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Diversity and Ecology of the Phytoplankton of Filamentous Blue-Green Algae (Cyanoprokaryota, Nostocales) in Bulgarian Standing Waters

Plamen Stoyanov*, Ivanka Teneva, Rumen Mladenov, Detelina Belkinova

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Abstract. The current study presents data about the diversity and ecology of filamentous blue-green algae, found in the phytoplankton of 42 standing water basins in Bulgaria. We identified 9 species from *Cyanoprokaryota*, which belong to 5 genera from order *Nostocales*. Ecological characterization of the identified species has been performed. Data about the physicochemical parameters of the water basins are also provided.

Key words: filamentous blue-green algae, standing water basins, morphology, ecology, distribution, Bulgaria.

Abbreviations used in the text:

L3 - Mountain lakes in Pontic province
L4 - Lowland or semi-mountain natural lakes end swamps in Pontic province
L7 - Black Sea freshwater coastal lakes
L11 - Large deep reservoirs
L12 - Small and medium-size semi-mountain reservoirs in Pontic province
L13 - Small and medium-size semi-mountain reservoirs in Eastern Balkans
L14 - Large lowland reservoirs with medium depth in Pontic province
L15 - Large lowland reservoirs with medium depth in Eastern Balkans
L16 - Small and medium-size lowland reservoirs in Pontic province

Introduction

The blue-green algae are characterized with high adaptive plasticity and quick adaptation to light, temperature or other physical and chemical changes in the environment. This is supported by their wide distribution (ANAGNOSTIDIS & DANIELIDIS, 1982; ECONOMOU-AMILLI *et al*., 1984; HALLFORS. 1984; KOMAREK, 1985; ANAGNOSTIDIS *et al*., 1985; SKULBERG & SKULBERG, 1985; KOMÁREK & KOVÁČIK, 1987).

TEMNISKOVA et al. (2005) summarized the studies of the algal flora in Bulgaria. Another synopsys on the phytoplankton in the Bulgarian reservoirs was done by STOYNEVA & MICHEV (2007) and BESHKOVA *et al*. (2007).

So far, the studies describing the hydrophilic blue-green algae and their

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distribution in Bulgaria are limited. Some contemporary studies on this subject are done by TSANEV & BELKINOVA (2009) and TENEVA *et al*. (2010a, 2010 b, 2011).

The aim of the study was to identify the filamentous blue-green algae from different standing water basins in Bulgaria.

Material and Methods

For species identification of the filamentous blue-green algae 42 natural samples were collected from 42 Bulgarian freshwater basins in 2009. The typology of the water basins (dams, lakes and swamps) was defined according to System B (BELKINOVA et al., 2013).

The physicochemical parameters (temperature, oxygen saturation, pH and conductivity) in the water basins were measured *in situ* with calibrated field devices. Additionally, NH_4N, NO_2N, NO_3N, PO_4P, total phosphorus and nitrogen were analyzed by using a NOVA 60 spectrophotometer (MERCK) following adopted standards: ammonium nitrogen (ISO 7150/1), nitrite and nitrate nitrogen (EN 26777 and ISO 7890-1), total nitrogen (EN ISO 11905-1), phosphate phosphorus (EN ISO 6878) and total phosphosus (EN ISO 6878).

The taxonomic composition was determined by using a light microscope MAGNUM-T, with 10x40 magnification, equipped with 3Mpx digital camera and the guide of JOHN *et al*. (2003).

For each taxon the frequency of occurrence (FQ) was calculated (TEMNISKOVA & STOYNEVA, 2011).

Results and Discussion

The ecological analysis of the species includes total distribution of the identified species and information about their habitats in Bulgaria. The physicochemical parameters of the water basins and the range of their variability (for species, occurring in more than one water basin) are presented.

We have identified the following species from order Nostocales:

FAMILY *NOSTOCACEAE*

Anabaena affinis LEMMERMANN

Habitat: occurs in the plankton of standing water bodies, sometimes causing "blooming" of the water.

Distribution in Bulgaria: large, deep reservoirs (type L11). In the current study it was found and documented in reservoir Studen Kladenets.

Abiotic parameters: t^O – 25.2^{O}C; %pO_2 – 109; pH – 8.5; ε – 253; NH_4N –0.092; NO_2N – 0.002; NO_3N <0.20; PO_4P <0.010; total P – 0.029; total N – 1.9.

Anabaena scheremetievi ELENK.

Habitat: occurs in the plankton of lakes, reservoirs and rivers. Widely distributed species.

Distribution in Bulgaria: middle and small semi-mountain reservoirs in Pontic province; big large lowland medium deep reservoirs in Pontic province; small and medium lowland reservoirs in Pontic province (L12, L14, L16). In the current study it was found and documented in reservoirs Kula, Ogosta, Enitsa, Yastrebino, Krapets, Antimovo, Aheloy, Poroy, Daskal Atanasovo, Ovchi kladenets and Ovcharitsa.

Abiotic parameters: t^O – 9.2÷29.2^{O}C; %pO_2 – 44÷98.23; pH – 7.92÷9.5; ε – 201÷863; NH_4N <0.010÷1.55; NO_2N – 0.002÷0.052; NO_3N – 0.08÷0.28; PO_4P <0.010÷0.223; total P – 0.015÷0.246; total N <0.5÷2.1.

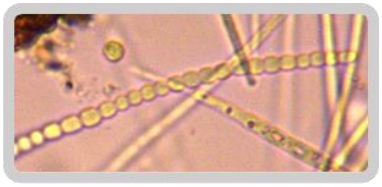
Anabaena spiroides KLEBAHN

Habitat: occurs in the plankton of standing freshwater basins. Widely distributed species.

Distribution in Bulgaria: Black Sea freshwater lakes and marshes, semi-mountain reservoirs in Pontic province, small and medium-sized lowland reservoirs in Pontic province (L7, L12, L16). In the current study it was found and

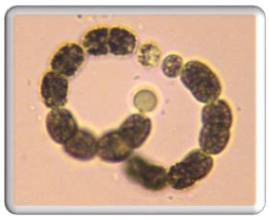
documented in reservoirs Enitsa, Poroy, Daskal Atanasovo, Ovchi kladenets, Ovcharitsa and in Durankulak swamp.
Abiotic parameters: t^O – 9.2÷29.2^{O}C; %pO_2 – 28.9÷95; pH – 8.02÷9.5; ε – 522÷1210; NH_4N – 0.019÷1.55; NO_2N – 0.005÷0.052; NO_3N <0.20; PO_4P <0.010÷0.223; total P – 0.044÷0.246; total N – 0.5÷2.1.

Anabaena variabilis (KÜTZING) BORNET ET FLAHAULT



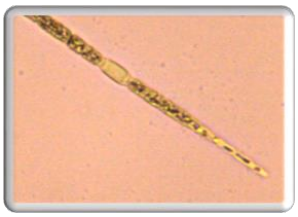
Habitat: occurs in standing freshwater and marine basins and sometimes even on wet soils.
Distribution in Bulgaria: middle-sized and small semi-mountain reservoirs in Pontic province; small and medium-sized lowland reservoirs in Pontic province (L12, L16). In the current study it was found and documented in reservoirs Antimovo and Ovchi kladenets.
Abiotic parameters: t^O – 24.9÷27.9^{O}C; %pO_2 – 95÷98.23; pH – 9÷9.5; ε – 536÷690; NH_4N – 0.016÷0.05; NO_2N – 0.002÷0.006; NO_3N <0.20; PO_4P <0.010÷0.034; total P – 0.062÷0.082; total N <0.5÷1.6.

Anabaenopsis arnoldii APTEK.



Habitat: Occurs in standing waters and rivers.
Distribution in Bulgaria: small and medium-sized lowland reservoirs in Pontic province (L16). In the current study it was found and documented in reservoirs Tri kladentsi, Enitsa and Krushovitsa.
Abiotic parameters: t^O – 9.2÷23.9^{O}C; %pO_2 – 44÷111; pH – 8÷9.29; ε –378÷609; NH_4N – 0.054÷1.55; NO_2N – 0.013÷0.052; NO_3N 0.16÷0.43; PO_4P – 0.009÷0.025; total P – 0.112÷0.195; total N – 0.8÷2.1.

Aphanizomenon elenkinii KISSEL.
Habitat: occurs in the plankton of standing water basins.
Distribution in Bulgaria: lowland and semi-mountain natural lakes and swamps in Pontic province; large deep reservoirs; middle-sized and small semi-mountain reservoirs in Pontic province; middle and small semi-mountain reservoirs in Eastern Balkans; small and medium-sized lowland reservoirs in Pontic province (L4, L11, L12, L13, L16). In the current study it was found and documented in reservoirs Rabisha, Rasovo, Dabnika, Devets, Telish, Valchovets, Yastrebino, Boyka, Antimovo, Aheloy, Poroy, Ovchi kladenets and Koprinka.



Abiotic parameters: t^O – 10.2÷27.9^{O}C; %pO_2 – 48.3÷121.4; pH – 7.92÷9.5; ε –195÷822; NH_4N – 0.019÷0.334; NO_2N – 0.002÷0.029; NO_3N – <0.20÷0.89; PO_4P <0.010÷0.044; total P – 0.017÷0.218; total N <0.5÷2.1.

Aphanizomenon flos-aquae [(LINNAEUS) RALFS] BORNET ET FLAHAULT

Habitat: occurs in the plankton of eutrophic freshwater and brackish basins. Causes "blooming" of the water. Widely distributed species. Toxin-producing populations are known to exist.
Distribution in Bulgaria: mountain type lakes in Eastern Balkans; lowland and semi-mountain natural lakes and swamps in Pontic province; Black sea freshwater lakes and swamps; large deep reservoirs; middle-sized and small semi-mountain reservoirs in Pontic province; middle and small semi-mountain reservoirs in Eastern Balkans; large lowland reservoirs in Eastern Balkans; small and middle lowland reservoirs in Pontic province (L3, L4, L7, L11, L12, L15, L16). In the current study it was found and documented in reservoirs Kula, Rabisha, Kovachitsa, Dabnika, Tri kladentsi, Barzina, Pancharevo, Krushovitsa, Telish, Valchovets, Kamenets, Yastrebino, Krapets, Beli Lom, Lomtsi, Kavatsite, Boyka, Antimovo, Aheloy, Mandra, Yasna polyana, Batak, Vacha, Pyasachnik, Daskal

Atanasovo, Ovchi kladenets, Ovcharitsa, Koprinka, Borovitsa, Studena, Pchelina and Durankulak swamp.

Abiotic parameters: t^{O} – 10.2÷29.2^{O}C; %pO_2 – 28.9÷214; pH – 6.66÷9.55; ε –62÷1210; NH_4N – 0.008÷0.39; NO_2N <0.002÷0.067; NO_3N <0.20÷0.89; PO_4P <0.010÷0.317; total P – <0.010÷0.364; total N <0.5÷4.1.

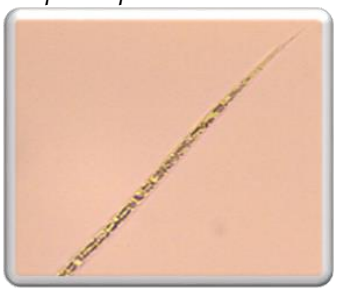
Cylindrospermopsis raciborskii (WOLOSZYNSKA) SEENAYYA & SUBB RAJU [Syn.: *Anabaenopsis raciborskii* WOLOSZYNSKA]

Habitat: occurs in the plankton of eutrophic lakes and reservoirs. Toxin-producing populations are known to exist.

Distribution in Bulgaria: middle and small semi-mountain reservoirs in Pontic province; middle and small semi-mountain reservoirs in Eastern Balkans; small and middle lowland reservoirs in Pontic province (L12, L13, L16). In the current study it was found and documented in reservoirs Drenovets, Smirnenski (Lomtsi), Kovachitsa, Telish, Valchovets, Kamenets, Boyka, Aheloy and Ovchi kladenets.

Abiotic parameters: t^{O} – 10.2÷27.9^{O}C; %pO_2 – 48.3÷123.3; pH – 8.1÷9.5; ε – 241÷813; NH_4N – 0.01÷0.39; NO_2N – 0.002÷0.02; NO_3N <0.20÷0.28; PO_4P <0.010÷0.044; total P – 0.047÷0.116; total N <0.5÷2.84.

Raphidiopsis mediterranea SKUJA



Habitat: occurs in the plankton of standing water basins.

Distribution in Bulgaria: lowland and semi-mountain natural lakes and swamps in Pontic province; middle and small semi-mountains reservoirs in Pontic province; middle and small semi-mountain reservoirs in Eastern Balkans; small and middle lowland reservoirs in Pontic province (L4, L12, L13, L16). In the current study it was found and documented in reservoirs Rabisha, Kovachitsa, Barzina, Asparuhov val, Enitsa, Krushovitsa, Valchovets, Kavatsite, Boyka, Baniska, Daskal Atanasovo, Ovchi kladenets and Pchelina.

Abiotic parameters: t^{O} – 9.2÷27.9^{O}C; %pO_2 – 44.÷214; pH – 8÷9.55; ε – 202÷1044; NH_4N – 0.019÷1.55; NO_2N – 0.002÷0.067; NO_3N <0.20÷0.81; PO_4P <0.010÷0.039; total P – 0.017÷0.202; total N <0.5÷4.1

The analysis of the results showed that most common taxa from the *Nostocaceae* family are genus *Aphanizomenon* (FQ=55%), followed by genus *Anabaena* (FQ=25%), *Raphidiopsis* (FQ=20%), *Cylindrospermopsis* (FQ=14%) and *Anabaenopsis* (FQ=13%).

The frequency of occurrence of the species is presented in Fig. 1. As can be seen from the figure, the species with the highest frequency of occurrence is *Aphanizomenon flos-aquae*, followed by *Aphanizomenon elenkinii* and *Raphidiopsis mediterranea. The rarest species was Anabaena affinis*, found only in reservoir Studen kladenets.

The species *Aphanizomenon elenkinii, Aphanizomenon flos-aquae, Anabaena spiroides* and *Anabaena scheremetievi* were found in water basins, which are included in protected territories. Thus, this study fulfills one of the 10 directions of the Bulgarian algological studies, suggested by TEMNISKOVA et al. (2005).

With the established in the current study 9 species from order *Nostocales* belonging to 5 genera, we enrich the existing information about the distribution of the filamentous blue-green algae in the standing waters in Bulgaria.

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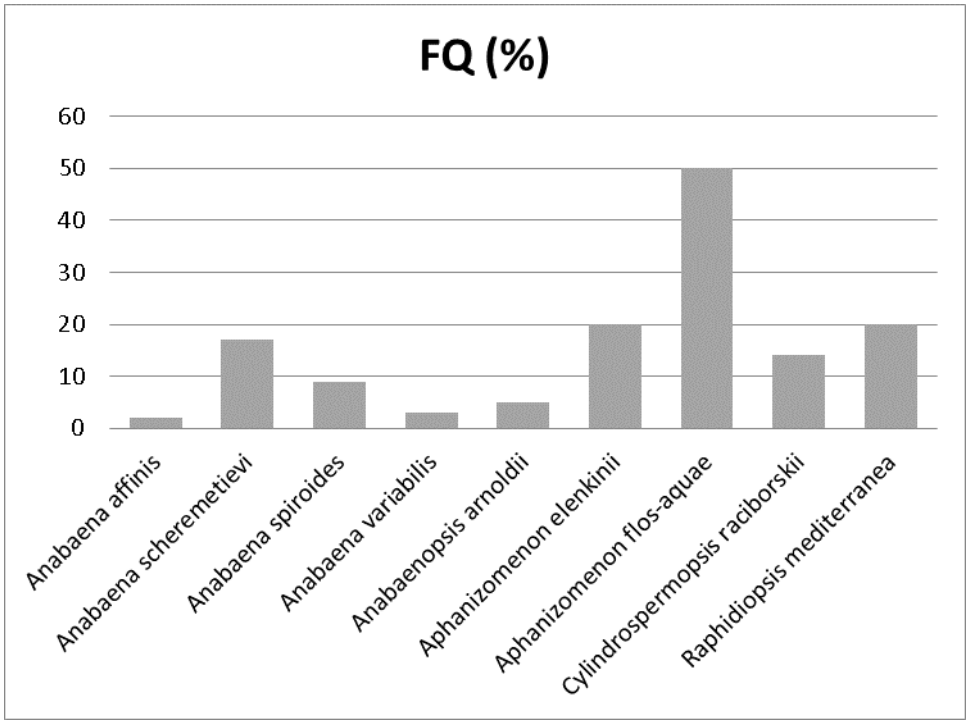

Fig. 1. Frequency of occurrence of filamentous blue-green algae from order *Nostocales* in Bulgarian standing water basins.

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New Data on the Vertical Distribution of Some Species of the Flora in Bulgaria

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Abstract. During field studies in different floristic regions of Bulgaria in the period 2006-2013, we found localities of *Stellaria alsine, Trifolium heldreichianum, Koeleria nitidula, Sieglingia decumbens, Stipa tirsa, Verbascum formanekii, Pedicularis leucodon, Saxifraga stribrnyi, Inula aschersoniana* and *Scilla bifolia* that expand our knowledge of the vertical distribution of these species in Bulgaria, and hence their ecological niche in the country.

Key words: plant species, vertical distribution, new data, Bulgaria

Introduction

The vertical distribution of each plant species provides valuable information about its ecological requirements and it is cited in all "Identification handbooks", "Synopses of the flora", "Checklists" and "Floras". Until 1967 vertical distribution of species of the flora of Bulgaria was approximately marked in the reference publications with digits from 1 to 3 (STOYANOV & STEFANOV, 1948; STOYANOV *et al*., 1966-1967). Digit 1 means that the species is spread in the lower or oak belt, which lies approximately between 0 and 1000 m altitude, digit 2 indicates that the species is spread in the mountain belt, roughly between 1000 and 2000 m altitude, and digit 3 indicates species distribution in highland (sub-alpine and alpine) zone, i.e. higher than 2000 m a. s. l. When the species occurs in more than one zone, combinations of digits are used. A dash before the digits 2 (-2) and 3 (-3) means that this species can be found below 2000 and 3000 m altitude, respectively, and a dash after the digits 1 (1-) and 2 (2-) indicates that the species is up and above 1000, or 2000 m altitude, respectively. These markings give approximate notion about the species' vertical distribution and for many species it had never been mentioned. In the multivolume edition of the Flora of Bulgaria (YORDANOV, ed. 1963-1979; VELCHEV, ed. 1982-1989; KOZHUHAROV, ed. 1995; KOZHUHAROV, ANCHEV, eds. 2012), the vertical distribution was indicated with real altitudes, but most often only the upper limit of species distribution was specified. "Identification Guide to higher plants in Bulgaria" (KOZHUHAROV, ed. 1992) and "Identification Guide to the plants in Bulgaria" (DELIPAVLOV & CHESHMEDZHIEV, eds. 2003, 2011) indicated altitudinal distribution of the species by specific numbers, because of dividing the altitude by 1000. Dashes were

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also used before or after the altitudes, like in the older literature sources; for some species the altitudinal range was listed as well.

Since 2001, a major and most accurate reference source for the vertical distribution of plant species has become the "Conspectus of the Bulgarian vascular flora", compiled by a group of Bulgarian botanists. Conspectus already had four editions, which is due to the rapid accumulation of information about the floristic richness and distribution of plant species in Bulgaria (DIMITROV, ed. 2001; 2002; ASSYOV & PETROVA eds. 2006; 2012). For example, the first edition included 3807 species of higher plants, while in the fourth edition they were 4102, i.e. 295 plant species have been added to the flora of Bulgaria, for only 11 year period. With a few exceptions, the "Conspectus" provides information about the range of altitudes (m) of distribution of each species. There are species, such as *Pseudotsuga menziesii* with no information about vertical distribution, because it has only recently been recognized as part of the adventitious flora of Bulgaria. (TASHEV *et al.*, 2012).

The purpose of the present work is to provide new data about the vertical distribution of some plants in Bulgaria, which could be considered in the next edition of the "Conspectus of the Bulgarian vascular flora" and in the publications elsewhere.

Materials and Methods

The data presented have been obtained during various floristic studies in the period 2006-2013. During our work, we paid special attention to the altitude, where plant species have been found, and if different from the well-known one, herbarium specimens were collected. The exact coordinates of localities and altitudes were scored by GPS. The plant names follow THE EURO + MED PLANTBASE – the information resource for Euro-Mediterranean plant diversity (2011) and ASSYOV & PETROVA (2012).

Results

Below we present descriptions of the new localities of species, found at altitudes not reported previously for the habitats of these species, thus representing new ecological niches. The following information is provided for each locality: floristic region and sub-region; geographic distribution, habitat, orography, exposition, slope, altitude, coordinates, date of collection and numbers of herbarium specimens in the Herbarium of the Institute of Biodiversity and ecosystem research – BAS (SOM). In some cases, floristic characteristic of the localities is provided. At the end we cite the references with altitudinal ranges of distribution different from these reported by us.

Caryophyllaceae
Stellaria alsine Grimm.

West Frontier Mountains. Mt Osogovo. locality Golyama chuka, in upland meadow, eastern exposition and inclination 5º, with *Luzula luzuloides* and *Lerchenfeldia flexuosa*, 1700 m a.s.l. (+200 m a.s.l.), 42°10'00.2"N, 22°35'56.5"E, 13.07.2011. coll. *Al. Tashev* (SOM 167596), *D. Dimitrov* (SOM 167613).

So far, in Bulgaria, the species has been known within the range of 1500 m a.s.l. (DELIPAVLOV ed., 2011: 75; ASSYOV & PETROVA, 2012: 399).

Fabaceae
Trifolium heldreichianum Hausskn.

West Frontier Mountains. Mt Osogovo. locality Golyama chuka, in upland meadow, eastern exposition and inclination 5º, together with *Luzula luzuloides* and *Lerchenfeldia flexuosa*, 1700 m a.s.l. (+100 m a.s.l.), 42°10'00.2"N, 22°35'56.5"E, 13.07.2011. coll. *Al. Tashev* (SOM 167595).

So far, in Bulgaria, the species has been known within the range of 1600 m a.s.l. (DELIPAVLOV ed., 2011: 217; ASSYOV & PETROVA, 2012: 421).

Poaceae
Koeleria nitidula Velen.

West Frontier Mountains. Mt Osogovo. Under peak Malak Ruen, in upland meadow, south-west exposition and inclination 2°, 2190 m a.s.l. (+1190 m a.s.l.), 42°09'43.0"N, 22°31'56.6"E, 13.07.2011. coll. *D. Dimitrov* (SOM 167620).

So far, in Bulgaria, the species has been known within the range of 900-1000 m a.s.l. (DELIPAVLOV ed., 2011: 501; ASSYOV & PETROVA, 2012: 247).

Sieglingia decumbens Bernh.

West Frontier Mountains. Mt Osogovo. Locality Kulin kamak., 1670 m a.s.l. (+170 m a.s.l.), 42°09'43.0"N, 22°31'56.6"E, 12.07.2011. coll. *D. Dimitrov* (SOM 167621).

So far, in Bulgaria, the species has been known within the range of 1500 m a.s.l. (DELIPAVLOV ed., 2011: 501; ASSYOV & PETROVA, 2012: 384).

Stipa tirsa Steven.

Rhodopi Mts (*Central*): Above the village Zhrebovo, on limestone rocks in the community of *Juniperus communis*. In the upper part of a slope, east-southeast exposition and inclination 15°, 1340 m a.s.l. (+240 m a.s.l.), KG-80, 41°34'19.8"N, 24°25'09.9"E, 7.09.2006. coll. *Al. Tashev, A. Vitkova* (SOM 164050, 164051).

This Euro-Asian species was reported from sea level to 700-1000 m a.s.l. (DELIPAVLOV ed., 2011: 515; ASSYOV & PETROVA, 2012: 401).

Scrophulariaceae

Verbascum formanekii Borbas ex Formanek

West Frontier Mountains. Mt Osogovo. Locality Mlachka reka, between a forest of *Fagus sylvatica* and a forest road on a slope, south-east exposition, inclination 15°, 1445 m a.s.l. (+445 m a.s.l.), 42°10'27.4"N, 22°36'34.8"E, 14.07.2011. coll. *Al. Tashev* (SOM 167599).

So far, this Bulgarian endemic species was reported up to 1000 m a.s.l. (DELIPAVLOV ed., 2011: 347; ASSYOV & PETROVA, 2012: 434).

Pedicularis leucodon Griseb.

West Frontier Mountains. Mt Osogovo. Under peak Malak ruen, on upland meadow dominated by *Alchemilla* spp., on a slope, south-west exposition, inclination 30°, 2192 m a.s.l. (+1192 m a.s.l.), 42°09'43.0"N, 22°31'56.0"E, 14.07.2011. coll. *Al. Tashev* (SOM 167600).

So far, this Bulgarian endemic species has been known only at altitude up to 1000 m a.s.l. (DELIPAVLOV ed., 2011: 353; ASSYOV & PETROVA, 2012: 307).

Saxifragaceae

Saxifraga stribrnyi (Velen.) Podp.

Rhodopi Mts (*Central*): Between village Bachkovo and town Asenovgrad, on limestone terrain, on the right bank of the Chepelare river (Chaya). 245 m a.s.l. (-265 m a.s.l.), KG-82, 41°59'31.2"N, 24°52'28.1"EO, 30.04.2008. coll. *Al. Tashev* (SOM 164668).

So far, this Bulgarian endemic species was reported at 500 – 2200 m a.s.l. (DELIPAVLOV ed., 2011: 169; ASSYOV & PETROVA, 2012: 368).

Asteraceae

Inula aschersoniana Steven.

Rhodopi Mts (*Central*): Above the village Zhrebovo, on limestone rocks in the community *Juniperus communis.* In the upper part of a slope, east-southeast exposition and inclination 15°, 1340 m a.s.l. (+240 m a.s.l.), KG-80, 41°34'19.8"N, 24°25'09.9"E, 7.09.2006. coll. *Al. Tashev, A. Vitkova* (SOM 163700).

So far, this Bulgarian endemic species was reported from sea level to 1000 m a.s.l. (DELIPAVLOV ed., 2011: 388; ASSYOV & PETROVA, 2012: 237).

Liliaceae

Scilla bifolia L.

The Tundja Hilly Plain:Ormana locality, close to the town of Yambol, in flooded forest in the community of *Quercus robur, Acer campestre, Cornus mas*, *Galanthus elwesii* etc. In a lowland forest, W-SW exposition and inclination 3°, 130 m a.s.l. (-870 m a.s.l.), 42°31'44.18"N, 26°31'13.3"E, 6.03.2013. coll.

Al. Tashev (SOM 169420, 169422).

In the herbarium of BAS (SOM) there are specimens of *Scilla bifolia,* without mentioning the altitude, but it is obviously far below 1000 m a.s.l. For example: *Strandzha:* By Tsarskoto kladenche, at the Veleka river, lands of the village Sinemorets. 23.03.1994. coll. *Ant. Petrova & T. Meshinev* (SOM 159188, 169422). *Black Sea Coast (South):* On the north bank of the Ropotamo river, opposite the natural landmark "Lion's Head". 13.03.1978. coll. *D. Peev* (SOM 137155).

The fact that this species has been known for all floristic regions of Bulgaria, many of which are located in lowlands, it expressively shows that it is distributed in many places below 1000 m asl. So far, this Pontic sub-Mediterranean species has been reported from 1000 to 2000 m asl (ASSYOV & PETROVA, 2012: 371).

Conclusion

This work presents new data on the vertical distribution of 10 species from the Bulgarian flora that can expand our knowledge of their ecological niche. The data presented can be used in preparation of new publications about the flora in Bulgaria.

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Content of Phenolic Compounds in the Genus Carduus L. from Bulgaria

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Abstract. Phytochemical screening of the content of total polyphenols, flavonoids, phenolic acids and anthocyanins in Bulgarian *Carduus* L. species was carried out. The plant materials (inflorescences) from all of the 14 species found in Bulgaria has been collected from natural habitats from different floristic regions, during the period 2011-2013. Chemical analysis of the specimens was carried out in accordance with 11 Russian and 7 European Pharmacopoeia. For some of the plant species the obtained results are the first published data about content of phenolic compounds. The content of flavonoids (1,8-3,2%) and total phenols(1,7-2,3%) was higher in comparison with this of phenolic acids (0,6-2,4%) and anthocyanins (0,5-1,5%). The highest content of total phenols and antocyanins was determined in the *Carduus thracicus.* The three species *Carduus thoermeri, Carduus nutans* and *Carduus candicans* ssp. *globifer* were characterized with the highest content of flavonoids. The highest content of phenolic acids was determined in the *Carduus armatus.*

Key words: *Carduus, phenolic compounds, phytochemical screening*

Introduction

Medicinal plants constitute one of the main sources of new pharmaceuticals and healthcare products (DEMIRAY *et al*., 2009). The role of medicinal plants in disease prevention or treatment been attributed to properties of their constituents, usually associated with a wide range of amphipathic molecules, broadly termed polyphenolic compounds (IVANOVA *et al*., 2005).

Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8,000 phenolic structures currently known (DAI & MUMPER, 2010). Plant phenolics include the groups of phenolic acids, flavonoids, antocyanins, highly polymerized substances such as tannins and some other compounds. These compounds exhibit a wide spectrum of medicinal properties, such as anti-allergic, anti-inflammatory, anti-microbial, anti-thrombotic, cardio-protective and vasodilatory effects (BALASUNDRAM & SAMMAR, 2006).

Flavonoids are the most abundant polyphenols in plants, sub-divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins (D'ARCHIVIO *et al*., 2007). Flavonoids possess a wide spectrum of biological activities in cardio vascular system and they are potential blood circulation enhancers in the

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brain (SHOHAIB *et al*., 2011). Antocyanins are water-soluble pigments, being the most reduced flavonoids. Anthocyanins occur in all tissues of plants, including leaves, stems, roots, flowers and fruits (ELOMAA *et al*., 2003). One of the most prominent property of the antocyanins is their excellent radical scavenging ability, intimately connected with their oxidation/reduction potential (YOUDIM *et al*., 2000). The role of anthocyanins in cardiovascular disease protection is strongly linked to oxidative stress protection too (KONG *et al*., 2003).

Phenolic acids, an other part of phenolic compounds, have attracted considerable interest in the past few years due to their strong antioxidant activities against free radicals (ACIKARA *et al*., 2013). Free radicals are the cause of many chronic diseases such as cancer, cardiovascular diseases, inflammation, brain dysfunction (GRUZ *et al*., 2011; KAMATOU *et al*., 2010). Phenolic acids also exhibit antibacterial, antipyretic, antirheumatic activities (MATTILA & HELLSTRÖM, 2007; MARKOWSKI *et al*., 1998). Ferulic and sinapic acids, like phenolic acids, are antioxidants (IBTISSEM *et al*., 2012) They exhibit direct antitumor activity against breast (GELINAS & MCKINNON, 2006) and liver cancer (BEEJMOHUN & FLINIAUX, 2007). Ferulic acid aslo may be effective in preventing cancer induced by exposure to carcinogenic compounds (QUINDE-AXTELL & BAIK, 2006).

The genus *Carduus* is presented in the Bulgarian flora by 14 species, 5 of which are endemic (STOJANOV *et al*., 1967; DELIPAVLOV & CHESHMEDZHIEV, 2003; TUTIN *et al*., 1976). The species *Carduus acanthoides* (Plumeless thistle) is used in traditional medicine as a diuretic, cardiotonic, sedative and anti-hemorrhoidal remedy (PETKOV, 1982).

Of the secondary metabolites contained in *Carduus* species, the flavonoids are the group, which has been most studied, regarding mostly their qualitative composition **(**HARBORNE, 1977; AMER *et al*., 1985; HEDIN & WAAGE, 1986; BAIN & DESROCHERS, 1988; FERNANDEZ *et al*., 1991; EL-LAKANY et al., 1997; SIMMONDS & GRAYER, 1999; WILLIAMSON *et al*. , 1999; JORDON-THADEN & LOUDA, 2003; TERENTJEVA & KRASNOV, 2003; ZHANG *et al*., 2001; XIE *et al*., 2005; JEONG *et al*., 2008**)**. Phytochemical studies on *Carduus getulus* revealed the presence of steroids and triterpenoid constituents as well (ABDEL-SALAM *et al*.,1982). Other authors reported alkaloids and phenolic acids, lignans and coumarins for the species *Carduus acanthoides, Carduus crispus* and *Carduus nutans*. (FRYDMAN & DEULOFEU, 1962; ZHANG *et al*., 2001, 2002; TERENTJEVA & KRASNOV, 2003; XIE & JIA, 2004). Our previous studies of six different *Carduus* species showed significant levels of total phenols and flavonoids (ZHELEVA-DIMITROVA *et al*., 2011).

Considering the fact that the phenolic compounds contribute most commonly to the biological activity of various medicinal plants, the objective of the present work was phytochemical screening for the main phenolic compounds content, in particular total phenols, flavonoids, phenolic acids and anthocyanins in the Bulgarian *Carduus* species.

Material and Methods

Plant material. The plant material (inflorescences) was collected from natural habitats, during the period 2011-2013, from all fourteen species of the genus *Carduus*, found in Bulgaria (Table 1). Voucher specimens of the studied species were deposited in a herbarium at the Agriculture University of Plovdiv, Bulgaria (Herbarium SOA).

Quantification of total phenols. The determination of total phenols in the plant drugs was performed according to the EUROPEAN PHARMACOPOEIA 7 (2011a) involving Folin-Chiocalteu reagent and pyrogallol as standard. The analyses were carried out at 760 nm. The measurements were carried out using an Ultraspec 3300 pro UV/VIS spectrophotometer (USA). All determinations were performed in triplicate (n=3).

Quantification of flavonoids. The content of the flavonoids in the plant material was spectrophotometrically determined at 430 nm by creating a complex with $AlCl_3$

according to the RUSSIAN PHARMACOPOEIA 11 (1990). The content of flavonoids was calculated as quercetin. The measurements were carried out using a Ultraspec 3300 pro UV/VIS spectrophotometer (USA). All determinations were performed in triplicate (n=3).

Quantification of phenolic acids. Determination of the total phenolic acids in the plant material was performed according to the EUROPEAN PHARMACOPOEIA 7 (2011b),using rosmarinic acid as a reference. The analyses were carried out at 505 nm. The measurements were carried out using an Ultraspec 3300 pro UV/VIS spectrophotometer (USA). All determinations were performed in triplicate (n=3).

Quantification of anthocyanins. The determination of anthocyanins in the plant material was performed according to the EUROPEAN PHARMACOPOEIA 7 (2011c). The method comprises sequential extraction with ethanol and butanol. Cyanidin chloride was used as a standard. The analyses were carried out at 505 nm using an Ultraspec 3300 pro UV/VIS spectrophotometer (USA). All determinations were performed in triplicate (n=3).

Table 1. Collection locality, altitude and voucher specimen of the studied *Carduus* species

Species	Collection locality, Floristic region in Bulgaria	Altitude (m)	Voucher specimen in Herbarium SOA-Plovdiv
C. acanthoides L.	Trigrad, Rhodopes Mountains (middle)	1100 m	059719
C. acicularis Betrol. (*C. argentatus*)	Tzarevo, Black Sea Coast (southearn)	30 m	059650
C. armatus Boiss. & Heldr. (*C. tmoleus ssp. armatus*)	"Balgarka" Nature park, Balkan Range (middle)	1415 m	059781
C. candicans Waldst. & Kit. *ssp.globifer* (Velen.) Kazmi	Starosel, Thracian valley	349 м	059723
C. carduelis (L.) Gren. (*C.alpestris*)	Yundola, Rila Mountains	1510 m	059779
C. crispus L.	Ivanski, Northeast Bulgaria	92 m	059725
C.hamulosus Ehrh.	Narechen, Rhodopes Mountains (middle)	588 m	059644
C. kerneri Simonkai *ssp.austro-orientalis* Franco (*C.scardicus*)	Beglika, Rhodopes Mountains (westearn)	1550 m	059651
C. nutans L.	Arkutino, Black sea Coast (southearn)	30 m	059660
C. personata (L.)Jacq.	"Balgarka" Nature park, Balkan Range (middle)	815 m	059780
C. pycnocephalus L.	Carevo, Black Sea Coast (southearn)	30 m	059649
C. rhodopaeus Velen. (*C.adpressus ssp.r.*)	Rozhen, Rhodopes Mountains (middle)	1431 m	059776
C. thoermeri Wienm. *(C. leiophyllus)*	Rozhen, Rhodopes Mountains (middle)	1465 m	059777
C. thracicus Hayek.	Tzatzarovci, Rhodopes Mountains (middle)	956 m	059773

Results and Discussion

Content of total polyphenols. The amount of total polyphenols (including all water soluble phenolic compounds in plant) was expressed as pyrogallol equivalent in % (Fig. 1). The amounts ranged from 1,72% to 2,25%. Species, characterised by higher contents of these substances were *C. thracicus* (2.25%), followed by *C. armatus* (2,12 %) and *C. nutans* (1,97 %). In most of the investigated species contents of total polyphenols between 1,7 – 1,8% was established.

The established quantities of total phenols in the studied genus *Carduus* were similar to those reported for the closely related genera *Silybum* (WOJDYŁO *et al.*, 2007) and *Centaurea* (EROL-DAYI *et al.* 2011). Relatively lower values were determined by NAZARUK (2008) – 0,012 -0,8% and YIN *et al.* (2008) – 0,022 % for species of the genus *Cirsium.* The obtained results revealed that

the Bulgarian *Carduus* species have good content of these valuable biologically active ingredients, confirming our previous data for six *Carduus* species (ZHELEVA-DIMITROVA *et al*., 2011), as well as the fact that the environmental conditions of growth location do not significantly affect the accumulation of common water-soluble polyphenols in a genus *Carduus* (ZHELEV *et al*., 2011).

Content of flavonoids. The phytochemical analysis for flavonoid content showed that this group of phenolic compounds was predominant in the studied *Carduus* species - from 1.78% to 3.20% (Fig. 2**).** *C. thoermeri* showed the highest flavonoid content of 3.20%, followed by *C. nutans* (3.00%) and C. *candicans* ssp. *globifer* (2.95%). Other species, with flavonoid contents more than 2% in descending order were *C. pycnocephalus, C. crispus, C. acanthoides, C. kerneri ssp. austro-orientalis* and *C. thracicus*.

Among the investigated phenolic compounds in the studied plant species the presence of flavonoids was established as predominant, although they are part of the total phenolic compounds group. This might be explained by the different extracting solvents - the polyphenols determination was carried out in the water extracts where the most flavonoids are not soluble. For quantification of the flavonoids 90% ethanol was used. More than a half of the studied species demonstrated significant levels of flavonoids, which confirm our previously reported results, especially for C. *thoermeri, C. nutans* and *C. candicans* ssp. *globifer* (ZHELEV *et al.,* 2011; ZHELEVA-DIMITROVA *et al*., 2011). The quantitative accumulation of total flavonoids is influenced not only by the species differences, but also by the conditions of growth locations (ZHELEV *et al*., 2011). In comparison, study of NAZARUK & SZOKA (2009) on species from the related genus *Cirsium* show significant lower values in range from 0.016 to 0,07%, compared to 2.31% flavonoids in *Cirsium japonicum* (Yin *et al.*, 2008).

Content of phenolic acids. Research on the quantity of phenolic acids has been carried out for the first time for the genus *Carduus.* The obtained results showed that this group of phenols varies in wide range 0,57% - 2,43% (Fig. 3). Among the investigated plans with higher content (more than 2%) were established to be *C. armatus* (2,43%), *C. personata* (2,15%), *C. hamulosus* (2,03%) and *C. carduelis* (1.96%). In comparison lower values of phenolic acids were reported by NAZARUK & SZOKA (2009) in species of the genus *Cirsium* 0,15% - 1,31%.

Content of anthocyanins. Anthocyanins, the most reduced flavonoids, were presented in *Carduus* inflorescences in range from 0.47% to 1.45% (Fig. 4) as *Carduus thracicus* was the species with the highest established amount of these purple or blue pigments. Among

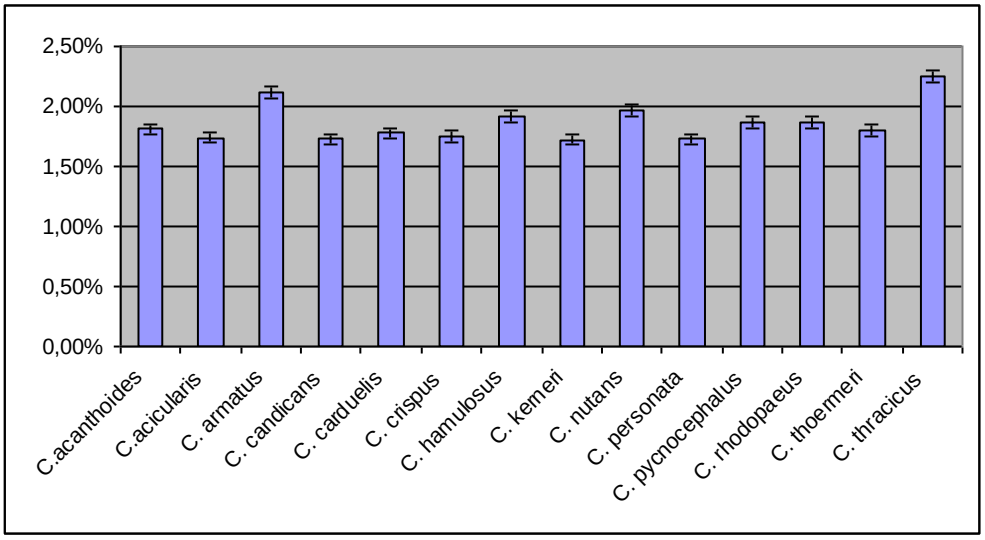

Fig. 1. Content of total water soluble polyphenols in Bulgarian *Carduus* species (%).

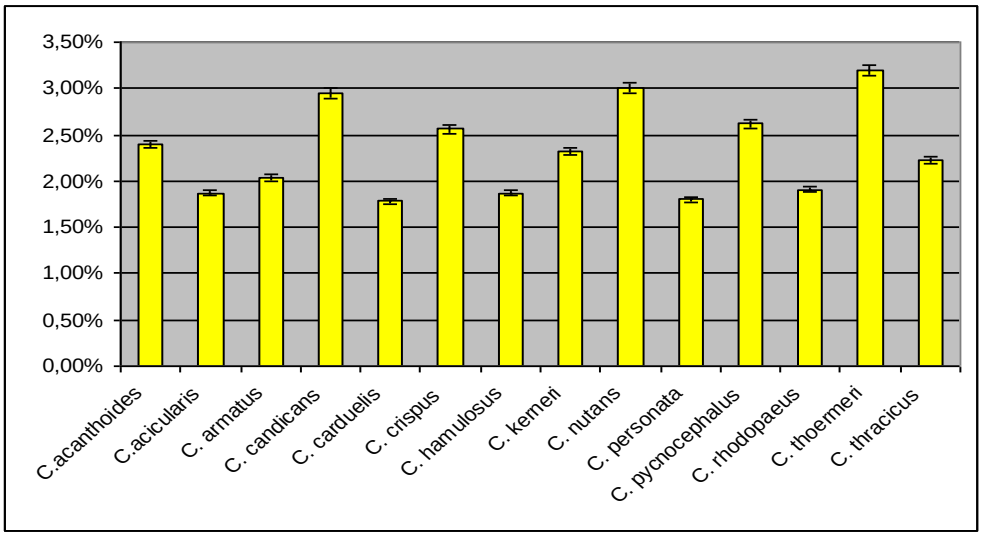

Fig 2. Content of flavonoids in Bulgarian *Carduus* species (%).

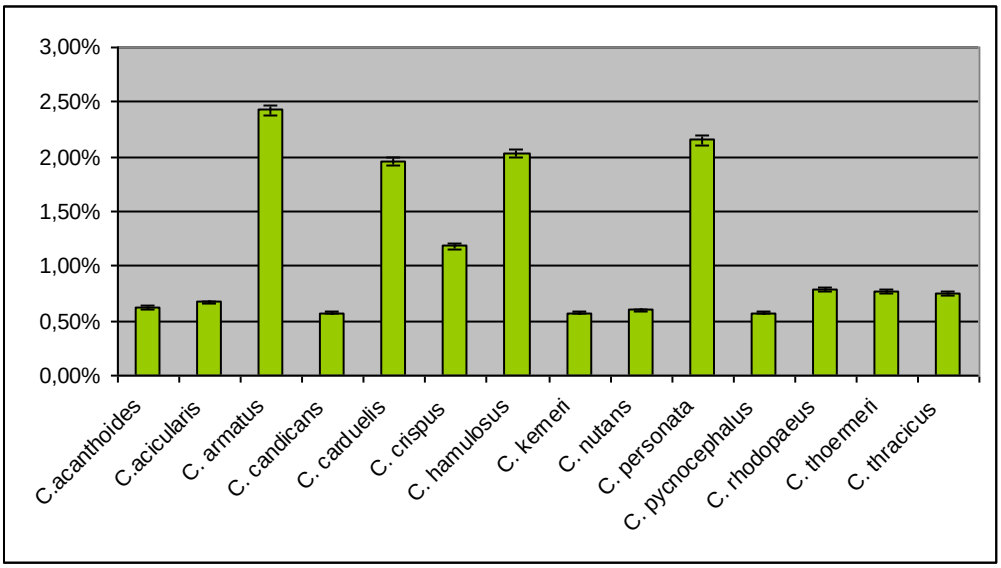

Fig. 3. Content of phenolic acids in Bulgarian *Carduus* species (%)

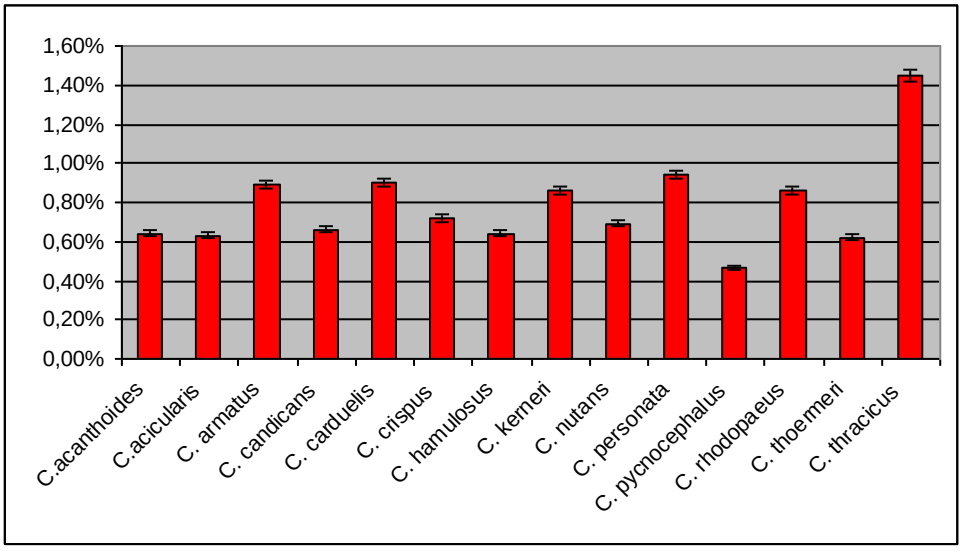

Fig 4. Content of anthocyanins in Bulgarian *Carduus* species (%)

the investigated plants with anthocyanins content in range of 1% were *C. personata* (0.94%), *C. carduelis* (0.90%), *C. armatus* (0.89%), *C. rhodopaeus* and *C. kerneri* (0.86% both).

It is notable that plant species from mountain areas at the above 800m altitude, contained higher quantities of these substances. However, comparison of the results is not possible, because there are no previous data on anthocyanins content in *Carduus* genus and related genera at all.

The investigations of other researchers conducted on phenolic compounds content were mainly related to their good antioxidant properties (DEMIRAY *et al*., 2009; DAI & MUMPER, 2010; MIHAYLOVA *et al*., 2013). Our previous study on six *Carduus* species showed that all tested extracts exhibit significant antioxidant activity (ZHELEVA-DIMITROVA *et al*., 2011). The antioxidant activity values for *C. thoermeri, C. candicans* ssp. *globifer* and *C. nutans* were similar compared to the standard compound (L-ascorbic acid). This could be an objective for further investigations on the qualitative composition and biological activity of the investigated groups of phenolic compounds in the species of this genus.

Conclusions

In summary, as a result of the conducted phytochemical screening of genus *Carduus* in Bulgaria in terms of main phenolic compounds content, their presence in the *Carduus* inflorescences was established. Flavonoids and total phenols were revealed as dominant constituents, followed by phenolic acids and anthocyanins. Species with the highest amount of these valuable biologically-active substances were *Carduus thoermeri, Carduus nutans*, *Carduus candicans* ssp. *globifer, Carduus thracicus* and *Carduus armatus.*

The present study provides for the first time scientific data for the quantity of the phenolic acids and anthocyanins occurring in all Bulgarian *Carduus* species. The total polyhenols and flavonoids content have been also studied for the first time for the mentioned species - *C. armatus*, *C. carduelis, C. hamulosus, C. rhodopaeus* and *C. thracicus*, respectively.

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Age Determination in Some Ophisops elegans Mènètriès 1832 (Sauria: Lacertidae) Populations Living in the Vicinity of Çanakkale and Akşehir-Eber

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Abstract. In this study, age determination was performed with the method of skeletochronology in 23 (13 ♂♂, 10 ♀♀) *Ophisops elegans* specimens collected from Çanakkale in the west of Turkey and 20 (10 ♂♂, 10 ♀♀) *Ophisops elegans* specimens collected from the vicinity of AkÇehir-Eber in the Central Anatolia Region. The mean ages of the specimens whose femur cross sections had been examined were determined to be 2.9±0.99 (1-4) in females and 2.85±0.68 (2-4) in males of the Çanakkale population and 3.9±1.19 (3-6) in females and 4.6±1.17 (3-6) in males of the AkÇehir-Eber population. The oldest age among the specimens was found to be six in two female and three male individuals of the AkÇehir-Eber population. When all specimens were evaluated altogether, the correlation between snoutvent length and age was computed as (r=0.572, p=0.008) in females and as (r=0.642, p=0.001) in males.

Key words: *Ophisops elegans*, skeletochronology, Çanakkale, AkÇehir, Eber.

Introduction

The Snake-eyed lizard, *Ophisops elegans*, one of the most commonly encountered lizard species in Turkey, is distributed in south-eastern Balkans, Turkey and Transcaucasia, on some islands of the Eastern Mediterranean Sea and the Aegean Sea, and in Syria, Palestine and the north of Africa (LANTZ, 1930; BODENHEIMER, 1944; BARAN, 1984; CHIRIO & BLANC, 1993; SCHLEICH *et al*., 1996; FRYNTA *et al*., 2000; SINDACO *et al*., 2000; GÖÇMEN *et al*., 2008). The studies on species *O. elegans*, which has been distributed over an extensive area in Turkey and whose taxonomic position has not been fully clarified yet, are generally on taxonomy and feeding biology (TOK, 1992; 1993; TOK *et al*., 1997; OLGUN & TOK 1999; AKKAYA & UĞURTAĞ, 2006; YILDIZ *et al*., 2012). In the morphology-based taxonomic studies, the need to make evaluations by dividing the animals into age groups was highlighted (CASTANET & SMIRINA, 1990). However, the age determination studies on reptiles are rather scarse in Turkey (OLGUN *et al*., 2005; MIAUD *et al.,* 2007; ÜZÜM, 2009; ÜZÜM & OLGUN, 2009; KUTRUP *et al.,* 2011; YAKIN *et al.,* 2012).

Today one of the most frequently used methods to determine the age of reptiles is skeletochronology. In this method, the diaphysial region which displays weak branching in the long bones of reptiles is the part that yields the best result (CASTANET *et al.,* 1993). The energy metabolism depends on temperature in reptiles, as in many other ectothermic living things. Throughout hibernation, reptiles live at low energy levels; therefore, their longevity increases depending on the duration and energy of

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hibernation (CASTANET, 1994). A resting line occurs in reptiles during hibernation. These structures are in the form of rings which indicate the local osteogenesis that stops temporarily (CASTANET et al., 1993; SMIRINA *et al*., 1986).

The current study aims to perform age determination with the method of skeletochronology on *O. elegans* specimens collected in the field studies carried out in the western and Central Anatolian regions of Turkey and to reveal the correlation between the parameters of age and snout-vent length (SVL) in two different populations. Another aim of the present study is to provide more reliable data about the age-SVL relationship in the morphological studies to be made regarding the species.

Material and Methods

Totally 43 (23 ♂♂, 20 ♀♀) wild-collected specimens during the field studies in the vicinity of Çanakkale and AkÇehir-Eber were examined with the method of skeletochronology. 20 (10 ♂♂, 10 ♀♀) specimens from the Çanakkale population and 23 (13 ♂♂, 10 ♀♀) specimens from the AkÇehir-Eber population were evaluated. In the study, the approximate ages of species *O. elegans* were determined, and the age-SVL relationship in the individuals of both populations was investigated. Body measurements of the specimens were done by using a digital compass sensitive to 0.01 mm. The measurement values were provided in millimeters.

After the morphometric measurements of the *O. elegans* specimens had been obtained, the left femora of the specimens were excised with scissors on both sides by opening a small incision in the femoral region so as not to damage the material. The surrounding tissues were cleaned off the femora. Later on, they were left in 5% nitric acid (HNO_3) for 3-5 hours for the procedure of decalcification. After the femora had been embedded in paraffin, 10-µm-thick sections were obtained. The preparations were stained with the Ehrlich's Hematoxylin and Olympus BX51 light microscope was used for the. They were photographed by using an Olympus Analysis LS program.

The t-test was applied for the SVL values and ages between sexes and localities, while linear regression and Spearman's correlation test were applied in order to reveal the correlation between age and SVL. The statistical analyses were made by using SPSS 15.0, and the significance level was considered to be 0.05.

Results

The Çanakkale Population. An age evaluation was made in 23 (13 ♂♂, 10 ♀♀) individuals of the Çanakkale population. The SVL values ranged from 40.54 to 50.52 mm in males, and the mean was calculated as 46.21±3.23 mm. In females, the SVL values ranged from 38.41 to 52.63 mm, and the mean was 46.53±4.01 mm (Table 1). No difference in SVL was determined between male and female individuals in the Çanakkale population ($p \leq 0.83$). Likewise, the values of age between male and female individuals of this population were found to be close ($p \leq 0.78$).

The Akşehir-Eber Population. An age evaluation was made in 20 (10 ♂♂, 10 ♀♀) individuals of the AkÇehir-Eber population. The SVL values ranged from 46.70 to 50.35 mm in males, and the mean was calculated as 49.19±1.05 mm. In females, the SVL values ranged from 46.62 to 53.47 mm, and the mean SVL was 49.22±2.01 mm (Table 2). No difference in SVL was observed between male and female individuals in this population ($p \leq 0.96$). The mean values of age between female and male specimens of the AkÇehir-Eber population were found to be close ($p \leq 0.19$). Between the Çanakkale and AkÇehir-Eber populations, a difference in SVL was determined among males ($p \leq 0.05$), whereas no difference was detected among females ($p \leq 0.07$).

The distribution of ages of *O. elegans* specimens by population is provided in Fig. 1. When the values of age for male and female specimens were compared between the populations, it was determined that the males were older ($p \leq 0.00$), but the females were at similar ages in the Central Anatolian specimens which had been collected almost in the same season ($p \leq 0.06$).

Table 1. Descriptive statistics of SVL (mm) and age (year) of investigated specimens and results of ındependent-samples T test (SVL: Snout-Vent Length; SE: Standard Error; SD: Standard Deviation).

		Çanakkale Population						Akşehir-Eber Population						
	Sex	N	Min	Max	Mean	SE	SD	N	Min	Max	Mean	SE	SD	P
SVL	♂♂	13	40.54	50.52	46.21	0.89	3.23	10	46.70	50.35	49.19	0.33	1.05	0.01*
	♀♀	10	38.41	52.63	46.53	1.27	4.01	10	46.62	53.47	49.22	0.63	2.01	0.07
Age	♂♂	13	2	4	2.85	0.19	0.68	10	3	6	4.60	0.37	1.17	0.00*
	♀♀	10	1	4	2.90	0.31	0.99	10	3	6	3.90	0.37	1.19	0.06

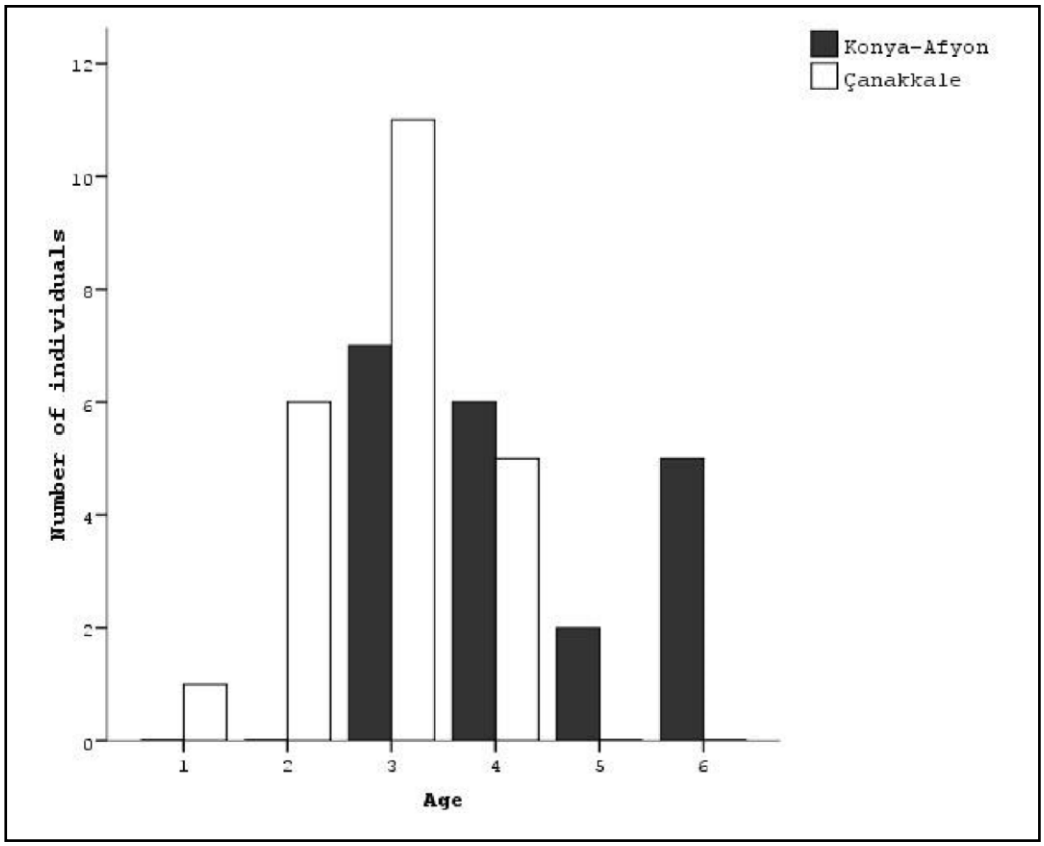

Fig. 1. Populations and age distributions of *O. elegans* specimens.

The graphs of the correlation between SVL and age in the Çanakkale and AkÇehirEber populations by sex are presented in Fig. 2 for males and in Fig. 3 for females. According to the linear regression analysis, a significant correlation between age and SVL was detected between the specimens of the Çanakkale population (male, ANOVA: F=6.31, df=1, p≤0.029; female, ANOVA: F=8.03, df=1, p≤0.021) and the specimens of the AkÇehirEber population (male, ANOVA: F=5.59, df=1, p≤0.041; female, ANOVA: F=6.02, df=1, p≤0.047). According to Spearman's correlation test, it was established that there was a moderately positive correlation between age and SVL (Çanakkale: male, r_s=0.52, n=13; female, r_s=0.56, n=10; AkÇehir-Eber: male, r_s=0.68, n=10; female, r_s=0.48, n=10). When all specimens were evaluated altogether, the correlation between SVL and age was computed as (r=0.572, p=0.008) in females and as (r=0.642, p=0.001) in males.

The approximate ages of the specimens were calculated according to the rings of age in the femur sections of the *O. elegans* specimens (Fig. 4).

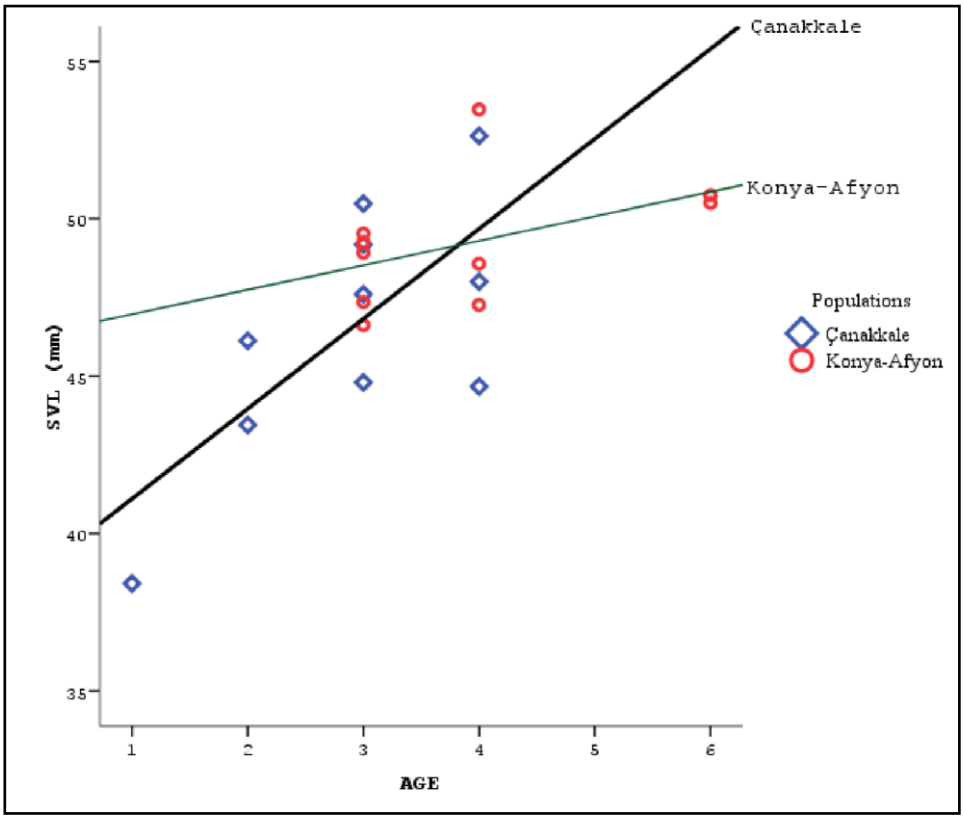

Fig. 2. The relationship between age and SVL of female *O. elegans* specimens.

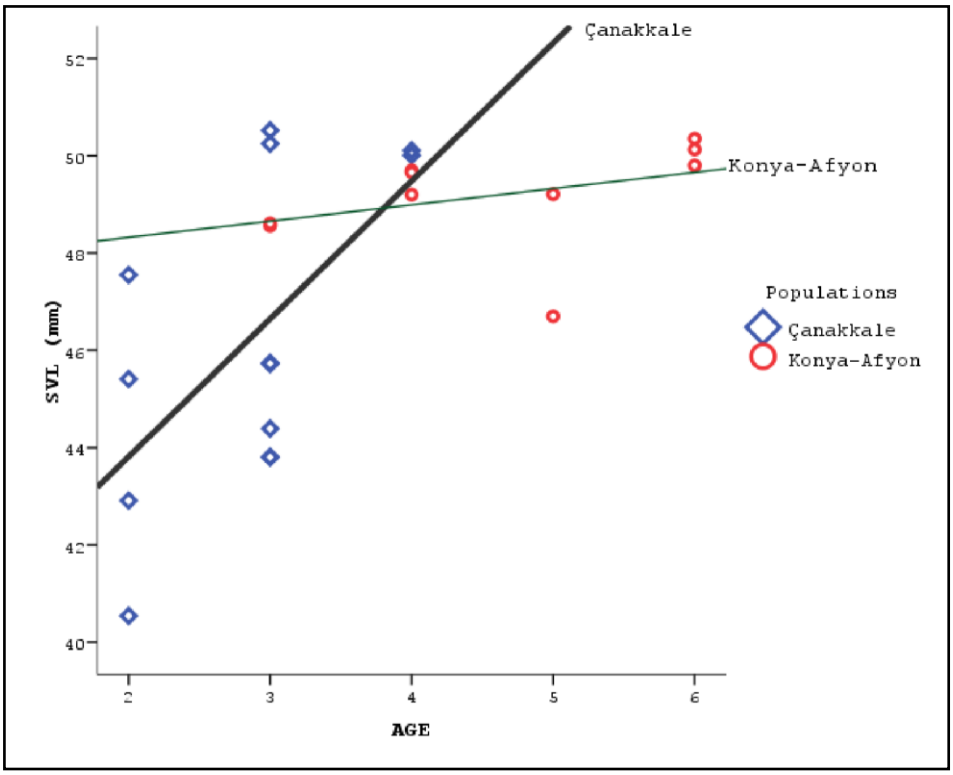

Fig. 3. The relationship between age and SVL of male *O. elegans* specimens.

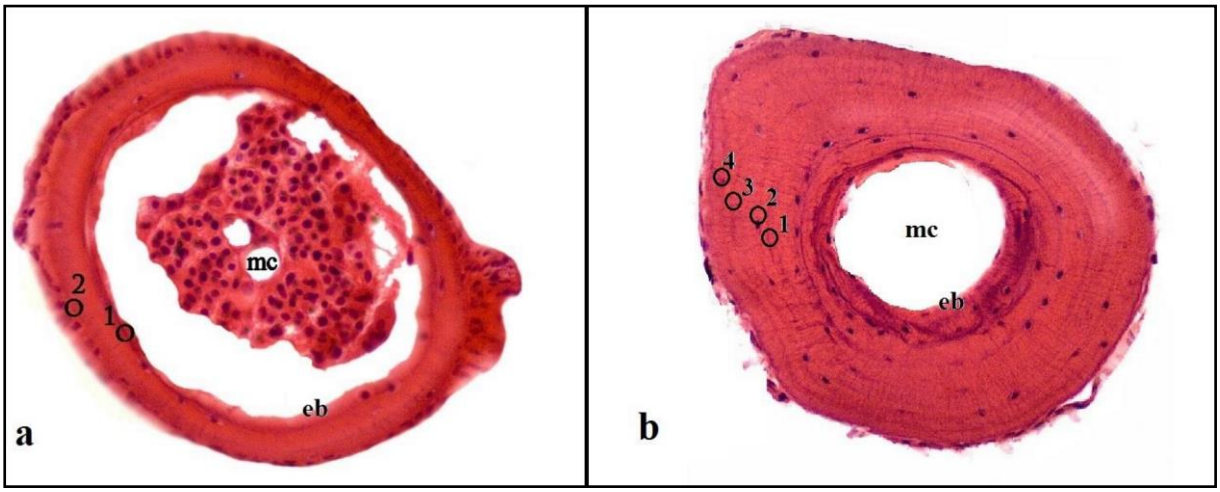

Fig. 4. Cross-sections of the femur of *O. elegans* a. 2-yr-old; b. 4-yr-old (mc: marrow cavity, eb: endosteal bone).

It was determined that endosteal resorption was present in the only one-year-old specimen of the Çanakkale population, and the SVL value was measured as 38.41 mm. In addition, endosteal resorption was detected in 4 three-year-old specimens of the Çanakkale population (57%), in 5 three-year-old specimens of the AkÇehir-Eber population (45%), and in three six-year-old specimens of the AkÇehir-Eber population (60%).

Discussion

The mean SVL of 25 juvenile and adult *O. elegans* specimens collected from west of Iran was reported 48.75 mm with no significant difference between males and females (GHARZI & YARI, 2013). According to TOK (1999) SVL was reported to be 46.99±0.29 mm in males and 46.77±0.31 mm in females in the 94 *O. elegans* specimens collected from the ReÇadiye (Datça) Peninsula. In our study, SVL was measured as 46.21±0.89 mm in males and 46.53±1.27 mm in females in the specimens of Çanakkale. The mean SVL values are in agreement with the study concerned. In another study, it was determined that the mean SVL values were higher in the 43 *O. elegans* specimens collected from the vicinity of Konya than those of the western population (TOK, 1992). However, the mean SVL was measured as 49.19±1.05 mm in males and as 49.22±2.01 mm in females in the AkÇehir-Eber specimens evaluated in our study (Table 1). As also stated in the literature, the specimens distributed in Central Anatolia have greater SVLs as compared with the western populations. Differences were determined for the male specimens in our study, which is in agreement with the literature (Table 1).

It was observed that generally no importance was attached to the distribution of ages when analyzing the morphological measurements in the majority of the taxonomic studies with reptiles. Nevertheless, it is stated that it is important to determine the ages of the specimens under examination in order to be able to make an accurate analysis (CASTANET & SMIRINA, 1990). When the specimens examined in our study were categorized according to the distribution of ages, it was determined that the majority of them were aged three (41.8%) and four (25.5%) years.

The difference in SVL values is thought to correlate with the fact that the maximum and mean ages were found to be greater in the male specimens from AkÇehir-Eber under examination.

Studies of age performed in some lacertid species by using the method of skeletochronology are known (CASTILLA & CASTANET, 1986; GUARINO *et al.*, 2010; KOLAROV *et al.*, 2010; GHARZI & YARI, 2013). In these studies, the maximum ages detected in the wildcollected lacertid species vary. In ARAKELYAN & DANIELYAN (2000) it was stated that the maximum age was seven in *L.*

armeniaca and six in *L. unisexualis*, *L. dahli* and *L. raddei* and that the oldest specimens in species *L. nairensis* were aged five years. In another lacertid study, however, the maximum age was found to be four in the males and five or six in the females of *L. derjugini* (ORLOVA & SMIRINA, 1983). In species *L. strigata* and *L. agilis*, longevity was computed as six or seven years (ROITBERG & SMIRINA, 1995). Nevertheless, longevity does not exceed four years in *Lacerta vivipara* specimens (PILORGE & CASTANET, 1981). Other lacertids, *Gallotia atlantica*, *G. galloti* and *G. stahlini*, can live for five, nine and eleven years, respectively (CASTANET & BAEZ, 1991). In GHARZI & YARI (2013) it was reported that maximum age in females and males was 5 and 4 years, respectively. In our study, the maximum age was found to be six in the specimens of the AkÇehir-Eber population and four in the specimens of the Çanakkale population. CASTANET (1994) as well, reported that the small lacertids living under colder environmental conditions have longer longevity. Semi-arid climatic conditions prevail in the vicinity of AkÇehir and Eber, located in the lower sections of Central Anatolia, and the Mediterranean climatic conditions, in which winters are warm and rainy, prevail in the coastal sections of Çanakkale, located in the Aegean sub-section where our specimens had been collected (ATALAY, 2002). We are of the opinion that the finding of older individuals among the specimens collected from the vicinity of AkÇehir-Eber with lower mean temperatures than the coastal sections of Çanakkale might relate to climatic conditions.

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Assessing the Influence of the Automobile Traffic on the Amphibians and Reptiles in the Buffer Zone of Biosphere Reserve "Srebarna" (NE Bulgaria)

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Abstract. Currently the problem of the effects of the road network and traffic on the amphibians and reptiles in Bulgaria is poorly studied. During the period March 2002 - March 2004 in the Buffer Zone of Biosphere Reserve "Srebarna" (NE Bulgaria) were built two anti-fire roads from the eastern and western side of the lake in area of grasslands of semi-steppe type, typical for north-eastern Bulgaria. The aim of the constructed roads is to provide access for fire vehicles to areas in and around the reserve. The current study aims to provide data on the impact of road traffic and the newly constructed road network and another previously existing road on the amphibians and reptiles inhabiting the buffer zone of the biosphere reserve "Srebarna". For the entire period of study in the three studied road sections a total of 15 dead specimens of amphibians belonging to 4 species (*Bombina bombina, Hyla arborea, Bufo bufo, Bufo viridis*) and 70 dead specimens of reptiles belonging to 8 species (*Emys orbicularis, Ablepharus kitaibelii, Lacerta viridis, Podarcis tauricus, Podarcis muralis, Natrix natrix, Coronella austriaca* and *Dolichophis caspius*) were recorded. Several "hot spots", where most cadavers were recorded are well described and possible conservation measures are discussed.

Key words: road traffic, road mortality, Amphibia, Reptilia, conservation, Srebarna, Bulgaria

Introduction

Building roads for the needs of automobile transportation greatly affects the environment (HAWBAKER & RADELOFF, 2004), because they serve as barriers or filters to some animal movement. Road width and traffic density are major determinants of the barrier effect, whereas road surface (asphalt or concrete versus gravel or soil) is generally a minor factor (FAHRIG *et al.*, 1995; FORMAN, 1995).

Existing road network with high intensity of traffic is virtually insurmountable barrier for small terrestrial animals, which include amphibians and reptiles. Particularly critical are road sections adjacent to ponds or wetlands crossed by ravines without the necessary equipment to protect small animals (BISERKOV *et al.*, 2005). A growing literature suggests that roads by wetlands and ponds commonly have the highest roadkill rates (FAHRIG *et al.*, 1995; ASHLEY & ROBINSON, 1996; VOS, 1997, CICORT-LUCACIU *et al.*, 2012). Reptiles and amphibians are among the fauna most negatively affected by poor transportation

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planning associated with wetlands alteration and their mortality can be significant (ASHLEY & ROBINSON, 1996; CLEVENGER *et al.*, 2001; SMITH & DODD, 2003).

In Bulgaria, this problem is poorly studied. With the exception of a few fragmentary reports of the road kill specimens of amphibians and reptiles in various parts of the country so far there are only two studies, which provide data on the impact of traffic on amphibians and reptiles in the city of Plovdiv (MOLLOV, 2005) and on a section of "Trakia" highway between Belovo Village and Plovdiv (KAMBOUROVA-IVANOVA *et al.*, 2012). Representative data from road sections located outside populated areas is extremely scarce.

During the period March 2002 - March 2004 in the Buffer Zone of Biosphere Reserve "Srebarna" were built two anti-fire roads from the eastern and western side of the lake in area of grasslands of semi-steppe type, typical for north-eastern Bulgaria. The aim of the constructed roads is to provide access for fire vehicles to areas in and around the reserve.

The purpose of the current study is to provide data on the impact of the road traffic and road network on some amphibians and reptiles inhabiting the buffer zone of the biosphere reserve "Srebarna" (NE Bulgaria).

Material and Methods

*Study area.*The lake of Srebarna is situated in North-Eastern Bulgaria on the right bank of the Danube River (44°05' n.l. and 27°07' e.l.) between the river kilometres 393 and 391. The total territory of the Srebarna Biosphere reserve is 902.1 ha including the shallow holo-polymictic Srebarna Lake with slightly alkaline to neutral waters and the Danubian island Devnya. The watershed basin covers area of 1,070 km^2 and is formed of the basins of the rivulets Srebrenska, Babukska and Kalnezha (MODEV, 1994).

Data collection. For purposes of the current study a series of observations were carried out in the period from March to October 2004-2006, in three road sections within the buffer zone of the biosphere reserve "Srebarna" (Fig. 1):

1. Anti-fire road in the western part of the buffer zone of the reserve (AFRBZ-W);
2. Anti-fire road in the eastern part of the buffer zone of the reserve (AFRBZ-E);
3. Section of the main road Ruse-Silistra (MRRS).

Surveying of road sections was done on foot in order not to miss any specimens. Observations were carried out mainly during the day from 9:00 am to 17:00 pm. Every recorded dead animal was determined visually using the field guide of ARNOLD & OVENDEN (2002). For each species are given a valid Latin and common name, place of death, age group (juvenile, subadult, adult). Probable date of death of recorded dead specimens was established the following way - the animals with daily activity presumed to have been killed in the day when we found them, and for animals with nocturnal activity – the day or night before. After recording each dead specimen it was removed from the road to avoid double counting.

The following measurements were taken for the studied road sections: cover type, width of the road (in meters), the volume of traffic (average number of vehicles passing per hour and average speed of passing cars) and data on climatic conditions (air temperature, cloud composition and rainfall) at the time of finding the animal (Table 1).

Statistical analysis. The differences in the number of killed amphibians and reptiles on the three types of roads were examined using t-test for independent samples. Because the data didn't have normal distribution it was normalized using the arcsine transformation (FOWLER *et al.*, 1998). The differences between the equal and the observed monthly distribution of road kills and the distribution of cadavers in relation of the climatic conditions were processed using χ^2 test. The Spearman correlation was used to find any dependence between number of killed animals and average 1-hour traffic intensity (number of vehicles per one hour) by month. For the statistical

processing of the data we used the software package "Statistica 7.0" (StatSoft Inc., 2004).

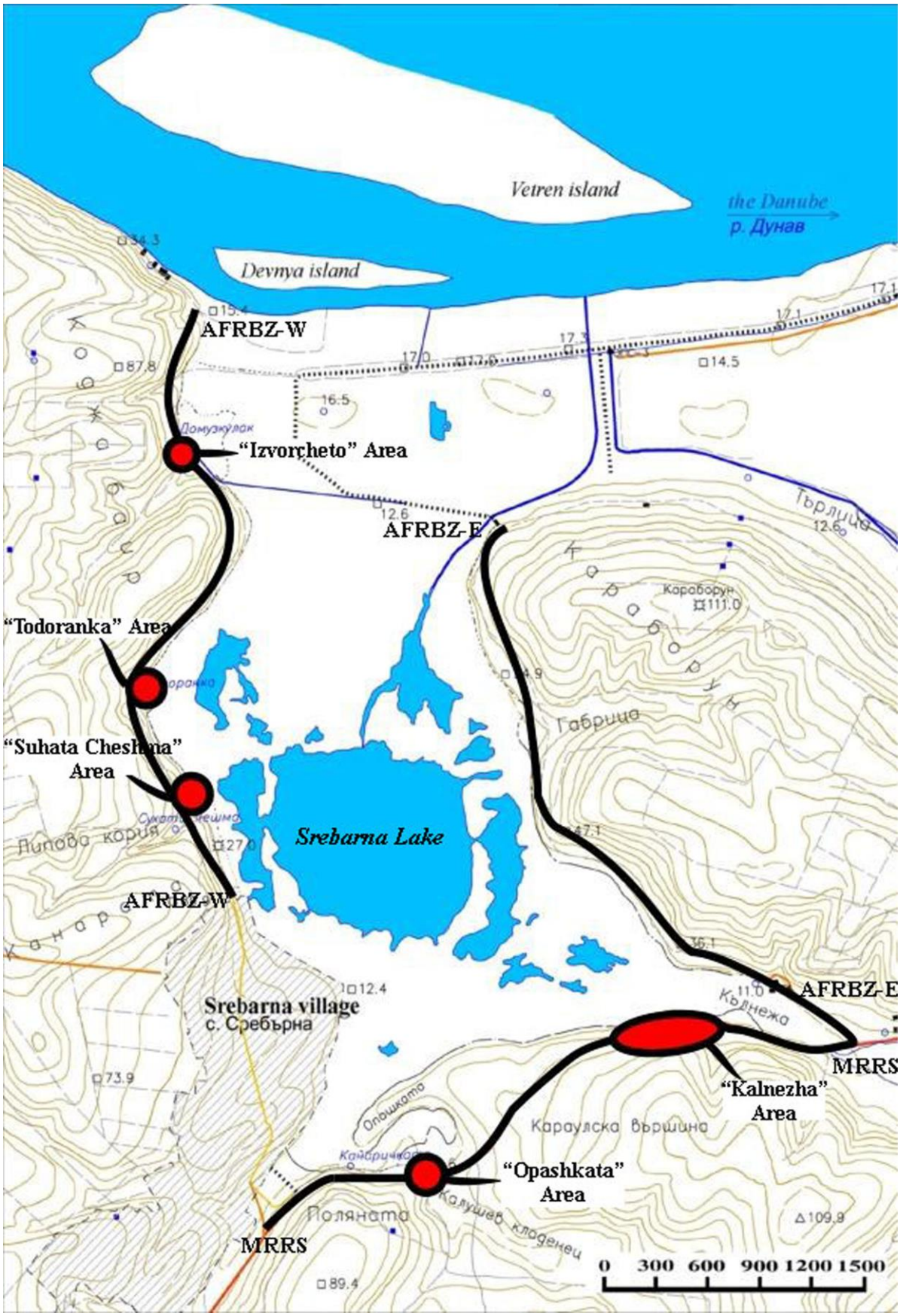

Fig. 1. Map of the location of the studied road sections in the buffer zone of Biosphere Reserve "Srebarna".

Table 1. Characteristics of the studied road sections.

Parameters / Road sections	AFRBZ-W	AFRBZ-E	MRRS
Cover type	grovel	grovel	asphalt
Width of the road (m)	4	4	6
Mean number of passing vehicles (number/hour)	1.91	1.09	63.09
Mean speed of the passing vehicles (km/h)	35	35	70

Results

The average number of recorded vehicles and other characteristics on the three types of roads for 1-hour per month during the study period is shown in Table 1. For the entire period of study in the three studied road sections a total of 15 dead specimens of amphibians belonging to 4 species (*Bombina bombina, Hyla arborea, Bufo bufo, Bufo viridis*) and 70 dead specimens of reptiles belonging to 8 species (*Emys orbicularis, Ablepharus kitaibelii, Lacerta viridis, Podarcis tauricus, Podarcis muralis, Natrix natrix, Coronella austriaca* and *Dolichophis caspius*) were recorded (Table 2).

Table 2. Distribution of the recorded dead animals by species in the three studied road sections in BR "Srebarna" Legend: t.c. – number of recorded dead specimens, %t – percentage within the class, %t.k. – percentage from the total recorded dead animals.

Species	AFRBZ-W			AFRBZ-E			MRRS		
	t.c.	*% t*	*%t.k.*	*t.c.*	*% t*	*%t.k.*	*t.c.*	*% t*	*%t.k.*
Amphibia									
Bombina bombina	2	11.11	4.08	-	-	-	-	-	-
Hyla arborea	3	16.67	6.12	-	-	-	2	50.0	6.67
Bufo bufo	4	22.22	8.16	-	-	-	-	-	-
Bufo viridis	9	50.0	18.37	-	-	-	2	50.0	6.67
Total Amphibia	**18**	**100**	**36.73**	**-**	**-**	**-**	**4**	**100**	**13.34**
Reptilia									
Emys orbicularis	1	3.22	2.04	-	-	-	-	-	-
Ablepharus kitaibelii	1	3.22	2.04	12	66.67	66.67	-	-	-
Lacerta viridis	9	29.03	18.37	1	5.55	5.55	8	30.77	26.67
Podarcis tauricus	9	29.03	18.37	-	-	-	-	-	-
Podarcis muralis	1	3.22	2.04	-	-	-	-	-	-
Natrix natrix	8	25.82	16.33	2	11.11	11.11	13	50.0	43.33
Coronella austriaca	2	6.46	4.08	-	-	-	-	-	-
Dolichophis caspius	-	-	-	3	16.67	16.67	5	19.23	16.66
Total Reptilia	**31**	**100**	**63.27**	**18**	**100**	**100**	**26**	**100**	**86.66**
Total animals	**42**	**100**	**100**	**18**	**100**	**100**	**300**	**100**	**100**

From the results displayed at Table 2 is evident that the majority of the dead animals in the buffer zone of the reserve were recorded at the western anti-fire road (AFRBZ-W) – 18 amphibians and 31 reptiles, followed by the main road Ruse-Silistra (MRRS) with 4 dead amphibians and 26 reptiles and the least cadavers were recorded at the eastern anti-fire road (AFRBZ-E) – 0 amphibians and 18 reptiles. Comparing the mortality of the amphibians between the two fire roads and the main road, no comparisons showed any

statistically significant differences (t-test, p>0,05).

We identified several areas, where regular amphibian and reptile migration on the three studied roads occur and we discovered most cadavers here (Fig. 1):

1. Anti-fire road in the western part of the buffer zone of the reserve (AFRBZ-W).

✓ "Suhata cheshma" area – a dry ravine with length of 3 km along arable land in the upper part. During the rainy season water drains through this ravine and flows into the lake. In this section of the road (located about 30 meters from the lake) there are header pipes, built in under the road, with diameter 1 m, through which rainwater drains to the lake and prevents flooding the road. This kind of set header pipes can act as ecological corridors for the amphibians and reptiles, but it seems the animals do not use them.

✓ "Todoranka" area - a dry ravine with length of 500 meters, located in acacia array. This mortality at this spot is lower, since at the upper part of the road, there are not many suitable habitats for amphibians and reptiles and the migration rate here is relatively low.

✓ "Izvorcheto" area - a dry ravine with length of 2 km of arable land in the upper part. During the rainy season water drains through this ravine and flows into the lake. In this section of the road, there are also two built-in header pipes, with diameter 1 m.

2. Main road Ruse – Silistra (MRRS)

✓ "Kalnezha" area – a small river with very low flow, which flows under the road into the southeastern part of the lake. Over the road, there are mixed tree plantations on limestone terrain that offers suitable wintering areas of amphibians and reptiles. During the breeding season, migrating to the lake, many of the amphibians are run over when crossing the road. In this section, there are no built-in header pipes.

✓ "Opashkata" area – part of the Srebarna Lake, surrounded from a small forest and arable land.

There is no correlation between the number of road-killed amphibians and reptiles on AFRBZ-E overall and average 1-hour traffic intensity by month. For AFRBZ-W we recorded a positive very strong, statistically significant correlation between the number of dead amphibians and traffic volume ($r_{Spearman}$=0.81) and low positive correlation for the dead reptiles ($r_{Spearman}$=0.34), but it was not statistically significant, so we discard it as accidental. For MRRS again we recorded a positive strong, statistically significant correlation between the number of dead amphibians and traffic volume ($r_{Spearman}$=0.67) and no correlation for the dead reptiles ($r_{Spearman}$=-0.04).

As shown on Fig. 2 the majority of the found dead animals were recorded in sunny weather at air temperature between 23-28°C (χ2=61.041, df=13, p<0.05). The species recorded in cloudy weather were *Podarcis muralis, Natrix natrix* and *Bufo bufo*. The only species recorded dead in rainy weather were *Bufo viridis* and *Natrix natrix*.

The monthly dynamic of the traffic induced mortality of the amphibians and reptiles (Fig. 3) shows that most of the cadavers are recorded in the spring time (April-May) and in the late summer – early autumn (July-September) (χ2=63.001, df=11, p<0.05).

Discussion

On the territory of the Biosphere Reserve "Srebarna" are recorded 12 species of amphibians and 15 species of reptiles (BESHKOV, 1998), which is why it is designated as important herpetological area (IHA) in Bulgaria (BISERKOV *et al.,* 2005). The registered by us amphibian and reptiles, killed by automobile traffic in the buffer zone of the reserve represent significant portion of the batracho- and herpetofauna occurring in the reserve.

The majority of the studies on the topic in Bulgaria so far show that the highest mortality among amphibians and reptiles is recorded at high intensity traffic roads (MOLLOV, 2005; KAMBOUROVA-IVANOVA *et al.*, 2012). Our results did not confirm this pattern. The reason for this is that we encountered several hot spots, where

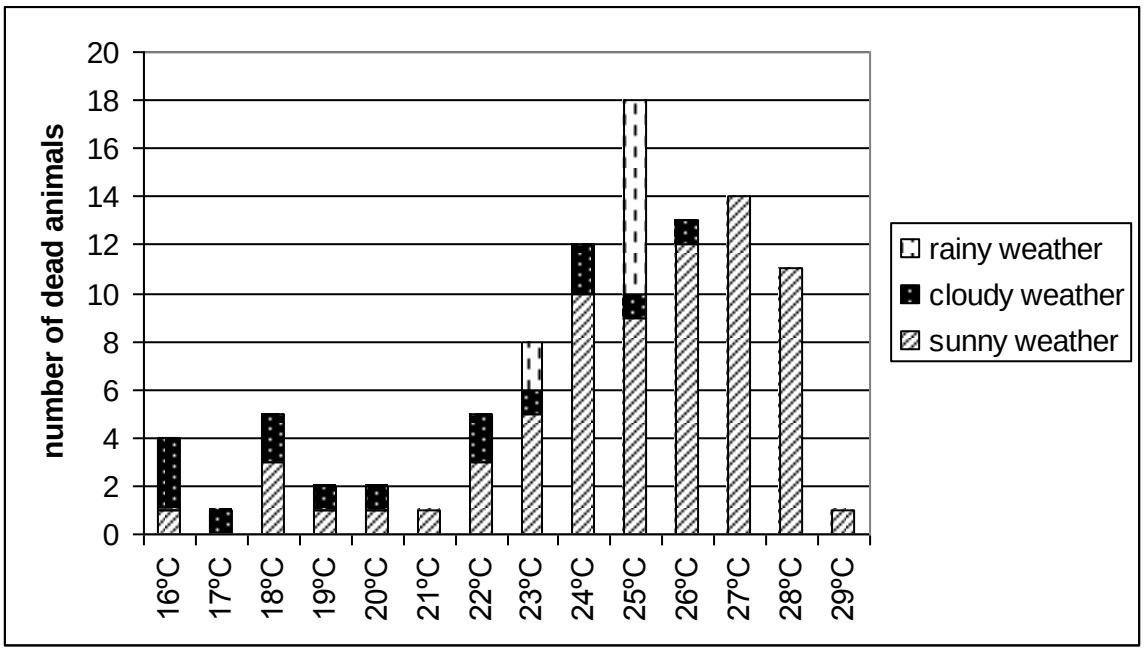

Fig. 2. Distribution of the registered dead amphibians and reptiles on the three studied roads according to the air temperature and the weather conditions.

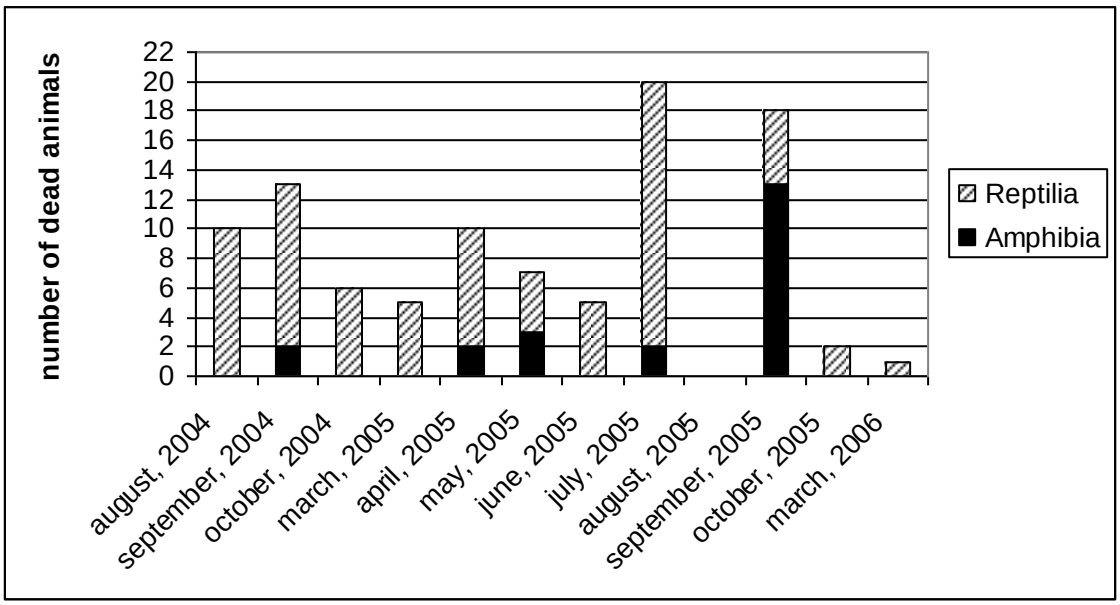

Fig. 3. Monthly dynamics of the registered dead amphibians and reptiles on the three studied roads during the whole period of study.

regular migrations of amphibians and reptiles between the lake and the surrounding terrestrial habitats occur (described in the following section). Namely on these hot spots most of the mortalities were recorded.

There are three such hot spots on AFRBZ-W, two at MRRS and none at AFRBZ-E. Furthermore, the reason for the registered low mortality at AFRBZ-E is the mounting of a barrier on this road in 2004.

Only certain authorized vehicles had access to this road from that point on.

The registered by us positive correlation between the traffic volume and number of killed amphibians is reported by other authors as well. MAZEROLLE (2004) reported that amphibian road mortality correlates with the variation in traffic intensity. According to MOLLOV (2005) the amphibian mortality in the city of Plovdiv correlates with the intensity of the traffic although the author did not processed his results statistically. KAMBOUROVA-IVANOVA *et al.* (2012) on the other hand did not register any correlation between the traffic intensity and the dead vertebrate animals (amphibians and reptiles included).

Almost all reptiles, especially the lizards are more active in sunny weather and that is the reason why there are more casualties from the traffic then. However, some amphibians like the toads are active and migrating in cloudy and rainy weather and sometimes during such migrations, more casualties from a certain species can be observed. CARR & FAHRIG (2001) pointed out that another determining factor of the amphibian's road mortality rate is their agility. Ervin and Fisher (2001) showed that the climate conditions also influence the road mortality rate of the amphibians, considering the fact that they are more agile at damp weather.

Our results for the monthly dynamic of the traffic induced mortality of the amphibians and reptiles; make perfect sense since in the spring all amphibians migrate to the lake from their hibernation sites for breeding and some of the reptiles migrate in the search of food. In the autumn begins the other migration from the lake to the surrounding dry land where the hibernation sites of the amphibians and reptiles are. KAMBOUROVA-IVANOVA *et al.* (2012) reported that the highest road induced mortality in amphibians is in May and September. They also recorded an astonishing autumn migration (hundreds of road kills) of the marsh frog (*Pelophylax ridibundus*) at a second-class road near Pazardzhik town. Oddly, we did not record any dead green frogs during the current study. Perhaps the low speed of the vehicles on the studied roads and the greater agility of these frogs, compared to the toads is a factor benefiting them and leading to their survival on the buffer zone in the reserve. KAMBOUROVA-IVANOVA *et al.* (2012) also reported highest road induces mortality among reptiles in September and the lowest in July. Our results confirmed these results, be we also recorded high reptilian mortality during the summer as well. In our opinion the climatic condition in the wetland are different than the area studied by the above mentioned authors and this may lead to a higher activity among the reptiles, especially *Ablepharus kitaibelii* and *Natrix natrix*, in the buffer zone of the reserve.

According BISERKOV *et al.* (2005) one of the main priorities for the conservation of herpetofauna in Bulgaria is set out for building protective structures (fences and transient tunnels) to the road network in areas at high risk of wild land animals. It is notable that the protective fencing associated with the transient tunnels under the roads act as ecological corridors at the micro level and are an integral part of ecological networks. Moreover, in most cases there are transitional tunnels, because these are built in the construction of roads for drainage of rainwater and small streams (JACKSON, 1996), but it remains to be building the protective fences.

Such drainage pipes lie on the shores of lake at an average of about 30-35 meters, and in some places, such as at the critical points (hot spots). The pipes drain the rainwater into the lake, and usually most of the year they are dry, but during the infusion of water from the Danube River, the pipes are permanently full with water for the period from March to July, and sometimes lasting until about September. Perhaps this is the main reason why the amphibians and reptiles rarely or never use these pipes to cross the roads. A new system of conservation measures is needed to take place in the buffer zone of the reserve in near future in order to protect the batracho- and herpetofauna. Also in our opinion in the conservation management plans of the protected area and the safety measures

taken for protection of rare and endangered amphibian and reptile species, the road density and the automobile traffic should be considered as an important factor influencing negatively their populations. On the spots where the roads divide the breeding sites from the feeding and the hibernation sites, the road mortality represent a great threat to the populations of many amphibian and reptiles species.

The organization of further, more serious and thorough studies on the influence of the automobile traffic on the amphibian and reptile populations in the country is needed.

Conclusions

1. For the entire period of study in the three studied road sections a total of 15 dead specimens of amphibians belonging to 4 species and 70 dead specimens of reptiles belonging to 8 species.

2. The majority of the dead animals in the buffer zone of the reserve were recorded at the western anti-fire road (AFRBZ-W) – 18 amphibians and 31 reptiles, followed by the main road Ruse-Silistra (MRRS) with 4 dead amphibians and 26 reptiles and the least cadavers were recorded at the eastern anti-fire road (AFRBZ-E) – 0 amphibians and 18 reptiles.

3. Several "hot spots", where most cadavers were recorded, are identified – three on AFRBZ-W, two at MRRS and none at AFRBZ-E.

4. For AFRBZ-W and MRRS we recorded a positive very strong, statistically significant correlation between the number of dead amphibians and traffic volume and low and no correlation, respectively for the dead reptiles.

5. The majority of the found dead animals were recorded in sunny weather at air temperature between 23-28°C. The species recorded in cloudy weather were *Podarcis muralis, Natrix natrix* and *Bufo bufo*. The only species recorded dead in rainy weather were *Bufo viridis* and *Natrix natrix*.

6. The monthly dynamic of the traffic induced mortality of the amphibians and reptiles shows that most of the cadavers are recorded in the springtime (April-May) and in the late summer – early autumn (July-September).

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Plants Species Diversity in Hyrcanian Hardwood Forests, Northern Iran (Case Study: Mazandaran Province)

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Abstract. In order to better understand and manage forest ecosystems, it is important to study the relationship between environmental factors and plants in these ecosystems. We investigated plant species diversity of three hardwood forest stands in the Hyrcanian forests, Sari, northern Iran. Our aim was to determine the effect of forest stand type on the diversity of plant species. One plot 150 × 150 m established at the center of each forest stand and in each plot, nine subplots 50 × 50 m were selected. Diversity values (Richness, diversity and evenness indices) were measured in five sample areas 0.01 ha per 50 × 50 m quadrates by estimating cover percentage of each species. The results showed that Geophytes (43.33%) had the highest life form spectrum among species. JACCARD'S similarity index revealed that the highest values exist between *Parrotia-Carpinus* and *Carpinus* stands. All herb layer species diversity indices varied significantly among different forest stands. Cover percentage significantly positively correlated with diversity indices in *Parrotia-Carpinus* stand. Diversity and richness indices of herb-layers plants were significantly negatively correlated with cover percentage in *Fagus* stand. Correlation analysis between all diversity measures and cover percentage in *Carpinus* stand wasn't significant. The result of the present study revealed that species diversity in temperate broad-leaved deciduous forest was significantly influenced by forest stand type.

Key words: biodiversity, broad-leaved trees, herb-layer species, Hyrcanian forests.

Introduction

Forest ecosystems give unequal share to the world's biodiversity (BATTLE *et al.*, 2000) and biodiversity maintenance is one of the major goals defined to attain forest sustainability (OSORIO *et al.,* 2009). In addition to economical importance, forests are significant as natural ecosystems and their biodiversity conservation, accordingly, is one of the main objects of forest management (PITKANEN, 2000). In most temperate forest ecosystems, herbaceous species play an important role in the overall plant species diversity (VON OHEIMB & HARDTLE, 2009). Forest ecosystems undergo several natural and human disturbances and due to major differences in terms of life form and regeneration, the way the herbaceous plants usually react to such disturbances is different from the way trees do (GILLIAM, 2007). In general, understory plants appears to be the largest element of temperate forest diversity and may be an important sign of site quality, upperstory regeneration patterns and preservation position (GRACIA *et al.*, 2007). The herbaceous layer is a significant and dynamic forest layer that contributes only to a small part of the total biomass of an ecosystem (GILLIAM & TRULLI, 1993). The species diversity dispersal is

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dependent on environmental factors and biological influence (WANG, 2006). Conservation of species diversity is an important object in sustainable forest management (LINDERMAYER *et al.,* 2000; POTTS *et al.,* 2005; ITO *et al.*, 2006). Forest operations that change site conditions to develop tree regeneration may result in changes in biodiversity patterns (ELLIOTT & HEWITI, 1997), consequently; the nature and distribution of biodiversity resources of the managed region is determined through biodiversity measures before forest management operations starts (SAGER *et al.,* 2003). According to AUBERT *et al.* (2003), the assessment of biodiversity in managed forest has become an important issue for studying ecosystems and their conservation. The herb-layer plants play important roles in the broadleaved forests for example in the competition among herbaceous species and seedling of upper-story dominant trees (GILLIAM, 2002). A major concern of forest managers in timber resources development is the maintenance of understory plant diversity (ELLUM *et al.,* 2010). Hyrcanian (Caspian) forests are positioned at green strip streching over the northern slopes of Alborz range and southern coasts of the Caspian Sea (HAGHDOOST *et al.,* 2011) and the vegetation is consisted typically of deciduous forests (AKHANI *et al.,* 2010). These forests were formed at the end of the third geological era (HOSSEINI, 2010). In Hyrcanian forest similar other temperate broadleaved forests, herb –layer species play an important role in the ecosystem. Plant species diversity in Hyrcanian forest have been addressed in some recent studies (ABRARI VAJARI *et al.*, 2012; POORBABAEI & RANJAVAR 2008; POORBABAEI & ROOSTAMI, 2006; SHABANI *et al.*, 2011; SOHRABI & HABASHI, 2011; ABEDI & POORBABAEI, 2010). The objectives of this study were to (1) characterize the effect of forest stand type on herb-layer species diversity indices and (2) determine the correlation between diversity indices and herb-layer species cover in each stand. This study is helpful to evaluate the plant species diversity in a given forest to design forest management plans.

Material and Methods

Study site. The study was conducted at three districts in forestry plan of Tajan watershed, located in Sari, northern Iran. In each district, one compartment was selected to study plant species diversity (Table 1, Fig 1). The region has a humid temperate climate, and dominant type of soil is forest brown soil.

Data collection. This experiment was conducted during the growing season of 2010. One plot 150 × 150 m established at the center of each forest stand (BAIDER *et al.,* 2001) and in each plot, nine subplots of 50 × 50 m (COX & ALLEN, 2008) were selected (Fig 2). Sampling plant species was carried out within five sample areas 0.01 ha per 50 × 50 m quadrate by estimating cover percentage of each species. For determining herb-layer diversity, herb-layer SR (species richness), Shannon-Wiener diversity index H' and evenness E [H'= – (Pi) (LnPi), where Pi = percentaged cover value; E=H'/ H' max; H' max: Ln(n), where n=herb-layer SR] were calculated. JACCARD'S similarity index was applied to evaluate ß diversity similarity among forest stands based on presence/absence of the species as follows: JI=a/(a+b+c); a: number of common species in samples, b: species that exist just in first sample, c: number of species exist just in second sample.

Data analysis. One-way ANOVA and S.N.K test post hoc were applied to distinguish herb-layer species diversity indices among different forest stand. The correlation calculated between herb-layer species diversity indices and its cover percentage with Pearson correlation coefficient. Differences obtained at a level of ≤ 0.01 were considered significant.

Table1. Characteristics of forest stand types in the study site.

Characteristic	Stand type		
	Parrotia-Carpinus	***Carpinus***	***Fagus***
Altitude(m a.s.l)	240-300	820-700	1335-1550
Area(ha)	44	59.57	39
Position	NW	NW	NW
Annual mean rainfall(mm)	808	900	858
Annual mean temperature(°c)	16-14	14-16	15
Slope(%)	30-0	50-80	70-0
Coordination	36° 26′N, 53° 07′E	36° 22′N, 53° 04′E	36° 13′N, 53° 27′E

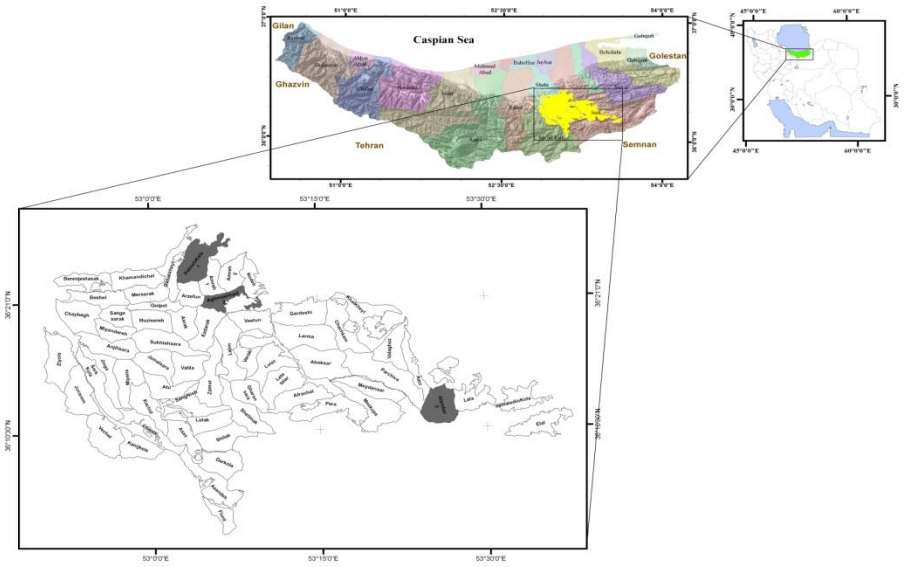

Fig.1. Location of study site in northern Iran, Mazandaran

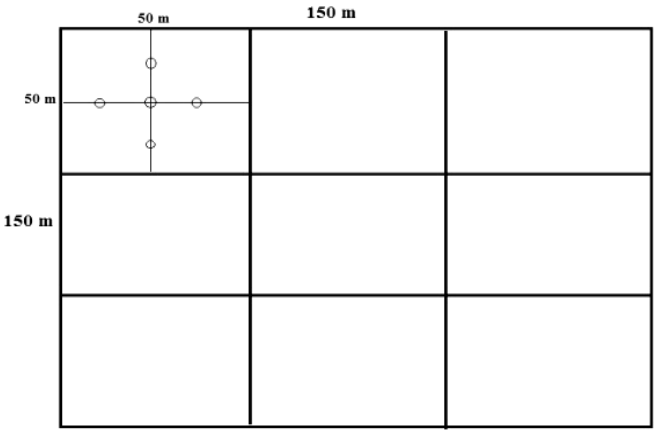

Fig 2. Location of sampling points of plant species in plots

Results

A total of 30 species representing 26 botanical families were identified within three forest stands in the study area (Table 2). The life form of these species is shown in Table 3, as the highest life form belongs to *Geophytes* (43.33%). JACCARD'S similarity index (Table 4) revealed that there are the highest values between *Parrotia-Carpinus* and *Carpinus* stands (0.625). All herb layer species diversity indices (Table 5, 6) varied significantly among forest stand types ($p \leq 0.01$). Correlation analysis (Table 7) showed that diversity and richness indices of herbaceous plants and cover percentage significantly positively correlated in *Parrotia-Carpinus* stand ($p \leq 0.01$). Diversity and richness indices of herb-layer plants were significantly negatively correlated with cover percentage in *Fagus* stand ($p \leq 0.01$). Correlation analysis between all diversity measures and cover percentage in *Carpinus* stand wasn't significant ($p \geq 0.01$).

Table 2. List of plant species in field

Species	Family	Life form
Carex sylvatica Huds.	Cyperaceae	He
Euphorbia helioscopia L.	Euphorbiaceae	Ge
Fragaria vesca L.	Rosaceae	He
Phylitis scolopendrium (L.) Newn.	Aspleniaceae	He
Hedera pastuchovii Woronow	Araliaceae	Ph
Urtica dioica L.	Urticaceae	He
Hypericum androsaemum L.	Hypericaceae	Ge
Viola alba Bess.	Violaceae	He
Lappa major Gaertn.	Compositae	He
Mentha sp.	Labiatae	Ge
Sambucus ebulus L.	Caprifoliaceae	Cha
Pteris cretica L.	Pteridaceae	Ge
Primula heterochroma Stapf.	Primulaceae	He
Rubus hyrcanus Juz.	Rosaceae	Ph
Smilax excelsa L.	Smilacaceae	Ge
Oplismenus undulatifolius (Ard.) L. P.	Graminae	Th
Trifolium repens L.	Leguminosae	Ge
Graminae sp.	Graminae	Ge
Pteridium aquilinum (L.) Kuhn	Dennstaedtiae	Ge
Sanicula europaea L.	Umbelliferae	He
Solanum dulcamara L.	Solanaceae	Ph
Polygonatum orientale Desf	Polygonaceae	Ge
Galium odoratum (L.) (Scope.)	Rubiaceae	He
Festuca rubra L.	Graminae	Ge
Allium sativum L.	Liliaceae	Ge
Poa nemoralis L.	Graminae	Ge
Polygonatum orientale Desf.	Liliaceae	Ge
Arum sp.	Araceae	Ge
Ruscus hyrcanus Woron.	Liliaceae	Ph
Cardamine impatiens L.	Cruciferae	He

He:Hemicryptopyte; Ph:Phanerophye; Ge:Geophyte; Th:Therophyte

Table 3. Frequency (%) of life forms for herb -layer species in site

Life form	Frequency(%)
Phanerophyte	13.33
Geophyte	43.33
Chamaephyte	3.33
Hemcryptophyte	30
Therophyte	3.33

Table 4. Jaccard 's similarity index of heb-layer species in research site

Stand	Index
Parrotia-Carpinus,Carpinus	0.625
Parrotia-Carpinus,Fagus	0.290
Fagus,Carpinus	0.435

Table 5. Analysis of variance of herbaceous plant diversity indices in different forest stands

Variables	MS	DF	F value
Richness	188.49	3	36.23**
Diversity	9.79	3	13.64**
Evenness	0.08	3	6.42**

**, Significant at $p \leq 0.01$.

Table 6. Means (± SE) for plant species diversity indices in different stand

stand	Richness	Diversity	Evenness
Parrotia-Carpinus	5.15±0.38^{b}	1.55±0.75^{b}	0.88±0.02^{a}
Carpinus	8.66±0.35^{a}	2.54±0.88^{a}	0.81±0.02^{b}
Fagus	8.73±0.24^{a}	2.5±0.06^{a}	0.80±0.01^{b}

Letter superscripts (a , b) in each column represent statistical differences using S.N.K test , SE: standard error of means.

Table 7. Pearson's correlation between herb-layer cover percentage and diversity indices in different stands

	Forest stand type					
	Parrotia-Carpinus		*Carpinus*		*Fagus*	
index	*R*	*P*	*R*	*P*	*R*	*P*
richness	0.754	0.000**	0.242	0.110 ns	-0.063	0.679 ns
diversity	0.620	0.000**	0.103	ns 0.499	-0.443	0.000**
evenness	0.003	0.984 ns	-0.064 ns	ns0.676	-0.642	0.000**

**, Significant at $p \leq 0.01$; ns: no significant.

Discussion

Difference among diverse stand types can be determined regarding to species richness, vegetation cover, diversity indices and the distribution of plant functional groups (MOELDER & SCHMIDT 2006). Biodiversity measures have been broadly used as indicator of ecosystem status, and take part in a vital task in studies dealing with the measurement of human effect on ecological systems (LEITNER & TURNER 2001). Results from different life forms within three forest stands included in this research showed that geophytes had the highest rate among various life forms whose overwintering buds are below the soil surface including *Euphorbia helioscopia* L., *Hypericum androsaemum* L., *Pteris cretica* L., *Allium sativum* L., *Polygonatum orientale* Desf. Ground vegetation differ in terms of their capability to penetrate into litter (SYDES & GRIME, 1981). SCHMIDT (2005) claimed that understorey flora and vegetation are suitable indicators for site conditions, human impact and forest dynamics. For example, *Galium odoratum* is indicators of suitable moisture, mild temperature, and high N, and *Sanicula europaea* show high relative humidity in beech stands (ABRARI VAJARI & AZIZI 2002). A comparison between the different functional groups showed that higher proportion belongs to forbs, while graminoids didn't respond to forest stand type. This may be caused by plant functional groups having diverse resource necessities (VOCKENHUBER *et al.*, 2011). The incidence of a particular plant life form could reveal the characteristic of the climatic condition in a specified region to which the species have adapted (BEENAKUMARI DEVI & SINGH 2011). In general, species richness (SR) was higher in the beech stand than in other forest stands. Plant species diversity in pure stands of beech and hornbeam was higher than mixed stand of Ironwood–Hornbeam and this in not consistent with BEATTY (2003) claimed that higher tree layer diversity might enhance herb layer diversity. Tree species can effect on the herbaceous layer by altering resource accessibility and environmental conditions in lower forest layers (BARBIER *et al.*, 2008). Soil conditions have a intense consequence on the herbaceous layer under maturing closed stands, Tree species composition can also be a factor in herbaceous diversity because of the different impacts on light regime and soil features of multi-species stands (KELEMEN *et al.*, 2012). Soil pH, nutrient status, and light conditions strongly affect on the species composition of the herbaceous layer in beech forests (BRUNET *et al.*, 2010). SCHMIDT (2005) declared that species richness of beech forests increases with increasing base-richness. Due to the lower Transmission of photosynthetically active radiation in beech canopies rather than canopies of other deciduous tree species, the establishment and development of shade-intolerant herb-layer and tree species are held back (SCHMIDT, 2005). An admixture of other tree species has a favorable influence on humus quality and herb species richness (GODEFROID *et al.*, 2005). Species richness significantly increased with pH when hornbeam and beech were combined but also the two species separately (KOOIJMAN & CAMMERAAT, 2009). According to TINYA *et al.* (2009) optimal light conditions are obviously different for the understory species. HARDTLE *et al.* (2003) showed that the impacts of light on the species richness of the understory depend on the type of the forest. Variations in understory diversity or composition in parallel with tree species composition are often seen as being a result of disparities in topsoil (BARBIER *et al.*, 2008). The multi-layered canopy produced much more heterogeneous light conditions in beech and hornbeam stands than Ironwood–Hornbeam stand. This resulted in the high diversity of herb-layer species. Due to the dominant position of trees in forests and their influence on different ecological gradients, the identity and composition of tree species can be expected to affect plant species diversity (BARBIER *et al.*, 2008). Understory composition usually varies noticeably among diverse forest types (LU *et al.*, 2011). Many factors influence the variations in species diversity and the proportion of forest related species groups,

including site history, management practice, time since cessation of management and the existing site conditions (SCHMIDT, 2005). Jaccard's similarity value is higher in *Parrotia-Carpinus, Carpinus* stands than other forest stands (Table 4) and this higher value show greater similarity between two stands, hence low ß diversity. According to MAGURAN (2004) a value close to 1 indicates greater similarity between stands and hence low ß diversity. In each of stands, these plots were located close to each other; it is thus reasonable that alike seed distribution mechanism were operational and that there may have been similar soil seed bank, regard to (OMORO *et al.,* 2010). The reason is that the two forest stands in good quality environmental conditions have high similarity. It explains these high values by the sampling method used: selection of stands developed on the similar soil, with the similar management and located in one forest may decrease factors of variability in plant communities (AUBERT *et al.,* 2003). Higher Beta diversity between *Pattrotia-Carpinus* and *Carpinus* stands and the lower Beta diversity between *Parrotia-Carpinus* and *Fagus* stands may be attributed to the magnitude of differences in species-individual ratio found there regarding to SAPKOTA *et al.* (2010). Beta diversity is used in biogeographic studies to generate results that better clarify the choice and plan of protected natural áreas (HERNANDEZ-SALINAS & RAMAREZ–BAUTISTA, 2012). Changing land use in forest systems certainly produces changes in biodiversity (LANGE *et al.*, 2011) this study indicates that the impacts of management may depend on the history of the particular forest system. The herbaceous layer is significant to the structure and function of forest ecosystems (GILLIAM, 2007) and understanding species diversity and distribution patterns is important for helping managers evaluate the complexity and resources of these forests. Variation in understory plants may be a useful indicator of overall response of biodiversity to forest stand type.

Conclusion

The understory vegetation diversity within different forest stands examined in this study demonstrated the significant difference among them. The present study indicates that beech stand has higher species richness and diversity values which may be due to biotic and abiotic factors. The high percentage of geophytes reveals high adaption capability of them to ecological parameters in these forest stands. In general, we can say that different herb species dynamics were related to different community types, reflecting differences in habitats in region. Many unharvested stands in Hyrcanian forests may be too dense for allowing the establishment of a variety of species in the stand. It can be recommending implementation of a seasonal monitoring of plant diversity in the three selected sites.

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Study on Seed Germination and Seedlings Growth of Bamboo (Dendrocalamus hamiltonii)

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Abstract. Bamboos are naturally propagating both sexually and asexually from seeds and rhizomes. In this study two experiments were carried out in a greenhouse to evaluate the influence of seed orientation (embryo-end-up, lay-flat and embryo-end-down) and sowing depth (3, 6 and 9 mm) of the bamboo *Dendrocalamus hamiltonii* on seed germination, seedling survival and growth after 60 days. Factorial design in two blocks containing 90 samples was used. Plastic pots filled with a mixture of sand, agricultural soil and peat (rate 3:1:1 and 2:2:1, respectively). Seeds were sown in two soil treatments. Seed germination and seedling survival rates in first soil (3:1:1) were 24 and 66.6 percent and in second soil treatment (2:2:1) were 31.1 and 85.7 percent, respectively. Mean seedlings height was higher at 6 mm depths and embryo-end-down in both soil treatments (11.5 and 27.5), it had significant difference in two soil treatments ($P < 0.05$). Mean seedlings height didn't show significant difference among seed orientation and sowing depth.

Key words: Bamboo, Seed germination, Seedling growth, *Dendrocalamus hamiltonii*

Introduction

Bamboos are tall perennial grasses with tree stature that grow up to about 30 m in height and 35 cm in diameter. They belong to the Poaceae (Gramineae) family and Bambuseae subfamily (OHRNBERGER, 1999). The main stem of the aboveground part of the plant is the culm, while the underground part constitutes the rhizome and root system. Most bamboo plants flower only once in their lifetime (14 to 50 years in most species) and then die soon. They emerge again from germinating seeds if the site is not severely disturbed by detrimental factors such as rodents, fire and etc. These phenomena were actually observed in bamboo forests (LIESE, 1985). Bamboo naturally propagates both sexually and asexually from seeds and rhizomes. Artificial propagation by vegetative methods includes planting of off-sets, culm cuttings, layering, and grafting of rhizome (EMBAYE, 2003). Most bamboos are found on sandy loam to loamy clay soils, derived from river alluvium or frequently from the underlying rock. *Dendrocalamus* species, after germination, produce a grass-like seedling the first year. The plumule, which appears as a conical bud covered by sheathing scaly leaves, develops rapidly into a thin wiry stem bearing single leaves, alternate at the nodes, the leaf bases covering the stem (HUBERMAN, 1956). *Dendrocalamus hamiltonii* Nees et Arn. ex

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Munro is a large caespitose bamboo, culms 10-20 m high, 10-16 cm in diameter, thin walled with 0.75-1.25 cm thickness (RAWAT, 2007), internodes 30-50 cm, sheaths stiff and persistent (HUBERMAN, 1956). Its seeds are like those of wheat, seed year happen in intervals of 30 years and flowering nature is gregarious and sporadic (AHLAWAT et al., 2002).

The aim of the current study is finding out the optimum sowing depth and seed orientation that could be recommended for successful seedling production and growth of the *Dendrocalamus hamiltonii* in greenhouse conditions.

PITTMAN (1965) records enhanced germination and early growth for corn seeds oriented with respect to magnetic lines of force. PATTEN & VAN-DOREN (1970) found substantially higher emergence and seedling growth of corn with embryo end-up than with embryo-end-down orientation. MAUN & RIACH (1981) studied sand deposition effects on seedling emergence of *Calamovilfa longifolia* at field sites and greenhouse plantings. They found that seedling emergence was negatively correlated with planting depth.

SANCHEZ & KING (1994) identify sowing depth as one of the most important factors that affect seedling emergence, survival and subsequent onward growth of acacia species from Ethiopia.

CHEN & MAUN (1999) sorted seeds of *Cirsium pitcheri* into three groups (small, medium and large) and buried at 2, 4, 6, 8, 10 and 12 cm depths in plastic pots filled with unsterilized sand, Data showed that percent seed germination and emergence of seedlings were not related to seed size. However, both variables were negatively correlated with depth. Seedling emergence occurred from a maximum depth of 6 cm with most seedlings emerging from 2 cm depth.

EMBAYE (2003) used a randomized complete block design to evaluate seedling emergence, subsequent survival and growth of bamboo (*Oxytenanthera abyssinica*). Seeds were sown in plastic pots that filled with mixture of sand and peat (rate 3 sand: 1 peat). Top of the soil surface and at 2.5 mm depth achieved faster and higher seedling emergence than those sown at 5 and 10 mm depths. However, mean seedling height and number of leaves per seedling were higher in 5 and 2.5 mm depths than surface and 10 mm depths. There were significant quadratic relationships between sowing depth and seedling height ($p=0.034$) as well as number of leaves per seedling ($p = 0.032$), both peaking around 5 mm soil depth. Lay-flat orientation, which was the most frequent position in broadcast sowing, was recommended at 5 mm sowing depth for the lowland bamboo based on overall performance in seedling emergence, survival and growth.

DAMIZADE (2004) planted seeds of *Capparis* deciduas in nursery for evaluating germination and survival rates of seedlings.

ZHAO et al. (2007) sorted seeds of *Nitraria sphaerocarpa* into three size-classes and buried at 2 cm depths in plastic pots filled with sand under controlled greenhouse condition. Two weeks after seedling emergence, seedlings were buried in sand to various depths of 0, 33, 67, 100 and 133% of their mean height. Seedling height, mass and absolute height growth rate in partial burial treatments were higher than those of the unburied and completely buried treatments. In each seed size class, with increasing burial depth, or in each burial depth, with decreasing seed size, there was a tendency that both biomass allocation to root and biomass allocation to belowground stem increased, while biomass allocation to aboveground stem decreased.

TABARI & TABANDEH (2007) investigated germination rate of Tilia platyphyllos at 1.5 and 3 cm of sowing depth and watering was done with two regulations: everyday and every other day. Results showed treatment composition of 3 cm sowing depth and everyday watering was the most germination rate than 1.5 cm sowing depth with every other day watering.

ZHANG & MAUN (2007) examine the effects of sand burial on seed germination, seedling emergence and establishment of *Panicum virgatum* L. on the foredunes of Lake Erie. In the field, all the seedlings established in one growing season had

originally emerged from sand burial depths of less than 12 cm. Within this burial range, seedlings from shallower burial depths had lower chances of establishment than expected, whereas those from deeper burial depths had higher probabilities of establishment than expected.

AZMY & APPANAH (1998) planted *Gigantochloa ligulata* at FRIM and results showed the seedlings developed in the second week after sowing. With 1:3 soil-sand rate and the germination was 76%.

Materials and Methods

This research is composed of literature review work and greenhouse experiment. Seeds were collected from Indian bamboo stands. Three experiments including soil, sowing depth and seed orientation were implemented. The experiments period was 60 days. Factorial design was used in the experiments: plastic pots were filled with a mixture of sand, agricultural soil and peat (rate 3:1:1 and 2:2:1 sand, agricultural soil and peat, respectively). There were two blocks that each of them consisted of 45 pots. Sowing depth experiment was determined based on average size of the seeds and consisted of one, two and three times of seed size i.e. tree treatments as 3, 6 and 9 mm at soil depth, respectively. It tried to select uniform size of seeds. The seeds were soaked with clean water for 12 hours and were sown in embryo-end-up, lay flat and embryo-end-down orientations at different soil depths. The amount of watering was adequate moisture to maintain in the soil to avoid seed desiccation while keeping it below field capacity to ensure adequate oxygen supply. The treatments were done through the greenhouse at 27ºC temperature and 80% relative humidity. Seedlings heights were measured every 20 days.

Results and discussion

First seed germination emerged 8 days after sowing and continued 8-14 days. Germination period of the species is expressed 8-28 days (AHLAWAT et al., 2002). The period for some species of *Dendrocalamus* is 2-3 weeks and about *Oxytenanthera abyssinica* is reported 14 days after sowing (EMBAYE, 2003).

Germination and survival rates in different soils are shown in Table 1.

Seed germination rate in soil 1 based on sowing depth after 60 days was in the order 9mm > 6mm > 3mm and survival rate followed 6mm > 9mm > 3mm and in soil 2 the amounts were 6mm >9mm > 3mm and 3mm > 6mm > 9mm respectively. In both of soils the most survival rates were related to shallow sowing depth and medium surface. CHEN & MAUN (1999) and ZHANG & MAUN (2007) approved the fact about other species, it maybe happens because of access to light quickly (PITTMAN, 1965) and water maintenance around the seeds (CHEN & MAUN, 1999). Differing results are documented on seedling growth in shallow sowing depth and significant difference between germination and survival of seedlings (MAUN & RIACH, 1981; SANCHEZ & KING, 1994; CHEN & MAUN, 1999; ZHAO et al., 2007). Seeds of *T. platyphyllos* were germinated in high deep soil better than shallow depth; the reason is probably for seed dormancy and physiological characteristics of seeds (e.g. seed size) (TABARI & TABANDEH, 2007). Seed treatment with water showed significant difference in seedling survival rate of *C. deciduas* (DAMIZADEH, 2004); we used the treatment in this study. Although, changing soil rates in different treatments were illustrated significant difference of survival rates about the species (DAMIZADEH, 2004).

Seed germination rate in soil 1 based on seed orientation after 60 days was 60% consisted of embryo-end-down at the 6 mm sowing depth and its survival rate was 100%.

Seed germination rate in soil 2 based on seed orientation after 60 days was 80% consisted of lay flat at the 6mm sowing depth and its survival rate was 100%.

The most survival rate about *O. abyssinica* was explained in lay flat orientation (EMBAYE, 2003). Growth rate is influenced by soil type (AZMY, 2008).

Germination and survival rates at different sowing depth and both soils are shown in Figures 1-6.

The most seedling height was 11.5 cm at age 60 days in embryo-end-down orientation and 6mm sowing depth in soil 1 (Table 4), highest seedling in soil 2 was 27.5 cm at the same orientation and sowing depth (Table 4). The most seedling height was reported at lay flat orientation for O.abyssinic (EMBAYE, 2003) and embryo-end-up for corn (PATTEN & VAN-DOREN, 1970).

The SPSS statistical software package was used to analysis the sets of data. Block means of the different treatments were applied for 3-WAY ANOVA calculations. Seedlings height between two soils were statistically significant differences until 40 days ($P<0.05$). After that the seedlings height growth were independent on soil type. Seed orientations and sowing depths didn't have significant differences on seedlings height ($P<0.05$) (Tables 2 - 4).

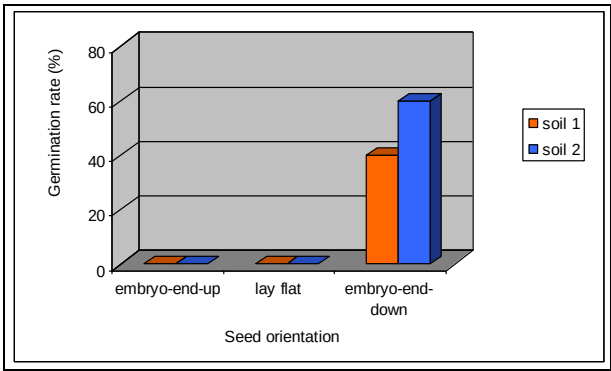

Fig 1. Germination rate in 3mm sowing depth

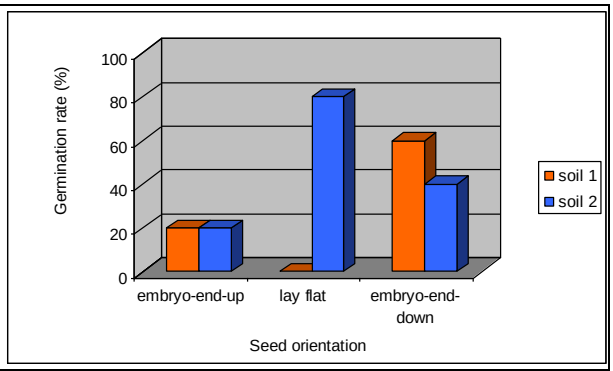

Fig 2. Germination rate in 6mm sowing depth

Fig 3. Germination rate in 9mm sowing depth

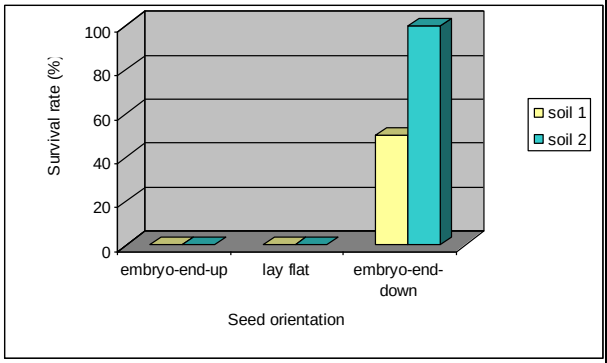

Fig 4. Survival rate in 3mm sowing depth

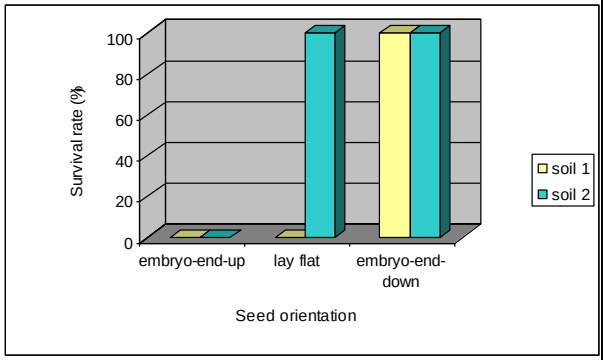

Fig 5. Survival rate in 6mm sowing depth

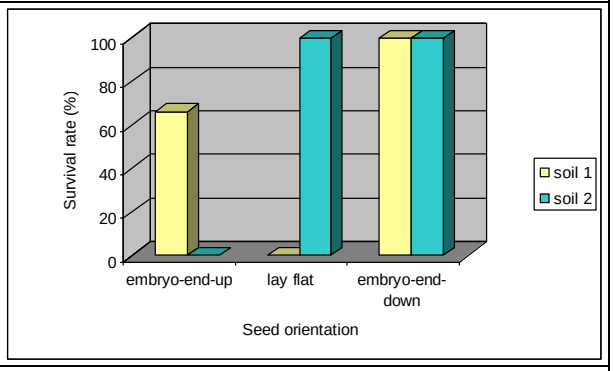

Fig 6. Survival rate in 9mm sowing depth

Table1. Germination and survival rates of two soil treatments

Soil treatment	Seed Germination rate (%)	Seedling survival rate (%)
3:1:1 (Soil 1)	24.4	66.6
2:2:1(Soil 2)	31.1	85.7

Table 2. Relationship of soil, sowing depth and seed orientation with seedling height 20 days after sowing

	Average seedling height								
	3 mm Sowing depth			*6 mm Sowing depth*			*9 mm Sowing depth*		
	Emberyo-end-up	Lay flat	Emberyo-end-down	Emberyo-end-up	Lay flat	Emberyo-end- down	Emberyo-end-up	Lay flat	Emberyo-end- down
Soil 1	0[a]	0[a]	1.4 [a]	3[a]	*0*[a]	3.43[a]	2.45[a]	2 [a]	0 [a]
Soil 2	0[a]	0[a]	4.77[a]	1[a]	*8.88*[a]	10[a]	2.5[a]	7.5[a]	0 [a]

Table 3. Relationship of soil, sowing depth and seed orientation with seedling height 40 days after sowing

	Average seedling height								
	3 mm Sowing depth			*6 mm Sowing depth*			*9 mm Sowing depth*		
	Emberyo-end-up	Lay flat	Emberyo-end-down	Emberyo-end-up	Lay flat	Emberyo-end-down	Emberyo-end-up	Lay flat	Emberyo-end- down
Soil 1	0[a]	0[a]	2 [a]	0[a]	*0*[a]	*6.67*[a]	*4.5*[a]	*0*[a]	*2.25*[a]
Soil 2	0[a]	0[a]	*12* [a]	0[a]	*13* [a]	*17.75*[a]	*0*[a]	*10.5*[a]	*0* [a]

Table 4. Relationship of soil, sowing depth and seed orientation with seedling height 60 days after sowing

	Average seedling height								
	3 mm Sowing depth			*6 mm Sowing depth*			*9 mm Sowing depth*		
	Emberyo-end-up	Lay flat	Emberyo-end-down	Emberyo-end-up	Lay flat	Emberyo-end-down	Emberyo-end-up	Lay flat	Emberyo-end-down
Soil 1	0[a]	0[a]	*10*[a]	0[a]	*0*[a]	*11.5*[a]	*9.5*[a]	*0*[a]	*5.5*[a]
Soil 2	0[a]	0[a]	*10.17* [a]	0[a]	*20.13* [a]	*27.5*[a]	*0*[a]	*15.75*[a]	*5*[a]

[a] *Data bearing same letters in a column do not differ significantly from each other at* $P < 0.05$.

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The European Eel Anguilla anguilla (Pisces, Anguillidae). Native or Alien in the Black Sea?

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Abstract. In 2006 the Scientific, Technical and Economic Committee for Fisheries (STEFC) of EU suggested the Black Sea region should be excluded from the list of natural areas, where the European eel (*Anguilla anguilla*) is distributed. The basic conception of this idea was that the eels caught in the Black Sea region represent escaped specimens from fish farming in some Danubian countries. This article illustrates an effort to be given an indirect answer on the question if Black Sea is the end of natural distribution of European eel. The species is present but never been abundant in the region and do not represent an object of commercial fishing.

Key words: European eel, *Anguilla anguilla*, Black Sea Region

Introduction

The life cycle of the eel was a mystery for a very long period of scientific history - more than 6500 publications about the eel, but still much of its life history is enigmatic.

Until 1893, larval eels — transparent, leaf like 5 cm creatures of the open ocean — were considered a separate species – *Leptocephalus brevirostris*. Italian zoologist Giovanni Grassi observed the transformation of a *Leptocephalus i*nto a round glass eel in the Mediterranean Sea, and French zoologist Yves Delage proved in a laboratory that both *Leptocephalus* and eels were the same species. Despite this discovery, the name *Leptocephalus* is still used for larval eel. A Danish professor - Johannes Schmidt from 1904 onwards, directed many expeditions in the Mediterranean Sea and the North Atlantic, largely financed by the Carlsberg Foundation. Finally in 1922, he ended up south of Bermuda in the Sargasso Sea, where he succeeded in catching the smallest eel-larvae ever seen. However, Schmidt was not able to observe the spawning directly, nor did he find ready-to-spawn adults. This area has not been confirmed by the presence of eggs, small larvae or spawning adults (BAKER, 1978). European eels are thought to spawn at ocean depths of 400-700 m in mid-water in late winter and early spring (BERTELSEN, 1967). The eggs are pelagic.

After hatchment the larval European eels travel by the aims of the Gulf Stream across the Atlantic Ocean and, after one to three years, reach European shores at a size of 45-75 mm. The common name for this stage is glass eel (based on the transparency of the body). Glass eels evolve into small eels before moving into freshwater basins. In fresh water they develop pigmentation, turn into elves (young eels) and feed on small crustaceans, worms and insects.

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Young eels spend their growth period in freshwater, males for 6-12 years (a length of 30-40 cm), females for 9-20 years (55-65 cm), before ending their metamorphosis. They grow up in 10 or 14 years to a length of 60 to 80 cm. In this stage they are called yellow (green) eels because of their pigmentation.

At the end of their growth period, they become sexually mature and migrate towards the sea, crossing even wet grasslands at night to reach the proper rivers.

Whether the adults can ever make the 6,000 km open ocean journey back to their spawning grounds remains unknown. The body undergoes other changes as well: the eyes start to grow, for optimal vision, and the body sides become silvery, for optimal mobility. These migrating eels are often called silver eels. According to SCHMIDT (1912) a travel speed in the ocean of 15 km per day can be assumed, so a silver eel would need 140 to 175 days to reach the Sargasso Sea.

Today our knowledge on the fate of the eels once they leave the continental shelf is based on three eels found in the stomachs of deep sea fish, a whale caught off Ireland and off the Azores and some experiments on five eels.

The European eel (*Anguilla anguilla*) is representing a highly valuable stock in view to fisheries and aquaculture, with final consequence the severe decline of its stocks. According to estimates from the International Council for Exploration of the Seas (ICES), the recruitment level (the number of baby eel produced each year) is only 1 % of what it was before the 1980s, and total reported landings of European Eels (to FAO) dropped to 43.5% from 1984–2000.

The species is included in the IUCN Red list of threatened species as Critically Endangered (A2bd+4bd) (RedList IUCN, 2912). In 2007, it was included in CITES App. II (CITES, 2011) that deals with species not necessarily threatened with extinction, but in which trade must be controlled. Since 1985 it is included in the Bulgarian Red Book (2011) as a critically endangered species. The distribution and stocks accordingly of this species in the Bulgarian water bodies during the last 50 – 60 years is decreased approximately more than 10 times (Fig. 1).

In the most relatively contemporary books, monographs, internet and others works concerning migrations and distribution of European eel we can find the next information concerning the species' distribution: Atlantic Ocean – Eastern Atlantic coast from Scandinavia to Morocco and rivers of North Atlantic, Baltic and Mediterranean Seas. There have been undertaken certain introductions to Asia, South and Central America, and Australia.

Development during life cycle with metamorphosis is typical for the representative species of order Anguilliformes (including the typical marine species). The unique biology of the species *Anguilla anguilla* is determined by the vast distances between places for spawn (Sargasso Sea) and the areas of distribution. In this case, according the strict sense (sensus stricto) of the term "native species", The European eel is not native for none of the European or other countries. These countries are part of the natural distribution areas for most of the species' developmental stages. To complete its life cycle the European eel should overcome distances between 10 000-12 000 km. Probably at first sight these enormous distances appear to be almost impossible for such a migration, but it should be considered, that this unique life cycle is a result of thousands of year's evolution.

In 2006 the Scientific, Technical and Economic Committee for Fisheries (STEFC) of EU suggested the Black Sea region should be excluded from the list of natural areas, where the European eel *Anguilla anguilla* is distributed. The basic conception of this idea was that the eels caught in the Black Sea region represent escaped specimens from fish farming in some Danubian countries. This article illustrates an effort to be given an indirect answer on the question if Black Sea is the end of natural distribution of European eel.

Materials and Methods

In order to register the species' presence in the Bulgarian Black Sea sector and

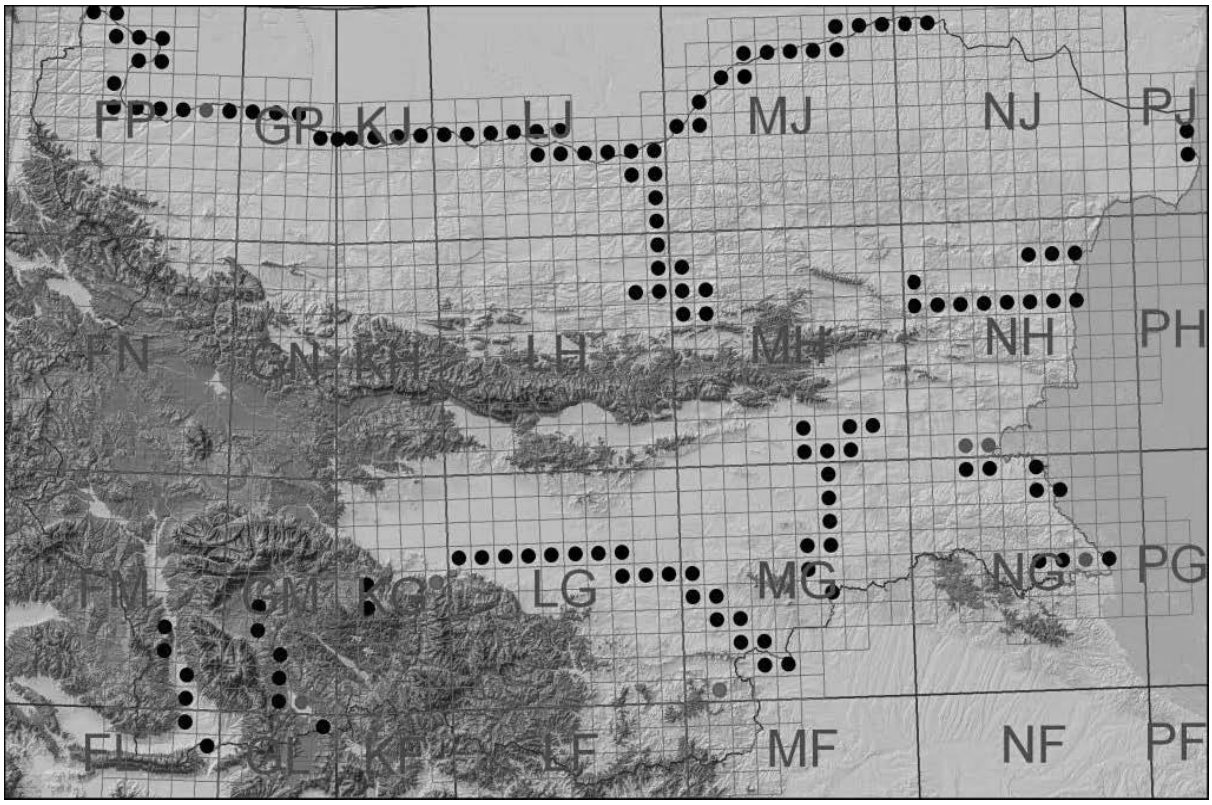

Fig. 1. Past and present distribution of the European eel in Bulgaria according to the Red Data Book of the Republic of Bulgaria, 2011, (Digital edition).

adjacent freshwater bodies during the period 2009-2012 the following methods have been applied: baited and unbaited net traps, underwater transects in both marine/brackish and fresh waters (the last when visibility permitted) – both during day and night, inquiries of local professional fishermen and fish markets. The survey covered almost the entire Bulgarian Black Sea sector, and adjacent sea shore lakes, lagoons, estuaries and rivers.

Results and Discussion

The results obtained from the questionnaires showed, that the European eel is present in most of the main freshwater bodies, connected directly with Black Sea, from north to south: Durankulak and Shabla/Ezerets coastal lakes, Varna lake complex, Kamchia River, Bourgas/Mandra coastal lakes system, Ropotamo and Veleka Rivers. It is not clear if inhabits rivers Karaagach, Diavolska and Rezovska, such information is lacking. From the other side the fishermen's statements have to be accepted as truly without doubt. Single specimens are also irregularly caught by professional pound nets along the entirely Bulgarian Black Sea coast.

Keeping in mind the cryptic and nocturnal life mode of the species, it is extremely difficult to be registered. In September 2010 a single European eel specimen has been captured by the authors, between Primorsko and Kiten towns (42° 14/ 32.84//, E 27° 45/ 59.04//) (Fig 2). The captured in Black Sea specimen occurred to be a yellow eel 92 cm long. It was discovered on rocky substrate hiding between rocks at a depth of about 2.5 m and represents the most recently documented registration along the Bulgarian Black Sea sector.

Native or alien?

In order to replay to the question "Is the Black Sea water basin part of the native area of distribution for European eel (*Anguilla anguilla*)", we should accept or reject the following two conceptions:

1. Black Sea water basin was artificially settled with eels from the humans – in this

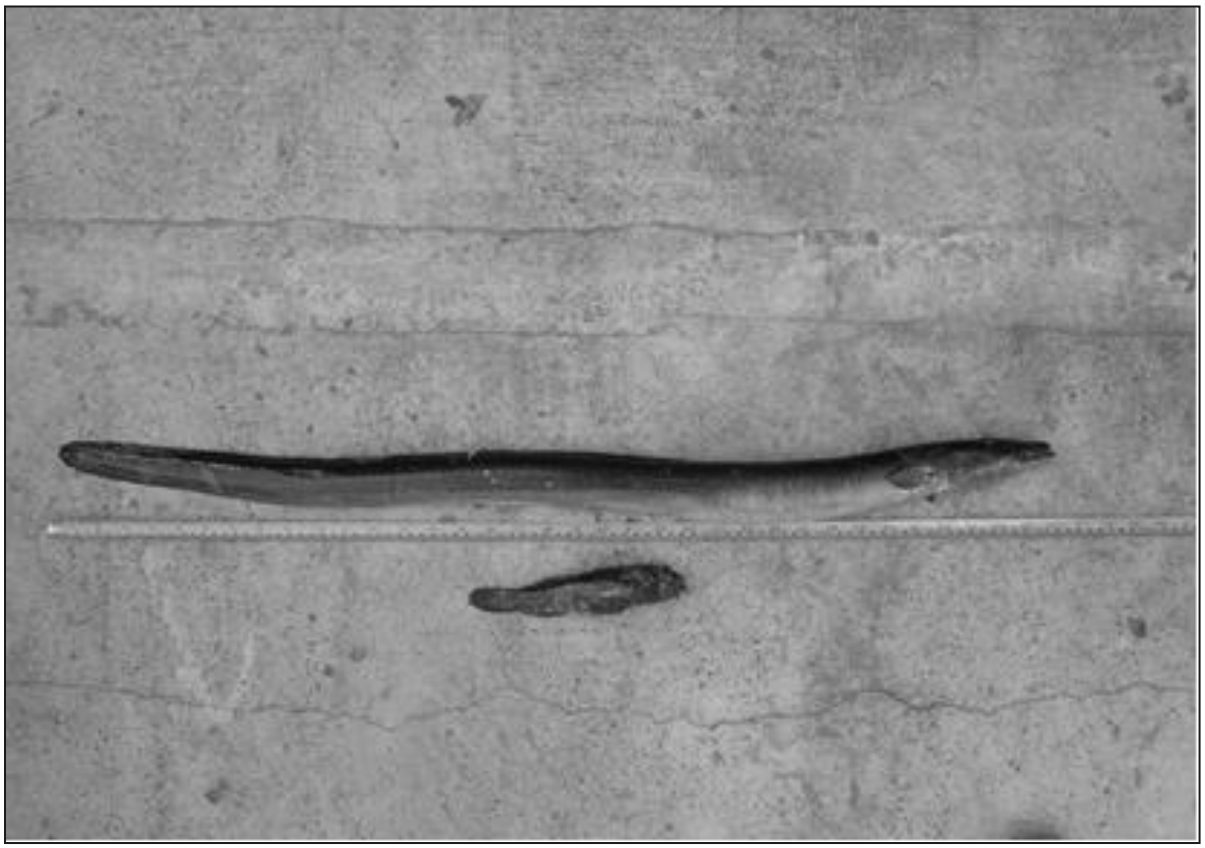

Fig. 2. The most recently documented European eel capture in Black Sea near Kiten town (N 42o 14/ 32.84//, E 27o 45/ 59.04//) in September 2009. Yellow eel, 92 cm, 806 gr.

case the Black sea water basin is not a native area of distribution.

2. The presence of eel in the Black Sea water basin is consequence of the natural distribution of the species trough Aegean Sea, Sea of Marmara and Bosporus.

In the first case the entrance of eel in the Black sea could happens only through the Danube river, as a result of aquaculture farming in the upper reaches of the river – in Germany, Austria and Hungary. Indeed the eel is farmed artificially in aquaculture (fatten to market size) in these countries from the beginning of the XXth century, in the beginning extensively and later intensively. The statement that aquaculture eel escapes in Danube river reaches could lead to settlement in Black sea water basin cannot be taken as a serious view. Pioneers in the eel aquaculture and artificial settlement of animal and plant species are considered to be the Romans, but still from those old times it is well known that to settle a certain species in new area and to ensure its survival it is necessary to perform successful acclimatization, in other words to establish spawning in the new area. Concerning the European eel, similar speculation is impossible, taking in mind what it is known about the biology of the species – after the entrance in the rivers and approaching maturity, the eel turns back to the sea, where undergoes migratory changes (coloration, behavior etc). The next stage is the long way to the Sea of Sargasso. In this way of thinking it should be considered, that except in the Danube River, Northern and North-Western part of Black Sea, the eel should not be found in the North, East and South parts of the Sea, in The Sea of Azov and in the other rivers of this particular water basin. This concept seems to be incorrect. The presence of the species in Black Sea and The Sea of Azov basins is proved by many scientific researches accomplished in the region and dated before the development of any aquaculture activities and building of artificial water

channels, connecting the basins of Black Sea and Baltic Sea.

During the 17th century the traveler scientist Marsilius (in BERG, 1961) announced presence of eel in the upper and middle reaches of Danube River. In 1774, the famous Russian ichthyologist GULDENSTADT (1791) described European eel in Dnieper River basin. Other Russian ichthyologist – KESSLER (1857) reported that this species is not rare in lower reaches in Dniester River. In the beginning of 20th century the eel is caught in most of the coastal lakes in Russia, Romania and Bulgaria. (ANTIPA, 1909; CHICHKOFF, 1912; MAKSIMOV, 1914; DRENSKY, 1923; BERG, 1949 etc.). For particular specimens capture in Black sea, Sea of Azov and the flowing nearby rivers is mentioned in the references of RYABKOV (1896); TROITSKYI (in BERG, 1949); BERG (1949, 1961); DRENSKY (1951); BANARESCU (1964); SVETOVIDOV (1964); MARTI (1980) etc.

Another evidence for the entrance of eel in Black sea trough Bosporus is the size of the discovered specimens – between 20 cm and 100 cm, i.e. that are eels (20-25 cm) which reached Black Sea and are ready to enter the rivers and others (over 50 cm) which are migrated from the rivers to the sea (fishermen's communications and recent capture). The case seems similar as another locality, where different sized specimens are caught altogether: the Baltic Sea, which is assumed as part of the eels natural areal. The species presence in Volga drainage (KOTTELAT & FREYHOFF, 2007) is also a pro argument for the second conception – it does not seem possible to travel in Black Sea via Danube from Baltic Sea, enter Caspian Sea drainage and then return to Black Sea for reproductive migration to the Sargasso Sea.

As an evidence for the ancient presence of European eel in Black Sea basin can also be indicated the original names of this species in some of the languages in the region, especially Bulgarian local name "Zmiorka", which does not have analogy among the other eel names as well Russian and Ukrainian name "Ugor". The usual practice during the introduction of alien (exotic) species is to take the origin name used in the country, from which the species is imported and not to be given a new one.

Actually, the Black Sea basin which is connected by Bosporus and Sea of Marmara with Aegean Sea (Mediterranean) Basin – is the end constituent of natural area of distribution of European eel (ANTIPA, 1909; KOVACHEV, 1922; Berg, 1949; DRENSKY, 1951; BANARESCU, 1964; SVETOVIDOV, 1964; MARTI, 1980. In this part of the area the European eel was never been abundant and respectively an object of commercial fishery. Probably because of this reason, it has not been an object of any careful studies and statistical data in the region.

Bulgarian rivers belong to both Black Sea and Aegean Sea watershed. In Bulgaria until 1940s – 1950s there were caught dozens of specimens by fishermen in the lakes and the rivers flown directly in the Black Sea (Kamchiya, Ropotamo, Veleka, Rezovska); in some marshes and rivers from the Aegean catchment (Struma / Strymon, Mesta / Nestos, Maritsa / Evros, Tundzha, Arda / Ardas); in the Danube River and marshes along the river (CHICHKOFF, 1912; KOVACHEV, 1922; DRENSKY, 1948, 1951).

During the last years the European eel is considerably rare caught in the lower parts of the rivers: Arda, Maritsa, Tundzha, Kamchiya, Veleka and in the Bulgarian stretch of Danube River (MIHAILOVA, 1965; KARAPETKOVA, 1974, 1975, 1976; KARAPETKOVA & ZIVKOV, 1995; MARINOV, 1978; VASSILEV & PEHLIVANOV, 2005 etc.).

Conclusion

Black Sea is the end of natural distribution of European eel. The species is not abundant in the region and do not represent an object of commercial fishing. At the present time, besides a natural penetration of eels through Bosporus, replenishment by fish farming is possible.

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Rehabilitation of Degraded Rangeland in Drylands by Prickly Pear (Opuntia ficus-indica L.) Plantations: Effect on Soil and Spontaneous Vegetation

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Abstract. In arid and semi-arid lands, the spiny prickly pear (*Opuntia ficus-indica*) is an outstanding plant for soil conservation and restoration. To determine the role of *Opuntia ficus-indica* on vegetation recovery process in desertified areas of Southern Tebessa (Northeast Algeria), we investigated the effect of prickly pear plantation age and some soil properties (grain size, pH, electrical conductivity, organic matter, total nitrogen, available phosphorus, and $CaCO_3$ equivalents) on native plant community. Vegetation cover and plant diversity were assessed by calculating the number of individual plants (N), species richness (S), their ratio (N/S), Shannon index, and Evenness in prickly pear plantation plots of different ages (control, 5 and 20 years). Even if surveyed soil parameters did not differ significantly among *O. ficus-indica* plantations, results of ANOVA testing the effect of *Opuntia* plantations on native vegetation traits revealed significant variation for plant abundance ($P < 0.0001$), N/S ratio ($P = 0.003$) and vegetation cover ($P < 0.0001$). Vegetation cover differed significantly with both prickly-pear plantation age ($P = 0.031$) and seasons ($P = 0.019$). Tukey's tests revealed that all vegetation traits were significantly higher on prickly pear plantations than in control plots. Multiple comparisons also showed that plant abundance, N/S ratio and vegetation cover were significantly different between both young and old plantations and the controls. Prickly pear cultures facilitated the colonization and development of herbaceous species by ameliorating the severe environmental conditions. In conclusion, the facilitative effect of *O. ficus-indica* has been clearly demonstrated for both abundance and cover of native vegetation.

Key words: rangeland, land rehabilitation, *Opuntia ficus-indica*, soil restoration, plant diversity, Algeria, drylands.

Introduction

Desertification is defined by the United Nations Convention to Combat Desertification (UNCCD) as being degradation or loss of productive capacity of land in arid, semi-arid and dry-sub-humid areas resulting from the combination of several factors including climate variations and anthropogenic activities (SIVAKUMAR, 2007). Desertified lands have an area of 3.6 billion worldwide, arid and semi-arid ecosystems represent over 25% of the earth (SMITH *et al*., 2000). Arid rangelands occupy an area of over 600,000 km² in North Africa covering, approximately 34% of Algeria, 31% of Libya, 19% of Morocco, 11% of Tunisia and 5% of Egypt (LE HOUÉROU, 1995).

Desertification indicators affecting vital ecosystem attributes are the deterioration of soil properties, including the reduction of

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water reserves and fertility, often up to critical regression of vegetation production (NEDJRAOUI, 2004; AIDOUD *et al*., 2006). These characteristics are a sign of huge vulnerability toward natural and/or man-induced changes (SIVAKUMAR, 2007; NEDJRAOUI & BEDRANI, 2008), which explains the complexity or impossibility to remediate damage occurring in these environments (BENABDERRAHMANE & CHENCHOUNI, 2010).

Rehabilitation of degraded rangeland habitats necessarily involves strategies; as the restoration of native shrubs to restore biodiversity and prevent both erosion and desertification processes (CARAVACA *et al*., 2003), or through manmade improvement, using even non-native plant species (NEFFAR *et al*., 2011). The use of drought-resistant plants in the rehabilitation of degraded semi-arid rangelands is very common, such as the cases of Acacias or Aloe (KING 2008). These plants can increase dynamics of biological resources and restore degraded vegetation, thus improving the severe conditions that limit the establishment of plant species (LE HOUÉROU, 1995). The fact that the presence of a plant species improves environmental conditions and provides favorable sites where less tolerant species can establish, is called facilitation (CALLAWAY, 1995). Through such positive interactions, plants facilitators can greatly influence composition, dynamics and diversity of the ecological community (WEZEL *et al*., 2000).

Besides its arid climate, characterized by erratic and insufficient rainfall with large seasonal and interannual variations, Algerian rangelands, extending over 20 million hectares (DJEBAILI, 1984), are intensively used for livestock activities. They feed 2/3 of Algerian populations of sheep and goat (DJEBAILI *et al*., 1989). Although these natural steppes play a key role in agricultural economy of the country they have been subject to rapid and severe degradation over the last three decades (SLIMANI *et al*. 2010), due to recurrent droughts and increasing human pressure mainly by clearing, overgrazing and inappropriate exploitation of unsuitable lands for crops (NEDJRAOUI & BEDRANI, 2008). This land degradation and desertification, when at its most advanced stage, reduces biological potential and breaks ecological and socio-economic balances (NEDJRAOUI & BEDRANI, 2008). Moreover, about 75% of the area of Algerian rangelands are being desertified or are at the desertification threshold (BENSLIMANE *et al*., 2008, BENABDERRAHMANE & CHENCHOUNI, 2010).

A shrub with low ecological requirements (SNYMAN, 2006) the prickly pear (*Opuntia ficus-indica*) is among the fodder plantations as well as *Atriplex* plantations, which are commonly used to combat desertification in Algeria (NEFFAR, 2012). The question that arises, can it act as a facilitator in degraded rangelands? Because arid-rangeland rehabilitation by plantations is known to have facilitative effects for (i) increasing and improving soil features (CARAVACA *et al*., 2003) and (ii) accelerating ecological succession processes (WEZEL *et al*., 2000), also since the identification of changes in soil properties is needed to understand the effectiveness of ecological reclamation and changes in soil nutrients in relation to plantation age (SHAO *et al*. 1996); we tested how prickly pear plantations, through their age, affect *in situ* edaphic factors of superficial horizons and traits of spontaneously "natural" vegetation. This study is an investigation that aims to assess changes in soil and native vegetation properties following the establishment of prickly pear plantations (*Opuntia ficus-indica* var. *amyclaea*) in an arid area located in Northeastern Algeria.

Materials and methods

Study Area. The study was conducted in Mzara (Tebessa, Northeastern Algeria) located at 780 m above sea level (34°51'08.4"N and 08°15'05.3"E; Fig. 1). Soils are skeletal and of sandy texture, poor in organic matter and are susceptible to degradation. Long-term climatic data (1990–2005) revealed that the Mediterranean climate of this region is arid-type with cold dry winters and severe hot dry summers. The region receives an average of 163 mm of rain per year. Average annual temperature

is about 14.1 °C with a minimum of 5.8 °C in January and a maximum of 23.5 °C in July. According the GAUSSEN and BAGNOULS' diagram (1954), the drought period extends over the entire year. During the study year 2008, the area had irregular monthly precipitation with several peaks of rainfall throughout the year except during summer "June-August" (Fig. 2). In addition, the index of De MARTONNE (1926) applied for the region revealed an arid climate (DE MARTONNE's index value = 6.76). According to NEFFAR (2012), overall the sparse-type vegetation of Tebessa steppes is represented by several native plant species such as *Stipa tenacissima* (L.), *Lygeum spartum* (L.), *Arthtrophytum scoparium* (Pomel.), *Artemisia herba alba* (Asso.), and *Astragalus armatus* (Willd.).

Sampling Design. The study was conducted in three types of *Opuntia ficus-indica* plantations: (i) plantations aged less than 5 years, (ii) more than 20 years and (iii) unplanted plots, taken as control. Three plots, of 2 – 4 ha in size and similar topographic properties, were selected for each plantation type as replicates located in separate plantations. The sampled plots, including control, were chosen close to each other to reduce site-variations of soil and climate. *Opuntia* plantation age was determined based on field investigations and confirmed by the High Commission for the Development of the Steppe (HCDS) in Tebessa.

Soil Analysis. In each surveyed plot, eight soil samples were collected using an auger on an average depth of 15–20 cm. All soil samples of the same plot type of plantation age "treatment" were pooled before processing. In the laboratory, physical and chemical analyzes were carried out on the fine earth (Ø < 2mm). The following edaphic parameters were measured repetitively six times on the pooled soils of each treatment:

– Grain size was determined by wet sieving method (AFNOR, 1990) once for each treatment. Then soil texture was derived by projecting fraction values of clay, silt and sand on the textural triangle according to USDA classification (DUCHAUFFOUR, 1977).

– The pH and electrical conductivity (EC) were measured at 1:5 soil-water suspension ratio.

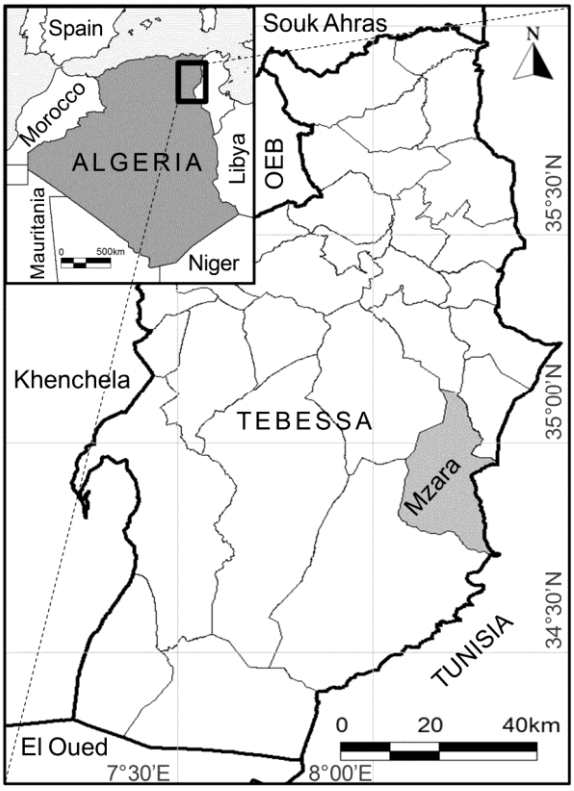

Fig. 1. Geographical localization of study area "Mzara" in Tebessa (Northeastern Algeria, North Africa).

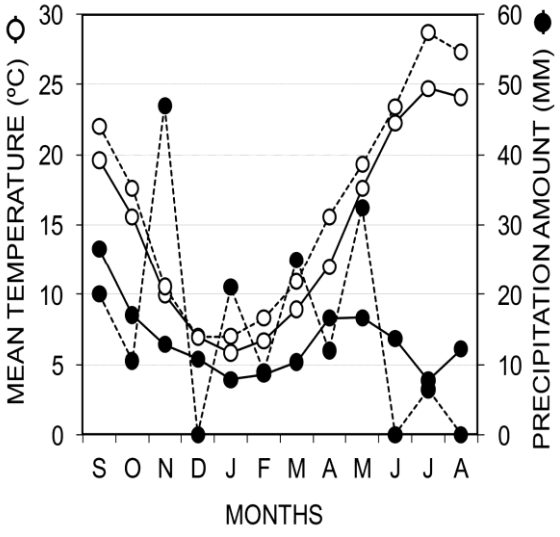

Fig. 2. Diagram of Gaussen and Bagnouls applied for Mzara (Tebessa, Northeast Algeria). Continuous lines represent data of 16–years period (1990–2005) and dashed lines symbolize climate data of 2008.

— The levels of calcium carbonate equivalent (CCE) were determined by volumetric calcimetry (DERMECH *et al*., 1982), which is based on decomposition of calcium carbonates by a strong acid (HCl) (V: 1/3) and measuring the volume of CO_2 released.

— DROUINEAU's method was used to determine the amount of active calcium carbonate equivalent (ACCE) (MATHIEU & PIELTAIN, 2003), which is based on calcium's property of combining with oxalate to give insoluble calcium oxalate.

— Organic carbon was determined by Anne method (BONNEAU & SOUCHIER, 1994) by oxidation of carbon with excess potassium dichromate ($K_2Cr_2O_7$) in sulfuric acid medium (heat source). The amount of non-consumed dichromate was measured back by Mohr's salt.

— The rate of organic matter (OM) was estimated by multiplying the percentage of carbon by 1.72 (MATHIEU & PIELTAIN, 2003).

— The total nitrogen was determined by the KJELDAHL method described by BONNEAU & SOUCHIER (1994). It consists of a mineralization of the nitrogenous organic matter by concentrated sulfuric acid in hot conditions. The transformed nitrogen into ammonia was determined by the amount of sulfuric acid in the form of ammonium sulfate.

— Available phosphorus (AP) was determined by Olsen method (MATHIEU & PIELTAIN, 2003) after extraction with a solution of sodium bicarbonate at 0.5 M.

Vegetation Sampling. Vegetation traits (vegetation cover, number of individuals and diversity) were estimated using linear transect or "line intercept" method (COOK & STUBBENDIECK, 1986) which consists in measuring the length covered by various species projected along a line graduated of 10 by 10 cm, and stretched through the vegetation, either at ground or on top of the dominant stratum for bushy and/or shrubby species. In total we have carried out three replications per season in each plot sampled. In every replication four line intercept measures were randomly carried out at the interline spacing between prickly pear plants and then these measures were averaged to have only one value per replication.

The tape length depended on the width of the interline spacing of *Opuntia* plantations, which varied between 2 and 13 meters. Along the stretched tape, the monitoring of vegetation parameters "number of individuals" was performed at one point every 10 cm where every individual touching a point of the line was noted. Points of the tape touched by the plant were counted, and then their percentage relative to the total number of points of the tape was calculated. The vegetation cover was evaluated by estimating continuously the percentage of ground covered by vegetation as the ratio of the length occupied by vegetation to the total length of the line. Although this method is not very precise, if the sampled transect was not representative, but at least has the advantage in rapid assessment (COOK & STUBBENDIECK, 1986).

While vegetation cover was measured seasonally (three months per season), the census of plant species and their abundance "number of individuals" were carried out during period of maximum growth of vegetation, which coincides with the month of April (mid spring: March–May). Species diversity was assessed by Shannon index (H') (FAURIE *et al*., 2003).

$$H' = -\sum_{i=1}^{S}[(\frac{ni}{N}) \times Log_2\,(\frac{ni}{N})]$$

where: n_i/N is the relative abundance, n_i is the number of individuals of each species, N is the total number of individuals of all species, and S the total number of species (species richness).

The evenness (E) is the ratio of Shannon index (H') and the maximum diversity value that is assessed for S species with equal population distribution. Evenness varies between zero and one (FAURIE *et al*., 2003).

$$E = H' / log_2 S$$

Statistical analysis. One–way analysis of variance (ANOVA) was carried out to test the effect of plantation age of prickly pear on soil parameters. The effects of prickly pear plantation age on the traits of

spontaneously vegetation "including the total number of individual plants (*N*), species richness (*S*), the ratio *N*/*S*, Shannon index, Evenness and vegetation cover" were analyzed using one–way ANOVA. Before computations, the normal distribution and homogeneity of data was verified by the SHAPIRO-WILK normality test, then all vegetation traits were log-transformed to normalize their distributions, and after that, we tested if the data meets the basic assumption of equal variance (homoscedasticity). We used a modified version of LEVENE's test, as described by GLANTZ & SLINKER (2000). The vegetation data were transformed by obtaining the absolute difference between each value and the corresponding group median (not the mean as in the original method), and then the test performed an analysis of variance on the transformed data (GLANTZ & SLINKER, 2000). Since the ANOVA makes a general statement about whether or not there are significant differences between the data groups, once the result is positive ($P < 0.05$) post-hoc tests were performed to find out which of the multiple comparisons that are possible for plantation age data set were significant. The post-hoc method performed multiple comparisons using TUKEY test, which requires computation of the so-called studentized range "*q*" (Rafter *et al*. 2002). The studentized range is the difference between the largest and smallest data in a sample measured in units of sample standard deviations.

In addition, two–way ANOVA was applied for testing the effect of plantation age, seasons and the interaction "age*season" on spontaneously vegetation cover. ANOVA-tests were considered statistically significant (*), highly significant (**) or very highly significant (***) for probability-value $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Results

Physicochemical properties of soils. Overall, the factor "plantation age" statistically had no significant effect on all studied soil parameters (Table 1). According to values of clay, sand and silt fractions, projected onto a texture triangle, studied soils belong to sandy class. The pH showed values exceeding 8, indicating an alkaline soils. The values of electrical conductivity (EC) were below 1000 µS cm^{-1} in all surveyed plots. Although EC variations were noticeable between unplanted and planted plots, there are no statistically significant differences for both variables (pH: $F = 2.40$, $P = 0.121$; EC: $F = 2.97$, $P = 0.082$).

The $CaCO_3$ equivalent showed average values, ranging between 12 and 15% in all samples with no significant difference among them. It is the same for the rate of ACCE that marked values between 4.81 and 6.69% in the planted plots and 7% in control plots. Planted plots showed an average rate of total nitrogen (0.06 to 0.12%) and that was similar to controls (0.12%). No significant difference was registered for available phosphorus concentrations that were slight and organic matter (OM) between planted plots (OM = 0.36 to 0.57%) and control plots (OM = 0.46%). The C/N ratio recorded mean values ranging from 2.72 and 1.75 in planted plots and 2.86 in control plots (Table 1).

Effect on vegetation characteristics. The highest value of spontaneously vegetation cover was observed in winter, followed by cover of spring and summer. Nevertheless, whatever the season, the planted plots (young and old plantations) showed a higher vegetation cover than controls (Fig. 3), which was statistically reported as significant ($P = 0.031$) considering plot-to-plot cover variation within treatment (Table 2). While the seasons had also a significant effect on vegetation cover variations ($P < 0.019$), the interaction "Plantation age*Season" was not significant ($P = 0.798$).

Indeed the lowest vegetation cover was observed during summer with 9% in controls and between 21% and 27% in planted plots, compared to winter in which the control plots recorded a value of 33% and in planted plots between 37 and 52%. In spring, vegetation cover varied between 39 and 45% among planted samples, and was 25% in unplanted plots (Fig. 3).

Shannon index (H') and evenness (E) were low in both young plantations (H' = 1.49 bits, E = 0.57) and control plots (H' = 1.26 bits, E = 0.54), and experienced a slight rise in old plantations (H' = 1.99 bits, E = 0.58). Regarding plant life forms, the area had 66% of therophytes in young planted plots, followed by 16% of geophytes and hemicryptophytes. In old plantations, the chamaephytes represented 42% followed by geophytes with 28%. While in control-plots, geophytes and chamaephytes occurred equally with 33%, followed by hemicryptophytes and therophytes with 16% for each (Table 3).

Regarding the effect of *O. ficus-indica* plantation age on natural plant species abundance and species richness (Fig. 4), both *Opuntia*-planted plots recorded an average plant abundances ranging between 19 and 21 individuals, slightly higher compared to controls (15 individuals), and the statistical analysis revealed a highly significantly deference ($P < 0.0001$). However even there was a high trend for species richness to be greater in *Opuntia*-planted plots compared to controls, its variation was not significant ($P = 0.052$). In addition, the ANOVA testing the effect of *Opuntia* plantations on native vegetation features revealed that N/S ratio and vegetation cover differed highly significantly, $P = 0.003$ and $P < 0.0001$, respectively (Table 4). The test was non-significant for plant diversity parameters including either Shannon's index ($P = 0.247$) and evenness ($P = 0.415$).

Multiple comparisons (TUKEY's test) revealed that all vegetation traits (plant abundance, N/S ratio and vegetation cover) were significantly higher on *Opuntia* plantations than in control plots. Multiple comparisons also revealed that plant abundance was significantly different between both young and old *Opuntia* plantations and the controls. While the *N/S* ratio was only significantly different between old plantations and controls, the test indicated also that vegetation cover was highly significantly different ($P < 0.0001$) between either young and old *Opuntia* plantations and the controls (but not between young and old plots). The *q*-values calculated for young and old plantations comparisons were the lowest (except for N/S ratio), indicating a slight range difference between these two treatments (Table 5).

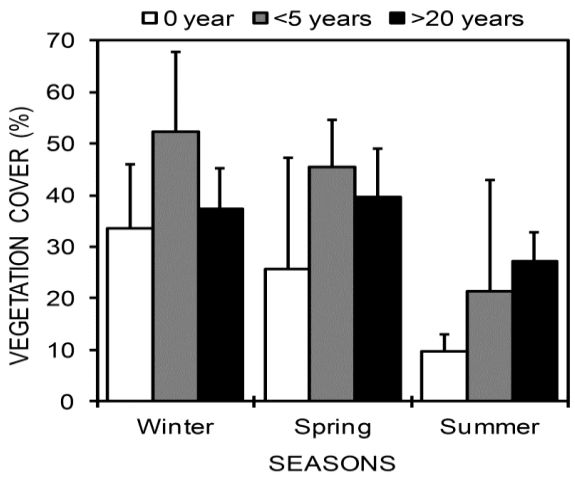

Fig. 3. Seasonal variation of vegetation cover according to ages of spiny prickly pear plantations.

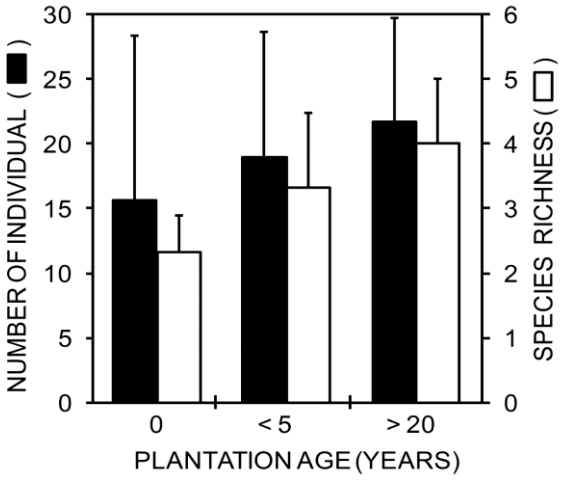

Fig. 4. Variation of the total number of plant individuals (N) and species richness (S) according to plantation ages of *Opuntia ficus-indica*.

Table 1. Mean values (± SD) of physicochemical soil parameters based on six measurements, followed by effect of the prickly pear plantation age on soil traits based on One–way ANOVA outputs. (EC: Electrical conductivity, CCE: calcium carbonate equivalent, ACCE: active calcium carbonate equivalent, AP: Available phosphorus).

Soil traits	Plantation age of *Opuntia ficus-indica*			ANOVA	
	Control (unplanted)	< 5 years	> 20 years	*F*	*P*
Sand (%)	85.40	79.71	87.78		
Silt (%)	4.29	6.76	4.07		
Clay (%)	10.30	13.52	8.14		
pH	8.07 ± 0.08	8.14 ± 0.03	8.09 ± 0.03	2.40	0.121
EC (µS cm^{-1})	753.66 ± 29.39	767.5 ± 27.13	826.16 ± 85.97	2.97	0.082
CCE (%)	15.30 ± 4.24	12.04 ± 3.90	13.98 ± 1.83	1.32	0.296
ACCE (%)	7.23 ± 2.39	4.81 ± 2.48	6.69 ± 1.32	2.11	0.155
Carbon (%)	0.27 ± 0.20	0.34 ± 0.26	0.21 ± 0.12	0.67	0.528
Total Nitrogen (%)	0.12 ± 0.05	0.06 ± 0.01	0.12 ± 0.07	2.60	0.108
Organic matter (%)	0.46 ± 0.35	0.59 ± 0.45	0.36 ± 0.21	0.66	0.530
C/N	2.86 ± 2.57	2.75 ± 1.93	1.72 ± 1.55	0.92	0.418
AP (ppm)	1.23 ± 0.46	1.80 ± 0.43	1.43 ± 0.66	1.42	0.271

Table 2. Results of two–way ANOVA testing for effects of prickly pear plantation age and season on for native vegetation cover.

Source	*Df*	*SS*	*MS*	*F*	*P*	
Age	2	1340.1	670.0	6.55	0.031	*
Plot	6	613.8	102.3			
Season	2	2408.6	1204.3	5.60	0.019	*
Age*Season	4	352.8	88.2	0.41	0.798	
Res. error	12	2578.6	214.9			
Total	26	7293.9	280.5			

Table 3. List and abundances of native plant species recorded in different plantation plots of prickly pear (*Opuntia ficus-indica*), with vegetation traits and diversity indices. (Ch: Chamaephyte, Geo: Geophyte, Hem: Hemicryptophyte, Th: Therophyte).

FAMILLY Species	Morphologic forms	Life forms	Opuntia Plantation age Control	< 5 years	> 20 years
POACEAE					
Cynodon dactylon L.	Perennial	Th/Geo	35	35	23
Hordeum murinum L.	Annual	Th	0	16	0
BRASSICACEAE					
Sinapis arvensis L.	Annual	Th	0	2	0
Raphanus raphanistrum L.	Annual	Th	0	1	0
PLANTAGINACEAE					
Plantago albicans L.	Perennial	Hem	3	2	26
LILIACEAE					
Asparagus stipularis	Perennial	Geo	0	1	1
FABACEAE					
Astragalus armatus Willd	Perennial	Ch	6	0	5
CHENOPODIACEAE					
Haloxylon scoparius Forsk	Perennial	Ch	1	0	1
ASTERACEAE					
Artemisia campestris L.	Perennial	Ch	0	0	3
ZYGOPHYLLACEAE					
Peganum harmala L.	Perennial	Hem	0	0	26
ASTERACEAE					
Echinops spinosus L.	Perennial	Geo	2	0	1
Abundance "*N*"			47	57	86
Species richness "*S*"			5	6	8
Ratio *N/S*			9.4	9.5	10.8
Vegetation cover (%)			25.6 ± 1.6	45.6 ± 8.9	39.8 ± 9.3
Shannon index "*H'*" (in bits)			1.26	1.49	1.99
Evenness "*E*"			0.54	0.57	0.58

Table 4. Results of ANOVAs testing for effect of plantation age of *Opuntia ficus-indica* on natural vegetation traits. Test for equal variance (*F*, *df* (numerator, denominator) and *P* values) was given for each vegetation variable.

Dependent variable (Test for equal variance)	Source	*Df*	*SS*	*F*	*P*	
Plant abundance "N"	Between groups	2	0.0160	79.92	<0.0001	***
($F_{2, 6}$ = 0.82, *P* = 0.485)	Within groups	6	0.0006			
Species richness "S"	Between groups	2	0.0016	5.06	0.052	
($F_{2, 6}$ = 0.45, *P* = 0.656)	Within groups	6	0.0010			
N/S ratio	Between groups	2	0.0402	17.13	0.003	**
($F_{2, 6}$ = 0.46, *P* = 0.651)	Within groups	6	0.0070			
Shannon index	Between groups	2	0.0176	1.78	0.247	
($F_{2, 6}$ = 1.32, *P* = 0.334)	Within groups	6	0.0297			
Evenness	Between groups	2	0.0000	1.02	0.415	
($F_{2, 6}$ = 1.02, *P* = 0.414)	Within groups	6	0.0000			
Vegetation cover	Between groups	2	0.0436	750.20	<0.0001	***
($F_{2, 6}$ = 3.58, *P* = 0.095)	Within groups	6	0.0002			

Table 5. Summary of multiple comparisons (Tukey's range test) examining the effect of plantation age of *Opuntia ficus-indica* on natural vegetation traits (Parameters which differed significantly in ANOVA: $P < 0.05$). (Age of *Opuntia* plantation plots: C: control, 5y: < 5 years, 20y: > 20 years)

Dependent variable	Comparison	Sorted treatment	Delta means	*SE*	*q*	*P*	
Plant abundance	5y * C	20y > 5y > C	0.103	0.006	17.87	<0.0001	***
	20y * C		0.054	0.006	9.38	0.001	**
	5y * 20y		0.049	0.006	8.49	0.002	**
N/S ratio	5y * C	20y > 5y > C	0.032	0.020	1.60	0.533	
	20y * C		0.155	0.020	7.83	0.004	**
	5y * 20y		0.123	0.020	6.24	0.011	*
Vegetation cover	5y * C	5y > 20y > C	0.148	0.003	47.45	<0.0001	***
	20y * C		0.148	0.003	47.43	<0.0001	***
	5y * 20y		0.0001	0.003	0.02	0.999	

Discussion

Results of this study revealed that plantations of prickly pear had no effect on soil parameters, but a variable effect is reported on spontaneously vegetation parameters.

Effects on soil characteristics. In general, the effect of prickly pear plantations on soil properties is not significant; the causes as well the result values can be explained as follows:

All plots showed an alkaline pH, included in the range of base (7.5 and 8.5) following the classification of BAIZE & JABIOL (1995). Soil pH is mainly affected by the parental material (REZAEI & GILKES, 2005) as well the organic matter transformed. Indeed, the soil of the station is classified as moderately calcareous (BAIZE & JABIOL, 1995), attributed to the calcareous origin of the steppe region (DJEBAILI, 1984) and the Mediterranean basin (NOUAIM & CHAUSSOD, 1995). The active fraction varies between 5 and 6%, which probably explains this alkalinity. According ROMANYA and ROVIRA (2007), this reduces the availability of phosphorus for plants and microorganisms, hence explains the low values observed for this element, reflecting a poor soil according to Olsen's method (MATHIEU & PIELTAIN, 2003).

Our results showed there was a trend for phosphorus content to be higher in planted treatments compared to control. This is probably because livestock (cattle, sheep, goats) graze in untreated plots, but avoid plots of the spiny prickly pear plants, which kept out livestock, in particular when *Opuntia* plantation was grown into dense shrubby bush. Knowing that livestock feces somehow contribute in extent nitrogen content (PEI *et al*., 2007), the latter factor has a strong trend to be lower in treated plots compared to unplanted areas (URIOSTE *et al*., 2006), which is attributed to plant uptake, so by the prickly pear in our case. LI *et al*. (2004), attribute this decline to drought. Indeed, the climate of study area is arid-type, where the index of De MARTONNE is 6.76 and rainfall is around 163 mm, making the mobility of nutrients such as phosphorus, very difficult.

Regarding the electrical conductivity, due to its average value less than 1000 µS cm^{-1}, it reveals slightly salty soil (MATHIEU & PIELTAIN, 2003). These values do not automatically imply an increase in salinity but probably reflect an increased concentration of mineral nutrients that is released during decomposition of organic matter (SU & ZHAO, 2003), which is fast under hot arid conditions.

The ratio C/N of a given soil in balance with its environment is about 10~12 and its variation is indicative of nitrogen variation in organic matter (ABULE *et al*., 2007). In the present essay, values of C/N were low in all surveyed plots, probably indicating a rapid mineralization of herbal plant litter (WEZEL

et al., 2000) or a loss of nitrogen than carbon, which means a high rate of nitrogen mineralization in relation to carbon (MARTÍNEZ-GARCÍA *et al*., 2011). It is clear that the ratio C/N was low because of the low rate of OM recorded in the study station. The latter may have originated from the litter of ephemeral vegetation developed after rain, or from the slight yield of biodegradation of Prickly Pear rackets, since their main composition are based of resistant mucilage (BRIGGS *et al*., 2007). Finally, this ratio fully depends on the nature of organic debris and maturation of humus (BENABADJI *et al*., 1996).

Effect on vegetation traits. Vegetation cover is considered as a key-indicator revealing the degree of restoration success because it clearly reflects changes in the rehabilitation process (YANG *et al*., 2006). With that in mind, this study reveals that vegetation cover and number of individual plants are greater in planted plots compared to the control, where vegetation cover is average (around 45%) and mainly composed of Poaceae (*Cynodon dactylon* and *Hordeum murinum*) and Zygophyllaceae (*Peganum harmala*). These plant species are ubiquitous and explain the homogenization of steppe flora. Indeed, *Cynodon dactylon* characterizes a crop-disturbed soil surface (LE HOUÉROU, 1996), while *Peganum harmala* is known as a degradation-indicator that replaces climax species such as *Stipa tenacissima* and *Artemisia herba alba* of Algerian rangeland ecosystems (LE HOUÉROU, 1995; NEDJRAOUI, 2004).

The decrease in vegetation cover and change in floristic composition are the elements that characterize regressive evolution of rangelands (NEDJRAOUI, 2004 ; SLIMANI *et al*., 2010). This is also seen through the Shannon index and evenness that showed low values, which did not differ significantly. This expresses low species richness with dominance of one or two plant species over others, which characterizes a pioneer community that still young and unstable within its environment. Considering the situation within rehabilitation context, biological recovery in a degraded land requires long time periods while taking into account the limiting factor "water", on which revegetation significantly depends (CAO *et al*., 2007 but see YANG *et al*., 2006). Although this is not always true, because it was reported a high degree of vegetation cover and a large recovery, while soil water was limited (YANG *et al*., 2006). It is recognized that the perennial species are the focus of restoration projects in arid environments because of their stability and effective soil protection capacity (OTT *et al,* 2011). However, OFTEDAL (2002) noted that annual plants are also important in term of plant biodiversity and ecological succession.

The biological spectrum is typical for the semi-arid Mediterranean bioclimate, with a well-marked presence of therophytes and chamaephytes. These life forms characterize plant adaptation strategy in drylands (HASHEMI, 2001). Indeed, the hyperarid climate and the instable soil structure promote the development of short-lived plants (therophytes, mainly annuals). The domination of therophytes and chamaephytes in the vegetation life forms of M'zara agrees closely with proportions of life forms recorded near a lake at the Algerian Sahara Desert (CHENCHOUNI, 2012) and in desert and sub-desert habitats of Saudi Arabia (El GHANIM *et al*., 2010). As for hemicryptophytes, if they are not abundant, it is because of the low amount of organic matter (GHEZLAOUI *et al*. 2011). PINK *et al*. (2010) and WEZEL *et al*. (2000) indicate the importance of soil texture, salinity and organic carbon for the composition and species richness of plant communities. According to ZHANG *et al*. (2010), organic matter is a pivotal soil fertility factor that can affect phytodiversity.

Following results of this study, in concordance to plantations of spineless prickly pear cactus (*inermis*) in another station of North Tebessa (NEFFAR *et al*., 2011), the facilitator effect of *Opuntia ficus-indica* was clearly highlighted for (i) native vegetation cover, (ii) number of plant individuals, and (iii) the ratio of the latter and species richness. The most important factor facilitating the establishment of several species, in particular herbaceous plants, inside *Opuntia* plots may be that the

spiny prickly pear plants keep out domestic herbivores, except at very young stage of the plantation or when the distance between prickly pear plants is large. According to NOY-MEIR *et al*. (1989) and MILCHUNAS and NOY-MEIR (2002) grazing by herbivores occurs selectively and thus causes differences among plant species. It alters notably species abundance by introducing the competition for light, water and nutrients.

The presence of perennial species such as *Stipa tenacissima* and *Artemisia herba alba* and ephemeral species may be a source of litter for agroecosystems like based-prickly plantations (AIDOUD *et al*., 2006). This shrub, as well as other species such as *Acacia* or *Aloe* (KING, 2008), may create under its canopy a microclimate for the installation of less tolerant species of barren environment conditions. By increasing its stock of plant seeds, it could create an island of fertility that represents a starting point for improving soil conditions and therefore a gradual increase of vegetation cover. Herbaceous litter is the main source of organic matter especially in these harsh environments. Not only it decomposes more easily than shrubs (LI *et al*. 2006), but could greatly improve soil structure and fertility without neglecting the great annual production of seeds (HUANG *et al*., 2007), whose rapid life cycle (growth and death) provides an important carbon pool for the soil and the nutrients (SU & ZHAO, 2003).

Opuntia ficus-indica in this case, even if not directly involved in the supply of soil organic matter in our study site due to the severity of climatic conditions, could have an indirect effect on this variable by improving harsh environmental conditions that will favorite the installation of grasses then the colonization and development of other less tolerant plant species.

Upon completion of this study, it is possible to argue that restoration of soil fertility induced by revegetation is a complex ecological process that is subject to simultaneous effect of biotic and abiotic factors (SU & ZHAO, 2003). However, the advantage of prickly pear plantations is its drought resistance (TEGEGNE *et al*., 2007), and its shoots can trap nutrient-rich dusts (WEZEL *et al*., 2000). Its roots have showing very high rates of mycorrhization (work in progress), reinforcing its environmental benefits.

Conclusions

This study reveals that after the establishment of *Opuntia ficus-indica* in arid rangelands of Tebessa (Northeast Algeria), the intended effect on the microenvironment was not statistically noticeable in soil properties, but by the concentration of plant species around the base of the planted shrub, which can only express an improvement in micro-environmental conditions. The results also show that the rehabilitation of degraded steppes can be achieved by developing a revegetation with herbaceous layer. Plantations can maintain vegetation cover in arid and semi-arid areas and over time contribute to the improvement of soil quality.

The use of prickly pear plantations in the Mediterranean basin should be encouraged not only for the restoration of biodiversity, but also to stop erosion and desertification processes in arid and semi-arid lands. The choice of this shrub as a modulator of rehabilitation returns to its power to stimulate the growth of vegetation points around it "fertility islands". On this basis, the expansion of prickly pear plantations is emerging as a promising strategy to raise the dynamic of resources and restore vegetation in arid and semi-arid rangelands.

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Natural Hybrid Posterities - Problem for the Existence of the Acipenseridae Family Species in Nature

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Abstract. During the period 2005 - 2010 in Bulgarian territorial waters of the Black Sea and the Danube River four interspecies hybrids of sturgeons were caught. Hybrid № 1 was defined as Ship sturgeon (*Acipenser nudiventris)* x Russian sturgeon (*Ac. gueldenstaedti*) - second or third generation. Hybrid № 2 - *Ac. stellatus* x *Ac. ruthenus*. Hybrid № 3 - *Huso huso* x *Ac. ruthenus* (bester). Hybrid № 4 was with morphological signs of Russian sturgeon (*Ac. gueldenstaedti*) and Sterlet (*Ac. ruthenus*). The simultaneous catch of more than one specimen at the same time and place, supports the view that the amount of obtained natural hybrids in populations of sturgeon species with drastically reduced numbers increases.

Key words: sturgeon hybrids, electrophoresis, Acipenseridae, Bulgaria

Introduction

The excess exploitation of the natural posterities of many species towards which humanity has a lasting interest in providing the necessary food resources, leads to the endangered status of their existence. Real examples in this aspect are the species from the *Acipenseridae* family. Species that from a historical point of view have been contemporaries of the dinosaurs are currently not only drastically reduced in number but also becoming extinct. Despite the efforts of the Convention on International Trade with Endangered Species of the Wild Fauna and Flora - CITES, to protect sturgeons, the number of natural populations of the remaining species of the family has reached abnormal reproductive values. The reduction in this number causes the lack of presence of specimens from both sexes of the same species - male and female in the area of the spawning sites. This leads to the following problems: reduced opportunities for the regular obtaining of heterogeneous posterities of one species; increasing the possibility of inbreeding, resulting in reduction of the species genetic diversity; changes in the resistance of posterities to traditional agents, causing diseases; increasing the possibility of obtaining fertile interspecies hybrid forms - genetic extinction of the species; outcome - extinction of the species in the wild.

Each of these problems affects the natural populations of these species both individually and in complex with the rest. The obtaining of natural hybrid posterities in species with similar hereditary mechanism would not show substantial effect on the purity of the hereditary material in species with a normal number of natural populations. The normal size of the population of the species maintains the genetic purity in its heterogeneity. With the reduced number of populations of species with similar hereditary material, however, there is a real danger for the genetic extinction of these species that have existed for millions of years. (DOBROVOLOV *et al.,* 2005; ENE & SUCIU, 2001; TSEKOV *et al.,* 2008).

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Material and Methods

During the period 2005 - 2010 in Bulgarian territorial waters of the Black Sea and the Danube River the following interspecies hybrids of sturgeons were caught:

Hybrid № 1. One specimen caught in October 2005 in the Danube River - 654 km near the village of Gorni Vadin. The specimen is weighing 130 g.

Hybrid № 2. Five specimens caught during the winter months of 2006 in the Black Sea – city of Varna, Galata cape. The specimens are weighing from 260 g to 450 g.

Hybrid № 3. Four specimens caught in February-March 2006 in the Black Sea near the city of Varna and the town of Sozopol. In April that same year, another specimen caught in the Danube River, near the town of Oryahovo (678 km). The specimens are weighing from 350 g to 470 g.

Hybrid № 4. One specimen caught in November 2010 in the Danube River near the town of Oryahovo (678 km), together with 12 Sterlet specimens. The specimen is weighing 580 g.

Electrophoretic studies by two methods were performed on materials from these hybrids (blood and skeletal muscle):

- Horizontal starch gel electrophoresis by the method of SMITHIES (1955), in modification of DOBROVOLOV (1973);
- Isoelectric focusing (IEF) on thin polyacrylamide ampholine gel with pH-gradient 3.5-10.

Results and Discussion

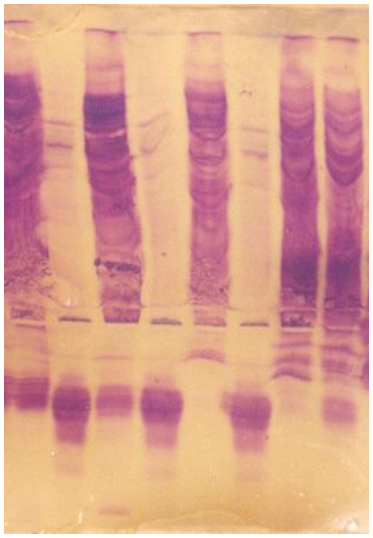
The conducted electrophoretic studies (Fig.1-3) allow the following characteristic of the referred hybrids:

Hybrid № 1 When caught was defined as Ship sturgeon (*Ac. nudiventris)*, but the starch gel electrophoresis showed also a heredity of Russian sturgeon (*Ac. gueldenstaedti*) - second or third generation (Fig. 4).

Hybrid № 2 The IEF of hemoglobin shows heredity of *Ac. stellatus* and *Ac. ruthenus* (Fig. 5).

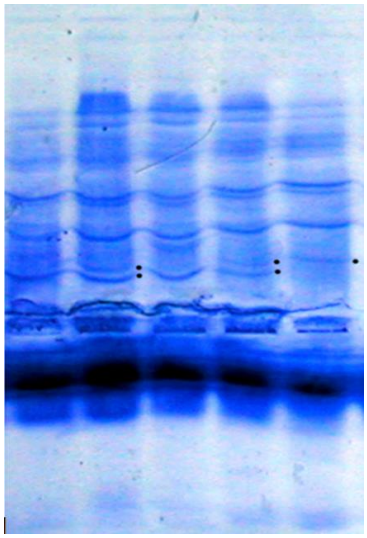
Hybrid № 3 The isoelectroforetic study shows heredity of *Huso huso* and *Ac. ruthenus* (bester) - also caught in the region of Varna, 2006 (Fig. 6).

Hybrid № 4 With morphological signs of Russian sturgeon (*Ac. gueldenstaedti*) and Sterlet (*Ac. ruthenus*) - caught the autumn of 2010 in the Danube River along with Sterlet specimens (Fig. 7).



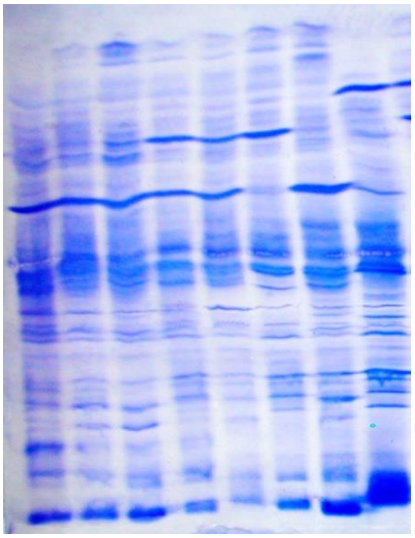
1 2 3 4 5 6 7 8

Fig. 1. Electropherogram of serum proteins: 1 – Russian sturgeon; 2, 4 and 6 – Hybrid № 1; 3 – Sterlet; 5 – Stellate sturgeon; 7 - Hybrid (Russian Sturgeon x Siberian sturgeon) and 8 – Siberian sturgeon.



1 2 3 4 5

Fig. 2. IEF of hemoglobin: 1 and 3 – Sterlet; 2 and 4 – Hybrid № 2; 5 – Stellate sturgeon.



1 2 3 4 5 6 7 8

Fig. 3. IEF of muscle proteins: 1, 2 and 3 – Russian sturgeon; 4 and 5 – Hybrid № 1; 6 - Sterlet; 7 – Hybrid № 3 and 8 – Great sturgeon.

Fig. 4. Photograph of Hybrid № 1.

Fig. 5. Photograph of Hybrid № 2.

Fig. 6. Photograph of Hybrid № 3.

Fig. 7. Photographs of Hybrid № 4.

Conclusions

The simultaneous catch of more than one specimen at the same time and place (four or five specimens), as are the cases of hybrids № 2 and № 3, supports the view that the amount of obtained natural hybrids in populations of sturgeon species with drastically reduced numbers increases.

The increased number of these fertile hybrids is the other factor, together with the excess catches, leading to the genetic extinction of the sturgeon species in nature.

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Review of the Distribution of the Family Gobiidae (Pisces) in the Bulgarian Danube Tributaries

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Abstract. The current study aims to give in detailed information on the actual distribution of the species from family Gobiidae in the Bulgarian Danube tributaries. All known literature has been revised and with the new data collected is given complete and actual information on their distribution. In the period 2010-2012 were sampled a total of 41 sites alongside each one of the Bulgarian Danube tributaries. The sampling started from the river mouths to upstream in order to discover what is the southern (upstream) distribution of each one goby species. Four goby species were recorded from the tributaries – the round goby (*Neogobius melanostomus* Pallas, 1814), the monkey goby (*Neogobius fluviatilis*), the racer goby (*Neogobius gymnotrachelus*) and the tubenose goby (*Proterorhinus marmoratus*). Further analysis showed preference of mixed substrates and silt in addition of homogenous ones. The occurrence of gobies in the studied tributaries decreased inversely proportional to distance from Danube.

Key words: freshwater gobies, distribution, invasive species, family Gobiidae, the Danube River tributaries

Introduction

The Danube River is the second longest river in Europe with length of 2850 km. Its springs are located in Germany. It flows through 10 countries, having more than 300 tributaries and mouths into the Black Sea. The Bulgarian sector of the Danube River is located between 845 and 375 river kilometers and is typical low river stretch. The major tributaries in Bulgarian sector are 12 (Fig. 1).

The saprobic characteristics of the different Bulgarian Danube tributaries in their middle and low sections vary between alpha to I or II beta-mesosaprobic conditions (RUSEV, 1994). The main factors that influence the saprobic index are the season, proximity to cities and villages, industrial and agricultural pollution.

Danube is one of the water bodies with highest biodiversity and ecological significance in Europe. The main reasons for this are the huge water flow, diverse ecological conditions and the geographical location (BĂNĂRESCU, 1964).The ichthyo-fauna of the Bulgarian sector of the Danube consists of 68 species (VASSILEV & PEHLIVANOV, 2005) and it is heterogeneous – consists of native pre-Pleistocene fish forms and post-glacial immigrants (BĂNĂRESCU, 1991).

The distribution of the freshwater gobies along the Danube is relatively well known, as the first researches on the topic

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for the Bulgarian section of the river have been done in the beginning of the XX century.

DRENSKY (1948, 1951) reported for the presence of the stellate tadpole goby (*Benthophilus stellatus* Sauvage, 1874) in the Bulgarian parts of the Danube – near the towns of Svishtov and Nikopol.

The monkey goby (*Neogobius fluviatilis* Pallas, 1814) was recorded for the first time in the Bulgarian Danube by DRENSKY (1921). Later the author confirmed the presence of the species in Danube (DRENSKY, 1948, 1951).

DRENSKY (1948, 1951) found the racer goby (*Neogobius gymnotrachelus* Kessler, 1857) in the Danube up to Ruse. Later GHEORGHIEV (1966) reported that the racer goby can be found up to Vidin.

The Kessler's goby (*Neogobius kessleri* Günther, 1861) was found in the past in the Danube up to the city of Vidin (DRENSKY, 1921, 1948, 1951), as the same author also stated that he got evidence for the presence of the species up to the city of Vienna.

The natural distribution of the round goby (*Neogobius melanostomus* Pallas, 1814) covered the lower sections of Danube River upstream to the town of Vidin (DRENSKY, 1948, 1951).

In the Danube the presence of the tubenose goby (*Proterorhinus marmoratus* Pallas, 1814) was registered by DRENSKY (1948). He stated that the species can be found upstream to Bratislava (1869 river km). Later GHEORGHIEV (1966) discovered the tubenose goby near the cities of Ruse, Svishtov and Vidin.

MARINOV (1966) reported the presence of 5 species of gobies in the Danube - *N. kessleri, N. fluviatilis, Pr. marmoratus, B. stellatus* and mis-determinated and currently not present in the Danube *Neogobius cephalarges constructor* Nordmann, 1840.

According to the latest data (VASSILEV & PEHLIVANOV, 2005; POLAČIK *et al*., 2008; VASSILEV *et al*., 2011) family Gobiidae in the Bulgarian sector of Danube is represented by three genera with six species: stellate tadpole goby, monkey goby, racer goby, Kessler's goby, round goby and tubenose goby. All of them are Ponto-Caspian relicts and are native in the Bulgarian section of the Danube. Recently there are clear indications for fast invasion of some of the gobies upstream (HEGEDIŠ *et al*., 1991; KAUTMAN, 2001; GUTI, 2004; PRASEK & JURAJDA, 2005; WEISNER, 2005; MANNÉ & POULET, 2008; POLAČIK *et al*., 2009).

The ichthyofaunistic studies of the Bulgarian Danube tributaries showed presence of gobies mainly in the lower sections of the rivers and in the river-mouths.

KARAPETKOVA (1985) reported the occurrence of *B. stellatus* in the Ogosta River, but only close to the river mouth in to the Danube.

According to DRENSKY (1932) *N. fluviatilis* was captured in lower and mid sections of the Osam River. Later DRENSKY (1948) discovered the monkey goby in the rivers Iskar, Osam, Yantra and Vit (up to Pleven, 41 km form Danube). DRENSKY (1951) found the species in Ogosta River. KARAPETKOVA (1985) report for the presence of *N. fluviatilis* in the Iskar, Ogosta, Vit, Rusenski Lom River, and in the Tsibritsa River near the village of Zlatiya (5 km from Danube). Later the species was reconfirmed for the Rusenski Lom River complex (MIHOV & KOEV, 2006). PEHLIVANOV *et al.* (2009) stated the presence of *N. fluviatilis* in the Vit River. According to another recent study (TRICHKOVA *et al.,* 2009) the monkey goby can be found in the rivers Vidbol (lower reaches), the Archar (Archar village), the Ogosta (near Mizia) and the Lom River (near Lom). The species is reconfirmed for the Yantra River (VASSILEV *et al.* 2009).

KARAPETKOVA & DIKOV (1986) reported for the presence of the racer goby in Vit River, after two years it was found also in the Rusenski Lom River complex (KARAPETKOVA & UNDZIAN, 1988). VASSILEV *et al.* (2009) confirmed the presence of the *N. gymnotrachelus* in Yantra River up to Polsko Kosovo village (52 km from Danube).

The Kessler's goby was registered in the lower sections of the Osam River by DRENSKY (1932) and in the Vit River by GHEORGHIEV (1966). KARAPETKOVA (1985) reported for the presence of the *N. kessleri* in

Ogosta. Some authors assume that in the past it reached upstream to the Iron Gates Dam (943 river km) in Serbia (BĂNĂRESCU, 1970, MILLER, 2003).

DRENSKY (1948) reported for the presence of the round goby in the Vit River up to Pleven (41 km from the Danube). Later the species was caught in the Iskar River (KARAPETKOVA, 1985). TRICHKOVA *et al.* (2009) found *N. melanostomus* in the Archar (close to Archar village) and the Ogosta River (found near Mizia village). The species was also found in the Yantra River (VASSILEV *et al.* 2009).

KARAPETKOVA (1985) reported for the presence of the *Pr. marmoratus* in the Ogosta River. The tubenose goby was registered for the Archar, Vidbol, and Skat (found near Galitsche village) and in the Ogosta River was confirmed for two locations – near Mihailovo village and near Hayredin village (TRICHKOVA *et al.*, 2009). Tubenose goby was registered for the Yantra River by Vassilev (VASSILEV *et al.* 2009).

Materials and Methods

The ichthyofaunistic survey was carried out during March, 2010 – April, 2012. A total of 41 sites were sampled alongside each one of the Bulgarian Danube tributaries (Fig. 1, Table 1, 2).

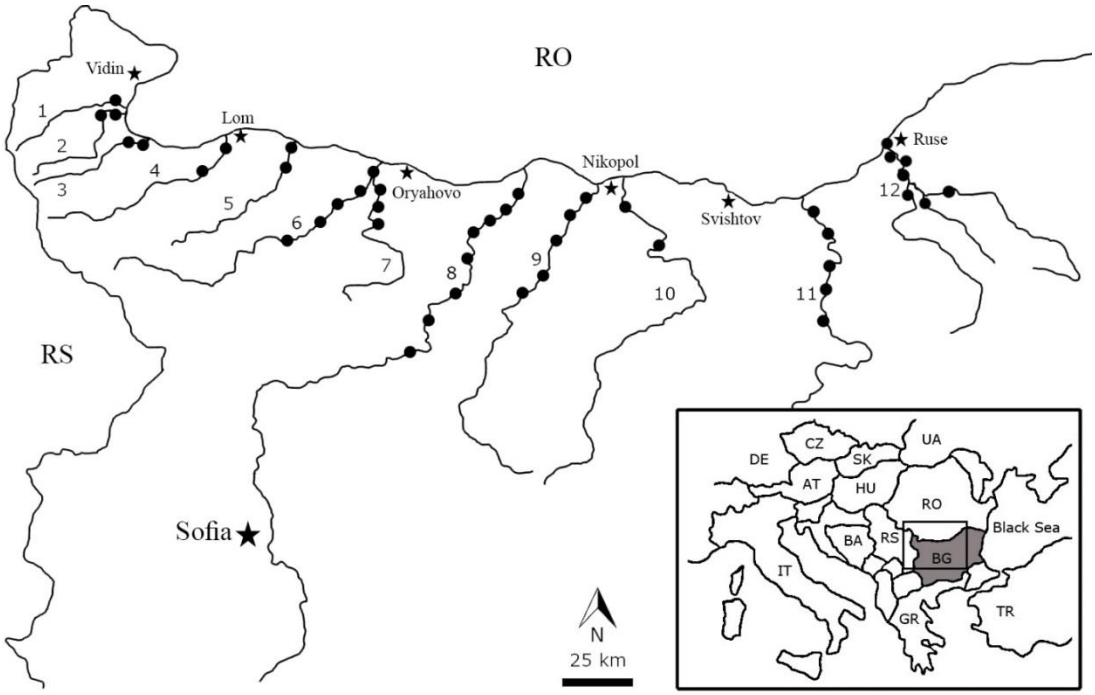

Fig. 1. Map of the Bulgarian Danube tributaries with the sampling sites. In the presented map the Danube River is water border between Bulgaria and Romania. The numbers correspond to the rivers as follow: 1 – Voynishka, 2 – Vidbol, 3 – Archar, 4 – Lom, 5 – Tsibritsa, 6 – Ogosta, 7 – Skat, 8 – Iskar, 9 – Vit, 10 – Osam, 11 – Yantra, 12 – Rusenski Lom River complex.

Two rivers were excluded of the sampling – the heavily polluted, water border with Serbia – Timok River and the Topolovitsa River, which has low water flow and is heavily vegetated and canalized. The sampling in each tributary started from the river mouth and continues upstream in order to give actual information about the southern distribution of the gobies in these rivers. Fishes were captured by standard portable electrofishing device. A transect of 100 m was sampled at each site, as if there were some obstacles in the river course we sampled the river section below and above them in order to complete the transect. Seine nets were used for sampling in the Ogosta Reservoir. The applied sampling methods have been focused on capturing gobies.

Almost all fish species were identified *in situ* and released back. Some of the captured specimens were preserved in 95% ethanol for further analyses. The percentages of occurrence on both the given substrate and given distance from Danube on the basis of the whole occurrences of each species on all registered substrate types and distances from Danube respectively, have been calculated.

Table 1. Sampling sites coordinates and recorded gobies for the period 2009 – 2012.The following abbreviations were used: Nf – *Neogobius fluviatilis*, Ng – *Neogobius gymnotrachelus*, Nm – *Neogobius melanostomus*, Pm – *Proterorhinus marmoratus*.

River	Site	Elevation	Substrate	Distance form Danube (km)	Gobies	River	Site	Elevation	Substrate	Distance from Danube (km)	Gobies
Voynishka	N 43.92624 E 22.80028	40	stone, gravel	3		Iskar	N 43.56292 E 24.2987	49	stone, sand	26	Nf, Nm
Vidbol	N 43.9033 E 22.80565	38	stone, sand	3		Iskar	N 43.51865 E 24.22502	48	stone, clay	35	Nf
Vidbol	N 43.901017 E 22.786767	52	stone, sand	5		Iskar	N 43.45607 E 24.23109	57	stone, sand	48	Nf
Archar	N 43.81265 E 22.91817	37	stone, gravel	1	Ng, Pm	Iskar	N 43.35268 E 24.16523	68	stone, clay	65	Nf
Archar	N 43.81119 E 22.90156	50	stone, gravel	3	Pm	Iskar	N 43.24875 E 24.04523	125	stone, clay	85	Nf
Lom	N 43.80059 E 23.24433	41	stone, sand	4	Pm	Iskar	N 43.18782 E 23.97203	149	stone, clay	102	
Lom	N 43.44492 E 23.11125	57	stone, sand	15		Vit	N 43.63378 E 24.6982	27	stone, clay	7	Nf, Nm
Tsibritsa	N 43.79763 E 23.50196	30	sand, silt	2	Nf	Vit	N 43.57147 E 24.6344	38	stone, clay	15	Ng, Nm
Tsibritsa	N 43.7499 E 23.48806	39	stone, sand	10		Vit	N 43.49338 E 24.5644	69	stone, clay	32	Nf, Nm
Ogosta	N 43.69185 E 23.82625	35	stone, sand	7	Nf, Nm, Pm	Vit	N 43.40639 E 24.51908	62	stone, clay	42	Ng
Ogosta	N 43.63233 E 23.71578	48	stone	20	Nf, Pm	Vit	N 43.33977 E 24.42632	101	stone ,clay	56	
Ogosta	N 43.5754 E 23.63588	54	stone, sand	32	Pm	Osam	N 43.62907 E 24.85043	29	stone, clay	9	
Ogosta	N 43.52543 E 23.53873	79	stone, sand	45		Osam	N 43.51005 E 25.00729	42	silt	32	
Ogosta	N 43.51877 E 23.44722	76	stone, sand	55		Yantra	Krivina	21	silt	5	Nf, Ng, Nm
Ogosta Reservoir	N 43.394133 E 23.185833	192	sand	85	Nf	Yantra	Beltsov	28	gravel, silt	25	Ng, Nm
Skat	N 43.62753 E 23.85772	46	stone, sand	16	Pm	Yantra	Byala	32	gravel, sand	60	Ng, Nm , Pm
Skat	N 43.55843 E 23.86247	73	stone	30		Yantra	Polsko Kosovo	34	gravel	70	Ng, Nm , Pm
						Yantra	Petko Karavelovo	44	gravel, sand	100	Nm , Pm
Skat	N 43.69399 E 23.84452	33	sand, silt	51		RusenskiLom	Ruse	19	stone, silt	2	Nf
						RusenskiLom	Basarabovo	25	stone, silt	11	Nf
Iskar	N 43.64363 E 24.42472	33	silt	11	Nf, Nm	RusenskiLom	Krasen	40	stone, silt	24	Nf
						RusenskiLom	Ivanovo	48	stone, silt	42	Nf
Iskar	N 43.58192 E 24.35618	40	stone, clay	20		RusenskiLom	Koshov	60	stone, silt	50	
						RusenskiLom	Nisovo	67	stone, silt	55	
						RusenskiLom	Pisanets	99	stone, silt	69	

Results

Four goby species from two genera were recorded from the studied sites – *N. fluviatilis, N. gymnotrachelus, N. melanostomus* and *Pr. marmoratus* (Tab. 2). In the most western studied rivers – the Voynishka and the Vidbol River we did not found any goby species. In the Archar River the presence of two species was registered – *N. gymnotrachelus* and *Pr. marmoratus*. In the Lom River we found only *Pr. marmoratus.* Two sites were sampled along the Tsibritsa River and in the closest to the river mouth-sampling site we found *N. fluviatilis*. In the Ogosta River we sampled 5 sites and we found gobies (*N. fluviatilis, N. melanostomus* and *Pr. marmoratus*) in only three of them. We also sampled in the Ogosta Reservoir, which is located further upstream, and we confirmed the presence of the *N. fluviatilis* in it. In the Skat River we found only *Pr. marmoratus.* In the longest river in Bulgaria – Iskar we sampled 8 sites and registered the presence of two goby species – *N. fluviatilis* and *N. melanostomus.* In the Vit River we recorded the presence of three goby species – *N. fluviatilis, N. gymnotrachelus* and *N. melanostomus*. In the Osam River we took samples from two locations but did not found any gobies. For the Yantra River we compared our data (Table 2) with a previous study on the ichthyofauna of this river (VASSILEV *et al.*, 2009), and did not found any changes. In the Rusenski Lom River complex we sampled 7 locations (Fig. 1, Table 2). We registered the presence of the monkey goby in 4 of them – up to the Ivanovo village (42 km from the Danube).

The calculated percentages of occurrence of the registered gobiid species on a given substrate on the basis of all types of substrates where the species is discovered showed that all of them prefer mixed substrates or the border between different substrates, rather than one type of substrate (Fig. 2). A deviation has been established for silt bottoms, where the percentage of occurrence for three species is rather higher than on gravel, sand or rock.

From the other side, as distance increases upstream from the Danube River, the occurrence of gobies decreases respectively (Fig. 3). There is a gap between 70 and 80 km from the Danube, where gobies have not been established. The registrations between 85 and 100 km have been accomplished in the Iskar and the Yantra Rivers.

Table 2. Distribution of the gobies in the Bulgarian Danube tributaries.

River	Goby species				Additional species
	Ng	Nf	Nm	Pm	
Voynishka					
Vidbol		X		X	
Archar	X	X	X	X	
Lom		X		X	
Tsibritsa		X			
Ogosta and Ogosta Reservoir		X	X	X	Bs, Nk
Skat				X	
Iskar		X	X		
Vit	X	X	X		
Osam		X			Nk
Yantra	X	X	X	X	Nk
Rusenski Lom	X	X			

The following abbreviations were used: Bs – *Benthophilus stellatus*, Nf – *Neogobius fluviatilis*, Ng – *Neogobius gymnotrachelus*, Nk – *Neogobius kessleri*, Nm – *Neogobius melanostomus*, Pm – *Proterorhinus marmoratus*.

Discussion

The species inhabiting the tributaries of the Danube River are determined in general by the Danubian fish fauna, which is the most abundant ichthyocomplex in Europe consisting of more than 100 species (BĂNĂRESCU, 1964) and 136 species for the Danube delta. The gobies inhabiting the Danube are freshwater or euryhaline, autochthonous Ponto-Caspian relicts. Their distribution is relatively well known and all the available literature on this topic was studied.

For the Yantra River we also summarized the data from KARAPETKOVA (1972) and VASSILEV *et al.* (2009).

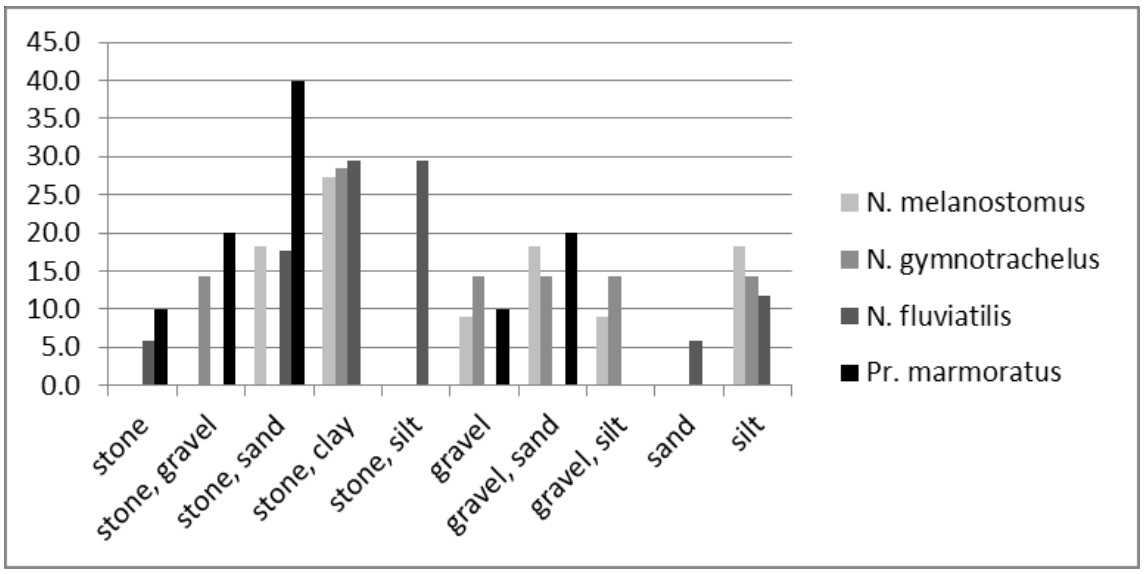

Fig. 2. Occurrence of the four discovered gobiid species in relation to the type of the substrate, where each given species is registered. On the y axis is illustrated the percentage of occurrence in given substrate, on the basis of the total number of occurrences.

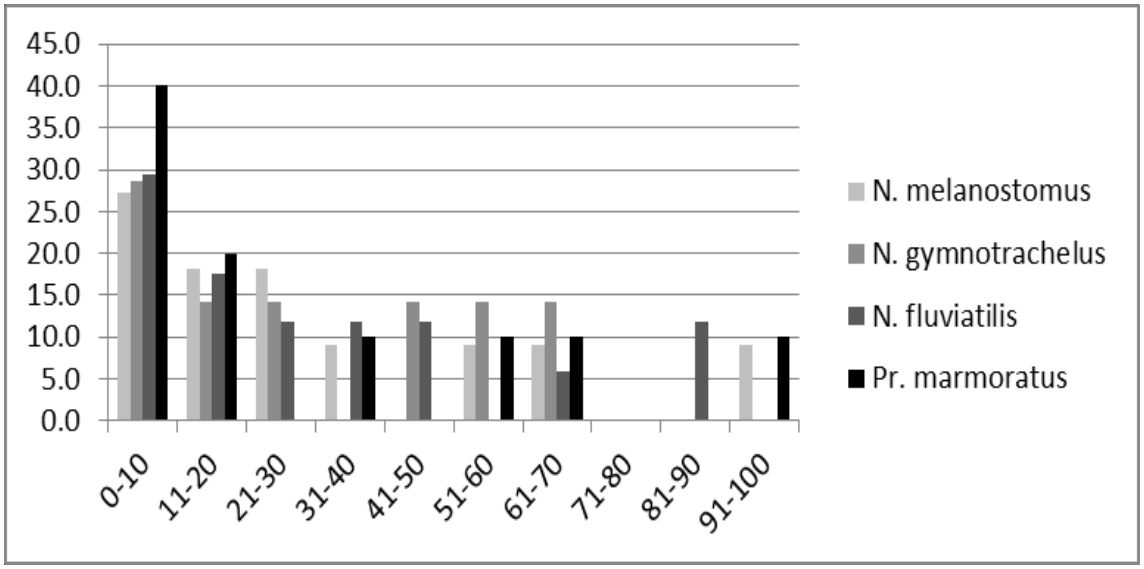

Fig. 3. Occurrence of the four discovered gobiid species in relation to the upstream distance from the Danube River, where each given species is registered. On the y axis is illustrated the percentage of occurrence in given distance, on the basis of the total number of occurrences.

KARAPETKOVA (1972) reports for the presence of *N. fluviatilis* and *N. kessleri,* while VASSILEV *et al.* (2009) found all four (*N. gymnotrachelus, N. fluviatilis, N. melanostomus, Pr. marmoratus)* gobies in different locations along the river (Table 2).

The southern points in these tributaries, in which we registered the presence of goby species, were in the Iskar River – 85 km upstream from the Danube (125 m a.s.l.) and in the Yantra River – 100 km from the Danube (44 m a.s.l.).

The calculation of percentages of occurrence on given substrate based on all substrates where given species registered showed preference for mixed substrates, rather than homogenous, with exception of silt bottoms. It is obvious that a more heterogeneous bottom discloses more opportunities for hiding/escaping from predators and finding food items. A rather

increased occurrence of three species on silt is not surprising-in such substrates are mostly appropriate for small benthic invertebrates on which the gobies prey, but do not give protection to gobies.

From the other side as distance from Danube decreases, the occurrence of gobies in the Danube tributaries decreases. This happens earlier in smaller rivers (Tsibritsa, Lom, Russenski Lom), in addition to the bigger Danube tributaries (the Iskar and the Yantra). The gobiid presence in the Ogosta reservoir should be considered as an isolated case; most probably *N. fluviatilis* have been introduced by accident in this particular water body.

Three of the studied gobies (*N. fluviatilis, N. kessleri* and *Pr. marmoratus*) are included in the IUCN Red List of threatened species with the status Least concern (LC) which indicates that they are not directly endangered. They are also included in the annex III (Protected fauna species) of the Bern Convention – "Convention on the conservation of European wildlife and natural habitats" which includes species that are in need of protection but can be hunted or exploited with regulations. Meanwhile these species are considered invasive, expanding their natural distribution rapidly and colonizing new territories far away from their native range.

In the past when the industry in Bulgaria was highly developed, the saprobic index of the rivers was worst. Now when most of the factories are not operational the saprobic index shows better values. This can be one of the reasons for the observed recent spreading of the gobies upstream in the tributaries – the ecological conditions are better and many of the pollutants are no longer present in the water.

There is also fast expansion upstream the Danube of the gobies recorded in the last two decades. The reasons for this are not still clarified. Some authors report that the global climate change can influence the invasion success (HARKA & BÍRÓ, 2007; KORNIS *et al.*, 2012), other the increased transportation and unregulated release of ballast waters (SAPOTA & SKÓRA, 2005; BROWN & STEPIEN, 2008; HAYDEN & MINER, 2009), some the changed environmental condition in the rivers and canalization (HARKA & BÍRÓ, 2007) and last but not least reason is given to the natural dispersion and migration capabilities of this species (WOLFE & MARSDEN, 1998; BRONNENHUBER *et al.*, 2011). Most probably, it is a combination of all these factors.

From their widespread distribution and recent invasions in new territories we can assume that in Bulgaria they are not in an urgent need of protection, as they are not endangered, but are very important part of the aquatic ecosystems in the Bulgarian Danube tributaries and lakes alongside the Danube like the world renowned Srebarna Biosphere Reserve (PEHLIVANOV, 2000) and are important study object for understanding the ecology of the invasive species and the reasons that leads up to this.

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†Associate prof. Dr. Milen Vassilev suddenly passed away on 30.05.2013. He left the article and much of his work uncompleted. His colleagues will continue his efforts.

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Histochemical Effects of "Verita WG" on Glycogen and Lipid Storage in Common Carp (Cyprinus carpio L.) Liver

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Abstract. We aimed in the present work is to study the effects of fosetyl-Al and fenamidone based fungicide ("Verita WG") on glycogen storage and expression of lipid droplets in common carp (*Cyprinus carpio*, L.) liver. Concentrations of the test chemical were 30 mg/L, 38 mg/L and 50 mg/L under laboratory conditions. We used PAS-reaction for detection of glycogen storage and Sudan III staining for detection of lipid droplets in common carp hepatocytes. Hence, we found that the amount of glycogen and the fat storage in the liver increased proportionally with the increased fungicide concentrations. We also found conglomerates of accumulated glycogen in certain hepatocytes at all used concentrations. Overall, the results demonstrated enhanced glyconeogenesis and fat accumulation in the common carp liver, exposed to the test chemical.

Key words: pesticides, histopathology, PAS-reaction, fatty degeneration, liver, common carp

Introduction

Water pollution may be defined as any impairment in its native characteristics by addition of anthropogenic contaminants to the extent that it either cannot serve to humans for drinking purposes and/or to support the biotic communities, such as fish. All water pollution affects organisms that live in these water bodies. It occurs when pollutants are discharged directly or indirectly without adequate treatment to remove the harmful constituents (AGRAWAL *et al*., 2010). Thus, pesticide contamination of surface waters have been documented worldwide and constitute a major issue that gives rise to concerns at local, regional, national and global scales (HUBER *et al*., 2000; CEREJEIRA *et al*., 2003).

"Verita WG" is a systemic and contact fungicide, effective against plant diseases, caused by fungi of the class *Oomycetes*. The active substances are fosetyl-Al and fenamidone. Fosetyl-Al belongs to the phosphonates, which constitute a relatively new class of systemic fungicides (COHEN & COFFEY, 1986). Fenamidone belongs to the chemical group of imidazolinone and isopropanol, respectively (PEST MANAGEMENT REGULATORY AGENCY, 2003).

Fish are used extensively for environmental monitoring (LANFRANCHI *et al*., 2006), because they uptake contaminants

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directly from the water. Fish liver is rich in antioxidants and plays a major role against toxic compounds which induce free radical production (VAN DYK *et al*., 2007). Therefore, liver serves as an ideal organ in histopathological studies for different pollutants effects, due to its susceptibility to morphological damage (STENTIFORD *et al*., 2003; FEIST *et al*., 2004). Morphological, histological and histopathological alterations related to pesticides presence in the fish liver have been studied, showing that these substances cause a severe damage to the liver cells (KOEHLER, 2004). The main advantage of histochemistry lies in the analysis of biological phenomena in the "particular cells". Histochemical techniques help to analyze not only the localization of lipid and glycogen at cellular level. Histochemical tests reveal the localization of chemical product of cellular activity. Intensity of staining can be used for comparing the lipid and glycogen contents present in the liver cells of the normal fish compared to treated fish with different toxic compounds (PATHAN *et al*., 2009).

Data from studies carried out on the effects of fenamidone and fosetyl–Al based fungicides on the fish liver are relatively scarce. Therefore, the main objective in the present experiment is to study the histochemical effects of the "Verita WG" on glycogen and lipid storage in common carp (*Cyprinus carpio* L.) liver.

Materials and Methods

Test organisms

Common carp is an economically important fish species, which is widely spread through Europe. It is also used as a bioindicator for estimation of the health of freshwater ecosystems because it is relative insensitive and could survive and accumulate contaminants at heavily contaminated sites (SNYDER *et al*., 2004; REYNDERS *et al*., 2008).

Forthy healthy common carps were obtained from the "Institute of Fisheries and Aquaculture" in the city of Plovdiv, Bulgaria. They were of the same size-group (mean std. length 16.3 cm ± 2.7; mean body mass 10.17 g ± 1.4) with no external pathological abnormalities. After transportation, the fish were moved in aquaria with chlorine-free tap water (by evaporation) to acclimatize for a week. After acclimatization the fish were divided into four groups (n = 10). Fish were not fed 48 hours before the experiment.

Chemicals and experimental setup

The fungicide "Verita WG" was used in the experiment. It contains the active substance fosetyl-Al (Aluminium tris-O-ethyl phosphonate) 667 g/kg and fenamidone (1-anilino-4-methyl-2-methylthio-4-phenylimidazolin-5-one) 44 g/kg, and it was provided by Bayer CropScience, Germany. Three groups of fish were exposed to fungicide at concentration of 30 mg/L, 38 mg/L and 50 mg/L, representing 50, 40, 30 times dilution of the stock solution, prepared according to the instructions of the manufacturer. The fourth fish group served as a control and the fish were kept in a tank with no added fungicide. The experiment last for 96 h.

All the aquaria had a permanent aeration with air pumps and the water was kept oxygen saturated. For the entire duration of the experiment, the animals were maintained under a natural light/dark cycle (12:12). Water physico-chemical characteristics such as pH, temperature, dissolved oxygen; oxygen saturation, were measured once per day according to a standard procedure (APHA, 2005). They were as follows: pH – 8.2±0.1; temperature – 24.5 °C ± 0.12; dissolved oxygen – 7.26 mg/L ± 0.2; oxygen saturation – 90.25% ± 3.3.

Histochemical analysis

Fish dissection was performed according to the international standard procedures given in the EMERGE Protocol (ROSSELAND *et al*., 2003).

Histochemical analysis was carried out in the laboratory at the Department of Anatomy, Histology and Embryology at Medical University of Plovdiv, Bulgaria. Cryostat (Leica, Jung Frigocut 2800 N) was used to cut the samples. Multiple carp liver sections (6 μm) of each specimen were prepared according to standard

methodology. Liver samples were also stained with Sudan III according to Daddy and by the PAS method according to McManus. All used histochemical techniques are described by PEARSE (1968, 1972)

Experimental set up was conducted in accordance with national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purpose (DIRECTIVE 2010/63/EU).

Liver histochemical alterations of all specimens, including control fish livers were appraised individually and semi-quantitatively by using the grading system. Positive PAS-reaction was presented in purple-magenta staining and positive Sudan III staining was expressed with small yellow and large orange fat droplets in hepatocytes cytoplasm. Evaluation of the histopathological changes was carried out and presented as an average value. Each grade represents specific histochemical characteristics and is categorized as follows: (-) – negative reaction of histochemical staining; (+/-) – very weak positive reaction of histochemical staining; (+) – weak positive reaction of histochemical staining; (++) –moderate positive reaction of histochemical staining; (+++) – strong positive reaction of histochemical staining in the hepatocytes.

Results

Intensity of PAS-reaction

We observed in the control fish liver positive PAS-reaction in separate areas of liver parenchyma (Fig. 1a). Intensity of the PAS-reaction was increased in direct proportion with the increase of the pesticide concentration, which was indicative of an increase in the glycogen amount in hepatocytes (Table 1).

We found a tendency towards an increased intensity of PAS-positive grain-like structures, which varied from fine to large grains in the three experimental fish groups, which suggests an increase in the amount of accumulated glycogen in hepatocytes.

There was an increase of PAS-reaction in all cryosections of fish, exposed to 30 mg/L pesticide, i.e. more-intense purple-magenta staining compared with the control group (Fig. 1b). Intensity of PAS-reaction was moderate. This suggests an increase in the amount of glycogen compared the control group.

We also detected moderate PAS-positive staining in the investigated fish, exposed to 38 mg/L concentration of pesticide. This indicates that the accumulation of glycogen in the hepatocytes was similarly to the test group, exposed to 30 mg/L pesticide (Fig. 1c).

Furthermore, we observed strong positive PAS-reaction in the fish group (Fig. 1d), which were exposed to the highest fungicide concentration (50 mg/L). Thus, more intense PAS-reaction demonstrated the highest amount of glycogen in the common carp liver.

Lastly, we detected conglomerates of accumulated glycogen in certain hepatocytes from all test fish groups (Table 2). Amount of the conglomerates was increased proportionally to the fungicide concentration. At the lowest concentration (30 mg/L) we determined conglomerates of accumulated glycogen in single hepatocytes (+/-). In fish liver from the test groups, exposed to 38 mg/L conglomerates were present in more hepatocytes (+) and respectively, the most pronounced presence of glycogen conglomerates was in the fish, exposed at concentration of 50 mg/L (++). We did not find conglomerates of accumulated glycogen in the hepatocytes from the control group.

Intensity of Sudan III staining

We found very weak positive reaction of Sudan III staining in control fish group. Intensity of Sudan III staining from all test groups increased with the increased concentrations of the test fungicide (Table 3).

Table 1. Intensity of PAS-reaction

Concentration of the fungicide	Control group	30 mg/L	38 mg/L	50 mg/L
Intensity of PAS- reaction staining	+	++	++	+++

(-) – negative reaction of histochemical staining; (+/-) – very weak positive reaction of histochemical staining; (+) – weak positive reaction of histochemical staining; (++) – moderate positive reaction of histochemical staining; (+++) – strong positive reaction of histochemical staining in the hepatocytes

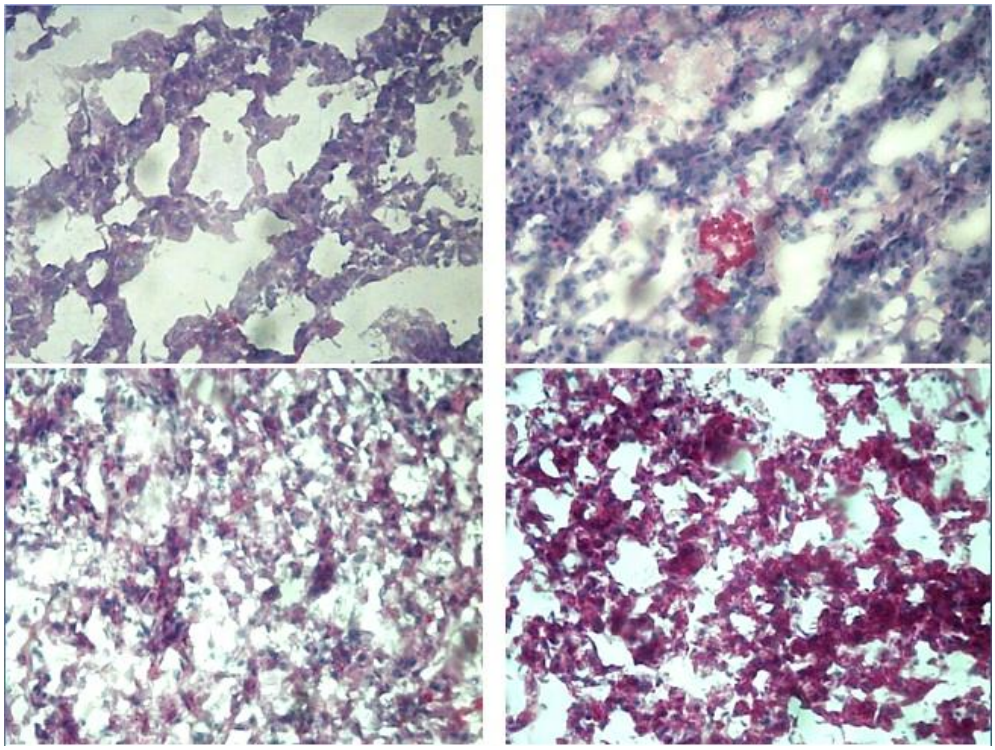

Fig. 1. Intensity of PAS-reaction in common carp liver. **a –** The intensity of PAS-reaction in control fish group, x200; **b –** The intensity of PAS-reaction and conglomerates of accumulated glycogen at concentration 30 mg/L fungicide, x200; **c –** The intensity of PAS-reaction and conglomerates of accumulated glycogen at concentration 38mg/L fungicide, x200; **d –** The intensity of PAS-reaction and conglomerates of accumulated glycogen at concentration 50 mg/L fungicide, x400.

Table 2. Conglomerates of accumulated glycogen in common carp liver.

Concentration of the pesticide	Control group	30 mg/L	38 mg/L	50 mg/L
Amount of glycogen conglomerates	-	+/-	+	++

(-) – negative reaction of histochemical staining; (+/-) – very weak positive reaction of histochemical staining; (+) – weak positive reaction of histochemical staining; (++) – moderate positive reaction of histochemical staining; (+++) – strong positive reaction of histochemical staining in the hepatocytes

Table 3. Intensity of Sudan III staining

Concentration of the pesticide	Control group	30 mg/L	38 mg/L	50 mg/L
Intensity of Sudan III staining	±	+	++	+++

(-) – negative reaction of histochemical staining; (+/-) – very weak positive reaction of histochemical staining; (+) – weak positive reaction of histochemical staining; (++) – moderate positive reaction of histochemical staining; (+++) – strong positive reaction of histochemical staining in the hepatocytes

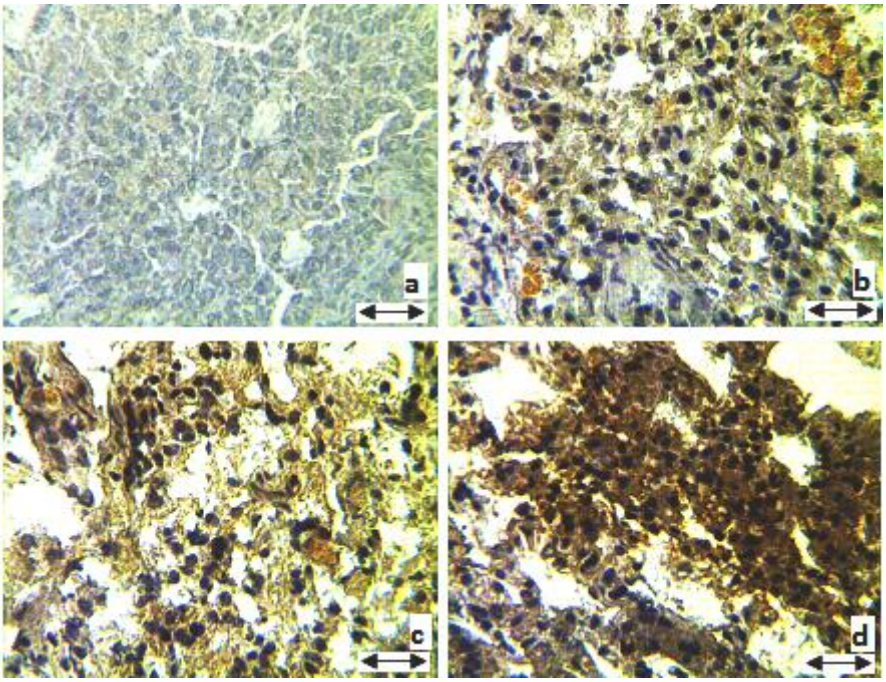

Fig. 2. Intensity of Sudan III staining in common carp liver, x 400. **a** – Control group; **b** – Intensity of staining and Sudan positive fat vacuoles at concentration 30 mg/L fungicide; **c** – Intensity of Sudan III staining at concentration 38 mg/L fungicide; **d** – Intensity of Sudan III staining at concentration 50 mg/L fungicide.

We observed in the control group in separate sections in the hepatocytes very weak yellow staining of Sudan III (Fig. 2a). Intensity of staining of the liver parenchyma of all specimens, exposed to the lowest concentration (30 mg/L) was weak (more intense yellow staining). Also, we found a slight increase of lipid droplets in the hepatocyte cytoplasm (Figure 2b). Moreover, we observed in the fish group, exposed to 38 mg/L of the test pesticide more pronounced lipid droplets in the hepatocytes, which were stained in yellow-orange (Fig. 2c). We found strong positive Sudan III staining at the highest pesticide concentration (50 mg/L), which indicates the increase of fat deposits in the cytoplasm (Fig. 2d). Lipid droplets were stained in orange.

Discussion

Our results indicate an increase in the amount of glycogen in the liver parenchyma. Similar to us FRANCHINI *et al.* (1991) found lipid accumulation in fish liver, which suggests changes in the liver function under the effluence of different toxicants. Other authors reported that exposure of subchronic doses of pesticides inhibit glycogenolysis and promote glyconeogenesis (SINGH *et al*., 1996). SPISNI *et al.* (1998) observed hepatic fat deposition. The authors described a disturbance resulting from an excessive or unbalanced

supply of dietary lipids which exceeds the physiological lipid conversion ability of liver, resulting in hepatic lipid accumulation as fat drops (triglycerides).

REZG *et al.* (2006, 2007) concluded that under the effect of subchronic dose of organophosphate insecticide malathion, the storage of glycogen in rat liver may be due to a stimulation of insulin secretion after the inhibition of acethylcholinesterase activity in pancreatic beta cells. These studies indicate that hyperglycemia is temporary, which is probably due to a stimulated glyconeogenesis that increases hepatic glycogen deposition in liver. The authors suggest that these results are due to the turnover of glucose by a succession between its release glycogenolysis and glyconeogenesis, which involves abnormal hyperglycemia, and its storage via glyconeogenesis in subchronic exposure to pesticide.

It is clear that other authors found in their studies increased amount of glycogen in the liver of different test species under the influence of many toxicants. We also found an increase in the amount of glycogen in the liver parenchyma. However, it would be difficult to comment in detail the mechanism for this accumulation. We, therefore, can only say that our results show that the changes in the common carp liver metabolism under the influence of the studied fungicide were expressed mainly in accelerating the process of glyconeogenesis and accumulation of lipids. In addition, intensity of accumulation of glycogen and lipids in the carp liver is proportional with the chemical concentrations.

Conclusion

Overall, we investigated an increase the intensity of PAS-reaction and accumulation of lipid droplets in fish liver, which correlates with the increasing concentration of the fungicide. We can conclude that the increase of the amount of glycogen and lipid droplets in the hepatocytes suggests a process of transforming the accumulated glycogen in fat (lipids). Moreover, we can associate this process with induced glyconeogenesis. Therefore, this could be considered a series of compensatory mechanisms in fish and liver metabolism in response to the toxic effects of pesticides and the stress they induce. Further investigations in this particular area need to be carried out to better understand the metabolic changes in the liver under the influence of organic contaminants.

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Data on the reproduction of a Caucasian Viper, Vipera kaznakovi Nikolsky, 1909 (Serpentes: Viperidae) from Hopa (Northeastern Anatolia, Turkey)

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Abstract. In the current study we report an observation of a Caucasian Viper, *Vipera kaznakovi* from Hopa (Artvin, Turkey) giving birth. During our field studies, we captured a female *Vipera kaznakovi* on July 21, 2012 from Esenkıyı village, Hopa (Artvin, Turkey), it was brought to the laboratory and kept in a terrarium for a period until May 28, 2013. The female (SVL= 483 mm, total length, TL = 541 mm) gave birth to eight young (mean SVL= 146 mm, mean TL= 161.4 mm, range= 155 – 172 mm; mean weight, W= 3.11 g, range= 2.6 – 3.4 g) on August 11, 2012. In six and a half months, the juvenile snakes had reached 163 mm SVL, 187 mm TL and 5.1g W and increased their size by approximately 15%.

Keywords: Caucasian Viper, *Vipera kaznakovi*, reproduction biology, Turkey

True vipers (subfamily: Viperinae) are distributed in Europe, Asia, and Africa and include generally ovoviviparous species (MALLOW *et al.*, 2006). *Vipera kaznakovi* is endemic to the Caucasus and listed as Endangered in the IUCN Red List due to its Area of Occupancy being less than 500 km^2 (TUNIYEV *et al.*, 2009a). The species range stretches along the Black Sea coast, covering the forested foothills of the Caucasus up to 1000m a.s.l, from Hopa (Artvin) in Turkey and the Suramsky pass in the east across Colchis up to the Mikhailovsky pass in the west (BAŞOĞLU & BARAN, 1980; BARAN & ATATÜR, 1998; ORLOV & TUNIYEV, 1990; SINDACO *et al.*, 2000; ANANJEVA *et al.*, 2006; TUNIYEV *et al.*, 2009a). The main threats to *V. kaznakovi* have been illegal overcollection for the international pet trade, habitat loss and destruction due to urban development, tourism, dam construction and agriculture (BARAN & ATATÜR, 1998; TUNIYEV *et al.*, 2009a).

The Caucasian Viper inhabits the forested slopes of mountains, in the bottoms of humid canyons, in post-forested clearings and meadows (BAŞOĞLU & BARAN, 1980; ORLOV & TUNIYEV, 1990; MALLOW *et al.*, 2006; TUNIYEV *et al.*, 2009a). Phenology of the species depends on altitude. The individuals on the Black-Sea coast are active from March to November, whilst in the highlands they are active in the second half of April - beginning of May to the end of

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September - beginning of October (ORLOV & TUNIYEV, 1990; TUNIYEV *et al.,* 2009a). Soon after emerging from hibernation, the period of mating occurs from the end of March up to mid-May and parturition time is at the end of August - early September depending on altitude (TUNIYEV *et al.,* 2009a).

On our field trip at the vicinity of Esenkıyı village, Hopa (Artvin, Turkey), we captured a female on July 21, 2012, the individual was brought to the laboratory and kept in a terrarium (120x40x50 cm, LxWxH) for a period until May 28, 2013. The female (snout-vent length, SVL= 483 mm, total length, TL= 541 mm) gave birth to eight newborn snakes on August 11, 2012, (Fig. 1) two died due to non-feeding in the terrarium. The average size of the newborn juveniles was 3.11 g (range=2.6–3.4 g) in weight, 140.6 mm (133-150 mm) SVL, and 161.4 mm (155–172 mm) TL (Table 1).

Coloration of the newborn juveniles was slightly different from that of the female. Dorsum ground colour is black, with two longitudinal red brown stripes forming an intervening zig-zag shaped or straight black dorsal band present as indicated by ORLOV & TUNIYEV (1990), HÖGGREN *et al.* (1993) and MALLOW *et al.* (2006). However, the dorsum ground color of the juveniles is more brightly coloured (ORLOV & TUNIYEV, 1990) than that of the female. ORLOV & TUNIYEV (1990) also reported that the head pattern of the juveniles is separated from the dorsal stripe by a light area as is shown in Fig. 1. The ventrum is black.

In captivity, females frequently give birth at night (ORLOV & TUNIYEV, 1990; MAMET & KUDRYAVTSEV, 1997). The cluch size of *V.kaznakovi* generally is between 3 and 5 (ORLOV & TUNİEV, 1990; MALLOW *et al.,* 2006). The newborns are 145 mm in SVL and weigh 4.1 g (ORLOV & TUNIYEV, 1990). MAMET & KUDRYAVTSEV (1997) reported that a female Caucasian viper [SVL= 484 mm, TL= 546 mm] gave birth to four newborn juveniles on July 9, 1993 at Moscow Zoo. The young individuals were measured as 152 mm for SVL and 4.11 g in weight by the authors. The cluch size of the closely related species *Vipera dinniki* and *V.darevskii* ranges from 3 to 7 and young snakes appear between August and September (ORLOV & TUNİYEV, 1990; MALLOW *et al.,* 2006; TUNİYEV *et al.,* 2009b). The mean size of newborn vipers is 131 mm for SVL, 3.1 g in weight (MALLOW *et al.,* 2006).

Newborn snakes first shed their skin on the second day from birth and twice before hibernating at the end of the October, 2012. ORLOV & TUNIYEV (1990) reported juvenile vipers generally shed twice before hibernation: first is second day after birth, second just before hibernating.

Six and a half months later, the average size of the juvenile Caucasian vipers had increased by approximately 16% (range= 11% - 20%) of the original length and the mean SVL had increased to 163 mm (148 – 173). The mean weight had increased to 5.10 g (4.4 – 5.8) and the rate of increase was about 61% (59% - 64%). ORLOV & TUNIYEV (1990) reported one year old individuals reach 200 mm in SVL and they had increased their size about 38% from birth.

In summary, our results confirmed previous data about reproduction of *V. kaznakovi* and range of clutch size was increased to 3 to 8. However, most of the available data was obtained from captive individuals. We urgently need to obtain data on the ecology of *V. kaznakovi* in the wild to help protect the species from future threats.

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Table 1. Summary statistics of juvenile Caucasian Vipers from Hopa.

	August 11, 2012			May 28, 2013		
	W	SVL	TL	W	SVL	TL
N	8	8	8	8	8	8
Mean	3.11	14.06	16.14	5.10	16.29	18.70
Range	2.6 – 3.4	13.3 – 15.0	15.5 – 17.2	4.4 – 5.8	14.8 – 17.3	16.8 – 20.2
SE	0.09	0.20	0.20	0.17	0.31	0.38
SD	0.26	0.57	0.58	0.49	0.88	1.08

Fig. 1. General view of female Caucasian Viper and newborns from Hopa.

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Contribution to the Knowledge on the Gastropod Fauna of the Natural Park "Balgarka" (Stara Planina Mts., Bulgaria)

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Abstract. A total of 35 species of snails were found: 6 freshwater and 29 terrestrial. One species was considered as local endemic and 5 species as endemics for Stara Planina Mts. One species was invasive.

Keywords: protected area, malacofauna, Bulgaria.

The malacofauna of the Bulgarian natural parks situated in Stara Planina Mts. is poorly known. Detailed research on the "Sinite kamani"park area was provided by Dian Georgiev from the Trakia University, Stara Zagora (GEORGIEV, 2008). A total of 23 species of terrestrial snails were found and data on their habitat distribution was represented.

Before description of the new stygobiotic species *Bythiospeum dourdeni* Georgiev, 2012 from the Chuchura cave near the village of Stantchov Han (Veltchovtsi area) by GEORGIEV (2012) there was no any other information on the malacofauna of the natural park "Balgarka" situated in the central part of the Stara Planina Mts., Bulgaria. As an associated mollusc species in the cave also a *Belgrandiella* (Wagner, 1927) species was recorded, determined as *B*. cf. *stanimirae* Georgiev, 2011.

The park area is consisted of steep mountain terrains occupied by broad leaf forests, meadows, streams, springs, and some small caves. Some anthropogenic areas as some villages and town vicinities (Platchkovtsi and Gabrovo surroundings) are also included in this protected area.

As it is well known that all the information on biodiversity supports its better conservation, in this paper I represent some original data on the species of snails (Mollusca: Gastropoda) which I found on the territory of this natural park.

Material and Methods

Most of the finds were made accidently during a large mammal study in the park area together with my colleagues from NGO "Green Balkans" in the periods between 3-7.7.2007 and 23.5.2012-13.4.2013 (Table 1).

The snails were collected by hand or a sieve for the soil samples. Species were determined mainly by DAMJANOV & LIKHAREV (1975), WELTER-SCHULTES (2012), and a reference shell collection of the author (deposited in the Regional Natural History Museum - Plovdiv).

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Table 1. Collection localities in the natural park "Balgarka" during present study.

№	Date	GPS coordinates	alt.	Area	Habitat
1	3-7.7.2007	N42 48 08.3 E25 34 40.0	558 m	cave Mokrata near vill. Stantchov Han	cave
2	3-7.7.2007	N42 45 50.0 E25 29 57.5		steep slope above Kokalenata cave near Balgarka Hut	*Fagus sylvatica* forest
3	3-7.7.2007	N42 49 E25 10	-	near the beginning of the road Partizanska Pesen hut	*Fagus sylvatica* forest near water source
4	23.5.2012	N42 50 19.1 E25 10 14.1	952 m	near Partizanska Pesen hut	*Fagus sylvatica* forest
5	24.5.2012	N42 47 23.8 E25 15 56.9	674 m	south of Zeleno Darvo vill.	spring near the river
6	25.5.2012	N42 47 38.9 E25 15 47.8	750 m	west of Zeleno Darvo vill.	broad leaf forest and bush
7	25.5.2012	N42 47 54.4 E25 16 16.5	720 m	vill. Zeleno Darvo	near a stream and buildings
8	25.5.2012	N42 49 11.7 E25 13 10.6	562 m	Todortchetata vill.	meadows and bush near a river
9	24.6.2012	N42 47 17.9 E25 28 31.9	662 m	Platchkovtsi town, Stoevtsi area	broad leaf forest near houses
10	6.4.2013	N42 48 14.0 E25 34 48.1	548 m	a hill near vill. Stantchov Han	bush areas,open terrains
11	6.4.2013	N42 50 30.6 E25 28 01.6	524 m	Dryanovska Reka River near Plachkovtsi town	rever
12	6.4.2013	N42 50 30.6 E25 28 01.6	524 m	along Dryanovska Reka River near Plachkovtshi town	*Alnus glutinosa* river bank forest
13	12.4.2013	N42 48 09.6 E25 34 43.4	555 m	at the entrance of Kilyikite cave near vill. Stantchov Han	*Fagus sylvatica* and *Carpinus* sp. forest
14	13.4.2013	N42 48 06.7 E25 34 45.3	511 m	the stream below Mokrata cave	stream
15	25.6.2012, 13.4.2013	N42 47 58.0 E25 34 23.7	573 m	cave Chuchura near vill. Velchovtsi	unkown, deposits inside the cave stream
16	25.6.2012	N42 47 58.0 E25 34 23.7	573 m	cave Chuchura near vill. Velchovtsi	the spring emerging from the cave
17	26.6.2012	N42 46 10.0 E25 33 07.2	859 m	Ktastets railway station	near a wall of abondoned building
18	26.6.2012	N42 44 53.5 E25 31 56.2	978 m	near Izvora hut	near a wall of abondoned building

Results and Discussion

A total of 35 species of snails were found: 6 freshwater and 29 terrestrial (Tab. 2). The species *Bythiospeum dourdeni* is known only from its type locality, so it is probably a local endemic. The other hydrobiids related to the genus *Belgrandiella* can be considered as endemics for the region of Stara Planina, and knowing the high levels of endemism of these Bulgarian species also the unidentified one from the entrance of the Chuchura cave can be included in this group.

From the terrestrial snails Bulgarian endemics are *Cattania balcanica* and *Alinda wagneri*. As an invasive species *Tandonia budapestensis* can be considered.

As most of the finds were made accidently it can be supposed that many other species (as for example the widely distributed in the country *Pupilla muscorum* (Linnaeus, 1758), *Sphyradium doliolum* (Bruguière, 1792), *Vallonia costata* (O. F. Müller, 1774), *Vallonia pulchella* (O. F. Müller, 1774), *Acanthinula aculeata* (O. F. Müller, 1774), *Cochlicopa lubrica* (O. F. Müller, 1774), *Zebrina detrita* (O. F. Müller, 1774), and many others) could also be found on the territory of this protected area. The rare slug species *Lehmannia horezia* Grossu & Lupu, 1962 found in the Shipka Pass very close to the park border (WELTER-SCHULTES, 2012) can also be expected. Detailed further research is recommended.

Table 2. Gastropod species found in the natural park "Balgarka" with their localities

№	Species	Locality №
1	*Belgrandiella angelovi* Pintér, 1968	5
2	*Belgrandiella stanimirae* Georgiev, 2011	15
3	*Belgrandiella* sp.	16
4	*Bythiospeum dourdeni* Georgiev, 2012	15
5	*Galba truncatula* (O. F. Müller, 1774)	14
6	*Ancylus fluviatilis* O. F. Müller, 1774	11
7	*Pomatias rivularis* (Eichwald, 1829)	10, 15
8	*Carychium minimum* O. F. Müller, 1774	12, 15
9	*Truncatellina cylindrica* (J. Ferussac, 1807)	7
10	*Chondrina* sp.	13
11	*Macedonica marginata* (Rossmässler, 1835)	4
12	*Laciniaria plicata* (Draparnaud, 1801)	12
13	*Alinda wagneri* (Wagner, 1911)	2
14	*Arion* (*Arion*) sp. cf. *lusitanicus*	7
15	*Arion* (*Mesarion*) sp.	1, 12
16	*Arion silvaticus* Lohmander, 1937	4
17	*Vitrea* sp.	13, 15
18	*Aegopinella minor* (Stabile, 1864)	12
19	*Oxychilus glaber* (Westerlund, 1881)	15
20	*Oxychilus* cf. *urbanskii* Riedel, 1963	12
21	*Oxychilus* cf. *inopinatus* (Ulicny, 1887)	13
22	*Tandonia budapestensis* (Hazay, 1881)	12
23	*Punctum pygmaeum* (Draparnaud, 1801)	12
24	*Limax maximus* Linnaeus, 1758 - complex	7, 17
25	*Deroceras turcicum* (Simroth, 1894)	7, 12, 13
26	*Cattania balcanica* (Kobelt, 1876)	4, 15
27	*Cepaea vindobonensis* (Ferussac, 1821)	10
28	*Helix lucorum* Linnaeus, 1758	7, 9, 12, 17
29	*Helix pomatia* Linnaeus, 1758	4
30	*Arianta arbustorum* (Linnaeus, 1758)	3
31	*Bradybaena fruticum* (Müller, 1774)	12
32	*Xerolenta obvia* (Menke, 1828)	6, 10, 18
33	*Perforatella incarnata* (Müller, 1774)	12, 13, 15
34	*Monacha carascaloides* (Bourguignat, 1855)	8, 12
35	*Euomphalia strigella* (Draparnaud, 1801)	6, 13, 17

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