there is a lack of available cartographic information with sufficient spatial resolution, obtaining a relationship with water quality of reservoir's tributaries allowing the development of strategies for conservation, sustainable management and restoration planning at watershed scale.

Materials and Methods

Study area

Río Tercero watershed located in Córdoba province (Argentina), has an area of 3000 Km^2 approximately and is divided in five sub-watersheds (*Fig. 1*). In last decades, natural forest of the watershed, characterized by dry woodland alternating with hard grasses (Cabrera, 1976), was replaced by urban expansion and agricultural activities, causing negative impacts in water quality of Río Tercero reservoir (Bonansea et al., 2015a). This reservoir, which is the largest artificial reservoir in the province, has an

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area of 46 km^2, a volume of 107 m^3 and maximum and mean depths of 46.5 and 12.2 m, respectively (Ledesma et al., 2013). The reservoir has multiple purposes such as water supply, power generation, flood control, irrigation, tourism and recreational activities (Bonansea et al., 2015b). According to Mac Donagh et al. (2009), rainfall is strongly seasonal, with dry winters and heavy rains during spring and summer.

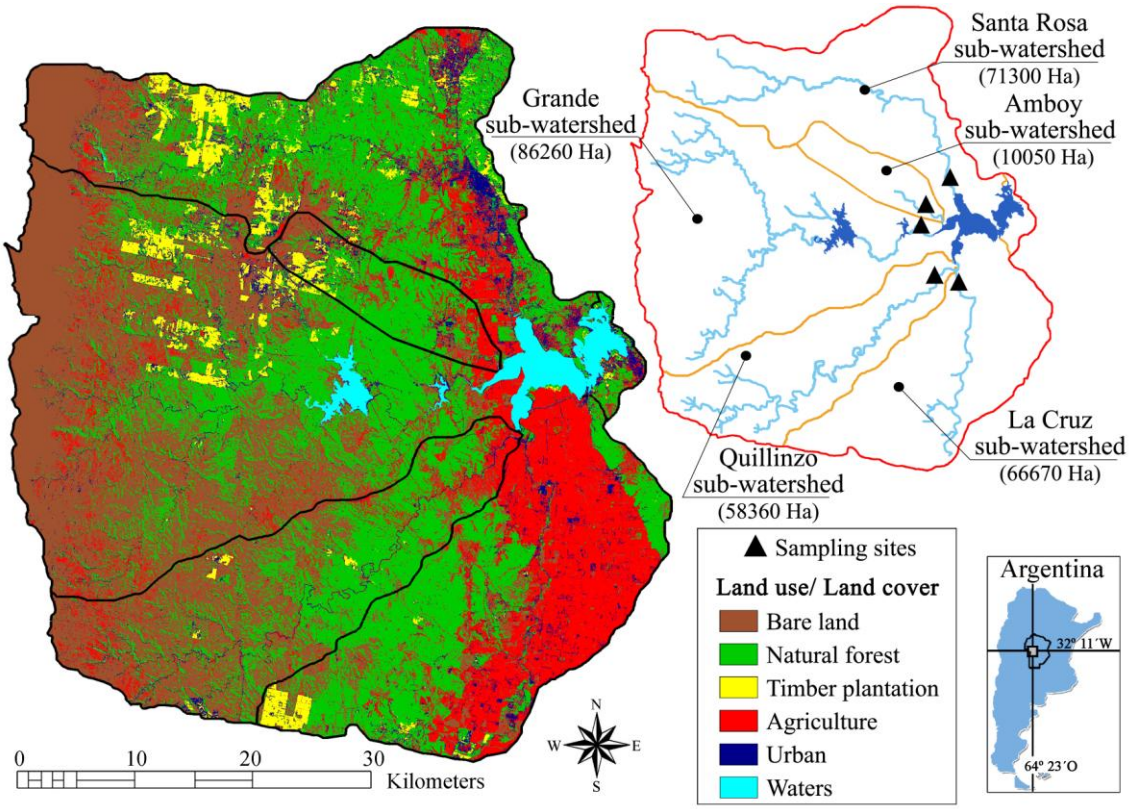

Figure 1. *Position of sampling sites, land use and land cover of Río Tercero watershed*

Field measurements

Each tributary of Río Tercero reservoir was analysed during 2006 bimonthly for physico-chemical variables, representing the dry season (April-September) and the rainy season (October-March) (*Fig. 1*). Coordinates of sample sites were recorded using a GPS device. In-situ, water temperature (WT), pH, dissolved oxygen, (DO) and stream flows (Q) were measured using portable electronic instruments. Total phosphorus (TP) and total nitrogen (TN) were determined in laboratory according to standard analytical methods and protocols (APHA-AWWA-WEF, 2000). Nutrient loads, defined as the total amount of TP and NT entering in the reservoir by each tributary during a given time were measured.

Land use and land cover analysis

The September 20th 2006 free of clouds image from Landsat 5 Thematic Mapper (TM) (Path: 229; Row: 82), downloaded from the USGS Global Visualization Viewer (http://glovis.usgs.gov), was used to determine LULC in the studied watershed. Six land cover categories were defined as (1) bare land, including gravels, bare ground and bare

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rocks; (2) natural forest, including natural shrubs, thickets and herb; (3) timber plantation, including coniferous (*Pinus taheda*, *P. Radiata*, *P. Insignias*, *P. Elliotis*, among others); (4) agriculture, including agricultural and livestock developments; (5) urban, including industrial and residential areas; (6) waters, including rivers, reservoirs, wetlands and sandy beach. The TM image was classified according to the resulting six land cover classes using the maximum likelihood algorithm (Chuvieco, 2002) (*Fig. 1*). This classifier has proven to be a robust and consistent classifier for multi-date classifications (Shalaby and Tateishi, 2007).

Statistical analyses

One-way analysis of variance (ANOVA) was employed as a first approach to analyse the significant differences between water quality variables ($p<0.05$; least-significance difference, LSD test). Pearson correlation coefficients and stepwise multiple regression analysis were carried out to investigate the relationships between water quality parameters and LULC ($p<0.05$). The Root Mean Square of the Error (RMSE), which gives an estimate of the error associated with the estimations was calculated according to Matthews et al. (2010).

Results and discussion

Water quality of tributaries

The basic statistics of water quality parameters collected in tributaries of Río Tercero reservoir are presented in *Table 1*. WT, Q and nutrient loads (PT and NT load) showed significant variations ($p>0.05$) between dry and rainy season. WT and Q were higher in rainy season, coinciding with normal variation of atmospheric temperature and rainfall. The higher load of nutrients also occurred in rainy season when river runoff was higher.

Table 1. *Mean values of water quality parameters measured in Río Tercero reservoir tributaries*

Parameter	Season	Tributary				
		Santa Rosa	**Amboy**	**Grande**	**Quillinzo**	**La Cruz**
WT (ºC)	Dry	20.2	18.1	17.2	16.0	14.4
	Rainy	26.5	27.0	29.3	29.5	18.9
pH	Dry	8.82	8.36	7.49	7.42	6.96
	Rainy	7.62	7.79	6.64	6.73	7.59
DO (mg/L)	Dry	8.5	7.2	8.0	8.7	9.5
	Rainy	9.1	7.5	8.6	9.3	9.8
Q (L/seg)	Dry	13.8	1.6	7.4	2.4	7.3
	Rainy	14205.0	567.7	696.3	5231.2	6469.5
PT (mg/L)	Dry	0.07	0.07	0.03	0.03	0.07
	Rainy	0.02	0.03	0.03	0.02	0.04
NT (mg/L)	Dry	3.1	1.1	1.7	1.5	1.7
	Rainy	0.9	1.1	1.4	0.9	1.5
PT load (T/year)	Dry	0.03	0.01	0.01	0.01	0.02
	Rainy	8.78	0.47	0.64	2.69	8.89
NT load (T/year)	Dry	1.35	0.06	0.39	0.11	0.39
	Rainy	403.17	19.69	30.74	148.47	306.69

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Land use and cover analysis

The TM image was usefull to classify LULC in the watershed of Río Tercero reservoir (*Fig. 1*). *Figure 2* shows that bare land and natural forest were the dominant land cover types. Bare land ranged from 12.1% in La Cruz sub-watershed to 54.6% in Grande sub-watershed. Timber plantation was comprised from 7.4% in Santa Rosa sub-watershed to 0.8% in Quillinzo sub-watershed. Natural forest covered from 29.5% in Grande sub-watershed to 56.5% in Amboy sub-watershed. Although agricultural activities were widely distributed across the basin but it mainly concentrated in La Cruz and Santa Rosa sub-watersheds, comprising around 46.0 and 28.5% of its land area respectively. Urban category ranged from 3.7 to 10.2% in Grande and Santa Rosa sub-watersheds respectively. Waters covered less than 0.1% of the sub-watersheds area except in Grande watershed (1.4% of its respective land area) where reservoirs are interconnected.

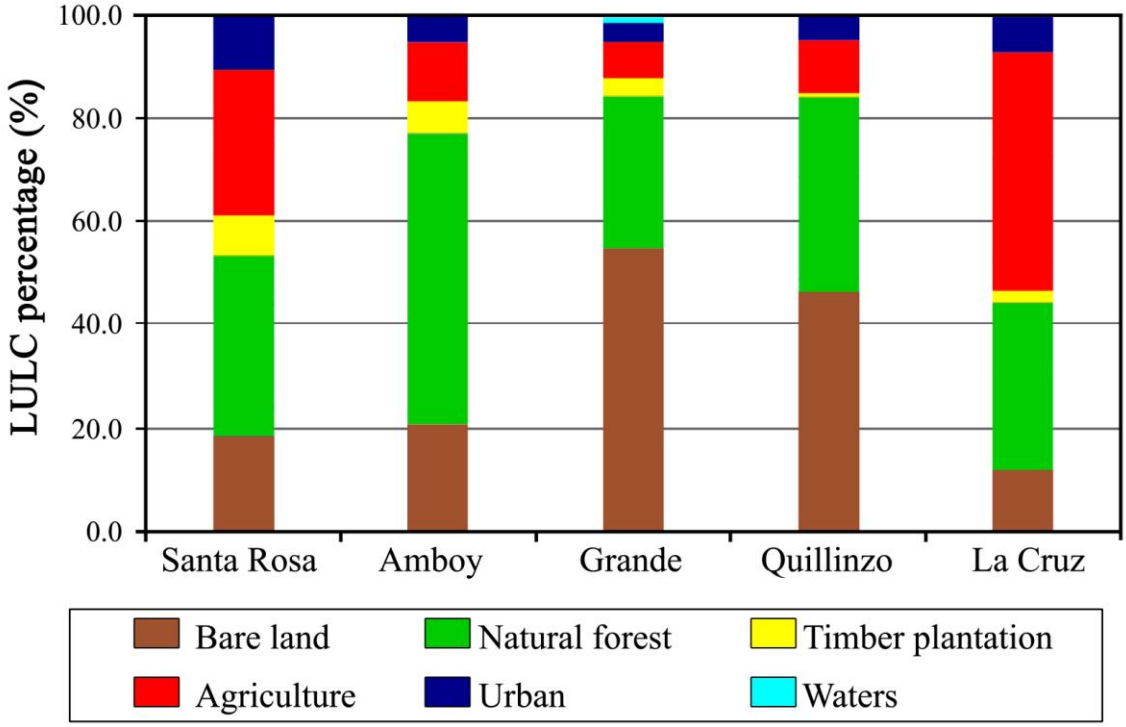

Figure 2. *Land use and land cover composition of Río Tercero reservoir sub-watersheds*

Relationship between LULC and water quality parameters

Correlation and regression analyses were performed by analysing the average values of water quality parameters and the area covered by LULC on each sub-watershed (*Table 2, 3*). These analyses indicate distinct influence of land use on water quality parameters. Only those correlation values higher than 0.5 were considered (*Table 2*). Results show that bare land presents significantly negative correlation with PT, and timber plantation has a strong positive correlation with NT. High and positive correlation was observed between nutrient loads and agriculture, which also contributes to WT, DO, Q, PT and NT. Natural forest has positive association with DO and NT, while urban has strong positive correlation to NT, NT load and Q, and also contributes to PT load and DO.

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Table 2. *Pearson correlation coefficients between LULC and water quality parameters in Río Tercero watershed*

Parameter	Waters	Bare land	Timber plantation	Agriculture	Natural forest	Urban
WT	0.30	0.41	0.28	**-0.75**	-0.02	-0.12
pH	-0.47	**-0.67**	0.41	0.01	-0.44	0.20
DO	-0.19	0.05	0.15	**0.81**	**0.71**	**0.64**
Q	-0.45	-0.28	**0.69**	**0.64**	0.49	**0.90**[b]
PT	-0.47	**-0.82**[b]	0.08	**0.56**	-0.42	0.15
NT	0.12	0.07	**0.93**[b]	**0.68**	**0.70**	**0.96**[a]
PT load	-0.46	-0.42	0.50	**0.94**[b]	0.45	**0.85**
NT load	-0.47	-0.36	**0.59**	**0.84**[b]	0.50	**0.91**[b]

Table 3. *Stepwise multiple regression analysis ($p<0.05$) for water quality parameters and LULC in Río Tercero watershed*

Parameter	Regression equation coefficients			R^2	RMSE
	Agriculture	Urban	Constant		
NT		$1.4*10^{-4}$	0.98	0.91	0.09
PT load	$1.6*10^{-4}$		0.05	0.88	0.64
NT load	$2.6*10^{-3}$	0.02	-21.03	0.88	18.86

Stepwise multiple regression analysis between water quality values and LULC indicated distinct influence of land cover on water quality parameters related with nutrients availability. As shown in *Table 3*, no single land cover type was able to describe the overall water quality, but some water quality parameters could be sufficiently predicted using one or two LULC according to the good fit between observed and estimated values ($R^2> 0.80$) and the lower and reasonable error associated with the estimations (RMSE). NT could be predicted by urban area ($R^2=0.91$). PT load was predicted by agriculture ($R^2=0.88$) and NT load by agriculture and urban activities ($R^2=0.88$). Results suggest that urban and agricultural activities could be the primary driving forces behind the variations in nutrient concentrations.

Discussion

Our results demonstrated that, larger sub-watershed where urban and agricultural activities were more frequents, displayed the highest values of nutrients concentration and nutrient loads, as the case of Santa Rosa and La Cruz sub-watersheds.

Comparing our results with the physiognomic vegetation map of the studied watershed published by Menghi and Luti (1982), we observed that natural forest showed the largest decline. During this period, natural vegetation revealed a general trend of a continuous reduction that in turn has led to an increase in human-induced types of land uses such as agricultural and urban residential developments. According to Schulz et al. (2010), in SouthAmerica the strong increases in agriculture activities has been stimulated by a combination of market liberalisation, incentives for new export-oriented crops, introduction of new irrigation technologies, and improvements in road infrastructure, while the expansion of urban areas coincided with population growth and the liberalisation of the urban land market.

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In last decades Río Tercero watershed has shown a reduction of natural forest, which has been replaced by agricultural activities and urban expansion which do not have adequate waste treatment facilities, directly discharging to water bodies, which resulted in decrease of water quality. Further, vegetation loss and degradation reduce precipitation infiltration and runoff regulation, which promotes soil erosion and has a negative impact on ground water recharge (Schulz et al., 2010). Currently, using a TM image we have estimated that 32% (937.9 km^2) of the entire watershed area is related with human-induced types of land uses. Because of the policies adopted by governments, increases in these activities over time are expected. Thus, efforts within the watershed should be focused to develop a planning process in which agriculture and urban interests with responsibility for water quality are shared. Addressing these issues together is essential to avoid short-term counterproductive conflicts and to develop a long-term vision for the watershed (Fisher et al., 2000).

In this study we have demonstrate that tributaries of Río Tercero reservoir whose watersheds were most affected by human activities, displayed the highest values of nutrient loads, as the case of Santa Rosa and La Cruz rivers. This is in agreement with different studies that suggest that the percentage of agriculture and urbanized areas at watershed scale are the primary predictor for nutrients (Ahearn et al., 2005; Li et al., 2008; Schulz et al., 2010). In addition, Chang (2008) and Li et al. (2009) found that these land uses trend to increase nitrogen and phosphorus non-points-source pollution. Thus, these parameters could be predicted using agricultural and urban activities. However different studies have demonstrated interactions between LULC and other water constituents of inland water which are also complex and important. In this sense, Tong and Chen (2002) suggest that in addition to nutrients, fecal coliform bacteria have strong positive relationships with agricultural and urban use. Li et al. (2008) also measured suspended particle matter (SPM), potassium permanganate index (IMn) and dissolved phosphorus (DP), finding relationship with agricultural and urban land cover. Urban cover positively influences chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in the Han River basin, South Korea (Chang, 2008). Unfortunately, we do not have collected ancillary data such as fecal coliform, SPM, COD or BOD measures to related with our results. However, we recommend that when more information is known, this information should be used when it considerably improves estimates.

On the other hand, the effects of change in land use and the subsequent decrease of tributaries water quality, has also been observed in Río Tercero reservoir. Thus, Ledesma et al. (2013) have shown that in the reservoir chlorophyll-a concentration (Chl-a) was higher close to river inputs and decreased toward the rest of the reservoir, evidencing the presence of a longitudinal zonification. This relationship could be related with larger river runoff with higher nutrients availability generated by watershed washout or water runoff from agricultural fields after rainfall (Bonansea et al., 2015b). This is in agreement with several investigators that have studied the effect of discharge of nutrients in different water bodies causing algal blooms and eutrophication (Nishimura et al., 2002; Shukla et al., 2008). An opposite pattern was observed in water clarity expressed in terms of Secchi disk transparency (SDT) where river inputs provide the greatest loads of suspended materials and dissolved solids that decrease the penetration of light (Bonansea et al., 2015b). A similar spatial pattern of lower SDT near river inflows and increasing with distance was found by Bazán et al. (2005), Giardino et al. (2010), and Guan et al. (2011) in different water bodies.

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Like most previous studies of water quality in relation to LULC, we focus on only one watershed, but the transferability to other environments remains unknown. However, similar characteristics of other watersheds of the region suggest that future research may allow the extension of such methodologies to the regional scale, allowing the study of dynamics of many watershed that currently lack systematic studies of water quality.

Conclusions

Using a TM image we could classify different LULC in the watershed of Río Tercero reservoir. Statistical analyses were used to identify and quantify the magnitude, direction, and significance of relationships between water quality and LULC.

Results showed that land use was related to water quality parameters. Thus, human-induced types of land uses such as agricultural and urban cover were the primary driving forces behind the variations in nutrient loads. We expect that if agricultural activities and urban residential developments continue to increase in Río Tercero watershed, water quality at watershed scale will decline. Therefore, to assess water quality of Río Tercero reservoir, it is recommended the development of a global approach management plan at a watershed scale, taking account river runoff, land use and land cover, and allowing the implementation of strategies for ensure sustainable development and preservation of water supply.

This study can have direct application values to state or local agencies, city planners and water management authorities and decision makers for defining the impacts of land use on water resources and for implementing long-term water planning and management scheme.

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BIOLOGICAL DIVERSITY OF NEMATODE COMMUNITIES IN CONVENTIONAL AND ORGANIC OLIVE FARMING

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Abstract. The main objective of managing agricultural soil is maintaining soil fertility, in order to stimulate biological activity in the soil. The aim of this study was to determine variability in the presence and number of genera of nematodes, trophic structure and indices of biodiversity in the soil under conventional and organic olive farming. Research was conducted in the area of Porec in Istria, Croatia, through 5 years (from 2007 to 2011) in conventional and organic olive farming system. Total of 49 nematode genera were established in this study, 42 genera in conventional and 44 in organic farming system. Average number of genera over the investigated period in 100 g of soil were 14 in conventional and 17.10 in organic farming system and differ significantly in between. Trophic group analyses showed the dominance of plant parasitic nematodes in the conventional olive farming, and bacterivorous feeding nematodes in organic olive farming. Biological diversity indexes (H', N1 and λ) didn't differ significantly between conventional and organic farming.
Keywords: *nematodes genera, conventional and organic farming, Rhabditis, Tylenchus, olives*

Introduction

Soil fertility is the base for determinate productivity of all agricultural production aspects and it is defined as soil possibility to provide nutriment for crops (Watson et al., 2002). Poor soil management practies have degraded soil and only way to ensure future food demands is that producers must improved management prectices (Kapp et al., 2014). Different production strategies reflect soil fertility by stimulating biological activity which ensures the stability of ecological processes and conditions in the soil. Organic farming maintains favorable conditions for the development of soil organisms that are essential for processes such as regulation, availability and circulation of nutrients (Blumenthal et al., 2003) and improves soil structure (Caravaca et al., 2006). Differences of nematodes community in soil between organic and conventional farming are higher in autumn compare to summer period (Neher, 1999). Rasmann et al (2012) predicted that nematodes of different feeding guilds use host-specific cues for chemotaxis. Abundance and biomass of individual species of bacterivorous in organic farming of tomatoes vary during the growing season (Ferris et al., 1996). Landa et al.

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(2014) have evaluated the effects of soil type and different soil management systems in commercial organic olive orchards in the structure and diversity of bacterial communities. Palomares-Rius et al. (2015) established high diversity of plan-parasitic nematodes associated to olive that can exert different damage to olive roots depending on the olive variety and their abundance.

The aim of study was to determine whether there is variability in the presence and abundance of nematode genera, trophic structure and indices of soil biodiversity under conventional and organic farming systems in the olive production.

Material and methods

The study was conducted in the Porec area (45°13'33" N and 13°35'38" E) in region Istria, Croatia, over 5 years (from 2007 to 2011) in conventional and organic olive plantation. Olive plantation in conventional farming system is located on the site of St. Anne, in the area Cervar`s, Porec. It was planted in 1980 and 1981. Planting distances within rows and between rows are 7 x 5 m. Plantation included three varieties of olives: Picholine, Leccino and Pendolino. Olive plantation in organic farming system is located on the Larun, near Porec, planted in 1960. with planting distances within rows and between rows are 6 x 4 m. Plantation included two varieties of olives: Leccino and Pendolino. Growth form is multi-conical shape on ruddle soil.

Soil samples were collected in conventional and organic farming system in the following periods: June 2007, October 2008, April 2009, February 2010 and January 2011. Treatments of the study are marked as conventional farming (Con07, Con08, Con09, Con10, Con11) and organic farming (Org07, Org08, Org09, Org10, Org11). The main reason for sampling in different seasons throught years was the fact that the aim of investigation was to compare different farming system (organic and conventional), not to compare differences between years. Soil samples were collected in four replications, each on Leccino cultivar, for each treatment and the 40 soil samples were examined. Laboratory analysis of samples of nematode communities was carried out in the Laboratory of Entomology and Nematology, Faculty of Agriculture in Osijek, Croatia.

Extractions of nematodes from soil was carried by Erlenmeyer method (Seinhorst, 1956). Determination of nematodes according to the morphological characteristics to the genera level is determined according to: Andrassy 1984, 1988, 1993; Bongers, 1994; Hunt, 1993; Mai and Lyon, 1975 and Zullini, 1982. Number of genera, trophic groups (Yeates et al., 1993), indices of soil biodiversity: Shannon-Weaver index of diversity (Shannon and Weaver, 1949), Hill index - N1 (Neher et al., 2004) and Simpson index - λ (Simpson, 1949) were analized. Analysis of variance (ANOVA) was preformed using the program Statistics 6.

Result and discussion

Total of 49 nematode genera were found in this study. In the conventional olive farming were established 42 and in organic olive farming 44 different nematode genera. Average number of genera occurred in 100 g of soil was 14 and 17.10, in conventional and organic farming respectively.

Statistical analyse of nematode genera showed significant differences between conventional and organic olive farming ($p<0.05$, 2.4023).

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Most dominant genera in conventional and organic farming system were Rhabditis and Tylenchus.

The list of nematode genera occurred in both, conventional and organic farming systems were: Acrobeloides, Acrolobus, Alaimus, Anatonchus, Aphelenchoides, Aphelenchus, Apocerlaimellus, Clarkus, Criconema, Dipterophora, Ditylenchus, Enchodelus, Eucephalobus, Eudorylaimus, Fictor, Filenchus, Helicotylenchus, Heterocephalobus, Malenchus, Mesodorylaimus, Metateratocephalus, Microdorylaimus, Monhystera, Mylonchulus, Ogma, Panagrolaimus, Paramphidelus, Paratylenchus, Plectus, Pratylenchus, Prionchulus, Prismatolaimus, Psilenchus, Rhabditis, Rotylenchus, Tylenchorynchus, Tylenchus, while Basiria, Crossonema, Rhabdolaimus, Trypilla, Xiphinema occurred just in conventional olive farming and Ciloplacus, Panagrobelus, Prodorylaimus, Pungentus, Turbatrix, Tylencholaimellus, Tylopharinxs occurred just in organic olive farming.

In conventional farming system plant parasitic nematodes was dominant group, while in organic farming system bacterivorous and fungal feeding nematode dominated (*Table 1.*)

Table 1. *Statistical analyses of nematode community trophic structure under conventional and organic olive farming (2007-2011).*

	Bacterial feeding %	Plant parasitic feeding %	Fungal feeding %	Omnivorous %	Predators %
Lsd test	(p<0.05, 9.7921)	(p<0.05, 10.455)	(p<0.05, 6.1558)	(p<0.05, 5.0184)	(p<0.05, 1.6451)
Conventional	24,3 a*	51,6 a	16,6 a	5,6 a	1,9 a
Organic	34,7 b	30,2 b	23,8 b	8,7 a	2,6 a

* Values marked by different letters in the column are statistically different (P <0.05, LSD)

Differences in the way of farming can lead to different nematodes communities that are prevalent in the soil (Sanchez-Moreno et al., 2006). Briar et al. (2007) reported a higher proportion of bacterial feeding nematodes in organic production, while fungal feeding nematodes, omnivores and predators were significantly different between organic and conventional farming only in a certain period of investigation.

Similar proportion of nematode trophic groups in organic and conventional farming of asparagus in Greece was reported by Tsiafouli et al. (2005). In our study fungal feeding nematodes were represented in higher percentage in organic olive farming. Similar results report other researchers (Girvan et al., 2004, Liu et al., 2007), although some authors observed the opposite results (Clark et al., 1998, Berkelmans et al., 2003).

Analysis of biodiversity in conventional and organic olive farming was conducted by using Shannon-Weaver index (H'), the number of abundant genera (N1) and the index of dominance (λ) (*Table 2*).

Shannon-Weaver index (H') in organic olive farming through the investigation period showed less fluctuations of biodiversity in compare to conventional olive farming.

Statistical analysis of the H', N1 and λ indexes showed no significant differences between conventional and organically olive farming (*Table 2.*). Van Diepeningen et al. (2006) established similar results with no statistically significant differences between indices of biodiversity between conventional and organic farming.

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Table 2. *Statistical analyses of nematode under conventional and organic olive farming (2007-2011) by community index H', N1 and λ.*

	H'	N1	λ
Lsd test	(p<0.05, 0.1180)	(p<0.05, 0.2813)	(p<0.05, 0.0705)
Conventional	1.025 **a***	2.864 **a**	0.411 **a**
Organic	1.127 **a**	3.110 **a**	0.360 **a**

* Values marked by different letters in the column are statistically different (p<0.05, LSD)

Similar results of diversity indices were obtained in Spain in the production of olives in conventional and organic farming (Garcia-Ruiz et al., 2009). Neher (1999) concludes that the best time to measure the trophic diversity between conventional and organic agricultural farming is in the autumn, because then the differences are the most obvious.

Conclusion

Number of genera and trophic group structure proved to be an excellent bioindicator of difference between conventional and organic olive farming. Both parameters statistically distinguished differences between production systems. Diversity indices (H', N1 and λ) did not differ significantly between conventional and organic olive farming in this specific investigation, but still have to be consider as important in future similar research.

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ANALYSIS OF CYANOBACTERIAL DIVERSITY OF SOME HOT SPRINGS IN AFYONKARAHISAR, TURKEY

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Abstract. The cyanobacterial diversity of Ömerli, Akkuş-Gazlıgöl, and Hüdai-Sandıklı hot springs in Afyonkarahisar District in Turkey were analysed and compared using cultivation and cultivation-independent methods, including DGGE (denaturing gradient gel electrophoresis) and cloning of PCR-amplified fragments of 16S rRNA genes. Cultivation studies revealed that a total of 74 isolates had 6 different ARDRA (Amplified Ribosomal DNA Restriction Analysis) profiles and they were identified at the genus level and all cyanobacterial isolates were phylogenetically related to *Fischerella, Geitlerinema/Leptolyngbya laminosa* and uncultured *cyanobacterium* genus. A total of 97 clones from 16S rRNA gene library was analysed by ARDRA. 16S rRNA sequence analysis of these clones revealed that the cyanobacterial clones were related to 16S rRNA gene sequences retrieved from environmental samples, *Geitlerinema* and *Leptolyngbya laminosa* genus members. DGGE analysis revealed *Geitlerinema*, *Cyanobacterium,* and *Phormidium* genus, and 16S rRNA gene sequences retrieved from environmental samples. High-throughput 16S rRNA gene sequencing with DGGE analysis showed that the most frequent sequences in Ömerli, Akkuş-Gazlıgöl and Hüdai-Sandıklı samples were affiliated with *Geitlerinema*. This work highlights the cyanobacterial diversity of Afyonkarahisar hot springs.
Keywords: *thermophilic cyanobacteria, hot spring, Afyonkarahisar, cloning, DGGE (Denaturating Gradient Gel Electrophoresis)*

Introduction

Cyanobacteria are Gram-negative bacteria which have the ability of oxygenic photosynthesis. They are the most adaptive photosynthetic organisms and live in almost every habitat on earth. They are found in fresh water, marine water, soil in thermophilic and psychrophilic conditions. Cyanobacterial morphology varies from unicellular to multicellular. Cyanobacteria that can develop over 45°C are called thermophilic cyanobacteria. These cyanobacteria have a more limited distribution area than mesophilic cyanobacteria (Whitton and Potts, 2000). Thermal hot springs are found in different geographical areas with different physical and chemical features which severely limit the survival of photoautotrophic organisms in them. Thermal environments make the cyanobacteria living there endemic (Castenholz, 1996; Papke et al., 2003; McGregor and Rasmussen, 2008).

Thus, except a few cosmopolitan thermophilic cyanobacterial species (i.e. *Mastigocladus laminosus* Cohn) (Castenholz, 1996; Miller et al., 2007), most thermophilic cyanobacteria are new operational taxonomic units (OTUs) (Ward et al., 1998; Taton et al., 2006; McGregor and Rasmussen, 2008).

Cyanobacteria are the most commonly reported microbial groups constituting thermophilic mats and considered as the major primary producers in these habitats (Castenholtz, 1973). Other bacteria living in the same environment have also important

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roles within these microbial communities (Ward et al., 1990; Weller et al., 1992; Moyer et al., 1995).

Afyonkarahisar is a district in western Turkey (*Figure 1*) with well known hot springs. Although several hot springs in different regions of Afyonkarahisar have been in use for many years, their cyanobacterial diversity has not yet been investigated by molecular phylogenetic approaches.

Figure 1. *Sampling locations in Afyonkarahisar (shown with circles on the map). GPS coordinates of the sampling points are 38°56'09.43N-30°29'48.11E (Akkuş-Gazlıgöl); 38°25'59.26N-30°10'54.89E (Hüdai-Sandıklı); 38°50'24.45N-30°25'01.08E (Ömerli). (Satellite imagery: Google/Google Earth).*

In this study, we applied the 16S rRNA gene analysis both for isolates and environmental DNA to determine the cyanobacterial community structure of three most popular hot springs in Afyonkarahisar.

Materials and Methods

Study site and sample collection

Three hot springs Ömerli (98°C), Akkuş-Gazlıgöl (64°C), and Hüdai-Sandıklı (68°C) in Afyonkarahisar were selected as our study sites (*Figure 2*).

Water and mat samples were collected as study material. Water samples were taken from the epilimnion at a depth of 1 m using sterile glass bottles and tubes during January and October 2012 and were kept on ice baths until they were analysed. Microscopic analyses of water samples were performed with Olympos microscope BX51 equipped with digital microphoto-camera DP70. Morphological analyses were carried out at the genus level based on the identification systems proposed by Geitler, 1932 and the "form-genus" approach of Castenholz, 2001. The samples were cultivated with or without nitrogen in BG-11 medium (Rippka et al., 1981; Boutte et al., 2005; Cuzman et al., 2010) supplemented with 50 mg l^{-1} of cycloheximide to avoid eukaryotic cells. Cultures were grown at 55°C for several weeks until a green active biomass became visible and purification process was then performed. Subsequent cultures were incubated in the BG11 solid medium at 55°C.

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(a)

(b)

(c)

Figure 2. *(a) Ömerli (b) Akkuş-Gazlıgöl (c) Hüdai-Sandıklı hot springs*

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Chemical analysis of water samples

Chemical analysis of all of the water samples from the hot springs (*Table 1*) was performed with the Spectroquant® NOVA 60 photometer using Merc cell tests.

DNA extraction from pure cultures, mat and water samples

The bacterial genomic DNAs from purified cultures were prepared using the protocol described by DNeasy® Plant Mini Kit (Qiagen). 500 ml water samples were filtered on 0.2 µm-pore-size filters (Millipore) to obtain the total bacterial genomic DNA. The filters and mat samples (0.5 g) with yellow, green and dark-green layers were obtained from 3-5 mm below the mat surface using a clean razor blade and placed in 15 ml sterile tubes containing 2 ml lysis buffer (40 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris HCl pH 8.3). Each sample was frozen at -20°C (Giovannoni et al., 1990). For the enzymatic lysis step, a volume of 50 µl lyzozyme (50 mg/ml) was added to the filters and incubated for 20 minutes at 37ºC. After then 100 µl SDS (10%) and 43 µl proteinase K (14 mg/ml) was added and incubated 2 hours at 37ºC. 2 ml of phenol/chloroform/isoamylalcohol (Merck, Germany) (25:24:1) were added and incubated for 10 minutes at 56ºC. The nucleic acids were precipitated from the supernatant (divided in Eppendorf tubes) by adding 2 volumes of ethanol and kept for 2 hours at -20ºC. Then, the tubes were centrifuged for 20 minutes at 16000 g. After extraction, DNA was subjected to purification step using the Wizard DNA Clean-Up System (Promega). To check the quality of nucleic acids, they were run in 1% agarose (LE, FMC Products, Rockland, ME) gel and visualized under UV light after ethidium bromide staining. The purified DNA were stored at –85°C (Wilmotte et al., 2002; Boutte et al., 2005).

Cloning of 16S rDNA and ARDRA (Amplified Ribosomal DNA Restriction Analysis)

The water samples were used for cloning. PCR amplification of the cyanobacterial 16S rRNA gene plus ITS and 5'end of 23S was made in a 3 x 50 µl reaction mixtures. Set of primers used was CYA 359F (5'-ggggaattttccgcaatggg-3') and 23S30R (5'-cttcgcctctgtgtgcctaggt-3') (Taton et al., 2003).

Applied Biosystems® thermal cycler was used for the amplification reaction contained 1 X of Q5® Reaction Buffer, 1 mg ml^{-1} of BSA (bovine serum albumin), 200 µM of dNTP mix, 0.5 µM of the forward and reverse primers, 1 U/µl of Q5® High-Fidelity DNA Polymerase (New England Biolabs, Inc) with proofreading activity in a final volume of 50 µl. The PCR amplification cycle was 5 min at 94ºC; 10 cycles of 45 s at 94ºC, 45 s at 57ºC, and 2 min at 68ºC; 25 cycles of 45 s at 92ºC, 45 s at 54ºC, and 2 min at 68ºC; and a final elongation step of 7 min at 68ºC. PCR products were purified with Quantum Prep® PCR Kleen Spin Columns (Bio-Rad). Poly (A) extension was performed using Qiagen® A-Addition Kit according to manufacturer's instructions. Cloning of the PCR products was done with a TOPO® TA Cloning Kit (Invitrogen) according to manufacturer's instructions. White and light blue transformants were purified twice by streaking and then were screened by performing colony PCR with the primer pair CYA359F (5'-ggggaattttccgcaatggg-3') and CYA783R (5'-gactactggggtatctaatcccatt-3'). The amplification conditions were as follows: incubation for 10 min at 94ºC; 35 cycles of 1 min at 94ºC, 1 min at 60ºC,

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1 min 68ºC; and a final elongation step of 7 min at 68ºC (Wilmotte et al., 2002; Boutte et al., 2005).

Plasmid DNAs were extracted with a Quantum Prep Plasmid Miniprep kit (Bio-Rad) by following the manufacturer's instructions. The inserted 16S rRNA gene plus ITS was reamplified with CYA 359F (5'-ggggaattttccgcaatggg-3') and 23S30R (5'-cttcgcctctgtgtgcctaggt-3') primers as described above and subjected to ARDRA to screen the clone libraries.

Additionally, the 16S rRNA gene amplicons obtained from the genomic DNA of the culture were used for ARDRA with two different restriction endonucleases, MspI and MboI (MBI Fermentas). The preperation of the digestion reactions were as follows: add 10 µl of PCR product to 2 µl of 10 x reaction buffer (buffer R +-MboI, and Y+/Tango – MspI) and 0.5 µl of restriction enzyme and complete to final volume of 20 µl with water. Incubation was carried out for 3 h in the water bath at the optimal temperature of 37ºC (according to company's instruction). The reaction was stopped by incubating at 65ºC for 25 minutes. Electrophoresis was performed at constant 90 V/cm for 180 minutes. The gel was stained with ethidium bromide after the migration and just visualized with UV light. pBR322 DNA/Alu I Marker (MBI Fermentas) was used as a marker (Boutte et al., 2005). The ARDRA patterns were compared according to the band positions, and identical patterns were considered as the same group. Partial sequences of 16S rDNAs from representatives of each group were determined. For each ARDRA type, sequencing was made with primers 23S30R (5'-CTTCGCCTCTGTGTGCCTAGGT-3'), 1492R (5′-GTA CGG CTA CCT TGT TAC GAC-3′), and 1092R (GCG CTC GTT GCG GGA CTT) by Macrogen (Seoul, Korea) and then these sequences were assembled.

DGGE Analysis

Three filter samples and three mat samples from the hot springs were used for the DGGE. A semi-nested PCR was performed so as to increase the sensitivity and to facilitate of the DGGE analysis.

For the first PCR, the forward primer 16S359F (5′-GGG GAA TTT TCC GCA ATG GG-3′) and the reverse primer 23S30R (5′-CTT CGC CTC TGT GTG CCT AGG T-3′) were used. 0.5µl of the DNA was added to 49.5 µl of the amplification mixture, where the final concentrations of the components were 1 X of Q5® Reaction Buffer, 1 mg ml^{-1} of BSA (bovine serum albumin), 200 µM of dNTP mix, 0.5 µM of the forward and reverse primers, 1 U/µl of Q5® High-Fidelity DNA Polymerase (New England Biolabs, Inc) with proofreading activity in a final volume of 50 µl. The amplification procedure was as follows: one cycle of 5 min at 94ºC; Touch down 10 cycles of (6 cycles of 45 s at 94ºC, 1 min 60ºC, 1.5 min at 68ºC; 4 cycles of 45 s at 94ºC, 1 min 60ºC, 1.5 min at 68ºC) and the final elongation step was done for 7 min at 68ºC for the b1 reaction. For the a1 reaction, 5 min at 94ºC, 27 cycles of 45 s at 94ºC, 1 min at 54ºC, and 1.5 min at 68ºC and final elongation step was done for 7 min at 68ºC. The resulting PCR products (0.5 µl) were served as templates for the second PCR, which was performed with the forward primer 16S359F and reverse primers 16S781R (a) (5′-GAC TAC TGG GGT ATC TAA TCC CAT T-3′) and 16S781R (b) (5′-GAC TAC AGG GGT ATC TAA TCC CTT T-3′). A 38-nucleotide GC-rich sequence (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CC-3') was attached to the 5′ end of each of the reverse primers (Nübel et al., 1997; Boutte et al., 2006).

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The reaction conditions were the same as those described above except the amplification process which involved incubation for 5 min at 94°C, followed by 35 cycles (b2) /30 cycles (a2) of 1 min at 94°C, 1 min at 60°C, and 1 min at 68°C and then a final elongation step of 7 min at 68°C. Two distinct reactions were performed for each reverse primer. The negative control for the first PCR was used in the second PCR to check for contamination. DGGE was made by Ingeny system and performed as described by Nübel et al., 1997 with the following modifications. The PCR products obtained with primers 16S781R (a) and 16S781R (b) were applied separately to the polyacrylamide gel. The gel contained a linear 40 to 65% denaturant gradient, the pH of the TAE buffer was adjusted to 7.4, and the electrophoresis was applied for 22 h at 80 V and 60°C. After being stained with ethidium bromide, the gel was visualized under UV light and then photographed by Uvitec gel documentation system. The DGGE bands were excised with a surgical scalpel. Each small gel block was placed in 100 µl of sterile water for 2 h at room temperature. Each solution was used as a template for PCR amplification as described above. The PCR products were then electrophoresed to confirm the bands. 350-bp part of 16S rRNA gene was sequenced with primer 16S784R (5′-GGA CTA CWG GGG TAT CTA ATC CC-3′).

Nucleotide sequence accession numbers

Environmental 16S rRNA gene sequences from Afyonkarahisar hot springs are available at the GenBank with the accession numbers KJ461812-KJ461849, KT715745-KT715753, and KT793918-KT793927.

Analyses of sequence data

All sequences were compared to the sequences in the BLAST search program at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). 16S rRNA sequences of the hits for our sequences were obtained through the RDP (Ribosomal Database Project) site at Michigan State University (http://rdp.cme.msu.edu/). The top five hits as well as some additional relevant sequences were used for phylogenetic analysis. Sequences of partial 16S rDNA of cultures, clones and DGGE fragments were analysed by BioEdit v7.2.5 software. All sequences were checked for chimera formation using the DECIPHER (Wright et al., 2012). CHECK-CHIMERA software developed by the Ribosomal Database Project and the phylogenetic affiliations of their 5′ and 3′ ends were compared. Phylogenetic trees were constructed using the maximum likelihood treeing algorithm and Nearest-Neighbor-Interchange (NNI) method in the MEGA 6 (Tamura et al., 2007). The Distance Matrix was calculated using the Jukes-Cantor correction. Validity of the tree topology was checked using the bootstrap method (1000 replicates).

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Results

Chemical properties of the hot spring water samples

As shown in *Table 1*, waters of three springs have little difference in their properties.

Table 1. *Some properties of the water samples (mg/l)*

	Akkuş-Gazlıgöl (64°C)	**Hüdai-Sandıklı** (68°C)	**Ömerli** (98°C)
pH	8.3	7.06	7.06
Mg^{2+}	19.3	30	20.6
NO_3^-	<0.5	<0.5	<0.5
Mn^{2+}	0.14	0.10	0.13
NH_4^+	<0.5	<0.5	<0.5
P	0.4	0.4	1.7
N	<0.5	<0.5	0.0
Cl_2	0.18 *0.15	0.07 *0.07	0.13 *0.12
CSB/COD	<10	<10	14
NO_2^-	0.020	0.044	0.048
F^-	1.85	1.68	1.83

*amount of free Cl_2

Microscopic observation and cyanobacterial cultivation analysis of the water samples of the springs

Microscopical examinations of the water samples of the hot springs showed a dominance of filamentous cyanobacteria *(Figure 3).* The cyanobacteria found were described based on their morphologies. *Oscillatoria*-like cyanobacteria were dominant and *Gleocapsa*-like unicellular cyanobacteria were encountered less frequently. 74 pure isolates having 6 different ARDRA profiles *(Table 2)* were obtained from the hot springs. BLAST analysis of partial 16S rDNA obtained from the isolates showed that Ömerli hot spring has more cyanobacterial diversity than the other two. ARDRA profiles of the isolates and their closest Genbank matches are shown in *Table 3*.

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In our ARDRA results, 73% of the isolates belong to the group S1. The groups S2, S3, S4, S5, and S6 are 4.05%, 6.75%, 2.70%, 12.16% and 1.35%, respectively. The similarities among the isolates with the closest relative sequences in Gen Bank can be seen in *Table 3*.

The sequences of the isolates CY2, CY9, and CY16 have similar with the genus *Fischerella* sp. isolated from Costa Rica (Unpublished, Acc. Number DQ786171). The CY8 isolate sequence showed identity with the *Uncultured cyanobacterium* obtained from as-rich and DIC-limited geothermal waters of El Tatio, Chile (Unpublished, Acc. Number KP794044.1) and *Fischerella* sp. (Acc. Number HM636645). The sequences of CY32, CY20, CY11, CY13 and CY31 were similar with the genus *Geitlerinema* sp./*Leptolyngbya laminosa* isolated from Euganean thermal muds, Padova, Italy (Unpublished, Acc. Number FM210758).

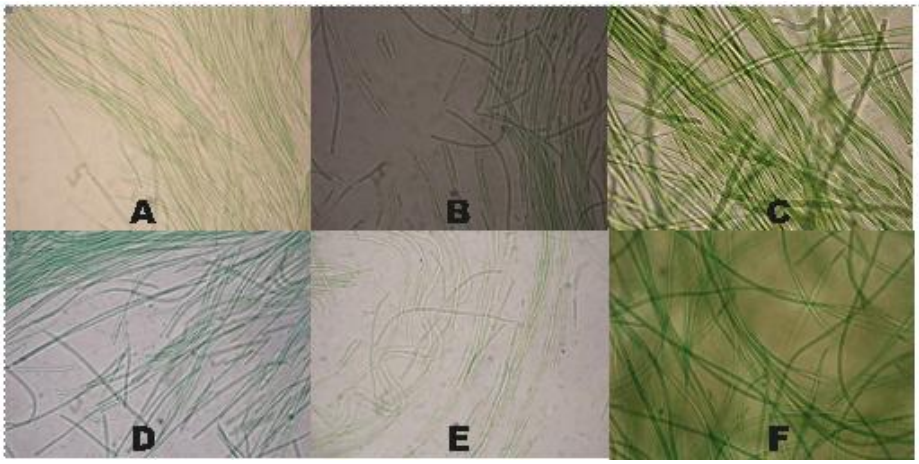

Figure 3. *Light microscopy images of some cultures (40X objective). A, B, C, D, E, F trichome morphology of Oscillatoria- like field colonies in BG-11 medium.*

Table 2. *Isolates, clones and the DGGE bands of Akkuş-Gazlıgöl, Hüdai-Sandıklı and Ömerli hot springs and their ARDRA groups*

	Isolates	**Clone libraries**	**DGGE bands**
Akkuş-Gazlıgöl	**14 isolates** Group S1 (9 isolates) Group S3 (5 isolates)	**30 clones** Group K1 (26 clones) Group K2 (2 clones) Group K6 (2 clones)	**2 DGGE (a2) band** (A.2.1, A.2.2) **7 DGGE (b2) band** (B.2.1, B.2.2, B.2.3, B.2.4, B.2.5, B.8.1, B.8.2)
Hüdai-Sandıklı	**17 isolates** Group S1 (15 isolates) Group S5 (2 isolates)	**32 clones** Group K1 (2 clones) Group K2 (22 clones) Group K3 (1 clones) Group K4 (7 clones)	**3 DGGE (a2) band** (A.5.3, A.5.4, A.5.5) **2 DGGE (b2) band** (B.6.1, B.6.3)

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Ömerli	**43 isolates**	**35 clones**	**2 DGGE (a2) band**
	Group S1 (30 isolates)	Group K1 (19 clones)	(A.1.2, A.1.3)
	Group S2 (3 isolates)	Group K2 (5 clones)	
	Group S4 (2 isolates)	Group K4 (8 clones)	**16 DGGE (b2) band**
	Group S5 (7 isolates)	Group K5 (3 clones)	(B.3.1, B.3.2, B.3.3, B.3.4, B.3.5,
	Group S6 (1 isolates)		B.3.6, B.3.7, B.3.8, B.4.1, B.4.2,
			B.4.3, B.7.1, B.7.2, B.7.3, B.7.4,
			B.7.5)

Table 3. *Cyanobacterial isolates, clones and their closest GenBank Matches.*

	ARDRA Group No	Sample Code (Accession no)	% of identity with the closest relative	Closest relative according to BLAST search/ Accession no/ Length
Isolates	S1	CY11 (KT715748)	99% 93%	*Geitlerinema* sp. (FM210758) / 1745 *Uncultured cyanobacterium* clone MSmat.3.11 (JQ612141) / 1745
	S1	CY20 (KT715750)	99% 93%	*Geitlerinema* sp. (FM210758) / 1780 *Uncultured cyanobacterium* clone MSmat.3.11 (JQ612141) / 1780
	S2	CY8 (KT715753)	99% 99%	*Uncultured cyanobacterium* (KP794044.1) /1087 *Fischerella sp.* (HM636645) / 1087
	S3	CY32 (KT715752)	99% 99%	*Geitlerinema* sp. (FM210758) /1089 *Uncultured Leptolyngbya* sp. clone Tsenher12otu4-1 (KT258783) / 1089
	S3	CY31 (KT715751)	96% 96%	*Geitlerinema* sp.(FM210758) /1526 *Uncultured Leptolyngbya* sp. clone Tsenher12otu4-1 (KT258783) / 1526
	S4	CY9 (KT715746)	99% 99%	*Fischerella* sp. (DQ786171) /1483 *Uncultured bacterium* clone B95 (AF407731) / 1483
	S4	CY2 (KT715745)	99% 99%	*Fischerella* sp. (DQ786171) /1491 *Uncultured bacterium* clone B95 (AF407731) / 1491
	S5	CY13 (KT715749)	98% 98%	*Geitlerinema* sp. (FM210758) /1531 *Uncultured Leptolyngbya* sp. clone Tsenher12otu4-1 (KT258783) / 1531
	S6	CY16 (KT715747)	97% 97%	*Fischerella* sp. (DQ786171) /1428 *Uncultured bacterium* clone B95 (AF407731) /

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				1428
Clones	K1	Clone 7 (KT793923)	97% 90%	*Uncultured bacterium* (AB757744) /1501 *Cyanothece* sp. (CP001344) / 1501
	K1	Clone 21 (KT793924)	99% 91%	*Uncultured bacterium* (AB757744) /1539 *Cyanothece* sp. (CP001344) / 1539
	K2	Clone 15 (KT793921)	99% 93%	*Geitlerinema* sp. (FM210758) /1737 *Uncultured cyanobacterium* clone MSmat.3.11 (JQ612141) /1737
	K3	Clone 12 (KT793922)	93% 93%	*Geitlerinema* sp. (FM210758) /1427 *Uncultured Leptolyngbya* sp. clone Tsenher12otu4-1 (KT258783) / 1427
	K4	Clone 62 (KT793926)	99% 91%	*Uncultured bacterium* (AB757744) /1470 *Cyanothece sp.* (CP001344) / 1470
	K5	Clone 46 (KT793925)	95% 88%	*Uncultured bacterium* (AB757744) /1623 *Oculatella atacamensis* (KF761587) / 1623
	K6	Clone 66 (KT793927)	93% 91%	*Leptolyngbya laminose* (FM210757) / 1646 *Uncultured cyanobacterium* clone MSmat.3.11 (JQ612141) /1646

Molecular Analysis

In addition to cultivation studies, we carried out culture-independent method cloning and Denaturating Gradient Gel Electrophoresis (DGGE) analysis for the determination of the biodiversity of Afyonkarahisar hot springs. To create a clone library containing 16S rRNA + ITS + 5 parts of 23S rRNA inserts, we used cyano-specific PCR primers which are CYA359F and 23S30R pairs. A total of 97 clones containing inserts of the right size were analysed. First, they were classified on the basis of their restriction profiles (*Table 2*). Akkuş-Gazlıgöl, Hüdai-Sandıklı, and Ömerli hot springs have 3, 4, and 4 different ARDRA profiles, respectively. These groups are K1, K2, and K6 for Akkuş-Gazlıgöl, K1, K2, K3 and K4 for Hüdai-Sandıklı, and K1, K2, K4 and K5 for Ömerli hot spring *(Table 2)*. The group K1 clones are the major ones in Akkuş-Gazlıgöl and Ömerli hot springs. One or two representatives from 6 different restriction groups were sequenced with the sequence primers 1092-1492-23SR. The BLAST analysis of the selected clones is shown in *Table 3*. 67% of the cyanobacterial clones were related to *Uncultured bacterium* (≥ 95-97% similarity) / *Cyanothece* sp. (90-91% similarity) or *Oculatella atacamensis* (88% similarity) as culture.

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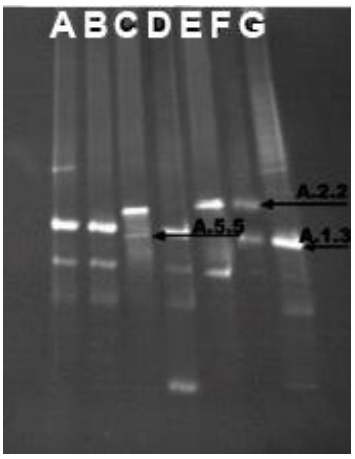
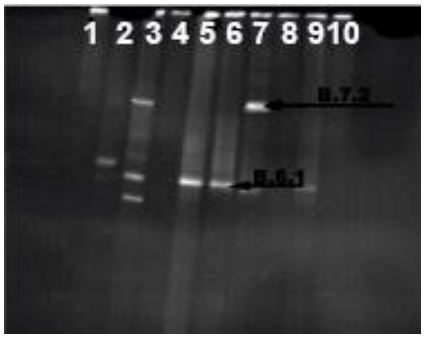
The similarities of the clone 7, 21, 62 sequences with *Uncultured bacterium* obtained from Padang Cermin Hot Spring Water (unpublished, Acc. Number AB757744) as environmental sample and with *Cyanothece* sp. (unpublished, Acc. Number CP001344) isolated from rice fields in Senegal as isolate were 90%, 91%, and 91%, respectively. Also, the similarities of clone 46 sequence with *Uncultured bacterium* (Acc. Number AB757744) and *Oculatella atacamensis* obtained from Atacama Desert (Karina et al., 2014, Acc. Number KF761587) as culture were 95% and 88%, respectively. The sequence of clone 12 and 15 were 93% and 99% identity with the genus *Geitlerinema* isolated from Euganean thermal muds, Padova, Italy (Unpublished, Acc. Number FM210758). Clone 66 sequence was 93% similarity with the *Leptolyngbya laminosa* from Euganean thermal muds, Padova, Italy (Unpublished, Acc. Number FM210757) *(Table 3).*

For the DGGE fingerprint analyses, we studied the 16S rRNA gene-defined community diversity in cyanobacterial water samples and mats from three hot springs. CYA359F and the CYA781R primer pair were used in DGGE method for the second PCR reaction. 25 and 7 positive bands were identified using DGGE (b2) and (a2), respectively. Representative samples of DGGE separations and some major bands of hot springs cyanobacterial 16S rRNA are presented in *Figure 4.*



DGGE (a2)

A) Hüdai-Sandıklı water, B) Akkuş-Gazlıgöl mat, C) Hüdai-Sandıklı mat, D)-E) Ömerli mat, F) Akkuş-Gazlıgöl water, G) Ömerli water



DGGE (b2)

B) (1)-(4) Ömerli water, (2) Akkuş-Gazlıgöl water, (3)-(7) Ömerli mat, (5)-(6) Hüdai-Sandıklı mat, C) (8) Akkuş-Gazlıgöl mat, (9) Hüdai-Sandıklı water

Figure 4. *DGGE gels (a2) and (b2) of the hot springs and some major bands*

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The DGGE band numbers from Akkuş-Gazlıgöl, Hüdai-Sandıklı, and Ömerli hot springs are shown in *Table 2*. The sequences obtained from most of the the bands (87.5%) yielded similarities to *Geitlerinema* with high percentages of similarity (99%/100%) that was isolated from thermal waters in İzmir (Unpublished, Acc. Number HQ197683.1) and one band (B.6.3) was related to the *Uncultured bacterium/Cyanobacterium* with similarity 99%/100%, respectively that was isolated from Yellowstone National Park, Fairy Springs (Boomer et al., 2009; FJ206395) and Yellowstone National Park, octopus spring cyanobacterial mat (Weller et al., 1992; Acc. Number L04709.1), two bands (A.1.2 and A.1.3) were related to the *Uncultured cyanobacterium* with similarity 99% and 94%, respectively (Unpublished, Acc. Number GQ480617), and one band (A.5.5) was related to the *Uncultured cyanobacterium/Phormidium* with similarity 99%/98% (Unpublished, Acc. Number EU728938.1 and DQ408370.1), respectively (*Table 4*).

In our DGGE results, Ömerli may have sequences belonging to the *Geitlerinema, Uncultured cyanobacterium*, Hüdai-Sandıklı may have *Geitlerinema, Uncultured bacterium/Cyanobacterium, Uncultured cyanobacterium/Phormidium* while Akkuş-Gazlıgöl may have only belonging to the *Geitlerinema* sp. sequences.

Table 4. *Obtained DGGE bands and their closest relatives in GenBank*

DGGE bands	% Similarity closest relative in BLAST search of Gen Bank
B.2.1, B.2.2, B.2.3, B.2.4, B.2.5, B.3.1, B.3.2, B.3.3, B.3.4, B.3.5, B.3.6, B.3.7, B.3.8, B.4.1, B.4.2, B.4.3, B.6.1, B.7.1, B.7.2, B.7.3, B.7.4, B.7.5, B.8.1, B.8.2, A.2.1, A.2.2, A.5.3, A.5.4	(99-100%) *Geitlerinema* sp. (HQ197683.1)
B.6.3	(100%) *Uncultured bacterium* (FJ206395) (99%) *Cyanobacterium* sp. (L04709.1)
A.1.2, A.1.3	(99%, 94%) *Uncultured cyanobacterium* (GQ480617)
A.5.5	(99%) *Uncultured cyanobacterium* (EU728938.1) (98%) *Phormidium* sp. (DQ408370.1)

Phylogenetic analysis

Sequences obtained from the isolates, clones and DGGE bands were aligned with the closest strains and retrieved from environmental samples obtained from RDP II and NCBI. Phylogenetic trees for cyanobacteria constructed based on partial 16S rRNA sequences were shown in *Figure 5*, *6,* and *7*. Aligned partial 16S rRNA gene sequences corresponding to *E. coli* sequence positions 388 to 1442 for the isolates, 551 to 1525 for the clones and 415 to 717 for the DGGE bands were used but the indels were not taken into account.

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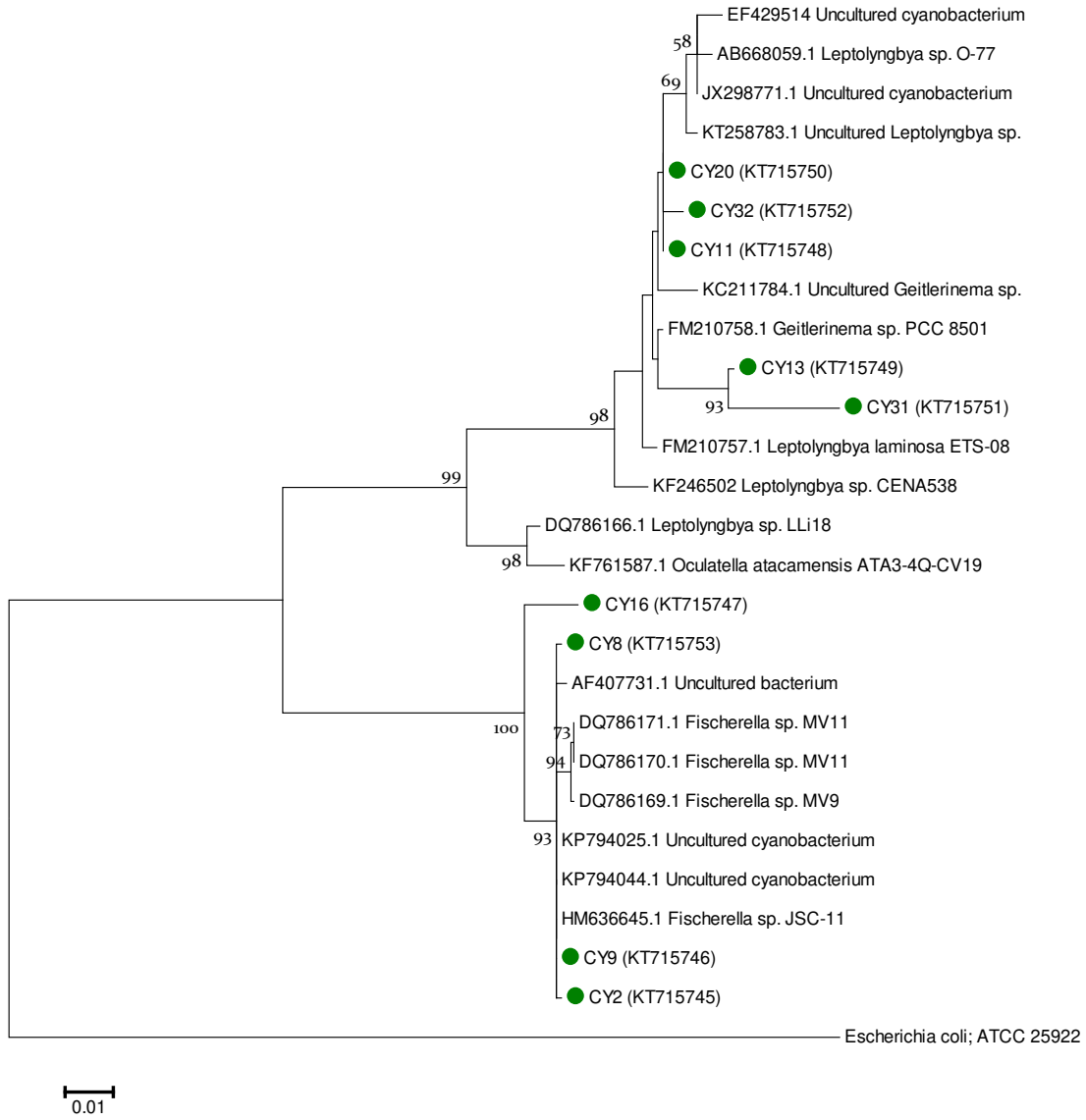

Figure 5. *Phylogenetic inferences based on 16S rRNA gene sequences from isolates (indicated by green circle). Sequence of the E.coli was used as outgroup. Scale bar represents expected number of substitutions per site. Bootstrap support values below 50% were not included in the figure.*

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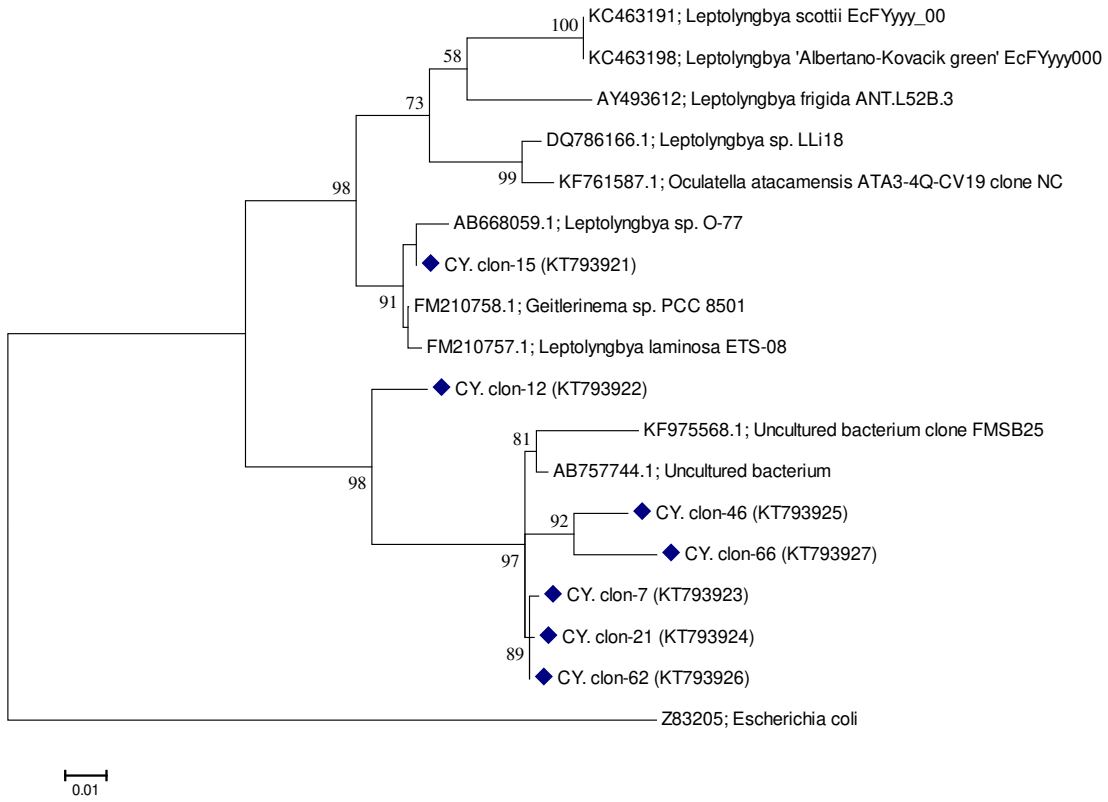

Figure 6. *Phylogenetic inferences based on 16S rRNA gene sequences from clones (indicated by blue diamond) belonging to the cyanobacteria. Bootstrap support values below 50% were not included in the figure.*

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Figure 7*. Phylogenetic inferences based on 16S rRNA gene sequences from some DGGE bands (indicated by violet trigon) belonging to the cyanobacteria. Bootstrap support values below 50% were not included in the figure.*

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Conclusions

The microbial diversity analysis has shifted in the last two decades from cultivation-dependent approaches to 16S rRNA-based cultivation-independent approaches, which led to the discovery of many novel microbial taxa. Nevertheless, this new approach also has limitations and is often restricted 16S rRNA clones through sequence similarity. Therefore, cultivation is still the method of choice to understand fully the physiology and complex ecological interactions in which microorganisms engage (Gunde-Cimmerman et al., 2005). The biodiversity of thermophilic cyanobacteria in terrestrial geothermal locations is fairly well documented, where they occur as part of a microbial mat community at temperatures up to ~65°C, and in biofilms at higher temperatures up to 75°C (Ward et al., 1998). The restriction fragment length polymorphisms (RFLPs) of particular PCR products can provide signature profiles specific to the genus, species, or even strain. Genetic characterization of cyanobacterial strains has been undertaken using RFLPs of the 16S rRNA gene (16S-ARDRA) (Lyra et al., 1997) and of the intergenic transcribed spacer region (ITS-ARDRA) (Lu et al., 1997; West and Adams, 1997).

Furthermore, amplification of the 16S–23S rRNA ITS, whose length and number was shown to be variable in cyanobacteria (West and Adams, 1997; Rocap et al., 2002; Iteman et al., 2002; Neilan, 2002, Laloui et al., 2002; Iteman et al., 2002) in cyanobacteria, can also be used as an identification tool. In this research, we used cultivation and molecular approaches, including DGGE analysis and cloning based on the 16S rRNA gene to reveal the cyanobacterial community composition of three Afyonkarahisar hot springs.

A large number of new thermophilic cyanobacterial isolates have been obtained for a long time by many researchers (Stetter, 1996; Casamatta et al., 2003). Our cyanobacterial isolates and previously cultured cyanobacteria, such as *Uncultured cyanobacterium/Fischerella* sp and *Geitlerinema* sp/ *Leptolyngbya laminosa* in Gene-Bank are very similar to each other. The sequence of our isolate CY31 had 96% similarity with the genus *Geitlerinema* sp. isolated from Euganean thermal muds, Padova, Italy (Unpublished, Acc. Number FM210758). 16S rRNA sequence similarity (96%) of CY31 indicates that this strain might be a novel species of the genus. Therefore, further studies, e.g. DNA: DNA homology should be carried out with different *Geitlerinema* species.

According to the cultivation results, the hot springs of Afyonkarahisar are inhabited by mainly thermophilic filamentous cyanobacteria including the *Fischerella* sp. and *Geitlerinema* sp., which are known to exist in thermal springs worldwide (Brock 1978; Ward and Castenholz 2000). *Spirulina labyrinthiformis* was earlier reported as the dominant organism in material collected by Aaronson from a 52 °C spring of Zerka Ma'in (Rayss 1944). However, we did not encounter *Spirulina* sp. in these hot springs. In addition, although one of the best-documented genera of thermophilic cyanobacteria is the genus *Synechococcus* (Ward et al., 1990, 1998; Miller and Castenholz, 2000; Ramsing et al., 2000; Papke et al., 2003). We did not determine any sequence related this genus in the hot springs.

The diversity and distribution of cyanobacterial groups in the springs were not high. The results obtained from Ömerli, Akkuş-Gazlıgöl and Hüdai-Sandıklı had some similarities with each other and those obtained from the other studies (Reysenbach et al., 1994; Ghosh et al., 2003; Komarek et al., 2003; Hongmei et al., 2005; Sompong et al., 2005; Jing et al., 2006; Sompong et al., 2006; Moro et al., 2007; Ionescu et al., 2009).

Cyanobacterial clones recovered from Ömerli hot spring were phylogenetically related to *Uncultured bacterium* and *Geitlerinema* sp. For Hüdai-Sandıklı hot spring, clones were phylogenetically related to *Uncultured bacterium* and *Geitlerinema* sp.

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Uncultured bacterium, *Geitlerinema* sp, and *Leptolyngbya laminose* sequences were also obtained in Akkuş-Gazlıgöl hot spring. Although Ömerli has the highest cyanobacterial diversity, Akkuş-Gazlıgöl has more cyanobacterial genus diversity. Contradictory results of same samples were obtained when analyzing the cyanobacterial community inhabiting the hot springs with the culturing. *Getilerinema* sp. was dominated in the culturing method while *Uncultured bacterium/ Cyanothece* sp. became major ones with the cloning.

16S rRNA PCR-DGGE is one of the most frequently used technique to assess the genetic diversity of microbial communities (Muyzer, 1999; Ercolini, 2004). This method allows the separation of small DNA fragments (maximum size of 1000 bp) of the same length but of different sequence according to their melting properties. Fragments with only one single base substitution can be separated with this technique (Nollau and Wagener, 1997). The analysis of these group-specific PCR fragments on a DGGE gel provides a valuable tool for monitoring the structure and dynamics of microbial populations over time or under the influence of environmental changes. Although limitations exist, such as the difficulty on band isolation and too short sequences retrieved to provide a robust phylogenetic analysis, this methodology has been revealed to be a valuable technique to monitor changes in bacterial community structure, both in relative abundance and in bacterial diversity (Lemarchand et al., 2005). We used the specific primers that have the advantage of giving a PCR product which corresponds to the variable regions V3 and V4, and contains significant information for phylogenetic assignments (Nübel et al., 1997; Boutte et al., 2006).

Casamatta et al., 2003 found various cyanobacteria like *Pleurocapsa, Phormidium, Anabaena, Synechocystis, Oscillatoria, Microcoleus* and *Pseudanabaena* with 16S rRNA gene sequences along with DGGE inhabiting Octopus hot spring. Similarly, *Geitlerinema* and *Phormidium* genus were also detected in our study. Bands that migrated to the same position in the DGGE gel and displayed no ambiguous differences in nucleotide sequences were considered to represent unique 16S rRNA sequence types. Moreover, identical sequences were obtained from different bands in the same lane. This is in agreement with the observation of Nikolausz et al., 2005 that dominant amplicons could be distributed at different positions in the same pattern. If several domains have similar melting properties, stochastic effects might cause one to denaturation before the other in a fraction of the amplicon population and could also explain the presence of different bands with the same sequence in one lane.

Consistent with cloning and DGGE, the 16S rRNA gene sequences from Ömerli showed that *Geitlerinema* sequences were recovered from both method and was dominated with DGGE. Similar to our cultivation results, *Geitlerinema* sp. was obtained with the cloning and DGGE analysis. But the *Fischerella* sp. could not be determined with cloning and DGGE sequences.

In this study, although high diversity emerged in ARDRA profile, low diversity in terms of genera was obtained from culture-dependent and culture independent assay results. Previous studies in thermal springs have shown an increasing diversity with decreasing temperature (Kullberg, 1968; Brock, 1978; Castenholz, 1978; Miller and Castenholz, 2000; Sompong et al., 2005). However, this result has some conflicts with ours. According to our results, the cyanobacterial diversity increased with the increasing temperature. Clearly, thermal stress is not a limiting factor since the hottest spring were most biodiverse. The cyanobacteria identified in this study do not follow a single pattern when compared with similar organisms from different thermal sites in the world. Some

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clones and isolates showed low similarities (93–97%) to those from other environments while others showed over 97% similarity to their counterparts.

Since thermophiles are notoriously difficult to culture, this study significantly expanded the known range of cultivated thermophilic cyanobacteria and provided a biological resource for future biotechnological exploitation. Furthermore, cultivation and characterization of axenic thermophilic cyanobacterial strains provided a further insight into the physiology of thermophiles and yielded a source of organisms for possible biotechnological exploitation.

It is intriguing that the unique environment of Afyonkarahisar hot springs has not been surveyed for cyanobacterial diversity before. This is the first study in which both culture-dependent and culture-independent techniques have been used simultaneously to target unique regions of the 16S rRNA +ITS gene in samples obtained from Afyonkarahisar hot springs.

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IMPACT OF EDAPHIC HYDROCARBON POLLUTION ON THE MORPHOLOGY AND PHYSIOLOGY OF PEA ROOTS (*PISUM SATIVUM* L.)

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Abstract. Exposure to persistent organic hydrocarbon pollutants can have deleterious effects on the growth, physiology and anatomy of plants. Sand collected at an oil-drilling quagmire in southern Algeria was analyzed by GC-FID and found to contain 18mg.Kg^{-1} hydrocarbons. *Pisum sativum* L. (pea) plants were grown in laboratory conditions in sandy soil from the site. Plants growing in hydrocarbon polluted sandy soil had shorter primary roots and fewer lateral roots than control plants growing in non-polluted sandy soil. However lateral root dry weight was 35% higher than control. Pollutant-induced oxidative stress on pea roots resulted in lipid peroxidation and accumulation of MDA, H_2O_2 and O_2^- in root tips. Enzymatic detox activities of superoxide dismutase and peroxidase were also over 40% higher in plants growing on polluted soil than in controls. The anatomy of pea roots was also affected by hydrocarbon-polluted soil, because xylem vessel differentiation was delayed and an unusual supplementary cell layer was formed in the endoderm. These data suggest pea plants adapt morphologically and anatomically to polluted soil.
Keywords*: hydrocarbons, pea roots, growth, oxidative stress, anatomy*

Introduction

Since the mid-1980s, hydrocarbon contamination has become a critical environmental problem worldwide due to its adverse effects on the environment and health (Li et al., 1993). In Algeria, petroleum is one of the main energy resources. Petroleum exploration can cause soil pollution because drilling mud is stored in quagmire spill sites so the soil surface becomes impregnated with total petroleum hydrocarbons (TPHs). TPHs are complex mixtures of various hydrocarbons that can be found at petrochemical sites and storage areas, waste disposal pits, refineries and oil spill sites (McElroy et al., 1989).

Plants are a dominant biotic component of ecosystems and as sessile organisms; they can be subjected to long-term pollution from hydrocarbons (McCarthy and Tschaplinski, 1991). Organic pollutants are able to penetrate plant organs through different mechanisms (Gao and Zhu, 2004). In plant tissues, organic pollutants can migrate from roots to leaves, and within the plant organs, they can be modified by conjugation, hydroxylation and by

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cytochrome containing monooxygenase enzymes (Korte et al., 2000). Plant growth is affected by decrease of biomass in oiled areas (Culbertson et al., 2008). For maize germination and growth in crude-oil polluted soil, the effect is proportional to the concentration of the crude oil in the environment (Ogboghodo et al., 2004). The considerable effect of polycyclic aromatic hydrocarbon fluoranthene (FLT) exposure was to inhibit germination of seeds, retard growth and affect root morphology (Kummerova et al, 2012). Anoliefo (1991) found evidence of cell disruption in roots and other organs and the presence of oil films in the epidermal and cortical regions of the root, stem, and leaves. Crude oil induced environmental stress in the seedlings causing inhibition of total amylase and starch phosphorylase activities and mitotic activity of root meristems (Achuba, 2006). The harmful effects of petroleum hydrocarbons in soils include inhibition of seed germination, reduction of photosynthetic pigments, slowdown of nutrient assimilation and shortening of roots and can disrupt the plant root architecture (Smith et al., 2006). Some other workers have also used anatomical changes to monitor environmental pollution (Gill et al., 1992). Petroleum hydrocarbons were reported to alter the shape and size of parenchyma tissue and reduce the intercellular space in the cortex of the stem and roots (Omosum et al., 2008).

In polluted soils, plants may experience a combined stress from nutritional deficiency and chemical toxicity. Indeed, abiotic stress such as that caused by polycyclic aromatic hydrocarbon exposure can also stress plants by generating reactive oxygen species (ROS) (Sun et al., 2002). ROS such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), are generated as by-products of normal metabolism in different subcellular compartments. Moreover, the imposition of biotic or abiotic stress may give rise to an excessive concentration of ROS, resulting in oxidative damage at cellular level that can be mitigated and repaired by a complex antioxidant system (Romero-Puertas et al., 2007). Stress induced ROS accumulation is counteracted by enzymatic antioxidant systems that include a variety of scavengers, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), glutathione S-transferase (GST), catalase (CAT) and non-enzymatic low molecular metabolites (Mittler et al., 2004).

The impact of hydrocarbon contaminants on plant roots, which are in direct contact with the pollutants, is not as well documented as the effect on leaves or photosynthesis. For this reason, we aimed to evaluate the effect of hydrocarbons from an edaphic pollution on the growth of pea roots (*Pisum sativum* L.) in controlled laboratory conditions. We observed changes in ROS (O_2^-, H_2O_2,) and ROS detoxifying enzymes, MDA levels were enhanced in pea roots growing in polluted soil. Furthermore an unexpected morphological adaptation was observed at the level of the endoderm cell layer.

Materials and methods

Soil

Soil samples were collected in the region of Hassi Messaoud-Ouargla province of southern Algeria. Contaminated soil was sampled at the site of a disused oil quagmire. Control soil was sampled at a site two kilometers from the quagmire. Three soil samples were collected at 0-30 cm depth using a stainless steel sampler. The three samples were mixed to form a single sample that was air-dried and then sieved (2 mm sieve) before analysis.

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Hydrocarbon analysis

Before hydrocarbon measurement soil samples were chemically dried, by adding Na_2SO_4. Analysis of hydrocarbons in soil was conducted in the ISSeP laboratory (Scientific Institute of Public Service, Colfontaine, Belgium) using techniques developed in the laboratory.

Polycyclic aromatic hydrocarbon (PAH) content in soil samples

The 16 PAH congeners listed as priority pollutants by the US Environmental Protection Agency (US-EPA) were analyzed in the soil samples. The extraction of these compounds was performed by the Accelerated Solvent Extractor (ASE) technology (Dionex ASE 350), allowing a solid/liquid extraction with dichloromethane at 150 °C at a pressure of 1500 psi. The dichloromethane extract was extracted with hexane and cleaned up with aluminium oxide. The sample was extracted with acetonitrile and the extract was concentrated at room temperature under a gentle stream of nitrogen. PAHs were then separated by ultra-performance liquid chromatography (UPLC) and detected using a fluorescence detector with appropriate excitation and emission wavelengths for the 15 PAH and a diode array detector for acenaphthylene. Quantification was performed by external standard calibration. Ultra performance liquid chromatography (UPLC) was performed with a 1.8 µm, 2.1mm ID × 100 mm AZE-PAH column at a flow rate of 0.4 ml/min. The mobile phase consisted of acetonitrile-water (50:50, v/v) for 9 min and 100% acetonitrile for 3.5 min.

Hydrocarbon index

The extraction of total petroleum hydrocarbons (TPHs) was performed by a ASE technology (Dionex ASE 350), allowing a solid/liquid extraction at 100°C under a pressure of 1500 psi, with a solvent mixture of *n*-hexane-acetone (50:50, v/v). The recovered extract was washed with an aqueous solution of hydrated magnesium sulfate to remove acetone and then cleaned up on a Florisil column (6 mm diameter,6 cm long). The eluate was then concentrated with a Syncore Analyst Evaporator to 0.5 ml to be used for analysis by gas chromatography (GC-FID) using a Column VF-5ht 15 m × 0.25 mm × 0.10 µm with a "splitless" injection technique (30 sec) and a pulse injection (10 psi for 1 min). The analyses were conducted under the following conditions: injection temperature, 300°C; injection volume, 1µl; carrier gas, helium; oven temperature program, 40°C for 5 min to 300°C and 300 °C for 5 min; flame ionization detector temperature, 330 °C. The following fractionation was performed: (C10-C12), (C12-C16), (C16-C21), (C21-C35) and (C35-C40). The limits of integration were placed at the corresponding retention times of *n*-alkanes (C12, C16, C21 and C35), areas of each fraction were measured and the calculation based on the total area C10-C40.

Plant growth and root growth analysis

Pea seeds (*Pisum sativum* L.)"Kelvedon wonder" were surface-sterilized with 0.1% sodium hypochlorite (NaClO) for 10 min, rinsed and soaked in distilled water at room temperature for 12 h. The seeds were germinated on water-imbibed paper in sealed plastic dishes. After three days, pea seedlings were transplanted into plastic containers containing 250 g of a mixture of peat and sand, polluted or control, (80:20, w/w). Plants were cultivated for 21 days under controlled conditions in a growth chamber with a 16 h

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light (90 μE) and 8 h dark cycle at a constant temperature of 25 °C and relative air humidity of 60%, watered by pure water according to usable water to field capacity calculated in gram of water per gram of sandy soil and after wards, every other day till the end of the experiment. The dry weight of roots, the number of lateral roots and the length of the primary root of plants were measured after 21 days of culture.

Hydrogen peroxide detection

Hydrogen peroxide was detected by a colorimetric method using 3,3 diaminobenzidine (DAB). DAB is taken up by living plant tissue and can be used to show H_2O_2 production when peroxidase activity is present (Thordal- Christensen et al., 1997). The root apices (excised 1 cm from the tip) were immersed in the dark for 12 h in a 1 $mg.ml^{-1}$ DAB solution in water at room temperature with gentle stirring. Hydrogen peroxide causes a redox polymerization with DAB molecule giving a stable brown precipitate at the reaction site.

Superoxide anion detection

The superoxide anion O_2^- is detected by colorimetric method using nitrobluetetrazolium (NBT) (Rao and Davis, 1999). Superoxide radicals reduce NBT to form a stable formazan blue blue-indigo (Beyer and Fridovich, 1987). Root apices (5 cm from the tip) were immersed in a 0.5 mg/ml NBT solution in 0.1 M sodium phosphate buffer pH (7.8) for 1 h at room temperature and in the dark. The root samples were rinsed in boiling 96° ethanol for 10 minutes. The root samples were stored in a glycerol-ethanol solution (1:4, v/v) until photographs were taken under a light microscope.

Determination of lipid peroxidation

Lipid peroxidation was determined as the amount of malondialdehyde (MDA) in roots. MDA is a thiobarbituric acid reactive substance (TBARS), which was measured according to Achary et al (2008). Root fragments were homogenized in 1.5 ml of reaction mixture containing 20% (w/w) trichloroacetic acid and 0.5% (W/V) thiobarbituric acid, heated at 95 °C for 30 min, cooled on ice then centrifuged 10 min at 13000 *g*. The absorbance of the supernatant at 532 nm and 600 nm was measured. The nonspecific absorbance at 600 nm was subtracted from that at 532 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM cm^{-1}.

Extraction of enzymes

Plant roots (100 mg) were homogenized in 2 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ethylene diamine tetraacetic acid (EDTA) and a small amount of polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 4°C for 20 min at 12000 × *g*. The resulting supernatant was used to measure peroxidase and superoxide dismutase activities. An aliquot of 0.1 ml was used to determine the protein content as per the method of Bradford (1976) using bovine serum albumin as standard.

Peroxidase activity

The peroxidase (POD) reaction solution (3 ml) contained 50 mM phosphate buffer (pH 5), 20 mM guaiacol, 40 mM H_2O_2 and 0.1 ml of enzyme extract. Changes in

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absorbance of the reaction solution at 470 nm were determined every 20 s. One unit of POD activity was defined as an absorbance change of 0.01 absorbance units per min. The enzyme activities were expressed relative to the protein content (Chance and Maehly, 1955).

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was assayed by measuring the ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The 3-ml reaction solution contained 50 µM NBT, 1.3 µM riboflavin, 13 mM methionine, 75 nM EDTA, 50 mM phosphate buffer (pH 7.8) and 20-50 µl of enzyme extract. The test tubes containing the reaction solution were irradiated by light. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction rate of NBT as monitored by absorbance at 560 nm (Giannopolitis and Ries, 1977).

Anatomical studies

To investigate the internal structure of pea roots, we made cross sections at two similar positions relative to the root tip (*Figure. 1*) to avoid the oscillation zone, a region of the spatial and temporal definition of lateral roots pre-branching sites (Jung and McCouch, 2013), and to have more differentiated tissues. Six plants were randomly selected from both soils. Roots were fixed in FAA (95° ethanol - 35% formaldehyde - acetic acid, 2:17:1, v/v) for 24 h, washed with distilled water several times then progressively dehydrated through an ethanol series up to 70° ethanol. For epifluorescence observations fresh samples were used. Fixed and fresh samples were cut with a vibratome in order to obtain 60-micrometer transverse sections. Staining with iodine green carmine was carried out as described in Locquin and Langeron (1978). Briefly, sections were placed for 10 to 20 min in sodium hypochlorite, then washed thoroughly with water, incubated in dilute acetic acid and stained with carmine-green iodine. After staining sections were washed with water. Microphotographs were taken by using a Zeiss Axioskop microscope equipped with an AxioCam camera MR (Zeiss) using ×50 and ×100 magnification objective lenses and the images were processed and archived with AxioVision software (Zeiss).

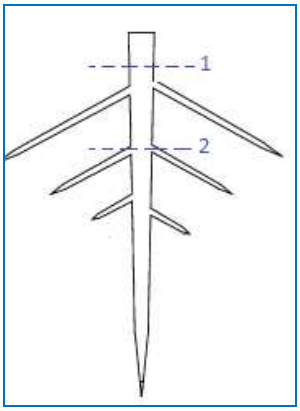

Figure 1. *Diagram of root scheme with location of sections used for anatomical studies*

To visualize lignified cells, sections were examined by using a digital imaging station comprising a motorized Zeiss Axio Imager Z1 microscope equipped with a light

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sensing device with automated color correction (Apotome, Zeiss). This system allows the observation of epifluorescence with a HBO mercury vapor light source type, which provides excitation light in the 340-700nm range.

Lignin autofluorescence was detected using the Zeiss HE DAPI filter set 49 (excitation, 3656 nm; emission, 420-470 nm). Digital fluorescence images were generated by an AxioCam MR Camera (Zeiss), using ×50 and ×100 magnification objective lenses. Images were processed and archived with AxioVision software (Zeiss).

Statistical analysis

All data presented are the mean values of five replicates ± standard deviation (SD). Statistical analysis was carried out by ANOVA analysis at a 5%, 1% and 0.1% significance level, using the statistical software package STATISTICA version 8.0.

Results

Soil analysis and hydrocarbon index

Samples of sandy soil were collected at a disused oil-drilling quagmire in southern Algeria was analyzed by GC-FID and UPLC. Control soil samples, collected from a non-industrial site 2 km away, had a similar sandy texture. Analysis showed presence of molecules of low and high molar mass. Gas phase chromatography showed the presence of 18 g of total petroleum hydrocarbon (TPH) per kg of polluted soil (*Figure 2A*). Fractionation of these hydrocarbons (*Fig. 2 A, C*) showed that they are mainly a mixture of C12-C21 molecules (*Fig. 2A*). No hydrocarbon was detected in control soil (*Fig. 2B*). Polyaromatic hydrocarbon (PAH) content of polluted soil analysis by UPLC showed the presence of fluorene (15.3µg kg^{-1}), phenanthrene (781.4 µg kg^{-1}), fluorenthene (30.9 µg kg^{-1}) and pyrene (282.5 µg kg^{-1}). Concentrations of other PAHs were below the detectable values.

Analysis showed an increase in soil moisture, total organic carbon, phosphorus and nitrate in polluted soil, but no significant differences for nitrite, pH and conductivity with control soil.

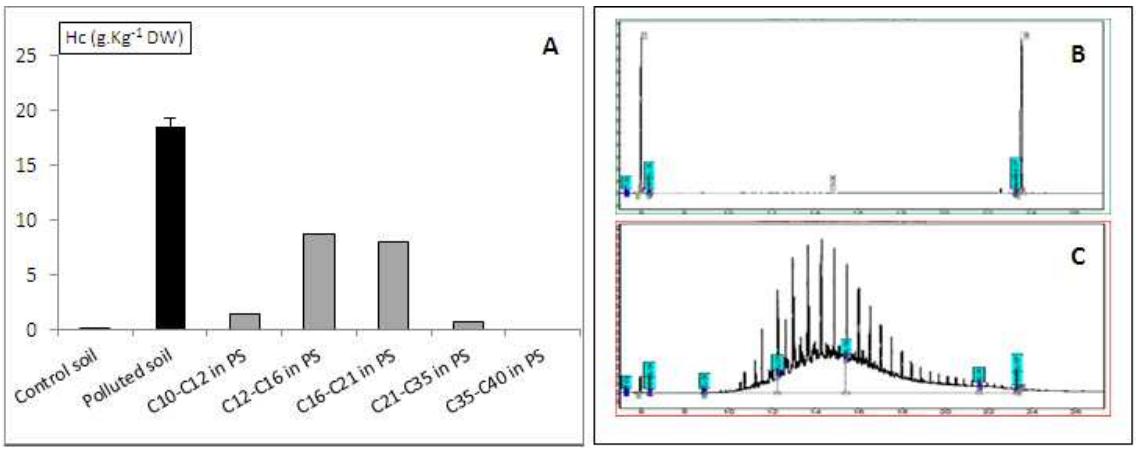

Figure 2. *(A) Hydrocarbon index in control (CS) and polluted soil (PS) and fractionation of hydrocarbons in (PS). Chromatogram of hydrocarbons from (B) control soil and (C) polluted soil.*

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Hydrocarbon pollution affects root growth

To investigate the possible deleterious effect of polluted sand on plant growth, *Pisum sativum* L. (pea) plants were grown in laboratory conditions in both control and hydrocarbons contaminated soil. We noted that 21-day-old pea seedlings on polluted soil developed all aerial organs like the control but were shorter (*Figure 3A*). To assess the effect of pollutants in direct contact with roots, we uprooted the plants and measured primary and lateral root lengths and number (*Fig. 3 B, C*). Primary root length is 37% shorter in pea plants grown in polluted soil compared with those of controls (*Fig. 3B*). Root architecture is a plastic phenotype being characteristic of individual species but also determined by the growth environment. Pea plants growing on polluted soil had 36% fewer lateral roots per plant (*Fig. 3C*). There was no significant difference in total root mass (including primary and lateral roots) in seedlings grown in both type of soil (*Fig. 3D*). In contrast, the dry weight of lateral roots from plants grown in polluted soil was 35% higher than that of lateral roots in control soil (*Fig. 3D*).

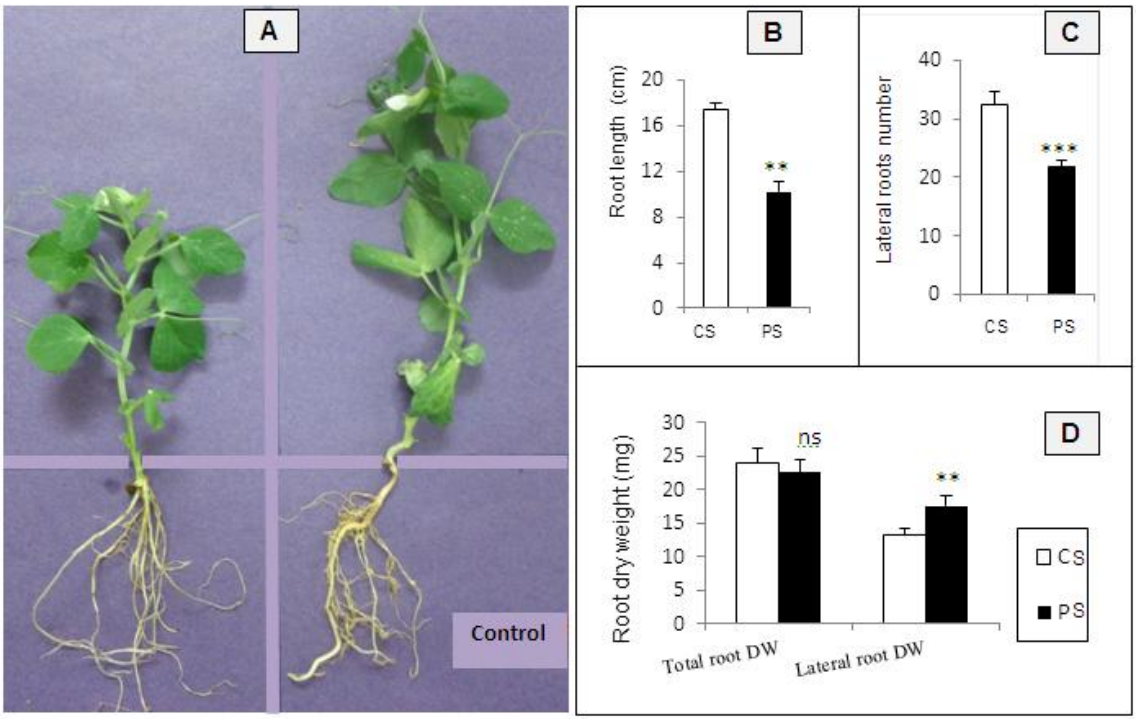

Figure 3. *(A) Morphology, (B) primary root length, (C) number of lateral roots and (D) dry weight of roots of pea seedlings grown in control (CS) and hydrocarbon polluted soils (PS).Values showing means quares from analysis of variance of data for each variable; ** and ***: significant at 0.01 and 0.001 level respectively, ns: not significant*

Effect of hydrocarbons on oxidative stress in pea roots

The adverse effects of polluted soil on primary root length and lateral root initiation and mass might be caused by direct chemical effects of the molecules or indirectly by affecting the physiology of plants, for example, by causing stress. We tested whether the polluted soil causes stress in pea roots by measuring known oxidative stress molecules.

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Reactive oxygen species (ROS) such as the superoxide radical O_2^- can be formed during stress by NADPH-oxidase enzymes or by the reaction of electron transfer chains, enzymes or metals with oxygen. NBT staining of pea seedlings grown in hydrocarbon polluted soil revealed that a large amount of superoxide accumulated along the length of the root from about 5 cm from the root tip to the tip itself (*Figure 4 A, B*). Hydrogen peroxide (H_2O_2), another ROS, is also formed in stressed tissues, either from superoxide metabolism or directly from water. H_2O_2 was revealed by DAB staining in roots (*Fig. 4 C, D*). The intensity of brown spots in stained roots indicates the relative accumulation of H_2O_2. In pea roots exposed to hydrocarbons the brown coloration is concentrated at the end of root tips (*Fig. 4 D*). In contrast in control roots, almost no staining with NBT or DAB was observed (*Fig. 4. A, C*). Membrane lipid peroxidation is often a consequence of damage that occurs when cells are exposed to superoxide or other ROS. Malondialdehyde (MDA) is an indicator of lipid peroxidation and MDA content reflects oxidative stress. The MDA content of roots grown in hydrocarbon-polluted soil was 22.7% higher than in control roots (*Fig. 4 E*).

Another indicator of oxidative stress is the activity of detoxification enzymes such as superoxide dismutase (SOD) and peroxidase (POD) that are able to catabolise superoxide and hydrogen peroxide respectively. Both SOD and POD activities were over 40% higher in roots from plants growing in polluted soil than in control roots (*Fig. 4 F, G*).

Our study reveals that pea roots growing on soil polluted with PAHs display symptoms of oxidative stress because both superoxide and hydrogen peroxide are produced and accumulate and detoxification enzymes are expressed suggesting a sustained physiological response to polluted conditions.

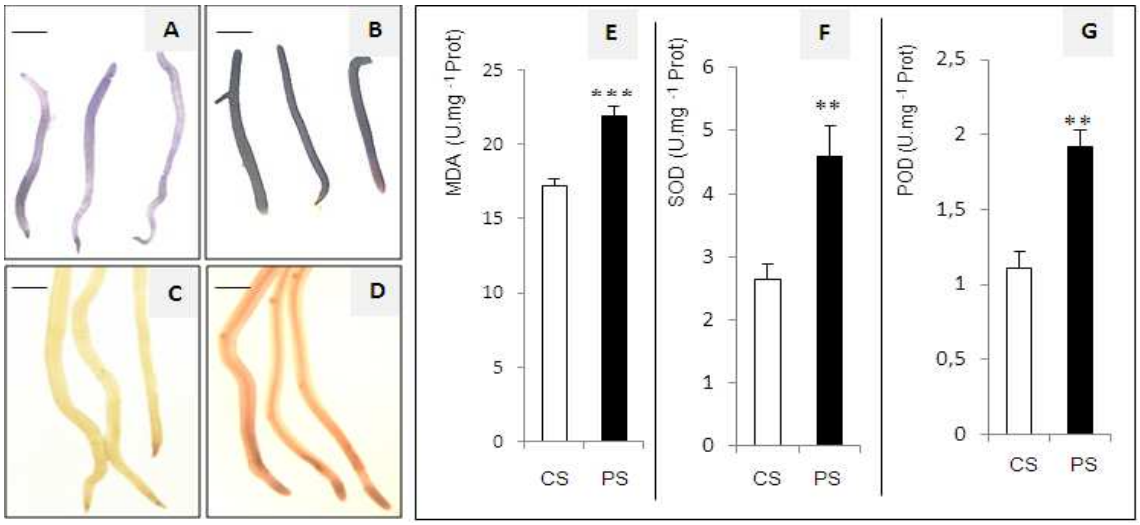

Figure 4. *NBT (A-B) and DAB staining (C-D) detect superoxide and hydrogen peroxide respectively. Pea root tips grown in control (A, C) or polluted soil (B, D). Scale bar = 1cm. MDA (E), SOD (F) and POD (G) activities in pea roots grown for 21 days in control (CS) and hydrocarbon polluted soil (PS). Values are significantly different at 0.01** and 0.001***, respectively.*

Anatomical studies

Our study suggests that the presence of hydrocarbons in soil affected many aspects of root growth. The overall root anatomy is similar in both polluted and control plant

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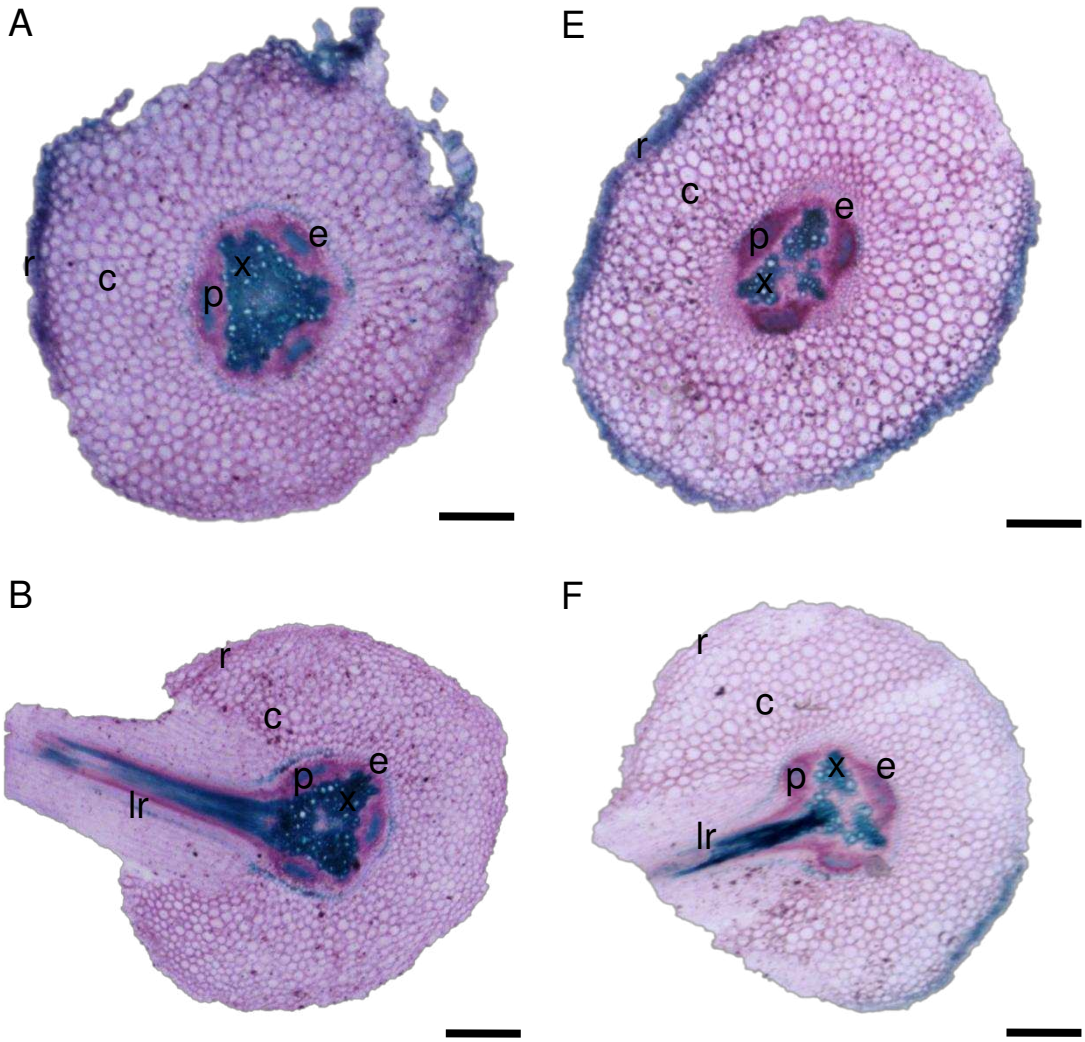
samples with cortex cells similar in size both types of roots samples. However there was a slight flattening of cortex cells in the primary roots that had been exposed to polluted soil (*Figure 5 A, E*). Lateral root initiation was observed in both samples (*Fig. 5 B, F*).

When xylem vessels were viewed more closely (*Fig. 5 C, G*), evidence of the centripetal differentiation of three primary xylem vessels was clearly observed in the primary root in both samples. However secondary xylem differentiation appears retarded in plants grown in the presence of hydrocarbons as much as less secondary xylem is present compared to control (*Fig. 5 C, G*).

Assuming the presence of hydrocarbons in soil caused a delay in the differentiation of secondary xylem, we looked for other signs that differentiation was affected. Lignin fluorescence was observed in cell walls under UV-fluorescent microscopy. Lignified xylem vessels are noticeably smaller in plants growing in polluted soil compared to control plants (*Fig. 5 D, H*). Most surprisingly in plants grown in polluted soil we observed a two-cell layered endodermis possibly adjacent to suberized cells (*Figure 5 H*, yellow circle). Roots grown in hydrocarbon-polluted soil therefore have unusual xylem and endodermis differentiation.



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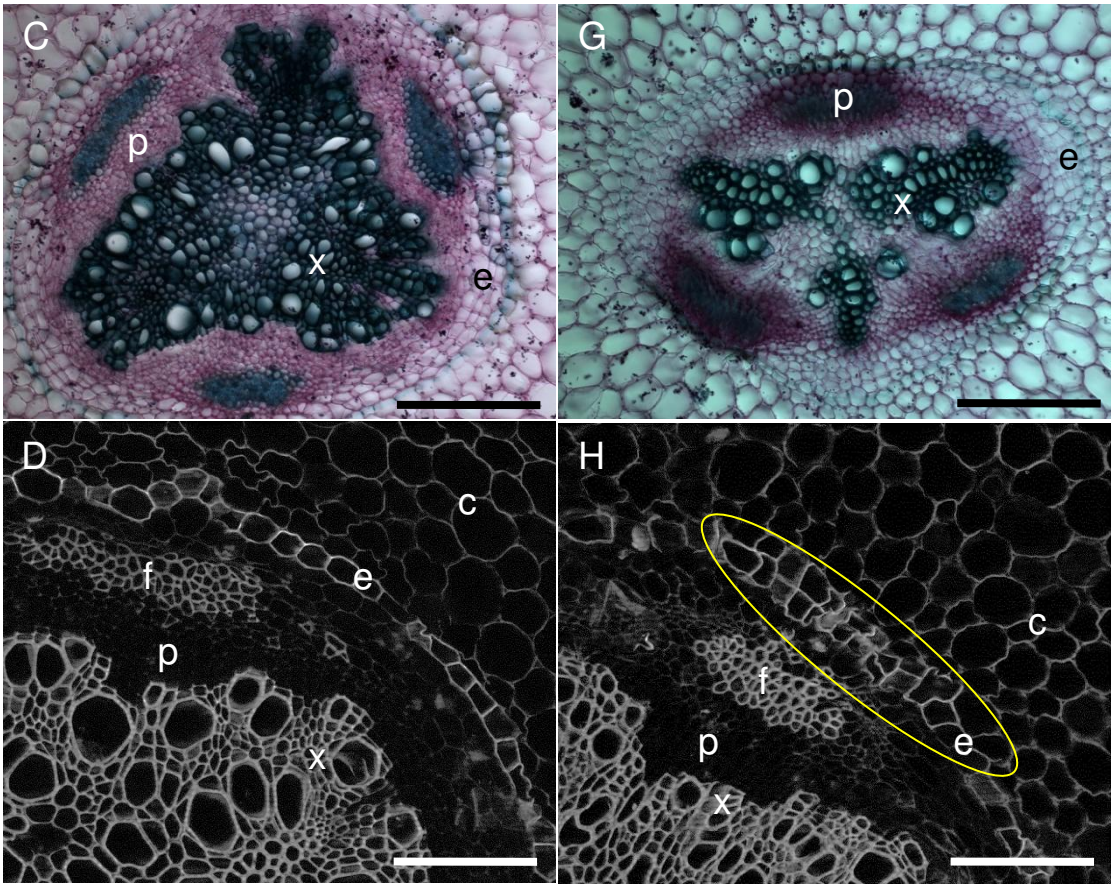

Figure 5. *Transverse sections of pea roots grown in control (A, B, C, D) and hydrocarbon polluted soil (E, F, G, H), observed by light microscopy (A, B, C, E, F, G) and by fluorescence microscopy (D, H). c, cortex; e, endodermis; f, fibers; lr, lateral root; p, phloem; r, rhizodermis; x, xylem. Scale bar, 500 μm.*

Discussion

Hydrocarbon in soil

Diesel fuel, on entering the terrestrial environment, will spread and seep into the soil. Michel et al (2002), state that petroleum constitutes a pollutant that can persist in the environment for a long period until the vegetation recovers completely, and its persistence can be explained by the slow biodegradation of hydrocarbons. The downward migration of diesel fuel through the soil profile however is limited due to the physical properties of the fuel (Adam and Duncan, 1999). Presence of total petrolium hydrocarbon (TPHS) in soil can cause damage in short and long term for the plant. The immediate toxic effect tends to be caused mainly by molecules of low molar mass that are quickly degraded. The chronic toxic effects, however, are due to molecules of high molar mass, generally aromatic, that present lower toxicity, but are persistent, causing a longer lasting effect (Spies et al., 1996).

Root morphology is disturbed in pea plants grown in polluted soil

Root growth and development are controlled by endogenous cues such as phytohormones (Casimiro et al., 2001). However exogenous factors such as water,

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salinity, nutrients or the presence of toxic metals have a considerable impact on the final root structure (Arduini et al., 1994). Many plant species are sensitive to petroleum contaminants (Huang et al., 2004). Hydrocarbons in the soil may prevent uptake of nutrients that are less mobile in contaminated soils (Atuanya, 1987). Water and nutrient absorption can also be limited by hydrophobic molecules, which can form a layer over the root when in excess in the soil (Quinones-Aquilar et al., 2003). Inhibition of plant growth parameters (germination, plant length, and biomass) can be caused by toxic compounds of petroleum hydrocarbons (Bossert and Bartha, 1985), such as low molecular weight hydrocarbons.We observed both inhibition of primary root growth and fewer lateral roots in pea plants growing on polluted soils. These results are reminiscent of the known inhibition of lateral root formation and initiation of root primordia by PAHs (Alkio et al., 2005; Baldyga et al., 2005).

There was no significant difference in the dry weight of total roots (primary and lateral roots). Interestingly pea plants grown on oil-contaminated fields also had a similar root dry weight as control plants after three weeks of growth (Xu and Johnson, 1995), although in older plants root weight was lower in polluted plants than in controls (Xu and Johnson., 1995). Generally, lateral roots appear thicker in the polluted samples.

Hydrocarbon pollution is associated with oxidative stress in pea plants

Many environmental stresses induce ROS production (Apel and Hirt, 2004). Their reaction with other molecules such as proteins or nucleic acids is often deleterious to the cells. Lipids when peroxidised lead to MDA accumulation and altered cell integrity (Apel and Hirt, 2004). ROS and ROS-detoxifying enzymes are more abundant in roots from pea plants grown in polluted soil. The presence of PAH in polluted soil might be directly responsible for ROS production, as it is generally observed that PAHs induce ROS production in plants, as seen with phenanthrene (Alkio et al., 2005) and N-heterocyclic PAHs (Paskova et al., 2006).

The observed increase in MDA in roots grown in polluted soil is suggestive of oxidative damage as a consequence of ROS accumulation. It indicates that hydrocarbon-induced stress alters biological membranes and affects cellular integrity. Phenanthrene alone can induce ROS generation, MDA production, and oxidative stress (Liu et al., 2009).

ROS abundance depends on rates of ROS generation and rate of ROS degradation and scavenging/neutralizing by antioxidants whether through enzymatic and/or non-enzymatic mechanisms (Amor et al., 2005). Plants have numerous detoxification mechanisms, such as glutathione S-transferases, POD, catalases, and SOD and non-enzymatic molecules like glutathione (Won et al., 2012). SOD activity and proteins increase in response to stress in plants (Shalini and Dubey, 2003; Song et al., 2006).

Detoxifying enzyme activity or abundance is induced by hydrocarbons, such as diethyl phthalate (Cheng and Cheng, 2012) and phenanthrene (Song et al., 2006) in greater duckweed *Spirodela polyrhiza*. We found that ROS detoxifying activities SOD and POD increased in roots of hydrocarbon-polluted pea plants suggesting that pea plants respond to environmental stress by producing detoxifying enzymes. This finding is broadly consistent with other abiotic stress responses, which quench excess ROS through enzymatic reduction to water, and oxidize electron-rich buffers such as ascorbate and glutathione (Apel and Hirt, 2004). However here not all of the stress-induced ROS are eliminated, leading to MDA accumulation.

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Anatomy

The results of this study support the idea that the presence of hydrocarbons in soil has affected not only the morphology and root development, but also their anatomical structure. Indeed we show that roots grown in polluted soils are delayed in xylem differentiation and have an additional cell layer in the endodermis.

Our results are in agreement with Kummerova et al. (2013), who showed that in pea and maize roots, the proportion of xylem vessels in the stele decreased when exposed to fluoranthene. Pea roots with less xylem in response to hydrocarbons in soil may be interpreted as an adaptation to minimize absorption of polluted water, because vessel number and diameter influence the amount of water flowing. Hernandez-Ortega et al. (2014) reported that values of hydraulic parameters diminished, but the loss of hydraulic conductivity was significantly enhanced as the diesel concentration increased. In addition fluoranthene exposure triggers changes in the cell morphology of other organs and tissues including the root tip, root cap, apical meristem and elongation zone (Kummerova et al., 2013). Similar abnormal development patterns of xylem have been also described in cotton grown in presence of high salinity (Reinhardt and Rost, 1995). Thus the xylem tissue seems to be particularly sensitive to external abiotic pollutant.

A single layer of endodermis in plants is defined by an evolutionarily conserved mechanism, where the SCARECROW (SCR) protein associated with the mobile SHORT-ROOT (SHR) protein delimits endoderm and pericycle founder cells around the quiescent center at the root tip (Cui et al., 2007). In our study, pea roots grown in polluted soils showed an additional division in endodermis. Observing a supernumerary cell division and cell differentiation in the developing root suggests that hydrocarbons modify the existing differentiation pattern. The endodermis is the innermost layer of the cortex and is characterized by the formation of Casparian bands in the anticlinal walls of its cells (Enstone et al., 2003). An extra cell layer might contribute to limiting exchanges between the cortex cells and the stele tissues reducing the import of hydrocarbons in the xylem flux. This might be another anatomical adaptation to pollutants like Casparian band and suberin lamellae thickening, increased suberization and lignification of endodermis cells (Zelko and Lux, 2004; Vance et al., 1980; Kalaji and Pietkiewicz, 1993; Shannon et al*.,* 1994; Schreiber et al., 1999).

Overall hydrocarbon residues found in sand samples extracted from the quagmire site profoundly modify plant growth and root architecture. ROS production and ROS detoxifying enzymes are induced in pea, most likely a consequence of physiological stress. We found that morphological and anatomical changes in pea roots exposed to anthropogenic pollution might be an adaptation to abiotic stress limiting the impact of the pollutant hydrocarbons on roots.

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THE FUNCTION OF PLANTATION FORESTRY IN LANDSCAPE CONNECTIVITY

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Abstract. Plantation forestry has been widely used for industrial purposes, creating vast extensions of culture forests. Although these plantations have a primary economic goal, they may indirectly serve other functions, such as landscape connectivity. Eucalypts are among the main plantation species used worldwide. In those areas in which they are not native, eucalyptus have been regarded as pernicious species for autochthonous forest and forest dwelling fauna. However, they may enhance connectivity between natural forest patches, thus favouring faunal dispersal. In Cantabria (Spain), *Eucalyptus globulus* were extensively planted in deforested areas mainly occupied by bushes and meadows. Here, we examine whether their massive introduction has modified landscape connectivity in the region. We used two indices based on graph theory and on the habitat availability concept (Integral Index of Connectivity and Probability of Connectivity), and applied them to the current forest network. Our results show how eucalyptus afforestation, principally based on temporary woodlands, has not improved forest connectivity in Cantabria significantly, though in the coast some of the new plantation areas may be locally important. Specific management actions targeted at these particularly relevant patches may enhance faunal dispersal and thus maintain biodiversity by reducing the fragmentation of these highly humanized areas.

Keywords: *Eucalyptus globulus, afforestation, graph-theory, fragmentation, forest management*

Introduction

Landscape connectivity is a key factor for the conservation of forest-dwelling species, since it guarantees genetic exchange among populations as well as increasing habitat availability (Taylor et al., 1993; Pascual-Hortal and Saura, 2006, 2008; Saura et al., 2011a; Blazquez-Cabrera et al., 2014). Deforestation reduces and fragments forest habitats, and consequently produces a loss of connectivity and increased habitat fragmentation (*sensu* Fahrig, 2003), which is a common spatial pattern in human-modified landscapes (Fischer and Lindenmayer, 2007; Harper et al., 2007).

The various industrial processes that use timber as a raw material have been, together with livestock and agriculture, a major factor in the deforestation of extensive areas worldwide (Anderson, 1990). Yet, logging has not always meant a loss of forest area, since timber demand has often extended forested areas by planting fast-growing species in areas which were already devoid of any tree cover. Indeed, in some countries during the last decades, the expansion of plantation forestry has reduced the rate of natural deforestation (Heilmayr, 2014). Despite this, modern forestry has radically altered the overall dynamics and structure of most forest ecosystems (Lindenmayer et al., 1999;

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Bergsten et al., 2013), especially when the establishment of forest plantations has involved the removal of native forest cover and its replacement by exotic species (Zurita et al., 2006; Aubin et al., 2008; Calviño-Cancela et al., 2012; Nahuelhual et al., 2012). More recently, plantations have occupied former traditional agricultural landscapes or mosaics, which are also valuable habitats for some species (Bennett et al., 2006; Santos et al., 2013).

It is generally accepted that natural forests offer better quality habitats for native forest species than plantation ones (Fabiao et al., 2002; Brockerhoff et al., 2008; Bauhaus et al., 2009; Calviño-Cancela and Rubido-Bará, 2013), since species are normally better adapted to the conditions of the native habitat in which they have evolved (Calviño-Cancela et al., 2012). Moreover, the rapid creation of plantations with characteristic intensive culture structures, added to their temporary nature implies ecological conditions that do not usually fulfill the habitat requirements of native fauna. For instance, short rotations in plantation forest can result in that vulnerable or more threatened species that require more stable forest networks may not be able to survive (Brockerhoff et al., 2003), or may be more easily predated upon than in habitats offering more permanent shelter conditions (Sanchez-Oliver et al., 2014). Despite this, plantations (even of exotic species), have been found to offer refuge and food for local faunas (Tellería and Galarza, 1990, 1991), and may even enhance the natural restoration of native forests when they acquire "old-growth" conditions (Humphrey, 2005) or when the physical and biological conditions of the native forest are modified positively (e.g. Geldenhuys, 1997).

These positive interactions especially occur when plantations are integrated in a landscape mosaic, together with the remaining natural forest network, thus benefitting both plantation purposes and the ecological services provided by the forest network (Brockerhoff et al., 2013). Moreover, this mosaic distribution can be useful for wildlife movements, by potentially expanding the availability of stepping-stones and movement paths in a given network (e.g. Nogués and Cabarga-Varona, 2014). Thus, plantation patches can enhance landscape connectivity acting as catalyzers of species movements between natural forest remnants (Hartley, 2002; Brockerhoff et al., 2008 and references therein), especially in those areas where natural forests are scarce, such as in densely urbanized areas (Kramer-Schadt et al., 2004). In this sense, patches which are more important from a connectivity perspective may need specific management practices such as protection figures or renaturalization measures (Nogués and Cabarga-Varona, 2014), so that the ecological services they provide are maintained.

The extent to which these patches may be utilized by the existing fauna will depend, among other factors, on their dispersal capabilities and the specific obstacles or permeability posed by the particular landscapes the patches are connecting (Fu et al., 2010; Gurrutxaga et al., 2011; Decout et al., 2012; Liu et al., 2014a; Hernández et al., 2015). In this sense, the threshold distance or the distance a species can cover in its dispersal movements is fundamental for species colonization at large scales, since it allows species migration and establishment in new habitat areas (Gil-Tena et al., 2013).

Eucalypt (*Eucalyptus globulus*) plantations were massively introduced in Cantabria (Northern Spain) in the late 40's and have since then expanded anthropically throughout the region due to increases in industrial activities. We analysed the role of these forest masses in landscape connectivity in Cantabria using two indicators based on graph theory. Specifically, we wanted to know whether these plantations have improved landscape connectivity in the regional forest network. We used a multi-species approach

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for terrestrial forest-dwelling species (see e.g. García-Feced et al., 2011) using various threshold distances.

Material and methods

Study area

Cantabria is a ca. 5,300 km^2 region located in the north of Spain (*Fig. 1*). Our choice of this region was not arbitrary, but motivated by its particular natural features, i.e.: its administrative borders present a fairly accurate adjustment to physical boundaries, since it limits to the north with the Atlantic Ocean, to the south with the Cantabrian Mountains, and to the east and west with the Deva and Agüera river basins. The mountainous relief and proximity to the sea determine a mild and humid Atlantic climate, with abundant orographic rainfall, where the predominant landscape should be temperate forests (composed principally by deciduous species such as *Quercus robur* and *Fagus sylvatica*, and evergreen ones as *Quercus ilex*). These natural forests are mainly located inland, and inhabited by some endangered faunal species such as the Iberian wolf (*Canis lupus signatus*), the brown bear (*Ursus arctos arctos*), or the Cantabrian capercaillie (*Tetrao urogallus cantabricus*), among others. Intensive forestry (almost entirely composed of *E. globulus*) and meadows characterize the landscape in coastal areas, which are also more densely urbanized and where the bulk of the road network lies.

Ancient forests disappeared due to various activities which took place from the modern age until the 19th century: ship construction, cannon and conventional foundries, mining, etc. Eucalyptus plantations began to be introduced during the second half of the 19th century (initially for mine lining and construction), especially in the vicinity of Torrelavega (Barreda, 1961). At this point, natural forests were already considerably reduced, especially in the coastal plain, and the dominant landscapes were rural areas consisting of farmlands and pastures, villages, and a deforested tree cover, restricted to a few small natural forest patches. The massive introduction of eucalyptus in the region was triggered by the operation of SNIACE (Spanish acronym for National Society of Industries for Applications of Spanish Cellulose), in 1944 (Nogués, 1987). The location of the cellulose plant in Torrelavega was mainly due to the favourable physical characteristics of the county of Torrelavega (high land availability, nearness to a river, and excellent communications). More importantly, the company was granted the possibility of replanting vast extensions with eucalypts through extensive land concessions. These plantations were mainly located between 0 and 350 metres of altitude, i.e.: principally along the coastal plain, and within the altitudinal limit of the eucalypts (MMA, 1966). Thus, the installation of the factory provoked a rapid increase in eucalypt plantation surface (annual 10.17% increase between 1953 and 1966, or from ca. 15,000 to 34,836 ha) (Barreda, 1961; Estadística Forestal de España, 1966), coinciding with an economic prosperity period which ended abruptly with the 1970's economic crisis. From 1960 to 2006, the annual increase rate in eucalyptus surface dropped to 1.04 %, and by 2006 eucalypts occupied 49,369 ha. Nowadays eucalypts dominate the coastal landscape in Cantabria, where they represent the main forest cover (*Fig. 1*).

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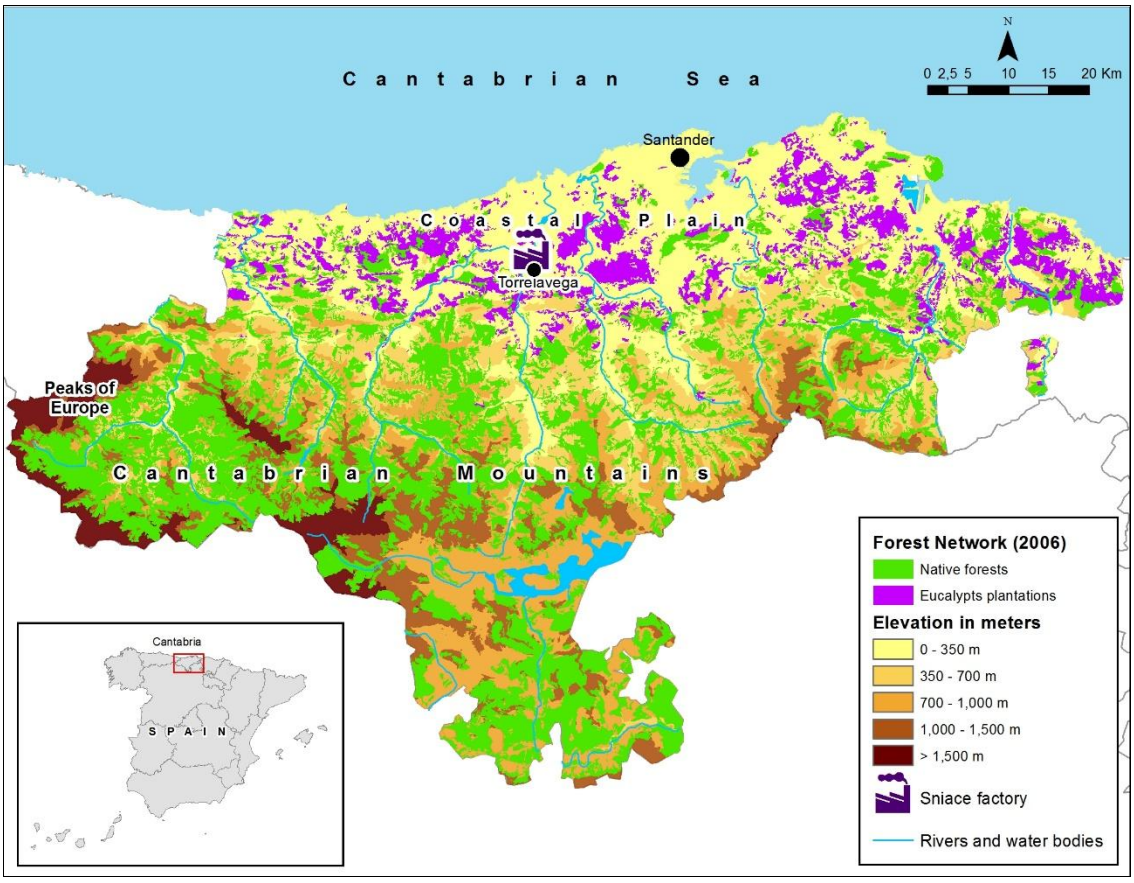

Figure 1. *Topography and forest cover (Spanish Forest Map) in Cantabria in 2006*

Data preparation

Forest cover data was based on availability and adequacy of the Spanish Forest Maps series to the aims of the study. We used the most recent (MMA, 2006) version of the Spanish Forest Map (1:50,000) as a cartographical base for our analyses. Given the complexity of the landscape network in our study area (i.e.: dense Atlantic forest cover, with numerous nodes and patches subdivided into multi-species polygons), and to avoid computational bottlenecks, we simplified the forest network by considering only those patches larger than 20 ha. Further, we aggregated adjacent patches (see e.g. Blazquez-Cabrera et al., 2014) and defined two types of forest patches: those in which native species accounted for more than 50% of the total patch cover (hereafter referred to as "natural"), and those in which eucalyptus predominated (>50%; hereafter referred to as "eucalyptus" patches). In this way, we reduced the number of patches from 1,130 to 519. This methodological adjustment resulted in networks with synthetic patches (natural vs. eucalyptus), and thus with a simplified structure and reduced species richness compared to the real ones. By considering only those patches ≥ 20 ha, we also minimized the possible effect of regular sequential forest logging due to eucalypt exploitation on patch availability, since usually smaller patches within these 20 ha are cut.

Landscape connectivity calculation

The role of plantations in landscape connectivity can be assessed using various connectivity metrics. Among them, those based in graph theory have been identified as

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the most adequate when analyzing connectivity related conservation problems at relatively large scales. These measures have modest data requirements and allow analyzing the potential functional connectivity for the fauna with reasonable detail (Calabrese and Fagan, 2004).

We used two complementary indices, the Integral Index of Connectivity (IIC) and the Probability of Connectivity (PC) index (Saura and Torné, 2009; García-Feced et al., 2011; Saura et al., 2011a; Gil-Tena et al., 2013). The integral index of connectivity uses a binary connection model of connectivity, while PC is probabilistic based, i.e.: it takes into account probabilities of direct dispersal between two habitat patches (Bodin and Saura, 2010). Thus, while the binary connection model considers any two patches as either connected or not (with no intermediate modulation, i.e.: graphs with unweighted links), the probabilistic model allows capturing the probability of direct dispersal between nodes as a decreasing exponential function of inter-patch distance (graphs with weighted links) (Saura and Pascual-Hortal, 2007a). Both indices integrate habitat patch area (or other patch attributes) and connections between different patches in a single metric (Pascual-Hortal and Saura, 2006). Therefore, the indices are based on two complementary concepts (Rubio et al., 2012), themselves useful for landscape connectivity analysis: graph theory, which allows modeling the relationships among nodes of a network (Urban and Keitt, 2001; Galpern et al., 2011), and the habitat availability concept, which considers a patch as a space where there is connectivity, implying that the larger the patch, the larger the connected area (Saura and Pascual-Hortal, 2007b; Pascual-Hortal and Saura, 2008).

The IIC and PC indices are computed following Saura and Pascual-Hortal (2007b) and Pascual-Hortal and Saura (2008), as:

$$IIC = \frac{\sum_{i=1}^{n} \sum_{j=1}^{n} \frac{a_i \cdot a_j}{1 + nl_{ij}}}{A_L^2} \quad \text{(Eq.1)}$$

$$PC = \frac{\sum_{i=1}^{n} \sum_{j=1}^{n} a_i a_j p_{ij}^*}{A_L^2} \quad \text{(Eq.2)}$$

Where a_i and a_j are the areas of patches i and j, respectively, A_L is the total landscape area (comprising both habitat and non-habitat patches), defined as the maximum product probability of all possible paths between patches i and j; nl_{ij} (IIC) is the number of routes in the shortest path (least-cost paths) between nodes i and j, and p_{ij} (PC) is the direct dispersal probability which includes the least-cost distances between each two patches at the different threshold distances established. Following Fu et al. (2010), we considered the same probability of dispersals between any two patches (i.e.: 0.5 for all threshold distances see below for more details on threshold distance).

Least-cost distances were calculated based on a friction matrix which was built according to the 2006 Spanish Forest Map (MMA, 2006). This friction layer or landscape matrix is a raster map where each cell represents the relative difficulty or cost of moving through that cell for a given species (Adriaensen, 2003). Least cost distances have been considered a better predictor of animal movement than Euclidean ones since they reproduce animal movements more realistically (Fu et al., 2010; Decout et al.,

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2012; Szabó et al., 2012; Ziólkowska et al., 2014). Due to processing capability limitations and considering computing time, we composed the landscape matrix at a cell size of 100 meters.

We proposed resistance values, based on our knowledge of the study area and values previously tested in the literature (e.g.: Fu et al., 2010; Gurrutxaga et al., 2011; Laita et al., 2011; Decout et al., 2012; Saura et al., 2011a; Clauzel et al., 2014). The lowest resistance values (0) were assigned to forest patches, which represent the node network in route calculation since they may function as a habitat or stepping stone (*Table 1*). Despite the fact that sometimes plantation forests pose greater resistance for species movements than native ones, considering the scale of our study and the overlapping of natural and planted species in several of our patches, we gave the same homogenous friction value to the whole forest network. In this way, we assumed homogeneity in the landscape connectivity role played by natural and plantation forests, assigning the same level of stepping-stone functionality to all types of patch, regardless of their composition. We also assigned low resistance values to bushes, given their presumable role as stepping-stone areas; and to agriculture land and meadows, which as an open field, also offer low resistance to dispersal. These landscape types are also often devoid of human-associated obstacles such as those appearing in urban areas. We assigned high resistance values to urban areas and water bodies (*Table 1*), to ensure that least-cost paths did not cross these barriers unless no other possibility of movement existed (see e.g. Decout et al., 2012; Ziólkowska et al., 2012). Finally, we added additional slope resistances to all cells, giving extra high values to those areas with very high slope conditions (*Table 1*). We edited all the land uses/covers in vector format and then converted them into a raster grid with 100 meters of cell size (see e.g. Ziólkowska et al., 2012). Management of the spatial data was performed using ArcGis 10.0 GIS software (ESRI ® ArcMap™ 10.0). Least cost paths were obtained using the ArcGis extension Linkage Mapper 1.0.2 (McRae and Kavanagh, 2011).

Table 1. *Resistance values assigned to the various landscapes to construct the movement resistance matrix*

Land cover / use	**Friction**
Forest	0
Bushes	4
Agriculture-meadows	8
Water bodies	100,000
Artificial (urban areas, roads)	10,000
Slopes-Values added to the remaining land covers	
0-15º	1
15-30º	2
30-45º	3
45-60º	4
>60º	250

Threshold distances were established using two criteria: their adequacy to the size of the study area, which is larger than 5,000 km^2 (100 km E-W by 50 km N-S), and the fact that our analysis was focused on terrestrial species with varying dispersal abilities such as medium and large sized mammals (e.g.: martens (*Martes martes*), badgers (*Meles meles*), wolves (*Canis lupus*), or bears (*Ursus arctos*). We chose values previously tested by other authors for these faunal groups (Fu et al., 2010; García-Feced et al.,

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2011; Gurrutxaga et al., 2011; Saura et al., 2011b; Zhao et al., 2014): 5,000, 10,000, 20,000, 30,000, 40,000, and 50,000 meters.

We based our connectivity analysis on the relative importance of the various patches found in our forest network. Following Pascual-Hortal and Saura (2006) and Saura and Pascual-Hortal (2007b), we used the simplified version of the IIC and PC indices, dIIC and dPC, respectively, which can be interpreted as the individual importance of every single forest patch in terms of percentage variation in the total degree of connectivity, as given by the IIC and PC indices.

Thus, patch importance is given by the expressions:

$$dIIC(\%) = \frac{IIC - IIC^i}{IIC} \cdot 100 \quad \text{(Eq.3)}$$

$$dPC(\%) = \frac{PC - PC^i}{PC} \cdot 100 \quad \text{(Eq.4)}$$

Where IIC or PC correspond to the overall index value calculated for a specific landscape (considering all habitat patches), and IIC^i or PC^i are the overall index values after removing patch i from the landscape. We applied the indicators to the current (2006) forest network excluding eucalypts. To evaluate the actual connectivity provided by each eucalypt patch to the natural forest network, we recalculated connectivity importance values using the "there are nodes to add" option (Conefor Sensinode 2.6, Saura and Torné, 2009). In this way, we calculated, on the one hand, the connectivity loss that the removal of each of the nodes of the original forest network (in our case, natural patches) would cause, and on the other, the connectivity improvement occurring as a result of the addition of each of the new nodes (i.e.: eucalypt patches), to the forest network. The importance of each potential (eucalypt) new node (dI) for improving landscape connectivity is calculated as:

$$dI(\%) = 100 \cdot \frac{I_{add} - I}{I} \quad \text{(Eq.5)}$$

Where I is the overall index value when all the initially existing nodes (excluding the eucalypts, in this case) are present in the landscape and I_{add} is the overall index value after the addition of each of the new eucalypt nodes to the landscape (Saura and Pascual-Hortal, 2007a).

Results

The complete (simplified) forest network was composed of 519 patches which occupied 37.57% (2,000.68 km^2) of the total study area (5,325 km^2), and concentrated mainly inland. Eucalyptus patches were distributed exclusively along the coast and amounted to 151, occupying 8.95 % of the total study area (476.63 km^2). The average eucalyptus patch size was 3.16 km^2, while that of natural patches was 4.14 km^2 (*Figs. 2 and 3*).

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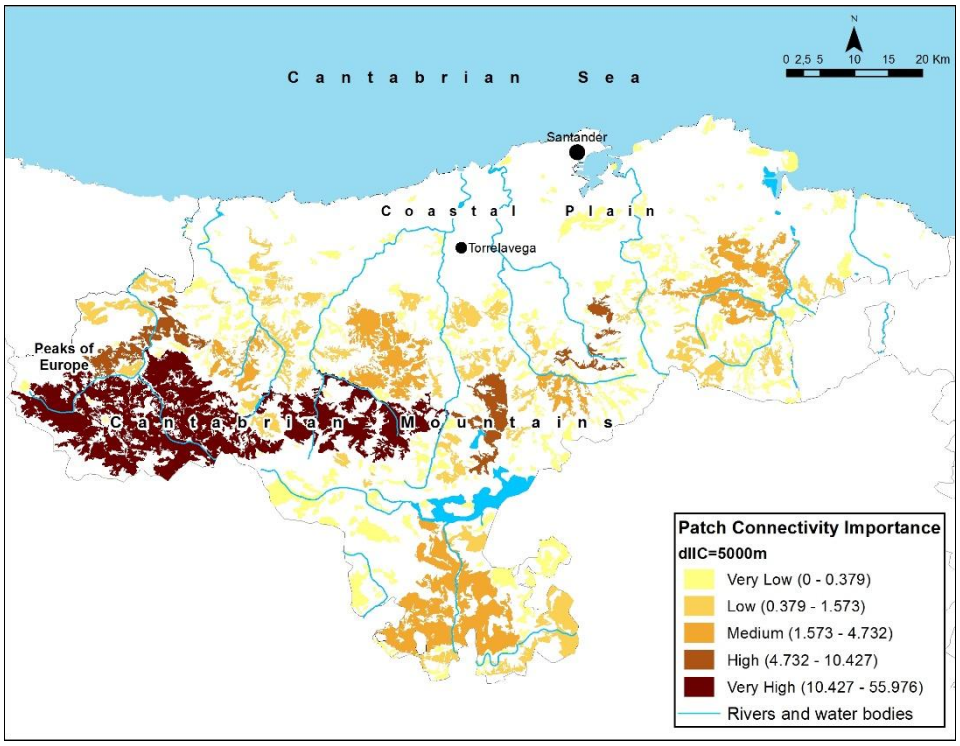

Figure 2. *Patch Connectivity importance (dIIC) at d=5000m in the natural network. Patch connectivity importance categories based on natural breaks*

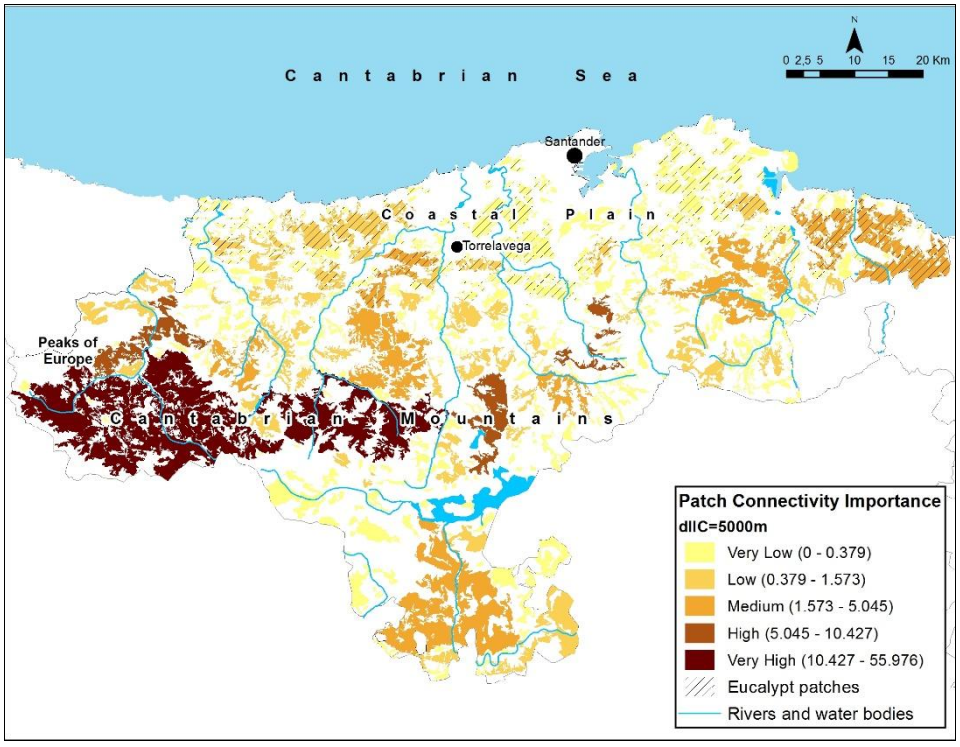

Figure 3. *Patch Connectivity importance (dIIC) at d=5000m using "there are nodes to add" for eucalypt patches (striped). Patch connectivity importance categories based on natural breaks*

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Results obtained with the dIIC and dPC indicators showed more or less the same trends for the whole patch network at the various threshold distances examined. The highest values in patch importance were obtained for the 5,000, 10,000 and 20,000 m distances and decreased thereafter (*Table 2*). The greatest relative increases in link number or connections between existing patches occurred between d=5000 and 20,000 m (*Table 3*).

Table 2. *Results of the dIIC and dPC indices for (1) the network without eucalypt patches, (2) natural and eucalypt patches and, (3) the added eucalypt patches, at the various threshold distances examined*

		Threshold distance (m)					
dIIC		**5000**	**10,000**	**20,000**	**30,000**	**40,000**	**50,000**
(1) Without eucalypt patches	Max	55.98	50.73	42.96	40.87	40.64	39.77
	Mean	0.61	0.54	0.53	0.51	0.51	0.51
	Sum	225.83	199.20	193.45	189.51	188.55	188.04
(2) Natural + eucalypt patches	Max	55.98	50.73	42.96	40.87	40.64	39.77
	Mean	0.48	0.45	0.46	0.46	0.46	0.46
	Sum	248.49	235.52	238.06	237.24	239.38	240.49
(3) Eucalypt patches	Max	5.05	5.28	6.02	6.00	6.65	6.80
	Mean	0.15	0.24	0.30	0.32	0.34	0.35
	Sum	22.67	36.32	44.62	47.73	50.83	52.45
dPC		**5000**	**10,000**	**20,000**	**30,000**	**40,000**	**50,000**
(1) Without eucalypt patches	Max	55.04	48.62	43.15	40.80	39.51	38.69
	Mean	0.75	0.75	0.70	0.66	0.64	0.62
	Sum	276.32	274.44	257.36	243.85	234.72	228.35
(2) Natural + eucalypt patches	Max	55.04	48.62	43.15	40.80	40.64	38.69
	Mean	0.57	0.60	0.59	0.57	0.46	0.55
	Sum	297.79	309.71	304.73	296.13	239.38	284.83
(3) Eucalypt patches	Max	5.44	5.14	6.43	7.45	8.02	8.38
	Mean	0.14	0.23	0.31	0.35	0.36	0.37
	Sum	21.47	35.27	47.37	52.29	54.89	56.48

There were differences in patch importance between patches of native and plantation forest (*Fig. 3*). The most connected landscape units were three large native patches of natural forest (*Figs. 2 and 3*), which accounted for less than 1% of the total number of patches. They concentrated 39.87% (dIIC) and 37.98% (dPC) of the total importance at a threshold distance of 5,000 m, respectively, and similar values at the remaining threshold distances (*Table 2*). The remaining forest network (whether natural or eucalyptus dominated) was mainly composed of patches attaining low connectivity values (mean values lower than 1). Only some patches, and among them some eucalyptus ones located in the eastern and western coastal fringe, reached intermediate importance values of up to 5 or 6, at specific threshold distances (*Table 2, Fig. 3*).

Table 3. *Number of links between nodes considering the natural and eucalypt patches*

d=m	# links natural+eucalypt patches network	% increment
5000	1218	-
10,000	2804	130.21
20,000	8085	188.34
30,000	15,669	93.80
40,000	24,693	57.59
50,000	34,473	39.61

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The maximum values in patch importance obtained for the natural network using the "there are nodes to add" option (i.e.: adding the eucalypt patches one by one), at all dispersal distances were equal to those obtained without eucalypts (*Table 2*). However, the addition of eucalypts did cause a slight decrease in mean values, as well as in the overall sum of connectivity importance (*Table 2*), which was higher when the plantation patches were included. Considering only the results obtained for the eucalypt patches (*Table 2*), they did not vary much at the various dispersal distances considered, but showed the inverse trend to that of the global network, i.e.: connectivity importance values increased the larger the distance between patches.

Discussion

Connectivity rises if for a given amount of habitat the connection status is improved, or for a given connection status, the amount of habitat increases (Fahrig, 2003; Laita et al., 2011). Since the expansion of eucalypts in Cantabria occupied previously unforested areas, indirectly at least, this should have entailed an improvement of forest connectivity in the area. However, our results indicate that the contribution of plantation forestry to landscape connectivity in Cantabria has been scarce and mainly confined to the coastal areas. Here, their importance may be even higher than that shown by our results, which were always calculated relative to the complete regional forest network. Indeed, these areas are highly fragmented due to human activities (see e.g. Gurrutxaga et al., 2011) and devoid of any large forest masses, other than the eucalypts and some very important relict patches of autochthonous vegetation,

In our study, the key connectivity providers were large natural forest patches located in the central-inland area of the region. Eucalypt patches were numerous but the majority was very small and located on the northern perimeter of the region (coastal area), which reduced their importance as connectivity providers within the whole forest network. The most important eucalypt patches were some relatively large ones connecting many routes or links, also with the isolated natural patches of high natural value close to the coast (*Fig. 4*). The dispersal of species inhabiting these isolated natural patches is most probably enhanced by the existence of the eucalypts (see e.g. Nogués and Cabarga-Varona, 2014).

Connectivity assessments should take into account the scale-dependent nature of habitat networks, since habitat patches can be defined at different spatial scales (from single patches to aggregations of them) (Blazquez-Cabrera et al., 2014). In our case, the severe forest network simplification conducted prior to the analyses may have had a bearing in our results, since a few very large patches agglutinated most of the connectivity importance. Thus, assessments using habitat availability and graph theory based indicators, such as dIIC and dPC, should always weigh the results obtained considering that they are highly dependent on the amount of surface which is connected, and thus in the size of the patches being evaluated. The prevalence of size dependent influences is particularly relevant in regions characterized by large and densely distributed patches, while in regions with low habitat cover the relative importance of the position of the patches within the forest network becomes more important (Rubio and Saura, 2012; Szabó et al., 2012; Zhao et al., 2014; Ziólkowska et al., 2014). Despite this, in our study the same patches were always identified as being the key connectivity providers, regardless of the landscape configurations and scales examined, and using

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two different indices (dIIC and dPC), which is in agreement with other studies in which similar patterns were found (e.g. Blazquez-Cabrera et al., 2014).

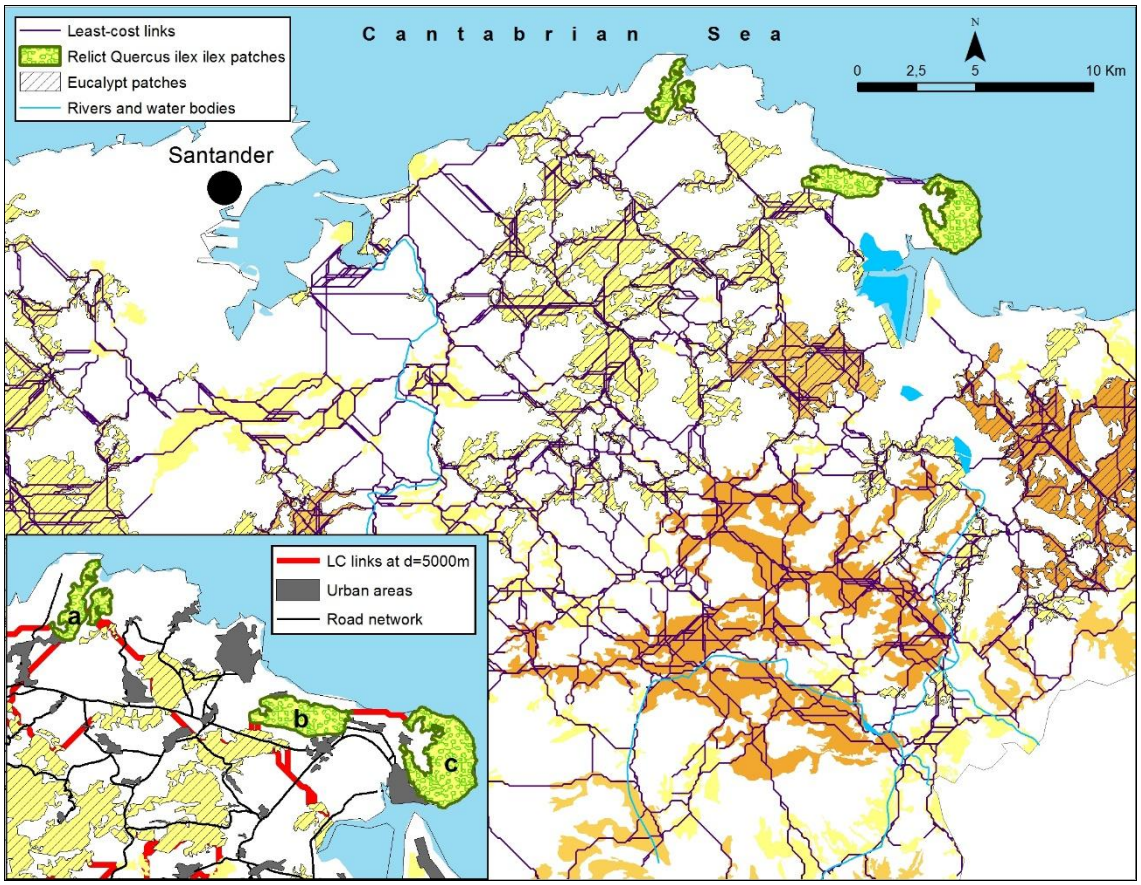

Figure 4. *Detail showing the relict Quercus ilex ilex patches (a,b,c) located in coastal areas and their connections (least cost links at all threshold distances) to the inland natural network through eucalypt patches. Note how for b or c the only possible connection route is through a single eucalypt patch. Patch Connectivity importance values and symbol categories same as in Figure 3*

In our study, the highest connectivity importance results were obtained at shorter distances when examining the natural forest network. This can be related with the denser structure of the natural network, in which the availability of patches and routes among them increased with distance, thus distributing importance so that individual patches obtained lower values. Also, it implies that the natural network is more vulnerable to connectivity alterations at shorter dispersal distances, which may be especially important for poor dispersers if nearby patches disappear or are modified substantially. Conversely, eucalypt patches obtained individually the highest importance values at large threshold distances, which might be explained by the fact that eucalypt patches were sparsely distributed and some (regardless of whether they were close or at longer distances), concentrated a large number of all possible dispersal routes, thus agglutinating importance for connectivity.

All in all, our results suggest that the best dispersers might be the most benefited by the introduction of plantations, since they may be able to use the patches as stepping

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stones to move among inland natural areas. Conversely, individuals with lower dispersal ability can use eucalypt patches to move among and colonize small natural remnants existing in the coast, but they probably have problems to cross unforested areas separating them from natural forest areas in more interior locations because of their high degree of humanization (see e.g. Di Giulio et al., 2009; Fu et al., 2010; Macpherson et al., 2011; Decout et al., 2012; Yu et al., 2012; Liu et al., 2014b).

Our study identified a series of patches (both natural and eucalypt dominated) as more important in terms of connectivity, indicating that the used indices may be relevant when attempting to conduct sustainable forest management schemes, in which the ecological importance of certain patches is considered. Once identified, those plantation patches which are more important for wildlife fauna dispersal (Saura et al., 2011b), can be preserved and/or restored into natural forest or at least managed considering wildlife needs and cycles (kept uncut or avoiding cleancut practices, respecting reproduction or dispersal periods, etc.). In this sense, it would also be interesting to identify which faunal species have been more affected (both positively and negatively) by the plantations, and which use them as habitat or only as stepping-stone. Further management measures could involve constructing mosaics of native and plantation patches, resulting in non-monospecific forest cultures (Carnus et al., 2006), which minimize biodiversity exchange and resilience. Eventually, many of these plantation areas could be restored into natural forest, especially in areas such as Cantabria, where the industrial activities causing the plantations are disappearing or decreasing their activity (Bowen et al., 2007; Onaindia et al., 2013; Hernández et al., 2015). These issues pose both a challenge and an opportunity for managers and practitioners intending to restore landscape connectivity in forested areas (García-Feced et al., 2011), since they introduce concepts such as ecological or environmental value of plantation patches which up until now have only been considered in terms of economic benefits.

Maintaining and restoring landscape connectivity is currently a central concern in ecology and biodiversity conservation (Saura and Torné, 2009; Tambosi et al., 2014; Zhao et al., 2014). We conducted an analysis of landscape connectivity by integrating methods of graph-based habitat availability (reachability) metrics and least-cost path matrices (Rubio and Saura, 2012; Ziólkowska et al., 2012), that allow the identification of key nodes providing connectivity. This is a valuable contribution for forest management tools, especially when the alterations of human disturbances to specific patches rendering some unsuitable, or reducing their ability to provide appropriate habitats for wildlife, are considered (Reza et al., 2013). In this way, forests managers and land planners can identify where management and conservation efforts should be concentrated and prioritized.

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EVALUATION OF GROWTH IN PIKE (*Esox lucius* L., 1758) USING TRADITIONAL METHODS AND ARTIFICIAL NEURAL NETWORKS

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Abstract. The present study was performed to assess the population structure and growth of pike in Mogan Lake using length-weight relationships, von Bertalanffy equations and artificial neural networks between February 2013 and March 2014. The age of *Esox lucius* caught in Mogan Lake ranged between I to VII years. The fork length of the fish ranged from 27.5 cm to 70.0 cm, and the body weight of the fish ranged from 200 g to 2820 g. The von Bertalanffy growth lengths were 130.30 for females, 122.70 for males and 126.50 for all individuals. The von Bertalanffy growth weights were 18889 for females, 14881 for males and 16688 for all pike. The results obtained using the artificial neural networks and regression technique were compared to those obtained using the growth rate of the fish caught from the natural environment and von Bertalanffy growth model. Artificial neural networks are an alternative to von Bertalanffy growth models.
Keywords: *growth, artificial neural networks, Esox lucius, von Bertalanffy, Mogan Lake*

Introduction

Pike (*Esox lucius* L., 1758) is a widely distributed species of fish in Turkey and one of the most valuable fish in inland waters (Çubuk et al., 2005). It is an essential element of the ecosystem as a piscivore and can tolerate a wide range of environmental conditions (Casselman and Lewis, 1996). This species is of high economic importance in the fisheries of Mogan Lake. Despite its importance, few studies have been conducted on the pike population in Mogan Lake.

There is a large body of literature regarding the growth properties of pike in various water reservoirs. Tanyolaç and Karabatak (1974) examined its growth properties in Mogan Lake, Aksun (1987) in Karamık Lake, Karabatak (1993) Akşehir in Lake, Treer et al. (1998) in Croatian Reservoir, Altındağ et al. (1999) in Kesikköprü Dam Lake, Lorenzoni et al. (2002) in Trasimeno Lake, Küçük and Güçlü (2004) in Çapalı Lake, Çubuk et al. (2005) in Karamık Lake, Gaygusuz et al. (2006) in Terkos Dam Lake, Erdem et al. (2007) in Uluabat Lake, Uysal et al. (2008) in Işıklı Lake, Yağcı et al. (2009) in Işıklı Dam Lake, Ziliukiene and Ziliukas (2010) in Lake Rubikiai, and Moslemi-Aqdam et al. (2014) in Anzali Wetland.

Much research has been performed regarding the prediction of future data using artificial neural networks (ANNs) because they exhibit better results than traditional methods found in the literature (Hill et al., 1996; Hamzacebi and Kutay, 2004;

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Suryanarayana et al., 2008; Türeli Bilen et al. 2011; Christiansen et al., 2014; Benzer, 2014). These studies showed that neural network models are significantly better than traditional statistical and human judgment methods when forecasting monthly and quarterly data (Desilets et al., 1992).

The present study investigated the growth of *Esox lucius* and provides information regarding the population structure (age, growth and sex ratio) of pike in Mogan Lake between February 2013 and March 2014.

Materials and methods

Study site

Mogan Lake is located approximately 20 km south of Ankara, the capital of Turkey, and lies within the coordinates 39º44'40" N and 39º47'45" N latitudes and 32º46'30" E and 32º49'30" E longitudes (*Figure 1*). It is near Gölbaşı town, which has become economically and socially developed and has had an increasing population and settlement in recent years. A large number of commercial establishments, such as restaurants, social clubs, and tea gardens, as well as summer resorts, have been built around the lake, which has become a popular site for sports, fishing, sailing, and rowing (Anonymous, 1989).

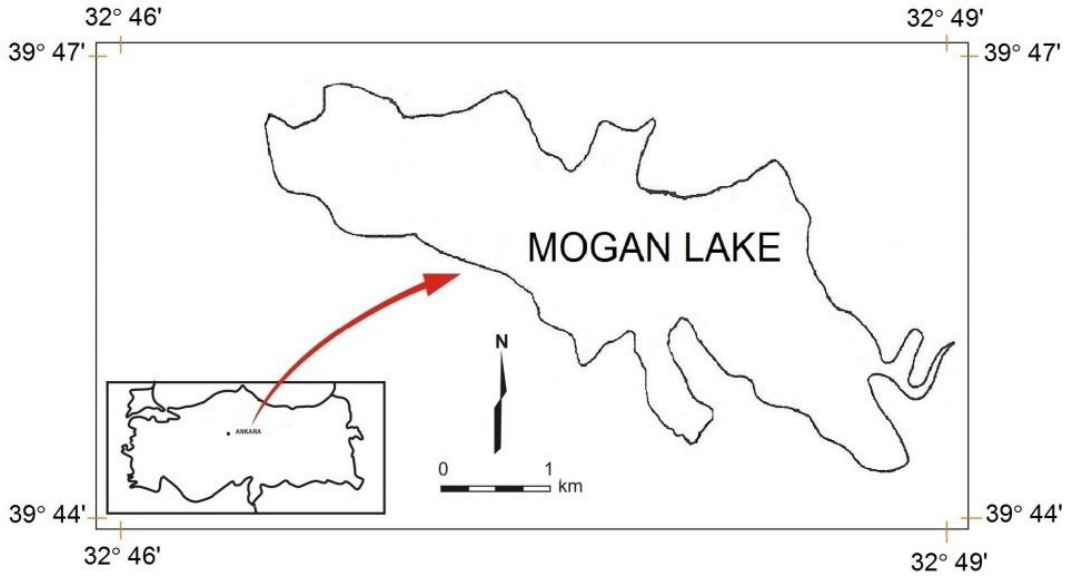

Figure 1. *Map of Mogan Lake*

Sampling / Data Collection

Fish samples (*Esox lucius* L., 1758) were collected from Mogan Lake. During the study, 431 fish specimens were caught between February 2013 and March 2014. The fish obtained from the lake were immediately transported to the laboratory. The length and weight (min-max) of the fish were 275 – 700 mm and 200 – 2820 g, respectively. Scales were sampled from each specimen to identify their age according to the method of Lagler (1966).

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Length-weight relations

The length-weight relationships were estimated from the formula $W = a L^b$, where W is total body weight (g), L is the total length (mm), and a and b are the coefficients of the functional regression between W and L (Ricker, 1973). The equation was log transformed to estimate the parameters 'a' and 'b'. When b is equal to three (3), an isometric growth pattern occurs, but when b is not equal to 3, an allometric growth pattern occurs, which is positive if >3 or negative if <3.

Von Bertalanffy equations

Growth was estimated using the von Bertalanffy growth curve model (Eq.1 and Eq.2) according to Sparre and Venema (1992).

$$L_t = L_\infty[1 - e^{-K(t-t_0)}] \quad \text{for length} \qquad \text{(Eq.1)}$$

$$W_t = W_\infty[1 - e^{-K(t-t_0)}]^b \text{ for weight} \qquad \text{(Eq.2)}$$

where L_t = the fork length (cm) at age t, L_∞= the asymptotic length (theoretical maximum length), k = the Brody growth coefficient (proportional to the rate at which L_∞ is reached), t = the age (years), t_0 = the age at zero length, e is the base of natural log (2.71828), W_t is the weight of the fish in g at age t, W_∞ is the asymptotic weight of (theoretical maximum weight) the fish in g and b is the constant in the length–weight relationship. The von Bertalanffy growth parameters were estimated for males and females independently, as well as for both sexes combined.

Artificial Neural Networks

The ANN provides a better model because it produces better predictions for lower values, and the normality of the residuals and their independence from the predicted variables are also improved. Several authors reported greater performances of ANNs compared to linear regressions (Sun et al., 2009). ANNs have another advantage in that the ANN modelling approach is fast and flexible (Brosse et al., 1999). In this study, the ANN is a new and alternative approach for predicting the growth and weight of pike compared to traditional methods.

ANNs are computational systems that simulate biological neural networks and can be defined as a specific type of parallel processing system based on distributional or connectionist methods (Hopgood, 2000). They appear to be more functional for predicting the future. ANNs can reveal the power relationships between unknown and unidentified data. By contrast, ANNs are simulations of biological nervous systems using mathematical models. They are networks with simple processor units, interconnections, adaptive weights and scalar measurement functions (e.g. summation and activation functions) (Rumelhart et al., 1986).

During the training of the network, the input data and input-output relationship between the learning of the network is provided. This method, generally called supervised learning, is a preferred method (Haykin, 1999). The supervised learning method trained with the network structure (back-propagation networks) was used to solve problems in this study.

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The sum squared error (SSE) and mean absolute percentage error (MAPE) were used in the study as the two performance criteria (Matlab, 2006). SSE was used as a criterion to determine training during the training of the network. In addition, comparisons were made involving more than one method because the MAPE of each provides information about the average relative size of their errors.

SSE and MAPE are described by equations Eq.3 and Eq.4, respectively.

$$SSE = \sum_{i=1}^{n} e_i^2 \quad \text{(Eq.3)}$$

$$MAPE = \frac{1}{n} \sum_{i=1}^{n} \left| \frac{e_i}{Y_i} \right| * 100 \quad \text{(Eq.4)}$$

where Y_i is the actual observation value, e_i is the difference between the actual value and the prediction value, and n is the number of total observations.

Data and identification models

The Neural Network Toolbox of MATLAB was used for the ANN calculations. This study was performed using 431 *Esox lucius* (157 females, 235 males and 39 immature individuals) caught between February 2013 and March 2014 in Mogan Lake. The data were divided into three equal parts, training, validation, and test sets. The MATLAB functions were used for "training", "testing", and "validation". They were used randomly, with 70% for training, 15% for testing, and 15% for the validation of fish.

Results

There were 36.43 % females, 54.52 % males and 9.05 % immature fish. The differences among different age groups were not statistically significant ($p > 0.05$). The age ranged between I to VII years.

The male:female ratio was 1.49:1 for the general population. The prevailing climatic conditions during the study were predicted to affect the sexual maturity, breeding, breeding time, egg development, and egg hatching (Hellawell, 1971).

Most of the samples studied in this study belonged to the III-year age group (*Table 1*). The females were longer and heavier than the males in the III, IV and VI-year age groups.

Table 1. *Age structure, parameters of the length–weight relationship (a and b), growth (L_∞, K, t_0) and CF of Esox lucius.*

Authors			Length Min-max	Age Range	a	b	r^2	L_∞ (cm)	W_∞ (g)	K ($year^{-1}$)	t_0 (year)	CF
Karabatak (1993)	♀	L cm	33.66-67.10	1-7	2.71	3.38	-	136.46	320.88	0.062	-3.59	0.83
	♂				2.22	3.08		160.46	379.00	0.048	-4.06	0.81
Treer et al. (1998)	♀♂	-	-	-	-	-	-	142.0	-	0.140	0.500	-
Altındağ et al.(1999)	♀	L cm	25.2-53.4	0-5	2.59	3.36	-	114.76	21942.81	-0.075	-3.349	0.863-
	♂				2.21	3.10		145.49	31915.62	-0.056	-3.318	1.005

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Lorenzoni et al. (2002)	♀♂	FL cm	-	-	-	3.04	0.99	162.76	-	0.089	0.291	-
Küçük And Güçlü (2004)	♀♂	-	-	-	0.226	2.719		48.84	-	0.416	1.449	0.877
Çubuk et al. (2005)	♀ ♂ ♀♂	FL cm	22.50-50.0	1-5	0.0060 0.0063 0.0059	3.10 3.07 3.10	-	117.0 123.1 121.6		0.089 0.098 0.092	0.74 0.74 0.75	-
Gaygusuz et al. (2006)	♀♂	SL cm	28.9-54.1	-	2.8931	1.0489	-	-	-	-	-	-
Erdem et al. (2007)	♀♂	FL cm	30.11-54.85	1-5	-	-	-	-	-	-	-	0.90
Uysal et al. (2008)	♀ ♂	FL cm	23-49.4	1-6	-	-	-	117.8 118.0	18003 21983	0.067 0.067	2.36 1.97	-
Ziliukiene and Ziliukas (2010)	♀♂	-	26.50-107.0	2-12	0.06	3.02	-	131.7	14870	0.150	0.040	-
Moslemi-Aqdam et al. (2014)	♀♂	FL cm	18.00-72.50	-	0.0037	3.21	0.98	-	-	-	-	0.949-1.106
Kahraman et al. (2014)	♀♂	Tl cm	40.2-70.3	-	0.0659	2.481	-	-	-	-	-	-
Present Study	♀ ♂ ♀♂	FL mm	29.23-63.50	1-7	0.00009 0.00007 0.00010	2.59 2.64 2.58	0.92 0.97 0.94	130.30 122.70 126.50	18889 14881 16688	0.039 0.043 0.044	0.77 0.69 0.65	0.8408 0.8113 0.8359

The length of a pike with known weight and the weight of a pike with known length were calculated with the logarithmic length-weight relationship using the regression coefficient of length and weight ($W = a L^b$) (*Table 2* and *Figure 2*).

Growth equations for the length of a pike at any age were calculated with the Von Bertalanffy growth equation using the age-length relationship growth data according to sex and length (*Table 3*). The proportional growth in both sexes and the age-length relationship curves are shown in *Figure 3 - 4*.

Table 2. *Length-weight relationship parameters, equations and correlation coefficients*

Sex	**Length-weight relationship parameters**	**Correlation coefficients (R)**
Female	$W = 0.00009657 \times L^{2.5926}$ Log W = -4.0152 + 2.5926 Log L	0.92
Male	$W = 0.00007 \times L^{2.6389}$ Log W = -4.1549 + 2.6389 Log L	0.97
Female + Male	$W = 0.00010328 \times L^{2.5773}$ Log W = -3.986 + 2.5773 Log L	0.94

Table 3. *von Bertalanffy growth parameters and equations*

Sex	**Growth parameters**			
	L_∞	W_∞	**K (year^{-1})**	t_o
Females	130.30	18889	0.039	-0.772
Males	122.70	14881	0.043	-0.693
Females + Males	126.50	16688	0.044	-0.654

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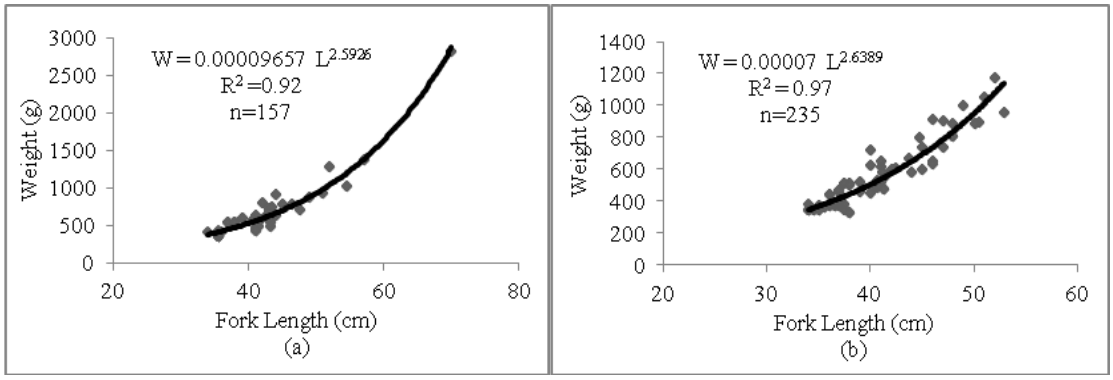

Figure 2. *Length–weight relationships in females (a) and males (b)*

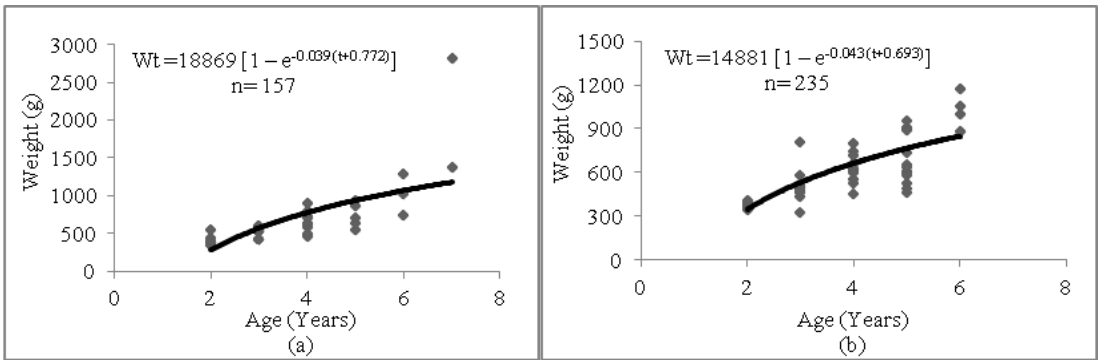

Figure 3. *Age–weight relationships in females (a) and males (b)*

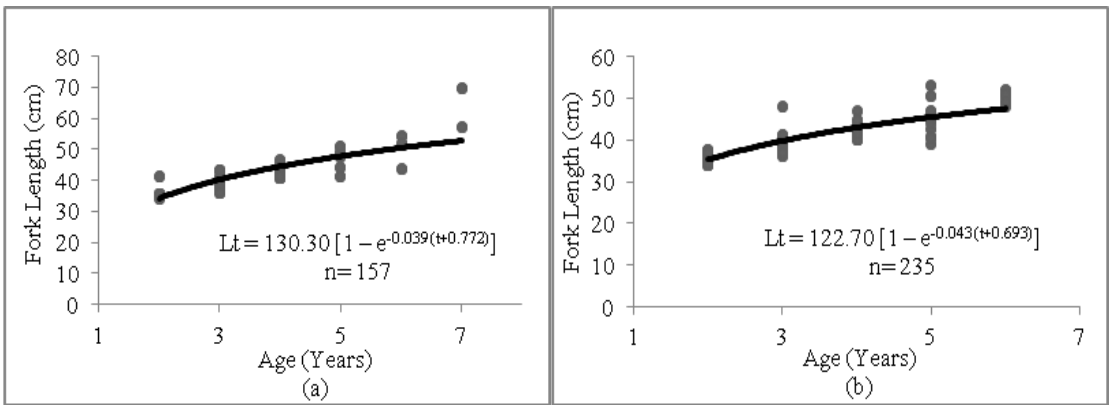

Figure 4. *Age–length relationships in females (a) and males (b)*

A multilayer feed-forward neural network was used for the ANN. A schematic representation of a typical ANN is shown in *Figure 5* and consists of 4 interconnected layers of 'nodes' or 'neurons', including an input layer containing 1 node per independent variable (i.e. ages, length, weight and sex of pike), a hidden layer, and

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finally, an 'output layer' with 1 node (i.e. the weight or length of the fish samples). *Figure 6* shows a graphical presentation of the fit between the actual and predicted values.

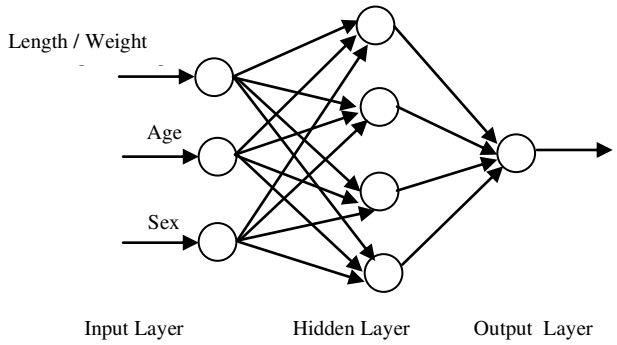

Figure 5. *An ANN consisted of an input layer with 3 nodes, a hidden layer, and an output layer with 1 node to be predicted*

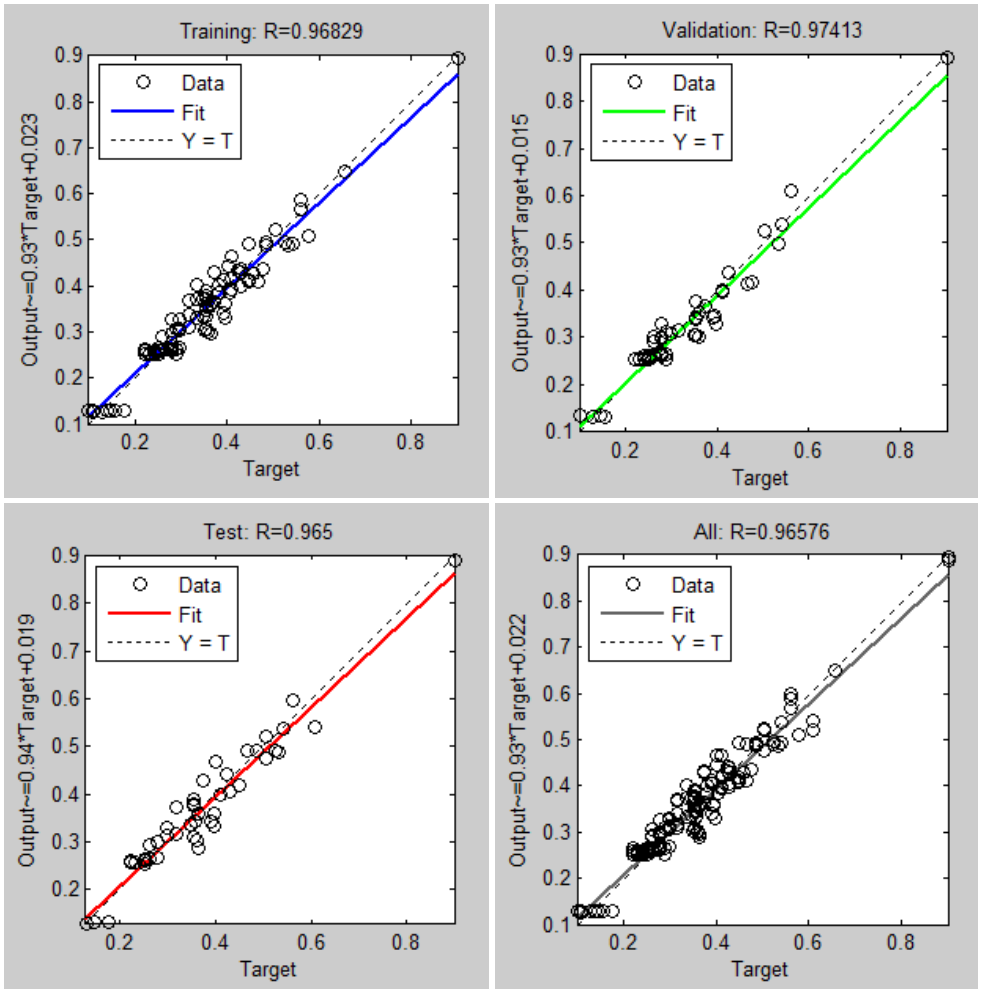

Figure 6. *Relationship between the actual data and forecast values for length.*

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The length-weight relationships for the *Esox lucius* living in Mogan Lake were $W= 0.00009657 \times L^{2.5926}$ for females, $W = 0.00007 \times L^{2.6389}$ for males and $W = 0.00010328 \times L^{2.5773}$ for females+males. The b value for the female-male group was 2.5773. The von Bertalanffy growth equations were $L_t = 130.30\ [1 - e^{-0.039\ (t+0.772)}]$ for females, $L_t = 122.70\ [1 - e^{-0.043\ (t+0.693)}]$ for males and $L_t = 126{,}50\ [1 - e^{-0{,}044\ (t+0{,}654)}]$ for all pike.

The condition factor (CF) of *Esox lucius* varied from 0.6014 to 1.1250 in males, from 0.6152 to 1.0798 in females and from 0.06014 to 1.1250 in all pike. The CF in both sexes was 0.8359 (*Table 1*).

Discussion

There were 36.43 % females, 54.52 % males and 9.05 % immature fish. The ages of the pike caught from Mogan Lake ranged between I to VII years. According to Nikolskii (1980), the age distribution is affected by various ecological factors and the prime factor is the availability of food. Factors such as the mortality rate and hunting pressure also play an important role. The age distribution in Mogan Lake is similar to that found by Tanyolaç and Karabatak (1974) in Mogan Lake.

The male:female ratio was 1.49:1 for the general population. This ratio was 1.22:1 in Kesikköprü Dam Lake (Altındağ et al., 1999), 1.10:1 in Apolyont (Uluabat) Lake (Erdem et al., 2007), 1.76:1 in Trasimeno Lake (Lorenzoni et al., 2002) and 1.68:1 in Işıklı Lake (Uysal et al., 2008). The climatic conditions during the study likely affected the sexual maturity, breeding, breeding time, egg development and egg hatching (Hellawell, 1971).

Although Altındağ et al. (1999), Erdem et al. (2007), and Uysal et al. (2008) found that most of the samples were in the II-year age group, Çubuk et al. (2005) found that most of the samples were in the I-year age group in their study site.

Uysal et al. (2008) found that females were longer than males in the I, II and III year ages groups, whereas females were heavier than males in the I, II, III and V-year age groups. Erdem et al. (2007) found that females were longer than males in the I, II, IV and V-year age groups, whereas males were longer than females in the III-year age group. It was found that females were heavier than males in the I, II, III and IV-year age groups (Erdem et al., 2007). Çubuk et al. (2005) and Küçük and Güçlü (2004) found that females were longer than males in all age groups.

The length-weight relationships for the *Esox lucius* living in Mogan Lake were $W= 0.00009657 \times L^{2.5926}$ for females, $W = 0.00007 \times L^{2.6389}$ for males and $W = 0.00010328 \times L^{2.5773}$ for females+males. The b value for the female-male group was 2.5773. The b values determined by different researchers were generally larger than the values determined in this study (*Table 1*). Kahraman et al. (2014) reported similar b values (2.481).

The slope (b) value of the length–weight relationship in both sexes was 2.58. The b value is often 3.0 and generally between 2.5 and 3.5. As the fish grows, the changes in weight are relatively greater than the changes in length due to approximately cubic relationships between fish length and weight. The b values in fish are species specific and vary with sex, age, seasons, physiological conditions, growth increment and nutritional status of the fish (Ricker, 1975; Baganel and Tesch, 1978).

Variations in fish growth in terms of length and weight can be explained as an adaptive response to different ecological conditions (Nikolsky, 1963). The von Bertalanffy growth equations were $L_t = 130.30\ [1 - e^{-0.039\ (t+0.772)}]$ for females, $L_t =$

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122.70 [1 - $e^{-0.043\ (t+0.693)}$] for males and L_t = 126,50 [1 - $e^{-0,044\ (t+0,654)}$] for all pike. The "K" value, known as the Brody coefficient, was 0.0772 in females and 0.043 in males. Munro and Pauly (1983) state that the "K" value is an indicator of the growth performance of the species. L_∞ was similar to that of Çubuk et al. (2005) and Ziliukiene and Ziliukas (2010), but was different from other studies (Karabatak, 1993; Treer et al., 1998; Altındağ et al., 1999; Lorenzoni et al., 2002; Küçük and Güçlü, 2004, Uysal et al., 2008).

The condition factor (CF) of *Esox lucius* varied from 0.6014 to 1.1250 in males, from 0.6152 to 1.0798 in females and from 0.06014 to 1.1250 in all pike. The CF in both sexes was 0.8359 (*Table 1*). The results are consistent with several earlier reports (Karabatak, 1993; Küçük and Güçlü, 2004), although they differed from studies performed by Altındağ et al., 1999 and Erdem et al., 2007. The condition factor provides important information about the effect of feeding habits and the population density of the fish on their growth and gonad development (Weatherley, 1972).

Gutierrez-Estrade et al. (2004), Türeli Bilen et al. (2011) and Benzer (2014) performed similar studies. Traditional growth models were also used for predictions together with ANN. The values obtained for the actual, ANNs, W-L relationship and von Bertalanffy data are presented in *Table 4*.

Traditional methods of statistical analysis (i.e. linear regression models, both single and multiple) may be inadequate for quantification (Maravelias et al., 2003). ANNs offer a promising alternative to traditional statistical approaches for predictive modelling when non-linear patterns exist (Joy and Death, 2004). Recently, ANNs have been used in biology and various disciplines of aqua-cultic ecology more than in physical or chemical sciences. Most of these studies demonstrated that ANNs perform better than classical linear regression and generalized additive models (Brosse et al., 1999).

Table 4. *Predicted and calculated values for ANNs, the length-weight relationship and von Bertalanffy*

Gender	Age	ACTUAL DATA		ANNs FORECAST		ANNs MAPE (%)		W - L Relationship CALCULATED		W - L Relationship MAPE (%)		von Bertalanffy CALCULATED		von Bertalanffy MAPE (%)	
		L	W	L	W	L	W	L	W	L	W	L	W	L	W
A	1	29.23	240.00	29.03	245.56	0.692	2.264	29.51	234.00	0.954	2.500	35.72	291.66	22.186	21.525
F		35.91	395.81	35.96	384.52	0.153	2.936	35.52	406.83	1.072	2.784	41.11	528.62	14.50	33.55
M	2	35.87	369.87	36.25	370.36	1.054	0.132	35.28	386.21	1.639	4.418	39.12	430.33	9.08	16.35
A		35.88	378.36	36.16	380.61	0.772	0.591	35.21	396.98	1.870	4.921	39.62	456.66	10.43	20.69
F		39.54	531.00	39.38	537.92	0.416	1.286	39.79	522.53	0.622	1.595	44.52	676.82	12.59	27.46
M	3	39.31	508.91	39.92	510.94	1.533	0.397	39.82	491.79	1.303	3.364	42.64	624.61	8.48	22.73
A		39.41	518.38	39.67	523.75	0.658	1.025	39.79	505.57	0.967	2.471	43.36	672.22	10.03	29.68
F		42.81	667.38	43.51	677.09	1.613	1.434	43.45	641.81	1.500	3.831	47.80	843.72	11.67	26.42
M	4	41.98	632.10	41.94	584.19	0.100	8.201	43.23	585.05	2.973	7.443	46.01	784.69	9.60	24.14
A		42.41	650.51	42.80	634.86	0.904	2.465	43.45	610.93	2.445	6.084	46.94	852.76	10.68	31.09
F		46.60	740.11	45.01	740.23	3.533	0.016	45.22	799.78	2.961	8.062	50.96	1028.62	9.35	38.98
M	5	47.12	688.47	44.88	710.09	4.980	3.045	44.65	703.37	5.232	2.164	49.24	961.68	4.51	39.68
A		47.96	702.82	44.95	719.61	6.687	2.333	44.78	730.37	6.623	3.920	50.37	1053.34	5.03	49.87
F		50.51	1019.00	52.42	1073.45	3.644	5.072	51.16	959.98	1.287	5.792	53.99	1344.00	6.90	31.89
M	6	49.72	997.80	50.55	1009.60	1.642	1.169	51.39	928.90	3.359	6.905	52.33	1154.45	5.25	15.70
A		50.31	1005.75	51.30	1035.14	1.924	2.839	51.46	934.29	2.280	7.105	53.64	1272.63	6.62	26.54
F	7	63.50	2100.50	62.62	2077.27	1.405	1.118	67.63	1783.97	6.504	15.069	56.91	1573.94	10.37	25.07
A		63.50	2100.50	62.62	2077.27	1.405	1.118	68.48	1728.78	7.843	17.697	56.78	1509.13	10.58	28.15
Average MAPE						1.840	2.080			2.850	5.900			2.36	9.24

F: Female, M: Male, A: Female+Male, AI: All Individuals

In this study, the application of the ANN was compared to a conventional linear approach. As a result, ANNs can be an alternative to the von Bertalanffy growth

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models. By contrast, the estimated growth parameters are model dependent; therefore, model selection uncertainty may be quite high in certain data sets. Ignoring model selection uncertainty may cause a substantial overestimation of the precision and estimation of the confidence intervals of the parameters below the nominal level. This uncertainty has serious implications, particularly when comparing the growth parameters of different fish populations. The set of candidate models should include at least the von Bertalanffy model, one or more sigmoid growth curves, and one or more non-asymptotic models (Katsanevakis and Maravelias, 2008). The von Bertalanffy model can be used to predict the features of a prospective older population from their sample caught when younger. The von Bertalanffy model provides better results with advancing age (Narinc et al., 2010).

In conclusion, the current study demonstrates that ANNs can be an alternative to von Bertalanffy growth models. In addition, it is recommended that the necessary steps should be taken as soon as possible to protect the *Esox lucius* population in the lake after investigating its stock situation and breeding and feeding behaviours.

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POSSIBILITIES OF PETROGEOTHERMAL ENERGY RESOURCES UTILIZATION IN CENTRAL PART OF POLAND

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Abstract. The current utilization of geothermal energy in Poland consists in development of geothermal waters for needs of heating systems and balneotherapeutic and recreational centres. In Poland, possibility of using the energy accumulated not in waters but in rocks deep-lying below the earth surface represents a new problem. The paper presents results of estimating the petrogeothermal energy resources accumulated in sedimentary rocks of central Poland. This region was distinguished as the most prospective in Poland for the possibility of locating systems that utilize petrogeothermal energy accumulated in sedimentary rocks, with regard to criteria of temperature and depth of occurrence of reservoir rocks.The carried out assessment of petrogeothermal energy resources accumulated in sedimentary rocks has shown a significant potential for using the heat from sedimentary rocks and has enabled indication of the best prospective locations for Enhanced Geothermal Systems. The most abundant resources occur in three locations of central Poland: Kutno area (the Lower Triassic reservoir), Pleszów (the Lower Carboniferous reservoir) and Konin area (the Upper Rotliegend reservoir). In these areas the potential for builiding system based on petrogeothermal energy was identified.
Keywords: *geothermal energy, enhanced geothermal system, petrogeothermal resources, Poland, sedimentary rocks.*

Introduction

Currently in Poland hydrogeothermal energy is utilized, for which warm groundwater produced from boreholes represents the energy carrier. On the other hand, petrogeothermal energy that constitutes heat resources of rocks, has not yet been utilized. At present geothermal applications involve space heating, balneotherapy, bathing and recreation, however, research on the possibilities of using geothermal energy for other purposes (including producing electricity) are carried out (Wójcicki et al. (eds.), 2013; Bujakowski and Tomaszewska (eds.), 2014).

The development of geothermal energy is driven by a number of interacting factors and the relationship between market and policy can be critical. For instance, electricity can be produced from geothermal resources through many different processes, and with varying efficiency. Where high-temperature hydrothermal resources are available, in many cases geothermal electricity is competitive with newly built conventional power plants. Binary systems can also achieve reasonable and competitive costs in several cases, but costs vary considerably depending on the size of the plant, the temperature level of the resource and the geographic location. EGS (Enhanced Geothermal System) cost cannot yet be assessed accurately because of the limited experience derived from pilot plants, but it seems to become competitive in a near future. Therefore, it seems important to have knowledge of existing geothermal resources. Effective used of geothermal energy is based on information about amount of resources (both hydro and petrogeothermal). In addition geothermal is associated with the geological risk. The geological risk exists especially at sites with only partially known subsurface

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conditions: the geothermal resource could be below expectations the fluid could be insufficient. Over the last 100 years, the production of geothermal energy has been concentrated in areas where rich hydrothermal resources are available. However, the development of advanced technologies has enabled the production of geothermal energy at low temperature in all European countries (EGEC, 2013).

Low-temperature systems (< 150°C) are more common and cover much larger areas in comparison to high-temperature ones. Also in Poland geothermal systems are characterized by low-temperature parameters. Poland has natural sedimentary-structural basins of diversified reservoir temperatures, from 20 to 80-90°C, in some cases even over 100°C. Geothermal energy resources in Poland are accumulated in underground reservoirs in various stratigraphic units and at various depths in the areas of the Polish Lowlands, the Carpathians and in some locations in the Sudety Mts and Carpathian Foredeep.

Geothermal resources in Poland are relatively well recognized as a result of a number of works and geothermal projects carried out by AGH University of Science and Technology in collaboration with other leading scientific centers. Recapitulation of the studies of the occurrence and utilization of geothermal waters and energy has been reflected in geothermal atlases of the Polish Lowlands (Górecki (ed.) et al., 1990; Górecki (ed.) et al., 1995; Górecki (ed.) et al., 2006a; Górecki (ed.) et al., 2006b) as well as geothermal atlases of the Carpathians (Górecki (ed.) et al., 2011; Górecki (ed.) et al., 2013a) and Carpathian Foreedeep (Górecki (ed.) et al. 2012) which represent unique works. Assessment of hydrogeothermal resources in different part of Poland has been the subject of numerous publications (e. g. Górecki (ed.) et al., 2013b; Sowiżdżał, 2010; Sowiżdżał and Górecki, 2013; Hajto and Górecki, 2010; Hajto et al., 2007; Hajto, 2011; Tomaszewska et al., 2010; Sowiżdżał, 2015) and allowed the identification of prospective areas and directions of geothermal energy development.

Petrogeothermal resources are the subject of global research on the technology of heat recovery for power generation in binary systems, often in combination with heat production. In case of sedimentary cover, reservoir rocks contain a small amount of groundwaters, so the utilization system is called EGS. EGS was defined as engineered reservoirs that have been created to extract economical amounts of heat from low permeability and/or porosity geothermal resources. EGS include conduction-dominated, low permeability resources in sedimentary formations. In EGS, the naturally occurring hot rock does not contain enough water and generally lies at greater depth than is typical of hydrothermal systems (Tester et al., 2006). In Poland, such systems do not yet exist. Since 2010, the work connected with assessment of analysis of the possibility of using petrogeothermal energy is carried out. A research team from the AGH University of Science and Technology conducted the work connected with analysis of the possibility of using reservoirs in sedimentary rocks for EGS. As the result of geological surveys (Wójcicki et al. (eds.), 2013) and petrophysical analysis of lithologies involves (Sowiżdżał and Kaczmarczyk, 2013; Sowiżdżał et al., 2013a) the central part of Poland was selected as one of the perspective areas for EGS development in sedimentary rocks (see *Figure 1*). The results of the project were presented in numerous publications (e. g. Wójcicki et al. (eds.), 2013; Sowiżdżał et al., 2013a; Sowiżdżał et al., 2013b; Sowiżdżał et al., 2014; Sowiżdżał and Kaczmarczyk, 2013; Sowiżdżał and Kaczmarczyk , 2014; Górecki et al., 2013b; Bujakowski et al., 2015).

An Enhanced Geothermal System is an underground reservoir that has been created or improved artificially. The concept of Enhanced Geothermal Systems is going greatly

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increase geothermal potential as it allows for the production of geothermal electricity nearly anywhere in Europe with medium and low temperature. This concept involves:

- Using the natural fracture systems in the basement rocks
- Enlarging its permeability through massive stimulation
- Installing a multi-well system
- Through pumping and lifting, forcing the water to migrate through the fracture system of enhanced permeability ("reservoir") and use the heat for power production.

A major effort to introduce EGS could create a substantial base-load electric power production, as geothermal energy is available independent from the time of day or year, of climate, weather, etc. A steady increase in geothermal power production could be expected in all EU countries (EGEC, 2013).

Geothermal energy from EGS represents a large, indigenous resource that can provide base-load electric power and heat at a level that can have a major impact on the many region of the world, while incurring minimal environmental impacts. With a reasonable investment in R&D, EGS could provide large amount of cost-competitive generating capacity in the next years. Further, EGS provides a secure source of power for the long term that would help protect against economic instabilities resulting from fuel price fluctuations or supply disruptions (Tester et al., 2006). The Polish economy is heavily dependent on fossil fuel prices. Diversification of energy sources as well as and increased use of more environmentally friendly sources, is vital in many region of Poland. Geothermal energy utilization is particularly justified in areas characterized by unique values of nature, tourist amenities and in towns exposed to the influence of gas and particulate pollutants as a result of burning of traditional energy carriers in local boiler plants and domestic furnaces.

Location of analysed area

In order to determine the most prospective area for EGS within sedimentary cover of Poland, based on international experiences (Brown et al., 2012; Tester et al., 2006; Tenzer, 2001; Sausse et al., 2007; Antkowiak et al., 2010), requirements for EGS in sedimentary rocks have been specified. Critical requirements for the EGS location comprise: thermal parameters of the rocks (temperatures >150°C), thickness of the reservoir (minimum 300 m), porosity and permeability of the reservoir rocks (as the lowest) and the depth of the reservoir (3-6 km). The maximum depth of reservoir was assess based on case studies (Baria et al., 2005; Tester et al., 2006). EGS will reach an optimum depth after which drilling deeper wells will not be more economical. However, studies by Tester and Herzog (1991) have shown that the optimal depth for minimum costs is on a fairly flat cost-versus-depth surface for most geothermal gradients. The insensitivity of project cost to depth, in the neighborhood of the optimal point, permits a range of economically acceptable depths. Lithology and mechanical properties of reservoir rocks are also important because of hydrofracturing. In case of sedimentary rocks the compact sandstones or limestones should be appropriate (de Graaf, et al, 2010; Tester et al., 2006).

The first step of the preliminary analysis was based on the existing geological and geothermal data, e.g., those collected in geothermal atlases (Górecki et al., 2006a; Górecki et al., 2006b) supplemented with new data. Complementary analyses of raw data and maps of surface heat flow density, subsurface temperatures, maps of gravimetric and magnetic anomalies allowed to determine several prospective locations.

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The most promising conditions (temperatures > 150°C in the depth of 5 km) occur in central part of Poland in an area covering the Mogilno-Łódź Trough region and a small part of the Kujawy Swell and Fore-Sudetic regions (see *Figure 1*). The area covers approximately 19 200 km^2 (Sowiżdżał et al., 2013b).

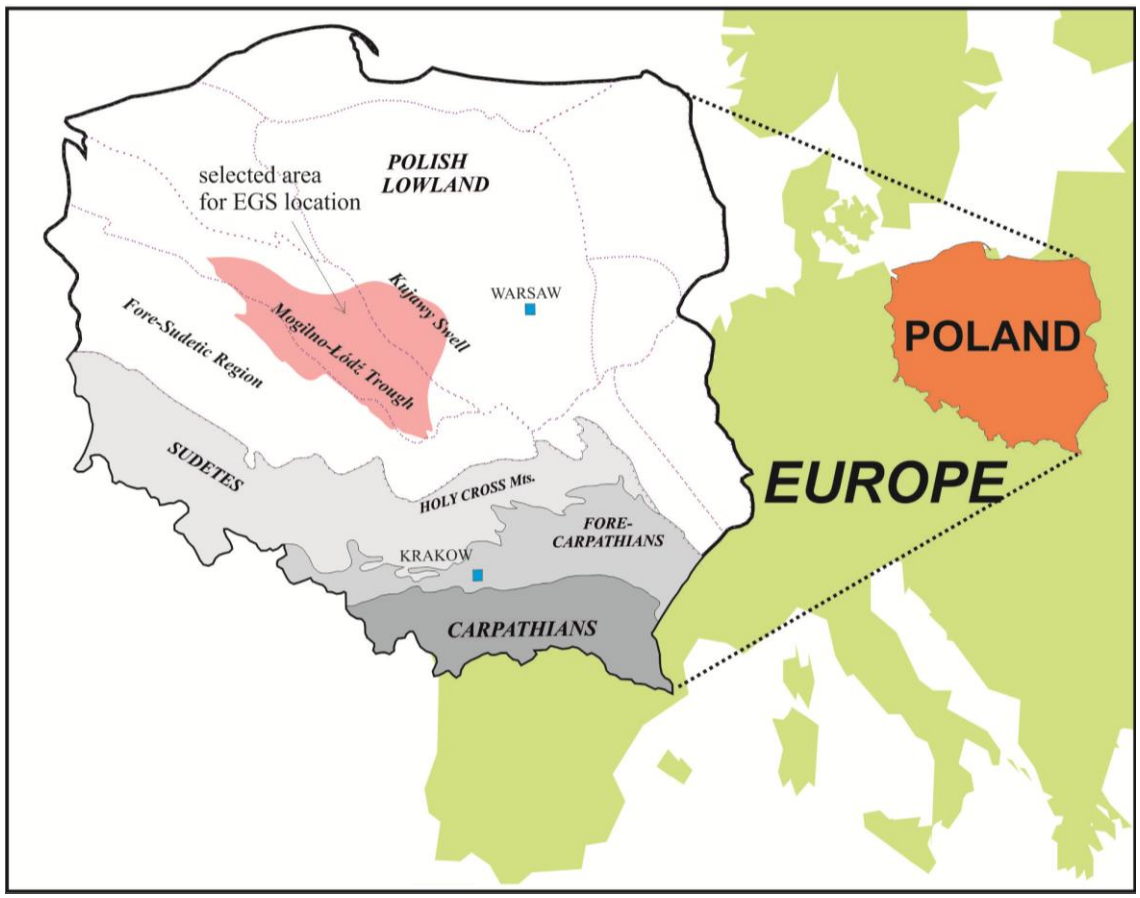

Figure 1. *Location of prospective areas for unconventional geothermal systems in sedimentary rocks.*

Geological background

In the area, preliminary analyses of petrophysical properties, including thermal ones, allowed to indicate the Triassic (mainly the Lower Triassic but locally also the Middle Triassic), the Lower Permian and the Lower Carboniferous formations as potential reservoir rocks for EGS systems. Stratigraphic information has been compiled on the basis of boreholes data as well as cited references. For interpretation of the distribution of subsurface temperatures, synthetic thermograms were employed, which were recorded in conditions of the stable thermal equilibrium (in the form of continuous measurements) (Sowiżdżał et al., 2013).

The Lower Carboniferous strata

The Lower Carboniferous strata are developed as follows: in the area of the postorogenic molasses (to the east and northeast of Poznań, and in the Konin, Sieradz, Łódź and Piotrków Trybunalski areas) – as the flysch lithofacies, so called exoflysch

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(debrites, turbidites); in the area of the Kujawy Swell – as sandstones, siltstones and claystones (pseudoflysch), and as sandstones and siltstone-claystone deposits (Karnkowski, 1999; Pożaryski and Karnkowski, 1992; Narkiewicz and Dadlez, 2008). Both the limited extent of occurrence and thickness of the Carboniferous formations (only locally exceeding the boundary thickness of 300 m) were decisive factors for claiming that better prospects of building the enhanced geothermal systems (EGS) will be related to the Lower Carboniferous strata. In the selected area, thickness of the Lower Carboniferous rocks varies from approximately 200 m to over 2500 m. Among the analysed reservoirs, the top of the Lower Carboniferous reservoir rests deepest, locally deeper that 6 km below sea level (b.s.l.). Assuming the depth of 6 km b.s.l. to be a criterion of the EGS profitability, the estimation of geothermal resources was performed only for areas where the reservoir rests shallower. Mean porosity of the Lower Carboniferous rocks ranges between 1.5 and 2.5%. In a major part of the chosen area, permeability is close to zero (about 0.1 mD), mean bulk density varies from 2.72 to 2.76 kg/m^3 and clay content ranges from approximately 56 to over 60%. It can be observed that temperature increases to the east where the top rests deeper than the established 6000 m b.s.l., therefore that zone was ruled out from further analyses. In the zone of shallower occurrence of the Lower Carboniferous strata, temperatures at the top range from 110 to 200°C (Wójcicki et al. (eds.), 2013). Analysis of petrophysical parameters, as well as analysis of the depth of occurrence of the Carboniferous strata with the predetermined thickness, have shown that the zone, where hot sedimentary rocks for EGS should be searched for, is located in the western part of the study area.

The Lower Permian (called Rotliegend)

The Lower Permian is represented by terrigenous deposits, originated from a desert, which were sedimented in the dry and hot climate. They typically form thick complexes of varigrained rocks which are diagonally bedded or lumpy. In the Polish Lowlands, the Lower Permian formations rest, with a distinct sedimentary gap, on basement rocks characterized by the Variscan and Caledonian consolidation. Among the Rotliegend formations, the Autunian effusive rocks (in the western part of Poland) play an important role. The Saxonian deposits are widely distributed and developed as facies of red clastic rocks (Pokorski, 1976). In the Saxonian profile, a number of sedimentary cycles can be distinguished, succession of which is sandstone – siltstone – claystone. The top of the Rotliegend strata is deepest in the eastern part of the analysed area (deeper than 7000 m b.s.l.). In the remaining parts, it is somewhat shallower (3500-6500 m b.s.l.). The accepted profitability criterion is the reason of elimination of the eastern part of the chosen area. The prospective Upper Rotliegend strata are thickest in the northern part of the area, where they exceed 750 m in thickness. Almost in the entire chosen area, the Upper Rotliegend thickness exceeds 300 m; only in marginal parts of the area it locally falls to smaller than 250 m. The Upper Rotliegend rocks are characterized by high (as for EGS) values of effective porosity (locally over 10%) and permeability (up to higher than 15 mD), which cause that they most frequently represent reservoirs of hydrogeothermal energy. Only locally in the central and eastern parts of the study area, porosity falls to less than 2%, which allows to infer that reservoirs of petrogeothermal energy may occur there. The decrease of porosity and permeability results from the depth of occurrence of the reservoir in these parts (deeper than 6000 m b.s.l.). Only in the Konin area, the top of the Rotliegend is shallower (approximately 5500 m b.s.l.). Also the clay content exceeds 50% there and density of rocks amounts to

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about 2.5 kg/m^3 (Wójcicki et al. (eds.), 2013). In consideration of prospects for locating the EGS systems within the Lower Permian reservoirs, a significant part of the originally chosen area has to be excluded due to the depth of occurrence of its top, its thickness, and reservoir parameters. The only prospective zone is situated in the vicinity of Konin where temperatures at the reservoir top reach 190°C.

The Lower Triassic

The Lower Triassic is represented by the Lower, Middle and Upper Buntsandstein rocks which in a major part of the Polish Lowlands are developed as lithofacies with predominance of claystone-siltstone deposits. In the Lower Buntsandstein of the southern part of the Polish Lowlands basin, sandy fluvial and (less frequently) eolian deposits occur. In the remaining area of Poland, the Lower Buntsandstein is developed as a monotonous complex of claystone-siltstone rocks with interbeds of oolitic limestones (except the eastern part of the Mogilno-Łódź Trough) and sandstones (Szyperko-Śliwczyńska, 1977; Szyperko-Śliwczyńska, 1979; Szyperko-Teller, 1982). The Middle Buntsandstein in the southern part of the basin is represented by sandstones and siltstones (Szyperko-Teller, 1997). In the Fore-Sudetic Monocline area, sandstones are dominant and toward the Mid-Polish Swell they pass into clayey sediments. The Upper Buntsandstein is analysed together with the Muschelkalk (T2+Tp_3) in consideration of its predominant development as carbonates, whereas sandstones of the Lower and Middle Buntsandstein (Tp_1+Tp_2) are treated as prospective formations of the Lower Triassic. In the distinguished study area, the top of the Lower Triassic strata rests at depths from deeper than 2000 m b.s.l. in the southwestern margin of the area down to approximately 6000 m b.s.l. in the eastern part. Within the extent of occurrence of the prospective Lower Triassic strata, the top rests at depths between 4000 and 6000 m b.s.l. (thus it meets the depth criterion everywhere). Practically in the entire distinguished area, total thickness of the Lower Triassic formations exceeds 300 m, acquiring the greatest values (over 2000 m) in its eastern part (the determined extent of the prospective Lower Triassic). Within the determined prospective extent, rocks are characterized by porosity ranging from nearly zero to 4%, low permeability (close to zero), bulk density between 2.6 and 2.7 kg/m^3 and clay content on the order of 50-60%. At the top of the analysed horizon, temperature is equal to 120 and 170°C, with the highest temperatures observed in the eastern part of the chosen area (east of Kutno), in places of the deepest top of the Lower Triassic (Sowiżdżał et al., 2013b).

The Middle Triassic

The Middle Triassic is represented by the Muschelkalk which can be divided into the Lower, Middle and Upper Muschelkalk. The Lower Muschelkalk of the Mogilno-Łódź Trough is developed as grey and beige limestones, often bedded and laminated with claystones and marls. In the northern part of the Kujawy Swell, marly and dolomitic limestones are dominant. The Middle Muschelkalk, represented by interbedding dolomitic claystones, dolomitic marls and anhydrites, reveals relatively homogeneous development over vast areas. As a rule, the Upper Muschelkalk is composed of limestones in the lower part of the profile and claystones with small limestone intercalations in the upper part. This lithologic type is characteristic of the Upper Muschelkalk in the Mogilno-Łódź Trough (Gajewska, 1997). In the area under discussion, the Middle Triassic is represented by the Muschelkalk that is divided into

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the Lower, Middle and Upper Muschelkalk (Senkowiczowa and Szyperko-Śliwczyńska, 1972). In the Mogilno-Łódź Trough, the Lower Muschelkalk is formed of grey and beige limestones, often bedded and laminated with claystones and marls. In the northern part of the Kujawy Swell, marly and dolomitic limestones are dominant. The Middle Muschelkalk, represented by intercalating dolomitic claystones, dolomitic marls, dolomites and anhydrites, shows relatively homogeneous development in large areas. The Upper Muschelkalk, as a rule, is composed of limestones in the lower part of its section, and of claystones with thin limestone interlayers in the upper part. Such lithology is characteristic of the Muschelkalk in the Mogilno-Łódź Trough (Gajewska, 1997). In consideration of the development as carbonates, the Upper Buntsandstein is analysed together with the Muschelkalk. Within the extent of the prospective formations, the top of the Muschelkalk rests at depths from 3500 to 4500 m b.s.l. These formations are characterized by relatively small thickness that is unfavourable for EGS, although areas appear with thickness exceeding the established 300 m. Rocks occurring within the determined extent are characterized by high values of effective porosity (over 2% everywhere), low permeability, bulk density on the order of 2.5-2.7 kg/m^3, and relatively low clay content, from 25 to 40% (Wójcicki et al. (eds.), 2013) .

Materials

In order to assess the geothermal energy resources, the authors used abundant materials gathered and analysed by a broad group of specialists during their research work. A key element was to determine thermal and petrophysical parameters of rocks that form the selected petrogeothermal reservoirs and were subjects of the resource assessment. A principal source of information on the subsurface thermal regime was represented by results of direct temperature measurements in deep wells. For interpretation of the subsurface temperature distribution, thermograms were used, for which analysis of measurement errors had been performed earlier, including corrections of subsurface temperature measurements, resulting from various reasons, technical and environmental in nature, e.g. effects of drilling mud. For evaluation of the quality and usefulness of gathered thermograms, it was cardinally important to determine whether the subsurface temperature measurements were really made in thermal stability conditions (after an appropriately long standstill). An essential indicator of the thermal measurement stability (honesty of the measurements) is consistency of results of temperature measurement in the near-surface zone with average values of the soil temperature in the near-surface zone (Wójcicki et al. (eds.), 2013). Sandstones and possibly dolomites and limestones should be potential reservoir rocks for locating the unconventional geothermal systems in sedimentary rocks. Among the analysed types of sedimentary rocks, sandstones are characterized by the best thermal parameters (specific heat, thermal conductivity). A significant feature of rocks that form petrogeothermal reservoirs is represented by their low porosity and permeability and high values of thermal parameters. The results of the examination of thermal properties, carried out on sedimentary rock samples collected from formations selected as potentially prospective for EGS, indicate considerable differentiation of both the thermal conductivity and specific heat of the analysed rock samples. Among sedimentary rocks, principally sandstones and limestones with reservoir parameters favourable for this type of systems are considered to be petrogeothermal reservoirs. As well the average thermal conductivity as the specific heat measured for carbonate

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rocks are characterized by lower average values in comparison with those measured for terrigenous rock samples, which does not mean that the latter cannot be considered to be prospective reservoirs of petrogeothermal energy. On the other hand, the relationship between thermal parameters of the rock and its porosity is unequivocally noticeable: the tighter is the rock, the better thermal parameters can be recorded (Sowiżdżał and Kaczmarczyk, 2014). Reservoir geometry is an essential element for the geothermal resource assessment. The reservoir layers should be at least 300 m thick to enable the reservoir fracturing. In potential areas with hot dry rocks (HDR), tectonic disturbance zones should not occur; if such zones do occur, throws of faults should be small and the faults should be local in nature. To assess the resources, results of structural modelling performed within the framework of the research work were applied (Wójcicki et al. (eds.), 2013; Sowiżdżał et al., 2013b). The distinguished reservoir horizons were characterized in detail from the point of view of their reservoir parameters. Analysis of petrophysical parameters, including thermal ones, performed on collected rock samples, allowed for determination of properties of hot dry sedimentary rocks (sandstones, limestones and dolomites), which are essential for modelling of their heat capacity. Results of petrophysical measurements and parametric modelling were used as the input material for assessment of petrogeothermal resources (Wójcicki et al. (eds.), 2013; Sowiżdżał et al., 2013b; Sowiżdżał et al., 2014).

Methodology of assessment of petrogeothermal energy resources

In order to estimate static geothermal resources that determine the total amount of heat accumulated in free water and rock matrix, with reference to the mean annual temperature at the ground surface, the methodology developed at the Department of Fossil Fuels, AGH University of Science and Technology was employed (Górecki et al., 1995). The methodology is based on principles of geothermal resource assessment used in European Union countries (Haenel and Staroste, 1988; Hurter and Haenel, 2002) and calculating the geothermal energy resources on the basis of a volumetric calculation model (Muffler and Cataldi, 1979).

Petrogeothermal resources are mostly related to the energy accumulated in rocks, and parameters of waters occurring there in small quantities are less important. Media (usually water) introduced through wells into heated rock formations (HDR) are heat carriers in systems that utilize this type of energy. For this reason, the resource assessment refers only to energy accumulated in the rocks. The assessment of the petrogeothermal energy resources accumulated in reservoir rocks of central Poland was carried out only for areas that meet the earlier discussed criteria of the reservoir geometry.

Static resources of geothermal energy

Static resources of geothermal energy are estimated according to the formula [1], with the first part of the formula referring to resources accumulated in the rock matrix and the second part determining the amount of heat accumulated in free water with reference to the mean annual temperature at the ground surface. If we dealt with nonporous rocks, by definition represented by hot dry rocks, it would be enough to estimate the thermal potential of the rock matrix. In case of reservoir rocks, we speak of EGS systems which use rocks with low porosity and permeability as reservoirs, locally

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containing small amounts of water. Such a small amount of water contained in the rock matrix, together with water pumped into the reservoir will represent the carrier of energy transferred to the surface in EGS systems.

The static resources E_{ZS} are calculated according to the following formula:

$$E_{ZS}= A*m*[(1-p_e)]*\varrho_s*c_s]*(T_s-T_o) \quad \text{(Eq.1)}$$

where:
- m - cumulative thickness of groundwater horizons in the reservoir [m];
- p_e - effective porosity [-];
- T_s - temperature at the top surface of groundwater reservoir [^oC];
- T_o - mean annual temperature at the Earth's surface [^oC];
- ϱ_s - mean density of rock framework [kg/m^3];
- c_s - mean specific heat of rock framework [J/ kg^oC];
- A - area of calculation block [m^2].

Static recoverable resources of geothermal energy

Static recoverable resources of geothermal energy represent a part of static resources of a given reservoir horizon, which can be exploited at the surface with regard to a given method of geothermal water exploitation. The recoverable part of geological resources is determined by so-called recovery factor of geothermal energy (Ro) for a given horizon or bed, which for exploitation through a geothermal doublet system is equal to:

$$Ro = \frac{As}{Ac} * \frac{Ts - Tz}{Ts - To} \quad \text{(Eq.2)}$$

where:
- As - cooled area of the dublet [m^2];
- Ac - total area affected by the dublet [m^2];
- Ts - temperature at the top surface of groundwater horizon [^oC];
- Tz - temperature of water injected back to the horizon (=25 ^oC);
- To - mean annual temperature at the Earth's surface [^oC].

In EGS systems, the ratio of cooled area versus total area affected by the geothermal doublet was assumed to be constant (0.5). The parameter was taken as empirical constant value based upon experience gained from the operating geothermal installations in the Paris Basin (France) (Górecki et al., 2006).

Static recoverable resources of geothermal energy were determined according to the following formula:

$$E_{ZSW} = R_o * E_{ZS} \quad \text{(Eq.3)}$$

- Ro - recovery index;
- E_{ZS} - static resources [J].

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The overall value of static, recoverable resources is a sum of recoverable energy accumulated in the all calculation blocks of given hyrogeothermal reservoir.

Results

As an effect of the calculations, unit and total resources of petrogeothermal energy were determined in the chosen study area for the Lower Carboniferous, Lower Permian, Lower Triassic, and Middle Triassic.

Lower Carboniferous petrogeothermal resouces

Total area of the resource calculation for the Lower Carbonifeorus reservoir amounted to 4100 km^2. Total static resources accumulated in this area are equal to **2.42 * 10^{22} J**. Unit static resources range from several to over 100 GJ/m^2. In the southwestern part of the analysed area, a zone with increased values of static resources is pronounced (locally over 70 GJ/m^2). The distribution of unit static and static recoverable resources is displayed in the map (see *Figure 2*). Total static recoverable resources amount to **6.89 * 10^{21} J.**

Lower Permian petrogeothermal resouces

Prospective formations of the Upper Rotliegend stretch over the largest area. Total area of their occurrence (with the layer thickness restricted with the minimum of 300 m), that is total area of calculation of static resources, amounted to 15,600 km^2; from this resulted the highest value of total static resources equal to **4.33 * 10^{22} J**. Unit static resources vary from several to over 40 GJ/m^2, with maximum values observed in the central part of the area (the Koło vicinity). The distribution of unit static and static recoverable resources is presented in the map (see *Figure 3*). Total static recoverable resources amount to **1.39 * 10^{22} J.**

Lower Triassic petrogeothermal resouces

Total area of calculation of static resources in the Lower Triassic reservoir amounted to approximately 3000 km^2. Total static resources accumulated in this area are equal to **6.87 * 10^{21} J**. Unit static resources vary in the range from several to over 80 GJ/m^2. The distribution of unit static and static recoverable resources is presented by the map (see *Figure 4*). Total static recoverable resources amount to **1.71 * 10^{21} J**.

Middle Triassic petrogeothermal resouces

Among the discussed reservoirs, the prospective Middle Triassic formations occupy the smallest area. It amounts to about 560 km^2 (a significant part of the area, about 340 km^2, was ruled out with regard to the criterion of minimum layer thickness equal to 300 m). The formations are characterized by the smallest thickness among the petrogeothermal reservoirs under discussion (in the considerable part of the area, the Middle Triassic thickness does not exceed 300 m). Total static resources accumulated in this area amount to **7.74 * 10^{20} J**. Unit static resources range from several to over 30 GJ/m^2. The distribution of unit static and static recoverable resources is displayed in the map (see *Figure 5*). Total static recoverable resources amount to **2.52 * 10^{29} J**.

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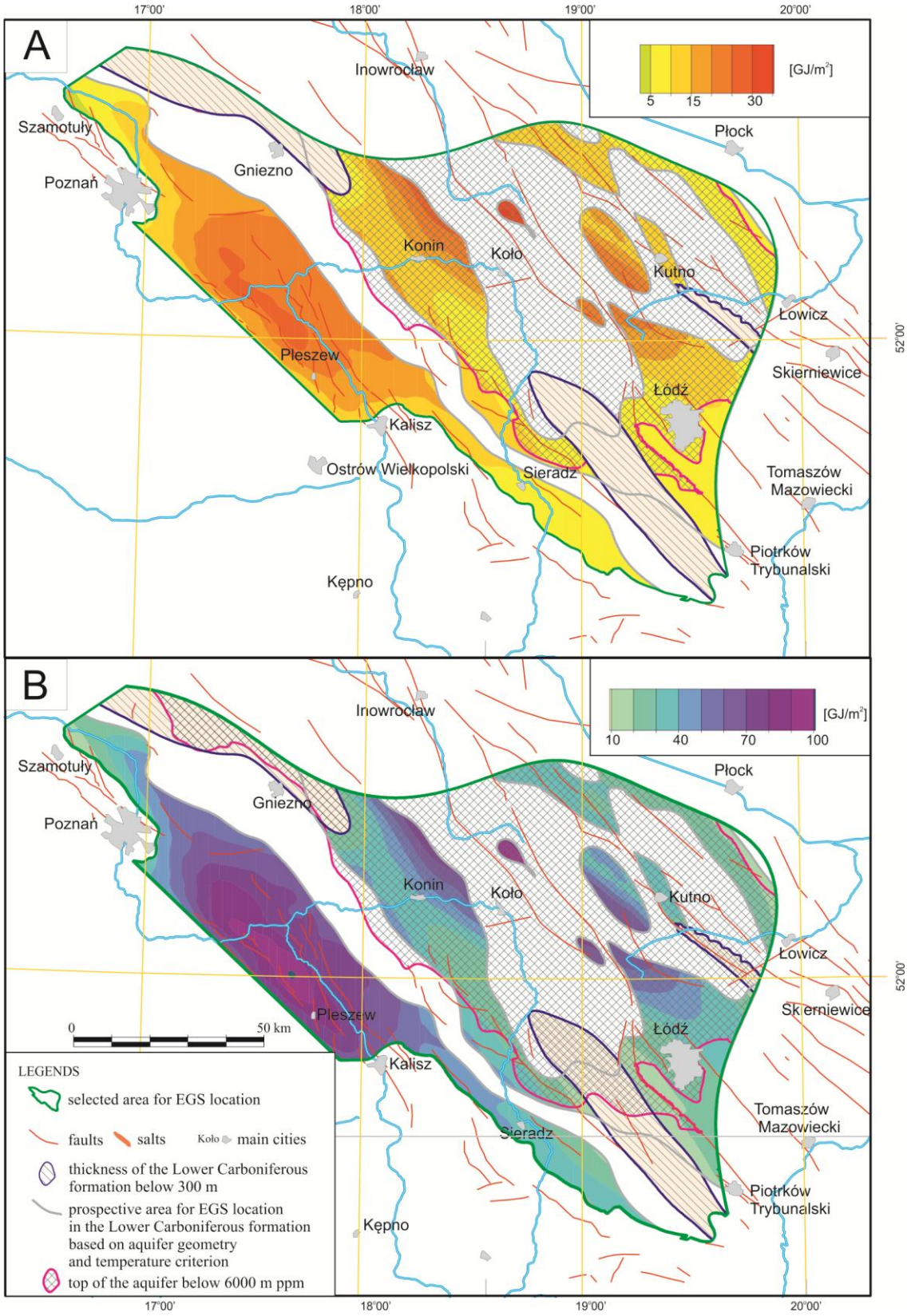

Figure 2. *Maps of unit petrogeothermal resources of the Lower Carboniferous aquifer in the selected area for EGS location: a –static recoverable resources, b- static resources.*

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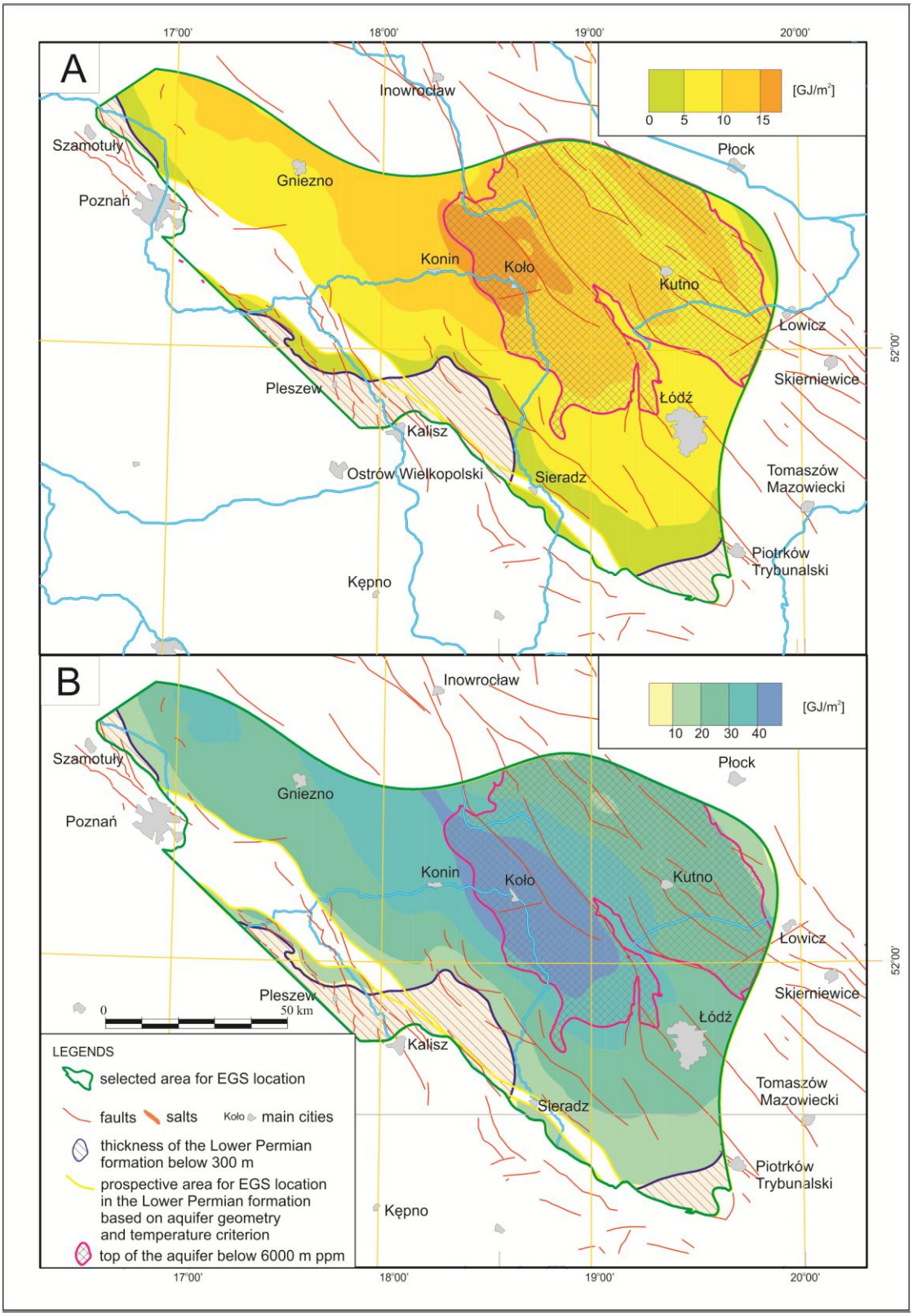

Figure 3. *Maps of unit petrogeothermal resources of the Lower Permian aquifer in the selected area for EGS location: a –static recoverable resources, b- static resources.*

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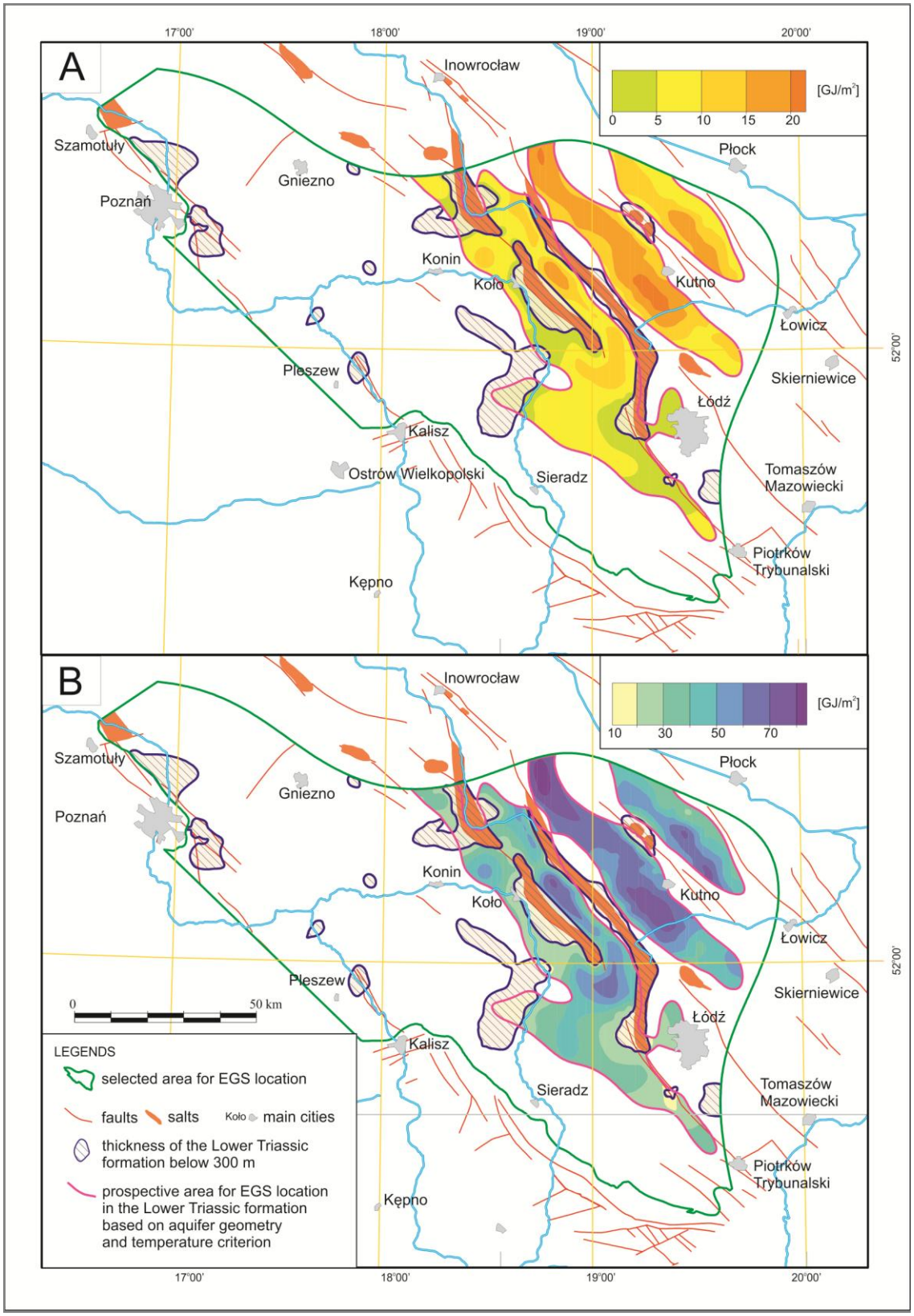

Figure 4. *Maps of unit petrogeothermal resources of the Lower Triassic aquifer in the selected area for EGS location: a –static recoverable resources, b- static resources.*

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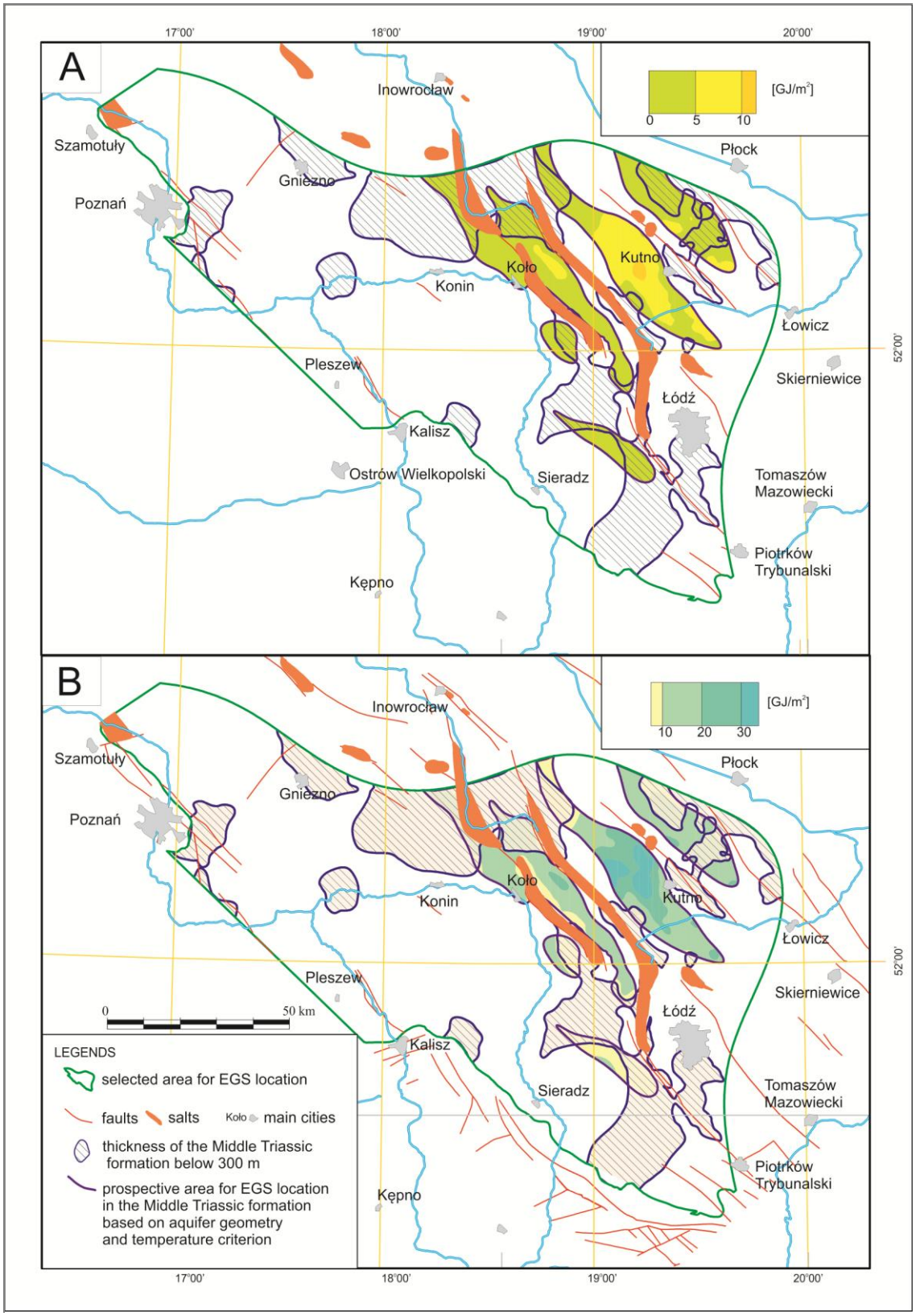

Figure 5. *Maps of unit petrogeothermal resources of the Middle Triassic aquifer in the selected area for EGS location:a –static recoverable resources, b- static resources.*

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Analysis of the results

The assessment of geothermal resources allowed for indication of the most prospective sedimentary reservoirs and locations for building the closed geothermal systems – enhanced geothermal systems (EGS) in the central part of Poland. Compilation of total static and static recoverable resources of geothermal energy in the analysed area is presented in *Table 1*. The geothermal resources were calculated with regard to the criterion of the reservoir rock thickness. In consideration of necessary fracturing in EGS systems, the minimum reservoir thickness was established at 300 m. For this reason, areas of the analysed geothermal reservoirs decreased to various degrees. The most significant decrease (almost 38%) was related to the Middle Triassic reservoir which is characterized by the smallest thickness among the analysed reservoirs. The Lower Triassic prospective area decreased by more than 13%. In case of the Lower Carboniferous and Lower Permian reservoirs, the decrease in area was insignificant (on the order of a few percent). Temperature at the top of the Middle Triassic, due to its relatively shallow occurrence is the lowest of the discussed reservoirs, which in conjunction with the smallest area of the resource calculation implicates the low values of geothermal resources (both static and static recoverable). The Lower Permian prospective formations are most widely distributed. The value of the calculated total geothermal resources is greatest there. Nevertheless, it should be kept in mind that a considerable part of this reservoir (like the Lower Carboniferous reservoir) rests deeper than 6000 m b.s.l., which has important effects on the resource estimate, at the same time imposing the techno-economic constraints on future exploitation. Total static recoverable resources represent 28.5% of total static resources in case of the Lower Carboniferous reservoir, over 34% in case of the Lower Permian reservoir, 24% in case of the Lower Triassic reservoir, and over 35% for the Middle Triassic reservoir.

Table 1. *Total value of geothermal energy resources in selected area for EGS.*

Aguifer	Total static resources [J]	Total static-recovery resources [J]	Area of calculation block [m^2]
Lower Carboniferous	$2.42 * 10^{22}$	$6.89 * 10^{21}$	4000
Lower Permian	$4.33 * 10^{22}$	$1.48 * 10^{22}$	15600
Lower Triassic	$7.87 * 10^{21}$	$1.89 * 10^{21}$	2600
Middle Triassic	$7.74* 10^{20}$	$2.76 * 10^{20}$	560

Considering the unit geothermal resources, three most prospective locations for the unconventional geothermal systems in sedimentary rocks were indicated. They are situated:

- in the Pleszów area where the Lower Carboniferous terrigenous deposits represent reservoir rocks; unit static recoverable resources amount there to approximately 20-30 GJ/m^2 and static resources reach up to approximately 100 GJ/m^2;
- in the Konin area where the Upper Rotliegend terrigenous deposits represent reservoir rocks; unit static recoverable resources

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- are on the order of several GJ/m^2 there and static resources range from 30 to 40 GJ/m^2;
- in the Kutno area where the Lower Triassic terrigenous deposits represent reservoir rocks; unit static resources exceed 70 GJ/m^2 there and static recoverable resources are on the order of 15-20 GJ/m^2.

In spite of the fact that the poorest prospects are related to the Middle Triassic formations, the most interesting area of this reservoir was observed in the Kutno vicinity (static recoverable resources of about 10 GJ/m^2) where several metres deeper hot Buntsandstein rocks rest, with reservoir parameters favourable for EGS systems.

Ultimately, it was decided that the best prospective reservoir horizon would be the thick Buntsandstein horizon resting shallower than the other prospective reservoir horizons, which is an extraordinary asset in consideration of costs and technical possibilities of the reservoir development. Analysis of thermal and petrophysical parameters of the Lower Triassic sandstones (Sowiżdżał et al., 2013b) together with the assessment of geothermal resources have demonstrated that it is just in the Kutno vicinity where this reservoir shows the best parameters.

For the most prospective EGS location research team from Mineral and Energy Economy Research Institute of the Polish Academy of Sciences performed an electricity production model. Numerical modelling was conducted using TOUGH2 code (Bujakowski et al., 2015). The modelled net power of an EGS plant operating in the Kutno area ranged from 1.3 to 1.6 MW depending on the permeability and volume of the fractured zone used for the circulation (Bujakowski et al., 2015). In the Konin area net power of an EGS plant was estimated to range from 1.9 to 2.2 MW, while in in the Pleszów area EGS plant net power was assessed at 2.2-2.5 MW (Wójcicki et al., 2013).

Discussion

The carried out assessment of geothermal energy resources have indicated the existence of a significant potential accumulated in deep-lying rocks of the sedimentary cover. At present this potential is not used. Considering the possibility of development of the existing resources of petrogeothermal energy, a number of aspects other than geologic ones should be taken into account, among others the technical, economic and organizational aspects of an investment. In case of sedimentary rocks, numerous problematical aspects of functioning of the system should be considered, resulting mostly from the absence of the world experience in making use of sedimentary rocks as reservoir rocks for unconventional geothermal systems (although experimental installations in such rocks are under construction now) and the associating variability of crucial parameters of a modelled system (e.g. susceptibility to fracturing). There exist a number of issues which may potentially affect the efficiency of EGS functioning, e.g. heterogeneity of reservoir rocks, presence of clayey material, occurrence of mineralized water, etc.

In Poland, the geothermal energy utilization develops slowly. In spite of the existence, in a number of regions, of adequate geothermal resources, due to numerous conditions the utilization of such energy represents a fraction of a percent in the total energy balance in Poland. The hydrogeothermal energy resources used to date have been relatively well recognized and documented. The present paper has shown that the research work aimed also at recognition of the petrogeothermal energy potential has started. The world predictions indicate that the petrogeothermal energy is the energy of

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future and in the coming years development of such energy sector can be expected. Also in Poland the occurrence of the petrogeothermal energy resources possible to be utilized in future has been observed.

Considering the resources accumulated in sedimentary rocks, it should be noted that the most abundant resources occur in three locations of central Poland (the Mogilno-Łódź Trough). The best prospective is the Kutno area where the Lower Triassic terrigenous deposits represent reservoir rocks and unit static recoverable resources are on the order of 15-20 GJ/m^2. In the Pleszów area, also significant potential have been shown; there the Lower Carboniferous terrigenous formations represent reservoir rocks and unit static recoverable resources amount to about 20-30 GJ/m^2. The Konin area is the third distinguished location, with the Upper Rotliegend terrigenous deposits representing the reservoir rocks and unit static recoverable resources amounting to several GJ/m^2.

Apart from the presented study results, other research teams have conducted work aiming at assessment of petrogeothermal energy resources in volcanic and crystalline rocks. Their results have shown that prospects for development of petrogeothermal energy from volcanic and crystalline rocks are even more optimistic than from sedimentary rocks, which allows to claim that in Poland the work also on development of such resources should be started, following the example of other countries.

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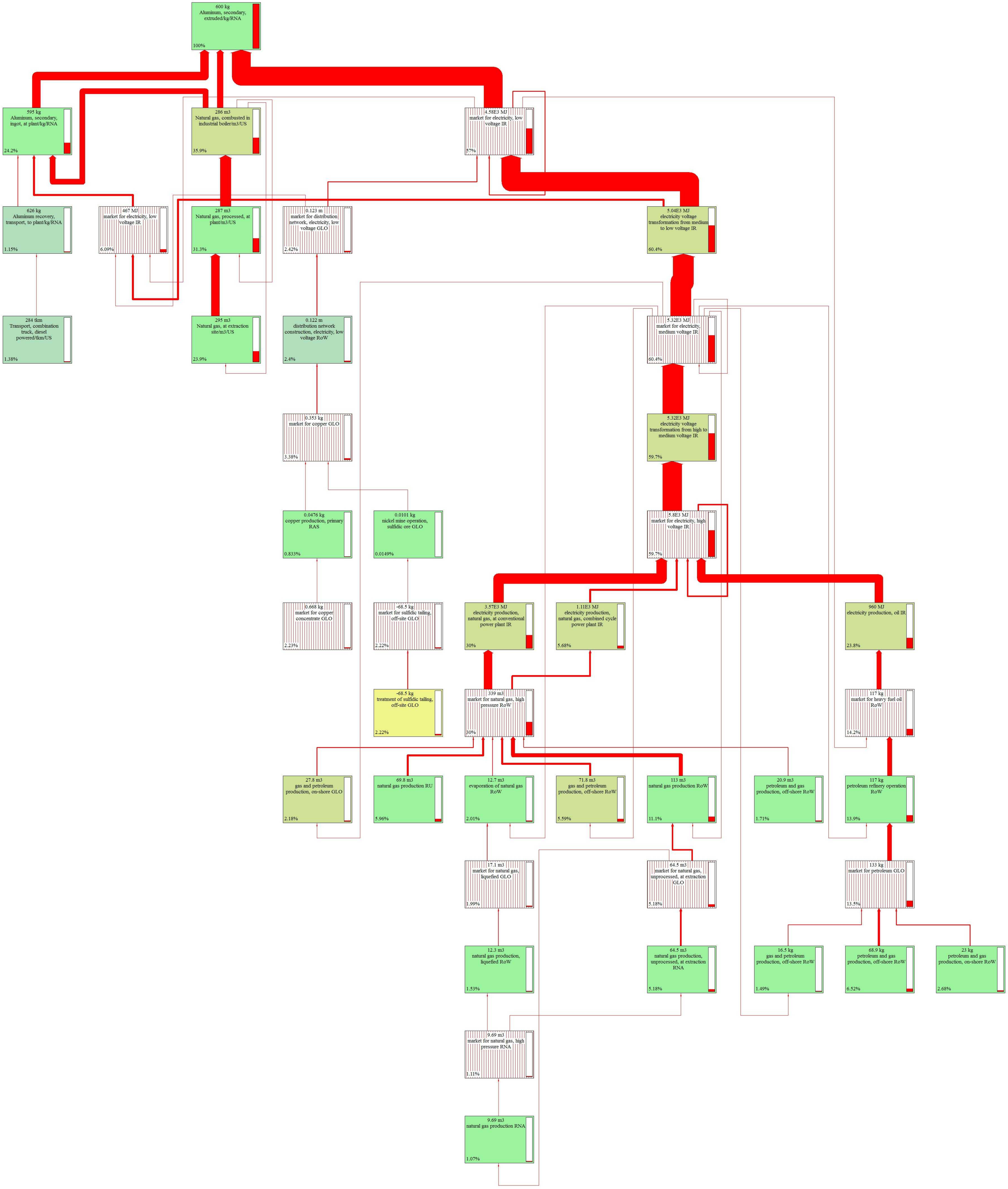
600 kg
Aluminum, secondary, extruded/kg/RNA
100%
595 kg
Aluminum, secondary, ingot, at plant/kg/RNA
24.2%
286 m3
Natural gas, combusted in industrial boiler/m3/US
35.9%
4.58E3 MJ
market for electricity, low voltage IR
57%
626 kg
Aluminum recovery, transport, to plant/kg/RNA
1.15%
467 MJ
market for electricity, low voltage IR
6.09%
287 m3
Natural gas, processed, at plant/m3/US
31.3%
0.123 m
market for distribution network, electricity, low voltage GLO
2.42%
5.04E3 MJ
electricity voltage transformation from medium to low voltage IR
60.4%
284 tkm
Transport, combination truck, diesel powered/tkm/US
1.38%
295 m3
Natural gas, at extraction site/m3/US
23.9%
0.122 m
distribution network construction, electricity, low voltage RoW
2.4%
5.32E3 MJ
market for electricity, medium voltage IR
60.4%
0.353 kg
market for copper GLO
3.38%
5.32E3 MJ
electricity voltage transformation from high to medium voltage IR
59.7%
0.0476 kg
copper production, primary RAS
0.833%
0.0101 kg
nickel mine operation, sulfidic ore GLO
0.0149%
5.8E3 MJ
market for electricity, high voltage IR
59.7%
0.668 kg
market for copper concentrate GLO
2.23%
-68.5 kg
market for sulfidic tailing, off-site GLO
2.22%
3.57E3 MJ
electricity production, natural gas, at conventional power plant IR
30%
1.11E3 MJ
electricity production, natural gas, combined cycle power plant IR
5.68%
960 MJ
electricity production, oil IR
23.8%
-68.5 kg
treatment of sulfidic tailing, off-site GLO
2.22%
339 m3
market for natural gas, high pressure RoW
30%
117 kg
market for heavy fuel oil RoW
14.2%
27.8 m3
gas and petroleum production, on-shore GLO
2.18%
69.8 m3
natural gas production RU
5.96%
12.7 m3
evaporation of natural gas RoW
2.01%
71.8 m3
gas and petroleum production, off-shore RoW
5.59%
113 m3
natural gas production RoW
11.1%
20.9 m3
petroleum and gas production, off-shore RoW
1.71%
117 kg
petroleum refinery operation RoW
13.9%
17.1 m3
market for natural gas, liquefied GLO
1.99%
64.5 m3
market for natural gas, unprocessed, at extraction GLO
5.18%
133 kg
market for petroleum GLO
13.5%
12.3 m3
natural gas production, liquefied RoW
1.53%
64.5 m3
natural gas production, unprocessed, at extraction RNA
5.18%
16.5 kg
gas and petroleum production, off-shore RoW
1.49%
68.9 kg
petroleum and gas production, off-shore RoW
6.52%
23 kg
petroleum and gas production, on-shore RoW
2.68%
9.69 m3
market for natural gas, high pressure RNA
1.11%
9.69 m3
natural gas production RNA
1.07%