ECOLOGIA BALKANICA

International Scientific Research Journal of Ecology

Volume 2 / December 2010

UNION OF SCIENTISTS IN BULGARIA – PLOVDIV

UNIVERSITY OF PLOVDIV PUBLISHING HOUSE

International Standard Serial Number
Print ISSN 1314-0213; Online ISSN 1313-9940

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Ecology of the Diatomic Flora in Thermo-Mineral Springs of Katlanovska Banja in the Republic Of Macedonia

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Abstract. Thermo-mineral waters have been attracting great attention in the world and in Republic of Macedonia lately, not only as a curative factor but also as an energy source. Thermo-mineral waters on the territory of the Republic of Macedonia are used for health-balneological purposes, and part of these since recently has been used in greenhouse economy.

Diatomic flora of the thermo-mineral waters in Macedonia is relatively poorly studied. Having in mind that the structure of diatomic flora of Katlanovska Banja has not been studied enough, and especially knowing that during the last two decades the taxonomy of diatoms has considerably changed, research of the diatomic flora of Katlanovska Banja has been started. Furthermore, these habitats are characterized by specific ecological conditions, which enable the development of characteristic diatomic micro-flora that is specialized for high temperatures (thermophilic species) and high content of salt (halophilic species).

The results of this research for the first time mention the presence of the genus *Nitzchia* in the thermal waters of Katlanovska Banja, not only as present, but also as dominant taxa. The discovery of the genus *Navicymbula* with the species *N. pusilla* is also very significant, because it was not known for the flora of the Republic of Macedonia.

Key words: diatomic flora, thermal springs, Katlanovska Banja, Macedonia.

Introduction

Diatoms are sensitive to many environmental variables, including light, moisture conditions, temperature, current velocity, salinity, pH, oxygen, inorganic nutrients (carbon, phosphorus, nitrogen, silica), organic carbon and organic nitrogen (e.g. KOLBE, 1932; VAN DER WERFF & HULS, 1957-1974; CHOLNOKY, 1968; WERNER, 1977). Therefore, they are considered to be powerful indicators for environmental changes, including acidification, eutrophication and climate changes, both in neo – and paleolimnological studies (e.g. DIXIT *et al.*, 1992; VAN DAM, 1993). Such studies often use autecological data, which are taken from the available literature. LOWE (1974) and BEAVER (1981) have compiled many data from the literature, but many important Western – European freshwater taxa are missing in these reports. The lists by DE WOLF (1982) and DENYS (1991) are containing a lot of useful literature data on the ecology of diatom taxa, but, as stated before, they do not cover all the diatom taxa in the Netherlands. The indices for the species recorded follow the list of the Netherlands freshwater diatoms, with ecological indicator values for those environmental variables which are

considered to be the most important ones in aquatic and wetland ecosystems in The Netherlands: moisture conditions, pH, trophic state and salinity (HIGLER, 1991; VERDONSCHOT *et al*., 1992; VAN BEUSEKOM, 1993). Measures against pollution by organic biodegradable material are a major issue in water quality management for many years (SMIT-KROES, 1989). Because diatoms are very susceptible indicators for such pollution and the associated changes in the oxygen balance (HUSTEDT, 1957; CHOLNOKY, 1968; SLADEČEK, 1986), we include indicator values for saprobity, organic nitrogen and oxygen as well. A unique eight – letter code (acronym) is assigned to each taxon to facilitate electronic date processing.

A thermal group of waters consists of mineral waters that that are distinguished from regular cold waters in their temperature and as such they have a number of applications in balneology, for heating settlements, greenhouses, and for getting electrical energy. This group of mineral waters is called thermo-mineral waters in literature. They are underground waters with temperature higher than the mean annual temperature of a particular location as high as 100°C.

The thermo-mineral waters in the Republic of Macedonia are used for health-balneological purposes: Bansko - Strumica, Banja - Kocansko, Negorska Banja-Gevgelija, Katlanovska Banja – Skopje, Kezovica – Stip, Proevska Banja – Kumanovo, Baniste and Kosovrasti – Debar (KOTEVSKI, 1987). For Katlanovska Banja the same author reported totally 23 taxa of 16 genera, while for Bansko - 29 species of 15 genera with greater representation of the taxa belonging to the genus *Navicula*. A slightly richer diatomic flora was reported in Debarska Banja where totally 34 diatomic taxa were determined. These thermo-mineral waters are dominated by the genus *Denticula* (STOJANOV, 1983). Likewise, the author listed several new taxa in the flora of Macedonia that are considered to be rare, i.e. their distribution in Macedonia is limited.

The aims of the current paper is to establish the structure of the diatomic flora of Katlanovska Banja; to establish rare or potentially new species in the flora of the Republic of Macedonia; to make valorization of habitats based upon the structure of diatomic taxa.

Material and methods

Study area. The tectonic structure of the terrain is of special significance for the formation of thermal, thermo-mineral, and mineral deposits of spring waters. In this sense the terrain neo-tectonics is particularly important. Most of thermal and thermo-mineral waters are found in the area of the tectonically unstable Vardar zone, and in the area of western Macedonia and regions directly connected with them. These tectonic faults are seismically active even today and, as such, they belong to areas with high level of seismic activity. Katlanovska Banja – Katlanovo belongs to Vardar zone.

Sampling methods. The algological material was collected from the stones, edges and bottom of springs and their flowing-out waters, from the rocks and scale across which thermo-mineral water flows out, from materials of concrete and wooden troughs of thermo-mineral taps, and from various objects immersed in water. At the same time, while collecting material, the environment reaction was measured - (pH-6,5-7).

Microscopic analyses were made by means of Microscope Nikon Eclipse E-800, and the micro-photographs were made with Nikon Coolpix camera. The valve of respective species is represented 1500 times enhanced.

Results

Taxonomic analyses

The analyses of the collected material showed the presence of the following species:

Genus *Navicymbula*

N. eriguga, N. cinata, N. Pusilla (Fig. 1-3)

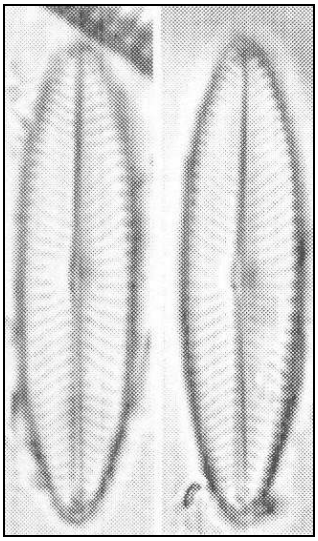

Fig. 1. *Navicula erifuga*

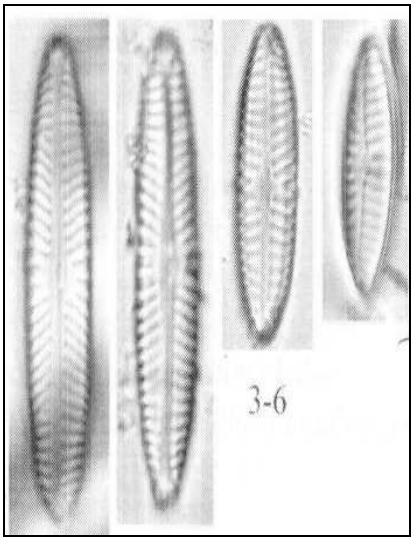

Fig. 2. *Navicula cinata*

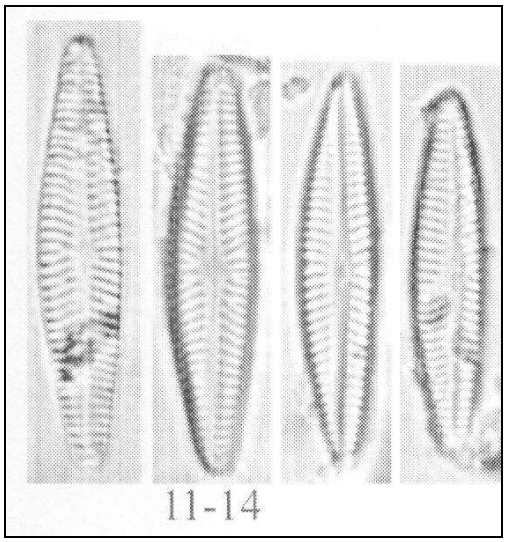

Fig. 3. *Navicymbula pusilla*

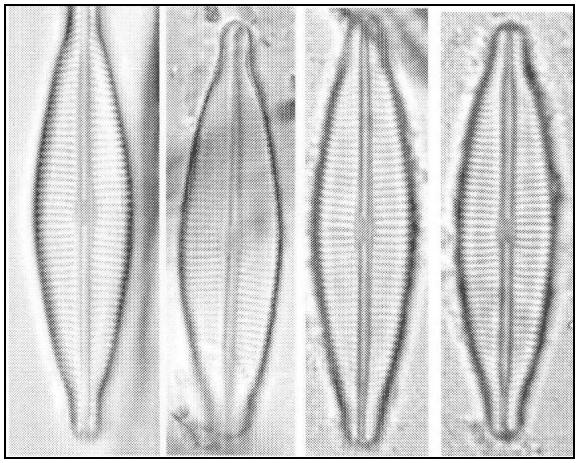

Fig. 4. *Craticula buderi*

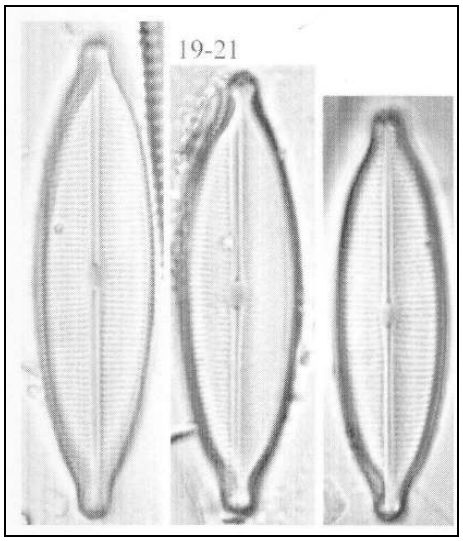

Fig. 5. *Craticula hlophilla*

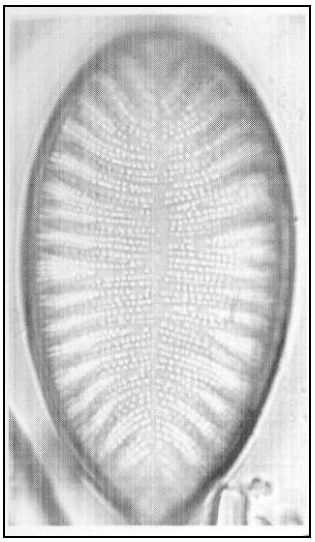

Fig. 6. *Surirella katlanovicensis*

Valves moderately dorsiventral, semi-lanceolate with moderately arched dorsal and convex or approximately straight ventral margin. Ends rounded, not protracted. Length 14-42 μm, breadth 3,9-7,2 μm, maximum length/breadth ratio 6. Axial area narrow, straight, linear or broadening gradually towards valve centre to form a narrow, lanceolate space.

Central area absent or not well expressed. Raphe filiform, proximal ends slightly expanded and barely ventrally displaced the ends dorsally deflected teminal fissures dorsally deflected following subterminal ventral deflection. Striae radiate, becoming parallel to slightly convergent towards the ends. Around the central nodule one to several striae distinctly shortened. Striae in the Lm very delicate, in the middle portion (dorsal) 15-18/10 μm, up to 20/10 μm towards the ends, lineolae (not recognized in the LM) 40-50/10 μm.

Genus *Craticula* (Fig. 4-5)
C. buderi, C. hlophilla

Distribution: Cosmopolitan, fairly common in the area covered by the flora. Its tolerance to trophy and saprobity is very broad, ranging from weakly meso- to hypereutrophic, and oligo- to α-mesosaprobic. It is also found in periodically wet habitats, e.g. moist meadows, and industrial effluent.

Genus *Surirella*
Surirella katlanovicensis (Fig. 6)

The surface of the valve is almost planar. Between the well developed traxapical undulations there are bi- or triseriate striae with one-sided areoles. The canal raphe is almost in the same plane with the surface of the valve itself. On the inside the canal is supported only by fibulas of Type 2 that bridge the distance between the face and the side of the valve. Between each two fibulas there is a big opening, portulaca, through which they communicate with the canal raphe. The apix (the wider end) of the valve has a continued raphe on the inner side, i.e. the proximal ends of the raphe are absent. Having in mind that raphe is quite slightly raised; there is no ridge, alar canals and punctae.

Discussion

According to the algological research in Katlanovo, STOJANOV (1982) found 23 taxa that belong to 16 genera. Eighteen of these are determined as species, and 5 are determined to be at the level of varieties. It was found that the genus *Navicula* represented with 5 taxa is most present in the thermo-mineral waters in Katlanovo, and 10 genera are represented with only one taxon, while the thermophilic species *Achnanthes gibberula* Grunow gives an essential characteristic to the Katlanovo thermae where it is the most dominant taxon. Frequent forms that were not previously reported in the algae flora in former Yugoslavia are: *Achnanthes gibberula, Navicula thienemanii*, *Caloneis hultenii*, *Rhopalodia giberula* var. *vanheurckii*, *Surirela margaritacea*. The diatoms in the thermo-mineral waters in Katlanovo are best developed in the sites with water temperature of 31°C where *Achnanthes coarctata* (Brébisson) Grunow and *Gomphonema parvulum* (Kutzing) Grunow are most frequent, and the poorest are the thermae with water temperature 54,5°C where only single samples were found of *Diatoma vulgare* Bory, *Gomphonema olivaceum* (Lyngb.) Kutzing and *Rhopalodia gibberula,* and slightly more often *Caloneis hultenii* Boye P., a typical inhabitant of warm springs up to the temperature of 45°C (STOJANOV, 1982).

The diatomic flora in the thermo-mineral waters of Bansko is richer than the one in Katlanovo thermae. In the much warmer thermae in Bansko 29 diatomic flora were reported (of 15 genera) of which 21 were determines as species, 8 as taxa at lower taxonomic level (6 varieties and 2 forms), and, unlike Katlanovo, all the reported diatomic forms belong to the class Pennatae (STOJANOV, 1982). The most represented genus in the thermo-mineral waters of Bansko is *Navicula,* represented with 6 taxa, then the genus *Pinnularia* with 4, and the genus *Achnanthes* represented with 3 taxa, and 10 genera represented with only one taxon each. The thermae in Bansko are particularly characterized by the genus *Denticula* with the species *Denticila elegans* Kützing, *D. thermalis*

Kützing, and *D. tenuis* Kützing as dominant in the researched area (STOJANOV, 1982). Some species were reported in the thermo-mineral waters of Bansko that were not previously reported in the algae flora in former Yugoslavia (*Pinnularia braunii* (Grun.) Cl. *Denticula thermalis*, *Nitzschia vitrea* Norman).

The research of the Katlanovo thermae area in February 2007 showed that thermal waters are characterized with constant reaction of pH 6,8-7,2 and temperature of 38˚C.

As a result of the algological research it was reported that the genus *Nitzschia* was found for the first time, which, according to Stojanov's research (STOJANOV, 1982; 1984), was never present in Katlanovo thermae, but was listed in the results of the algological research of the Debar thermae. Comparing the results from 2007 with the above mentioned Stojanov's results, the following was reported: Besides the genera listed by STOJANOV (1982) there are some new ones that have not been mentioned in the diatomic flora of Katlanovo thermae, such as: *Amphora, Cocconeis, Denticula, Navicymbula, Planothidium, Ulnaria* and species *Eolimna minima, Fallacia insociabilis, Tryblionella constric,* while in Stojanov's results (STOJANOV, 1982) there is *Pinnularia interrupta* f. *minor*, but was not reported the algological testing in 2007.

It is interesting that in 1982, Stojanov reported hat the genus *Nitzschia* was not present but in the algological results in 2007 it was reported to be the dominant species results (STOJANOV, 1982).

In comparison to the algological research of Debar thermae and Stojanov's statements in 1984 that *Nitzschia* was present in Debar and absent in Katlanovo thermae results (STOJANOV, 1984), according to the results of the research in 2007 it can be stated that *Nitzschia* is a common taxon of Katlanovo and Debar thermae. The reported species present in Katlanovo thermae but absent in Debar thermae are: *Mastoglioaeliptica sp., Navicymbulla pusilla, Planothidium lanceolatum, Rhopalodia* spec. *gibberula, Surirella ovalis, Tryblionella constricta.*

When compared to the algological results of Bansko thermae and Katlanovo thermae (by STOJANOV 1982, 1984) and to the results from February 2007, it was reported that instead of *Achnanthe* as dominant there was *Nitzschia* as dominant species, which, again by Stojanov, was not reported in Katlanovo thermae.

All upon mentioned diatom flora are results from several analyses made by STOJNOV (1982, 1984) *Surirella katlanovicensis,* is different from other *Surirella* sp.

Conclusions

According to the results concerning the area researched for the presence of diatomic flora in the thermo-mineral spring of Katlanovska Banja, the following conclusions can be made:

1. The final analyses of the results determined a total number of 40 diatomic flora. The determined representatives are mostly highly specialized for thermal and thermo-mineral waters, in other words, they mainly belong to the group of thermopile and hemophilic species. This research has determined a considerably large number of species in terms of the previous research; this is probably the case due to a large number of substrates and a more detailed microscopic analysis.

2. The presence of the genus *Nitzschia* was mentioned for the first time, not only as present, but as dominant species in the thermae of Katlanovska Banja. The following species are present in this genus: *Nitzschia communis, Nitzschia linearis, Nitzschia sigmoidea, Nitzschia amphibia-small, Nitzschia vitrea.*

3. Several taxa are determined that can not be identified on a genus level at the moment. These are considerably different in comparison to the already known species. It is supposed that these represent new species for the science. A typical example is *Surirella* sp. which belongs to the complex around *Surirella ovalis,* but it is considerably different in density and shape of the areoles.

4. The finding of the genus Navicymbula is also significant with the genus Navicymbula pusilla that has so far been unknown in the flora in the Republic of Macedonia. This result suggests that additional research of a large number of thermo-mineral waters in Macedonia is necessary in order to determine the total diversity of the diatoms, as well as

establishing highly-specialized species for life in these conditions. It is necessary to conduct detailed research in different weather seasons with more detailed electron-microscopic research.

5. Everything that is given in this part is a description on diatomic flora found at the place of research, there are several species like: *Nitzschia communis, Nitzschia linearis, Nitzschia sigmoidea, Nitzschia amphibia-small, Nitzschia vitrea,* and these taxa were not found another previous result from other researchers like dominant species.

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Received: 21.08.2009
Accepted: 14.05.2010

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ECOLOGIA BALKANICA

2010, Vol. 2 December 2010 pp. 7-13

Changes of Some Biochemical and Physiological Parameters in Capsicum annuum L. as a Consequence of Increased Concentrations of Copper and Zinc

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Abstract. Soil cultures of peppers were (*Capsicum annuum* L.*)* cultivated. The first leaves of young plants in the stage of forming were treated with excessive concentrations of $ZnSO_4 \cdot 7H_2O$ in four different concentrations (mg/kg): 1.0; 5.0; 10.0 and 20.0 while the control group of plants was treated with water only. Parallel to this, a part of the plants were treated with excessive concentrations of $CuSO_4 \cdot 5H_2O$ again in four different concentrations: 0.5; 1.0; 5.0 and 10.0, while the control group of plants was treated with water only. The material for the analysis was taken at the end of the vegetation period, in the phase of bearing fruit, and then it was dried to absolute dry mass at the temperature of 60-80^{o}C. The dry plant material is broken up into small pieces and used for analyzing the phenol compounds. Raw material is used for determining the contents of Chlorophyll and vitamin C.

Key words: heavy metals, copper, zinc, *Capsicum annuum* L , phenol compounds, chlorophyll

Introduction

Soil is fundamental and irreplaceable part of the environment and its pollution cannot be avoided. Even though the soil has great buffer capacity in relation to outer influences, the functioning of this capacity can be disturbed, and this in turn represents a considerable problem of today's modern society. Plants are an important indicator of soil pollution with heavy metals.

Heavy metals and their influence on mineral feeding of plants

The chemical substances that can be commonly found in nature every day (air, water, soil) are increasing in number. By origin, they can be natural products, but not rarely also synthetic, or they are the products of chemical transformation of natural products. Isolated as pure chemical substances they possess certain characteristics that make them important and are the basis of their everyday application. These substances are usually found in soil but in various quantities. One type of these matters is elements that are found in soil in such small quantities that they are called elements in traces or microelements. Other elements, found in soil in traces but not necessary to plants, in great quantities can be harmful and dangerous for plants, man and animals. This group consists of toxic elements, and the above-

mentioned groups are made up mostly of such heavy metals. These two terms comprise a group of metals that can pollute the soil and the environment. Unlike water and air pollution, soil pollution with heavy metals is not easy to determine and is different for different soil types.

Research goal

Interest in growing fruits of pepper, is on the rise, due to its high content of bioactive substances and antioxidants. The research showed the impact of heavy metals and different variation of several components with antioxidant properties. Through the concentration of synthesized substances with antioxidant capabilities can be observed the influence of toxic metals.

Starting from the previous, and having in mind the toxic influence of the excessive concentrations of heavy metals upon some morpho-physiological and anatomic parameters in a number of plant species, the goal of this research was do examine the effects of different concentrations of copper and zinc on the contents of photosynthetic pigments, vitamin C and bioflavonoid (anthocian and phenol).

Material and methods

The pepper culture (*Capsicum annuum* L.*)* cultivated in experimental conditions was used in the research. During the growing phase, the young leaves were treated with excessive concentrations of $ZnSO_4 \cdot 7H_20$ with 4 different concentrations (mg/kg): 1.0; 5.0; 10.0 and 20.0 while the control group of plants was treated only with water. Simultaneously a part of the plants were treated with excessive concentrations of $CuSO_4 \cdot 5H_2O$, again with 4 different concentrations: 0.5; 1.0; 5.0 and 10.0 while control plants were treated only with water. The material for analysis was taken toward the end of vegetation, in the phase of bearing fruit, and it was dried afterwards until it was dry mass at the temperature of 60-80°C. The dry plant material is chopped up and used for analysing the phenol compounds. The fresh material is used for determining the content of chlorophyll pigments and vitamin C.

Determination of the content of chloroplast pigments using spectrophotometric method

200 mg fresh leaf mass of pepper (*Capsicum annuum* L.) were measured on an analytic scale. They were then transferred into a porcelain mortar where it is macerated in the presence of 85% solution of acetone. The procedure is repeated until the leaf mass is completely decolorized. The resulting extracts are kept in the dark in order to prevent the destruction of the chlorophyll molecules. The extracts are photo-metrically measured at the wave length of:

Chlorophyll a (*Chl* a) - 665 nm
Chlorophyll b (*Chl* b) - 650 nm
Carotenods – 452.5 nm

A pure acetone solution is used as a blind sample.

The concentration (content) of chlorophylls in the solution is calculated with the following formulas *MECKINNEY* expressed in mg/l:

$Chl\ a = 16.5 * A\ 665 - 8.3 * A650$
$Chl\ b = 33.8 * A650 - 12.5 * A665$
$Chl\ (a + b) = 4.0 * A665 + 25.5 * A650$

Determination of Vitamin C content according to 2, 6-dichlorophenolindophenol method

First the available leaf mass for this analysis is measured on an analytical scale. The plant material is macerated with 5-6 drops of 3% threechlor acid solution; it is quantitatively filtrated in a 100 ml lab dish and is filled to the mark with 3% measured by means of a mensure and this is moved into solution of CCl_3COOH. Out of this solution 20 ml are an Erlenmeyer dish where it is titrated with 2,6-dichlorfenolindofenol until slight pink coloration occurs which lasts as long as one minute. The concentration of the titrants is 0,001 mol/dm^3. The solution mass of 2,6-dichlorfenolindogenol is calculated with the

following formula:

$$m = V \cdot C \cdot M$$

The mass got from the indicator is dissolved in a measuring 100 ml lab dish.

To calculate the percentage (%) of vitamin C in the plant material the following formula is used:

$$\%C = \frac{V \bullet 0{,}001 \bullet 176}{m}$$

V- volume of used ml of 2,6-dihlorfenolindofenol;

0,001 - concentration of 2,6-dihlorfenolindofenol;

176 - molar mass of ascorbic acid;

M - grams of the taken material for analysis.

Determination of the anthocianines content

The dried fruit mass used for anthocianine extraction is first measured and we take 1-10 g (depending on the anthocianine content). The quantity taken is transferred into a 100 ml dish and filled to the mark with 1% of HCL solution in methanol. After the extraction is finished (30 minutes in the dark), it is filtered through filter paper. From the resulting filtrate we take 5 ml and put it into the measuring dish of 50 ml and fill it to the mark with a buffer pH-1.

This solution is used for the analyses are measured in relation to the control of the spectrophotometer at the wavelength of 510 nm.

The following formula is used for calculation:

$$A = \frac{E - PH_1 \bullet V_1 \bullet V_2}{m_1 \bullet m_2 \bullet V_1}$$

V_1 - volume of the filtrate;

V_2 - volume of the extract;

m_1- fresh mass of the plant material;

m_2 - dry mass of the plant material.

Determination of phenol and flavonoid content according Folin−Ciocalteu method

The extraction of phenols and flavonoids begins with macerating of the fruit mass with 3ml 80% methanol and is incubated 30 minutes at 4°C in an ultrasonic bath. After that the extract is centrifuged for 10 minutes at 13700 rpm. After centrifuging 2 ml of the supernatant is collected into specially labelled test tube, and methanol is again added to the residue (grounds) and re-extraction and centrifuging is performed; then another 2 ml are collected. The procedure is performed after 1ml Folin−Chioclateau reagent and 800 µl 0.7 MNa_2CO_3 are added to 1 ml extract. The mixture incubates for 5 minutes at room temperature.

The absorption of the total phenols is measured at 765 nm and for flavonoids at 425 nm.

The solution of catehin (0.4 mg/ml) is used to prepare the standard curve.

The formula for calculating is as follows:

$$\text{A}\mu\text{g catehin} = \frac{\sum A_{st}}{\sum AC_{st}}$$

Aµg-catehin for total phenols (765 nm)=0.332
Aµg-catehin for flavonoids (425 nm)=0.161

Preparation of the original solution: 50 ml of dry plant material is dissolved in a 25 ml lab dish with several drops of 80% methanol (also possible 100%), and is filled to 25 ml with methanol -FV1. 0.5 ml is taken for determination. If concentration is high, it is diluted. The calculation is done according to the following formulas:

$$Cmg/L = \frac{Aproba}{A(1\mu g_{katehin}) \bullet FV} \bullet DF / 1000(\mu g / mg)$$

$$DF = \frac{FV}{V}.$$

Results and Discussion

The following results were got after the analyses made in pepper (*Capsicum annuum L.*) concerning certain biochemical-physiological parameters (Table 1).

Table 1. Content of photosynthetic pigments (mg/100g wet weight) in pepper plant material (*Capsicum annuum* L), treated with $ZnSO_4 \cdot 7H_2O$.

Treatment with $ZnSO_4 \cdot 7H_20$(mg/kg)	Chl a	Chl b	Chl (a+b)
Control	204.5	340.2	544.7
1.0	181.0	278.2	459.2
5.0	193.0	318.5	510.9
10.0	184.5	299.5	481.7
20.0	197.2	310.0	507.0

Zinc is a significant plant nutrient in limited quantities. If it is added in concentrations greater than optimum, it gets toxic. The presence of greater quantities of reductive metals such as Cu, Fe or Zn in plants causes oxidative damage (LUNA *et al.,* 1994) as well as lipid per-oxidation and anti-oxidative protection (GORA & CLIJSTERS, 1989). A negative effect can be noticed in our results caused in plants by Zn through the reduction of the chlorophyll a and b biosynthesis, when it is applied in concentrations exceeding the optimum.

Zn mainly acts as an inhibitor of the photosynthetic electronic transport (KAPPUS, 1985) causing reduction of the maximum efficacy capacity of PS2 (F_v/F_m), and in quantum gain of electric transport through PS2.

The same parameters were examined in *Lolium perenne* and it was noticed that bigger concentrations of Zn were first seen in its growth inhibition. The increased concentration results in concentration reduction of Ca, K, Mg and Cu, reduction in quantum gain from the electric transport through PS2, as well as the efficacy and photosynthetic energy conversion compared to the control plants (MAKSYMIEC & BASZYNSKI, 1996). However, it was noticed that in this plant only the biggest concentration of 50 mg/kg results in such an effect, which in turn shows that these plants have great power of protection from high concentration of heavy metals. Nevertheless, the number of plants that accumulate Zn well is small.

Table 2 present the results of the analysis of pepper (*Capsicum annuum L.*) that was previously treated with $CuSO_4 \cdot 5H_2O$.

Table 2. Content of photosynthetic pigments (mg/100g) in pepper plant material (*Capsicum annuum* L.)

Treatment with $CuSO_4 \cdot 5H_20$(mg/kg)	Chl a	Chl b	Chl (a+b)
Control	204.5	340.2	544.7
0.5	212.3	332.7	545.0
1.0	174.5	270.7	444.2
5.0	210.5	361.7	572.0
10.0	219.5	419.7	639.0

From the results in Table 2 of the analyses performed on pepper (*Capsicum annuum* L.) we can notice that in higher concentrations at the beginning of the experiment copper shows a stress effect on the treated plants which in turn leads to the reduction in chlorophyll a and b bio-synthesis; later, during the application of greater concentrations a kind of plant

adaptation occurs, probably because of the building in of the Cu into the compounds – participants in photosynthesis, mostly plastocyanine.

Here higher concentration of Cu leads to greater chlorophyll a and b synthesis. We can notice that, compared to zinc, pepper much better adapts to copper.

Copper is an important for plants and especially for photosynthesis as a process because of its role in the transport of electrons as a constitutive part of cyto-chrome.

It mostly leads to the inactivation of Rubesko and phosphoenol piruvat-carboksilase (PEPC), through an interaction with SH-groups (LIDON & HANRIQUES, 1991). It stimulates lipid peroxidation (SANDMANN & BÕGER, 1980), which continues with serous damage of tilacoid membranes. It mostly affects the reduction of the chlorophyll influencing its synthesis as well as its degradation (VANGROUSVELD & CLIJSTERS, 1994).

A great number of authors worked on the same analyses in different plants. *Hordeum vulgare* is one of the analysed plants where the toxic effect of copper was confirmed. Here it was noticed that Cu causes a strong lipid peroxidation (SANDMANN & BÕGER, 1980), which again results in the destruction of the tilacoid membranes and the reduced synthesis of chlorophyll a and b; the reduction is connected with the existing limitations of the tilacoid membranes (VASSILEV *et al.,* 2002). It was also noticed that the synthesis of ethylene is strengthened (LIDON & HANRIQUES, 1991), also noticed in spinach, rice, etc.

As for the content of ascorbic acid in the course of treating pepper with $ZnSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$, the following can be stated:

Table 3. Content of vitamin C in fresh pepper plant material (*Capsicum annuum L*)

Treated plants	Control	$ZnSO_4 \cdot 7H_20$ 20mg/kg	$CuSO_4 \cdot 5H_2O$ 10 mg/kg
(%)vitamin C	0.0911	0.206	0.1139
ml/100g	26.200	86.17	30.730

Looking at the results in Table 3 got from the pepper we can see that an increased synthesis of vitamin C occurs as well as of the enzymes taking part in protecting the plant from oxidation that is caused by high concentrations of copper and zinc. Ascorbic acid is the main anti-oxidant in the photosynthetic and non-photosynthetic tissues where it directly reacts with ascorbat oxidat.

Similar to this enzyme is ascorbat-peroxidase that catalyzes the de-toxication of H_2O_2 (NOCTOR & FOYER, 1998). We have already said that when a higher concentration of Zn or Cu is applied in plants it mainly causes lipid peroxidation of the membrane leading to freeing a great number of free radicals. The synthesis of ascorbic acid is used by plants for protection from the created free radicals.

The analysis made in *Lolium perenne* showed an increase in the activity of SOD-super-oxide desmutase which takes part in the degradation of the formed super-oxide radical as well as of ascorbat oxidase, monohydroascorbat reductase, dehydro-ascorbat reductase and glutation reductase. SOD increases up to 100% when $ZnSO_4$ reaches the concentration of 50 mM (OUARTLI *et al.,* 1997). It can be seen in our results that the highest concentration of Zn also mostly increases the concentration of vitamin C.

The results of the research analysis made on pepper concerning the concentration of anthocianines lead to the following:

Table 4. Content of anthocians (mg/l00g) in the fruit of red pepper treated with different concentrations of $ZnSO_4 \cdot 7H_2O$

	Control	1,0 mg/kg	5,0 mg/kg	10 mg/kg	20 mg/kg
Anthocians	42	96.98	84.095	45.91	47.938

Table 5. Content of anthocians (mg/l00g) in fruit treated with different concentrations of $CuSO_4 \cdot 5H_2O$

	Control	0,5 mg/kg	1,0 mg/kg	5,0 mg/kg	10 mg/kg
Anthocians	42	255.485	1032	1302	71.265

From the analyses made and the result in Table 4 it can be concluded that during treatment of plants with Zn there is a reversely proportionate dependence between the applied concentration of respective heavy metals and the anthocians content being synthesized; the least concentration leads to greatest synthesis; still, all the values are bigger than in control plants.

Copper that also enters the anthocianine synthesis increases the anthocianine synthesis much more compared to the control group of plants according to Table 5; however, in the last case we notice a great fall in the anthocianine content and we can come to the conclusion that the plant somehow stops fighting against the negative influence of heavy metals. When looking at the comparative results of the influence of copper and zinc in both Table 4 and 5 on the anthocianine synthesis it can be said that Zn does not cause significant stress effect in pepper.

Table 6. Content of phenols (mg/g) and flavonoids (mol/g)in plants treated with $ZnSO_4 \cdot 7H_2O$

	Phenols mg/g	Flavonoids mol/g
Control	21.540	7.910
20mg/kg $ZnSO_4 \cdot 7H_2O$	17.006	8.470

Anthocianines represent compounds that, among other, also show anti-oxidative characteristics, protecting the plants from the formed free radicals (Lee and Gould, 2002).

Anthocianine, compared to other components, represents the best indicator of the oxidative stress resulting in plants under the influence of heavy metals. As a mechanism protecting from the toxic influence of high concentrations of Zn and Cu it is synthesized in plants in great concentrations if compared to the control plant.

Table 7. Content of phenols (mg/g) and flavonoids (mol/g) in plants treated with $CuSO_4 \cdot 5H_2O$

	Phenols mg/g	Flavonoids mol/g
Control	21.540	7.910
10mg/kg $CuSO_4 \cdot 5H_2O$	18.270	8.810

Based upon these results shown in Table 6 and 7 we can state that, generally, both zinc and copper cause a fall in the total content of phenols (Table 6 and 7) compared with the controle, while the content of flavonoids (Table 6 and 7) and anthocianines (Table 4 and 5) is increased. There is a directly proportionate dependence between the concentration of heavy metals and the contents of flavonoids and anthocianines, and a reversely proportionate dependence with the total phenols.

Phenolsand flavonoids are important anti-oxidants. Szent-Györgyi, Nobel Prize winner who isolated the ascorbat demonstrated that flavonoids behave in the same manner as the ascorbat. Their synthesis can be induced by biotic and abiotic factors (DIXON & PAIVA, 1995). Phenols are considered tobe anti-oxidants the function of which is to help the primary ascorbat-dependent anti-oxidative system in plants (YAMASAKI *et al.*, 1999).

Conclusions

The results shown clearly indicate the negative effect that heavy metals have on plant material of *Capsicum annuum*. This leads to the following conclusions:

1. All the applied concentrations of heavy metals result in toxic symptoms in plants, and

the seriousness of the damage depends on the plant type, kind of the pollutant, manner of application, concentration, etc.

2. After treating with Zn and Cu a reduction of the photosynthetic activity was noticed, i.e. the chlorophyll pigments.

3. A rapid increase of the vitamin C synthesis was noted as well as of anthocianines, phenols and flavonoids because of an anti-oxidative defence of the plant from free radicals.

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Received: 01.05.2010
Accepted: 19.07.2010

Union of Scientists in Bulgaria – Plovdiv
University of Plovdiv Publishing House

ECOLOGIA BALKANICA

2010, Vol. 2 December 2010 pp. 15-18

Analysis of the Plankton in the Area around the Cape Maslen Nos, Bulgaria: Possibilities for Cultivation of Mediterranean Mussels (Mytilus galloprovincialis)

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Abstract. The aim of the study was to establish the species taxonomic composition and the quantitative characteristics of plankton in the Cape Maslen Nos area. Representatives of the Protozoa, Rotatoria, Annelida, Mollusca and Arthropoda predominated in the composition structure of zooplankton whereas members of Bacillariophyta, Chrysophyta, Dinophyta, Cyanophyta, Euglenophyta in that of phytoplankton. The comparative analysis of phytoplankton data shows that the highest mean biomass values were 24.76 – 33.33 g/m^3 and mean biomass values of zooplankton – 51.43 g/m^3.

Key words: plankton, cultivation, Maslen Nos, *Mytilus galloprovincialis,* Bulgaria

Introduction

Plankton is a key factor in the ecological equilibrium of Black Sea ecosystems and therefore, the knowledge of its structuring communities is of utmost importance (GEORGIEVA, 1998). The environmental effects of urbanization of Black Sea coast consist in a steady trend towards alteration of chemical and biological conditions (MAVRODIEVA *et al.,* 2005). Consequently, a misbalance in the successive development of phytoplankton societies has emerged (MAVRODIEVA *et al.,* 2005). The occurring changes could influence the next trophic levels and to change the organization of the whole biotic system at the Bulgarian Black Sea coast. This new cyclicity would have an impact on hydrobiont cultivation technologies.

In a number of European countries, an industrial-scale production of local and introduced mussel, oysters, shrimps and fish species is organized (STAYKOV, 2001). During the last years, there is an increased interest towards cultivation of Mediterranean mussels in Bulgaria. Several farms have been created in Sozopol, Kavarna, Balchik. Regardless of the technological differences in the cultivation of *Mytilus galloprovincialis* in current conditions, the presence of plankton and littoral is essential (KOLAROV *et al.,* 2005). In these zones, the strong tidal currents provoke an intensive ion exchange, which provides optimal

trophic background, saturation of water with oxygen and removal of metabolites (KOLAROV *et al.*, 2005).

The purpose of the study was to investigate the natural nutrient resources and therefore, the potential for cultivation of *Mytilus galloprovincialis* in front of the Cape Maslen Nos aquatory.

Material and methods

The investigations were carried out in 2007 at the Midia Experimental Base of the Faculty of Agriculture, Trakia University, Stara Zagora located at Cape Maslen Nos. The area is within the Black Sea biogeographical region and represents the eastern part of Strandzha that sticks out in the sea (GRUEV & KUZMANOV, 1994). It is located 8 km away from Primorsko (Fig. 1). The average altitude is 16 m, the atmospheric pressure ranges between 712–763 mm. The average annual temperature is 13° C, and the annual temperature amplitude – 20°C. The average temperature in July is 23–24°C, and August is the hottest month (VELEV, 1997).

For determination of the qualitative and the quantitative composition of plankton, samples were collected in April, July and October from various depths (0.5, 1.2 and 5 m from the bottom as per MAVRODIEVA *et al.*, 2005) with a bank bathometer for phytoplankton and at a depth of 5 m with plankton net for zooplankton (KONSULOV, 1994). Samples were conserved with 4% formaldehyde. Phytoplankton was identified to a genus level and zooplankton – to the species level institute of Fishing Resources, Agricultural Academy. Zooplankton samples were processed according to quantitative method of DIMOV (1959).

Fig. 1. A general view of Cape Maslen Nos.

Results and Discussion

The composition of the phytoplankton at Cape Maslen Nos consisted of 84 taxa from the following divisions: *Bacillariophyta* (45); *Dinophyta* (8); *Chrysophyta* (5); *Cyanophyta* (23); *Euglenophyta* (3). In the year of the study, the divisions *Bacillariophyta* and *Cyanophyta* were predominating. A similar trend towards increase of the *Bacillariophyta* share has been reported in 2000-2004 in the Sozopol bay (MAVRODIEVA *et al.*, 2004).

The mean biomass of the phytoplankton ranged between 24.76 –33.33 g/m^3, with highest values in the summer sample. In the area reported similar values (39.7 g/m^3 for *Acartia clausi*) by GEORGIEVA (1998).

Phytoplankton was mainly represented by diatom algae (*Bacillariophyta*), comprising more than 50% of phytoplankton biomass, with their share increasing during the autumn (Table 1). Co-dominant diatom genera were *Skeletonema, Cyclotella, Nitschia.*

The next group by biomass was dinoflagellates (Dinophyta) with predominating species *Prorocentrum* and *Peridinium.*

Table 1. Dynamics of phytoplankton (biomass in g/m^3) in front of the Cape Maslen Nos aquatory

Division	April	July	October
Bacillariophyta	56,61	58,76	69,41
Dinophyta	41,72	42,03	30,37
Euglenophyta	0,52	0,05	-
Chrysophyta	0,20	0,06	-
Cyanophyta	-	-	0,22
Overall biomass	99,05	100,9	100,0

The *Euglenophyta* and *Chrysophyta* genera were very poorly represented and during the autumn, they were not detected in samples. The group of blue-green algae *Cyanophyta* was found out only in the autumn sample. The observed tendency for domination of *Bacillariophyta* over *Dinophyta* was most probably due to the effect of ecological factors that were not identified in the present study. The causes for the abundance of diatom algae should be searched in the ratio of biogenic elements and silica (not subject of this study) that has a key role in the dynamics of plankton societies in the Bulgarian Black Sea area (MONCHEVA & KRASHEV, 1997 cited by MAVRODIEVA, 2005).

In the region of the mussel farm, the zooplankton was included representatives of *Protozoa*, *Rotatoria*, *Annelida*, *Mollusca* and *Arthropoda*.

From Protozoa, the dinoflagellate *Noctiluca scintillans* was extensively developed. *Rotatoria* was represented with the species *Synchaeta vorax*, while *Annelida* was represented with larvae of annelid worms. From molluscs (*Mollusca*) larvae of Mediterranean mussel, other mussels and snails. The *Arthropoda* type included lower crustaceans from subclass *Branchiopoda,* order *Cladocera* and representatives of subclass *Copepoda* – *Pseudocalanus elongatus*, *Centropages kroyeri* and *Oithona minuta*. According to KOVALEV *et al.* (1998, cited by STEFANOVA *et al.,* 2005) *Centropages kroyeri* is a rare species that in the early 1990-ties was almost not present in the Black Sea basin. In the view of authors, this was due to the increased anthropogenic eutrophication.

The average zooplankton biomass in the Cape Maslen Nos area was 51.43 g/m^3. During the spring it was 35.39 g/m^3, in the summer – 102.15 g/m^3 and in the autumn – 16.76 g/m^3. Because of the high counts of *Noctiluca scintillans*, it was included in the calculation of the total zooplankton biomass.

Table 2. Dynamics of quantitative structure of zooplankton (specimens/m^3) in the Cape Maslen Nos aquatory

Species	April	July	October
Noctiluca scintillans	42442	46920	22631
Synchaeta vorax	198	96	49
Pleopis polyphemoides	294	175	106
Podon leucarti	146	487	69
Acartia clausi	510	981	238
Acartia sp.	586	843	341
Acartia nauplii	488	765	674
Pseudocalanus elongatus	286	-	116
Paracalanus parvus	232	487	126
Oithona minuta	348	596	316
Copepoda nauplius	193	215	27
Polychaeta larvae	154	199	72
Lamellibranchia veliger	366	361	148
Gastropoda (veriger stage)	112	149	117
Penilia avirostris	-	487	117
Centropages kroyeri	-	291	-
Evadne tergestina	-	-	136

Among the represented zooplankton species, the dinoflagellate *Noctiluca scintillans* was extremely numerous, followed by the crustacean *A. clausi* and its larvae (naupilus and copepod). *N. scintillans* dominated with massive bloom. This event was specific for the

80-90-ties of the last century, when the Black Sea was subjected to strong eutrophication due to anthropogenic press (STEFANOVA *et al.*, 2005).

The analysis of phyto- and zooplankton in the Cape Maslen Nos area showed increased biomass during the summer months. We support the belief of STEFANOVA *et al.* (2005), that the extensive development of zooplankton and particularly of *N. scintillans* is consecutive to the spring development of phytoplankton. Our data about *N. scintillans* counts (46920 organisms/m^3) exceeded the average annual values for this species in Bulgaria. According to STEFANOVA *et al.* (2005) this is a specific process for Black Sea coastal zones (up to the beginning of the 1990-ties) when high amounts of phyto-and zooplankton have been detected.

Conclusions

The observed trends in the dynamics of plankton in the Maslen Nos region were similar to characteristic fluctuations for the Bulgarian Black Sea area. Regardless of the fact that after the mid 1990-ties the zooplankton structure was restored, there is still a possibility for competition between *M. galloprovincialis* and *Mnemiopsis leidyi* for food. The successful cultivation (intensive production) of *M. galloprovincialis* in the studied aquatory during the spring and summer months could rely upon the biomass of the observed groups of *Protozoa*, *Rotatoria*, *Annelida*, *Mollusca* and *Arthropoda*.

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Received: 13.05.2010
Accepted: 09.09.2010

Union of Scientists in Bulgaria – Plovdiv
University of Plovdiv Publishing House

ECOLOGIA BALKANICA

2010, Vol. 2 December 2010 pp. 19-23

Influence of Zinc on Gill Morphology of Gibelio Carp (Carassius gibelio)

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Abstract. The influence of increasing concentrations of Zinc sulfate (Zn SO_4x7H_20) on the hystostructure of Gibelio carp gills was investigated. Changes were observed even in the lowest concentration (0.1 $mg.l^{-1}$) – degenerating, cirulation and hyperplastic processes. With the increasing of the Zinc concentration, the hyperplasic processes were predominant over the degenerating and cirulation ones.

Key words: histopathology, zinc, gills, fish, Gibelio carp

Introduction

By the sub-lethal chronic heavy metal concentrations observations were made on the changes appearing in various cells, tissues, organs and processes in the hydrobionts.

This is connected to the early diagnosis of the pathologic changes which allows the use of certain groups of hydrobionts as test-objects to determine the level of heavy metal pollution of the water ecosystems.

As to its toxicity to fish, Zinc is of middle strength – less toxic than Mercury, Copper and Cadmium and more toxic than the Nickel and Lead. Its toxicity is expressed mainly in morphofunctional damage of respiratory organs (MOORE & RAMAMAURTI, 1987).

Although Zinc is a necessary microelement, the sub-lethal concentrations of this metal change the biochemical parameters in fish organism which can lead to a change of the normal cell function GIODA *et al*. (2007), TYAGI & SRIVASTAVA (2005).

Bioaccumulation of Zinc in the fish's gills is proven as well (SERRA *et al*., 1996; VELCHEVA, 2006; MURUGAN *et al*., 2008; KORI-SIAKPERE & UBOGU, 2008; ARNAUDOVA *et al*., 2008).

According to SAPPAL *et al*. (2009) the gills namely play an important role for the absorption of Zinc from the water and hence why physiological and morphological changes often appear in them (CAVAS *et al*., 2005; SAPPAL *et al*., 2009; DOBREVA *et al*., 2008).

The aim of this investigation is to trace *ex-situ* the histological changes in the gills of *Carassius gibelio* in water with increasing Zinc concentration.

Material and methods

Experimental set-up. In aquariums of 25 l filled with dechlorated tap water were set the

following increasing Zinc sulfate ($ZnSO_4.7H_2O$) (produced by MERC) concentrations - 0.1, 0.5 ,1.0, 1.5, 2 mg.l^{-1}. The chosen concentrations are below LC50 for this species and are in consideration with the LAC by the Bulgarian legislation.

As a control environment dechlorated tap water was used.

In each aquarium 10 specimens of the experiment fish from the species *Carassius gibelio* was used. The experiment species were with no exterior pathological changes and from the same size (10 – 12 cm) and age group (1 year old). The fish was acclimatized before the test for 1 week in clean dechlorated tap water. During the experiment the fish were not fed. The duration of the experiment for each concentration was 96 hours. In the process of the experiment the following parameters of the water were maintained – oxygen content – 8.3 mg.l^{-1}, pH – 7.0-7.5, temperature - 17.5-19.0°C and hardness 9dH for each of the experiment concentrations (BATHE & FREI, 1985).

Histological study. The biopsied material from the gills was fixed in a 10% neutral formalin solution for 12 hours. The samples were treated with increasing concentrations of ethyl alcohol (70%, 80%, 86%, 96%, 100%) and they were put in paraffin with a melting point of 54-56°C by the method described by ROMEIS (1989), 0.6 μm wide paraffin cuts were made and put for colouring in hematoxyline and eosin (H&E). The morphologic variations were examined through a light microscope (Olympus CX21), using as reference TAKASHIMA & HIBIYA (1995), and photographed using a digital camera.

Results

Compared to the control group, the changes (Fig.1a) in the gills were observed even under the influence of the lowest concentration (0.1 mg.l^{-1}). The following major changes were found out.

1. Degeneration of the secondary lamella.

The degenerative changes observed were caused only by the influence of lower concentrations (0.1 and 0.5 mg.l^{-1}). Such changes were not present by the higher concentrations.

A thinning of the secondary lamella walls, shortening in length and increasing of the distance between two contiguous lamellas was observed (Fig. 1 a, b).

2. Circulation changes

Changes of a hyperaemic type were found. In the capillary of some secondary lamellas tens of erythrocytes were observed (norm is 1-2) and the lacunas had disappeared. (Fig. 1c).

The circulation changes usually accompanied the degenerative but were seen by higher concentrations as well, but only in areas with no hyperplastic changes. Hyperaemic changes were found in the primary lamellas blood vessels, too.

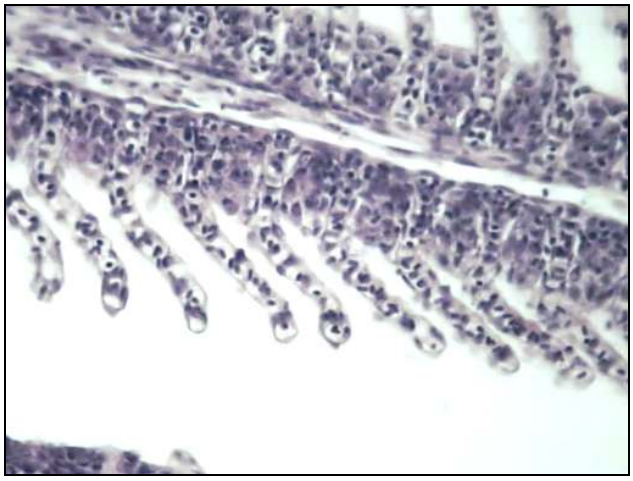
3. Hyperplasic processes

They were observed even under the influence of the lowest concentration (0.1 mg.l^{-1}) and were found in all other ones in a progressive manner. By the lower concentrations investigated (by 0.1, 0.5 and partly by 1.0 mg.l^{-1}), the hyperplasia revealed itself as proliferation of the intra-lamellas epithelial cells (Fig. 1d). Epithelia tissue growth was partial and it did not fill the intra-lamella space. By the 1.5 mg.l^{-1} concentration, the hypertrophy processes occurred in the distal part of the primary lamella (the so-called Club-shaped) which denotes the progressing nature of the damages (TAKASHIMA & HIBIYA, 1995) (Fig. 1e).

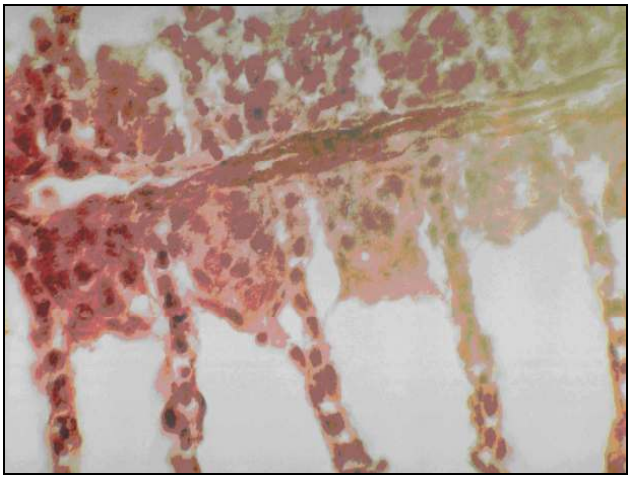
By the specimens treated with the highest concentration (2.0 mg.l-1) and partly with the lower ones (1.0 and 1.5 mg.l^{-1}) the proliferation of the intra-lamella epithelia cells causes full filling of the space between contiguous (fusion). Secondary lamellas had merged and the distance between them had disappeared (Fig. 1f).

Discussion

Our results show that probably the zinc influence is connected to an initial oxidative stress in fish.

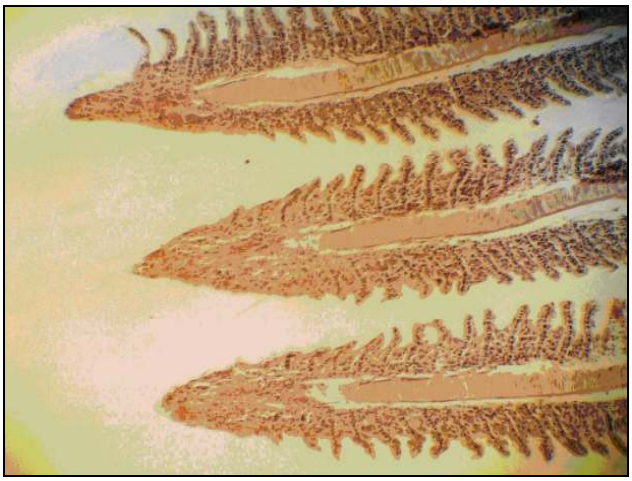


a. Normal structure. Control group. H&E x 400.

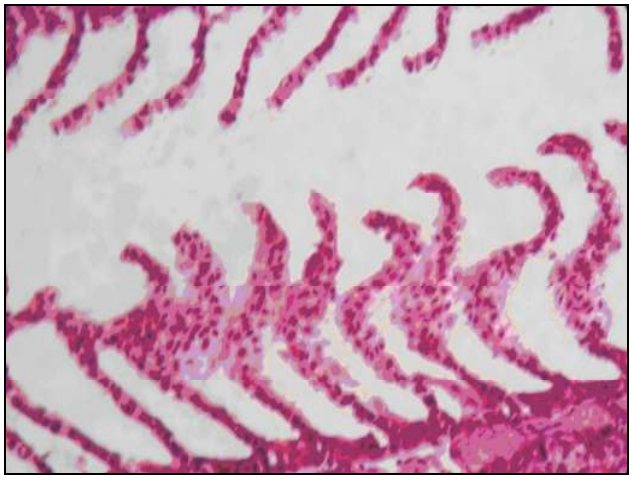


d. Proliferation of the intralamellar epithelial cells. 0.5 mg.l^{-1} Zn SO_4x7H_2O. H&E x 400.

b. Degeneration of the secondary lamellae. 0.1 mg.l^{-1} Zn SO_4x7H_2O. H&E x 400.



e. Club-shaped primary lamellae. 1.5 mg.l^{-1} Zn SO_4x7H_2O. H&E x 200.



c. Hyperemia of the secondary lamellae. 0.1 mg.l^{-1} Zn SO_4x7H_2O. H&E x 400.

f. Fusion of the secondary lamellae.2.0mg.l^{-1} Zn SO_4x7H_2O. H&E x 200.

Fig. 1. Morphological alteration in gills of Gibelio carp under influence of zinc

Verification for this are the results from some past investigations of ours (DOBREVA *et al*., 2008) where we found a decrease of breathing intensity of *Carassius gibelio*, decrease of its sustainability towards Oxygen deficit, as well as changes in the hematological indicators (ARNAUDOV *et al*., 2009). These processes lead to disorders of blood circulation and a full or big foreclosure of the gas diffusion between gills and water. Similar to our opinion, SKIDMORE & TOVELL (1972) show that the initial changes in the gill tissue under the influence of Zinc are typical for an acute inflammatory infection accompanied by blood circulation disorder and a death possibility at a longer exploitation.

The gills epithelia damage leads to impeding of other vital processes – the maintenance of the alkaline-acid balance, ion regulation and the excretion Nitrogen metabolites. This, combined with the caused hypoxia, is to us the probable reason for the high mortality which the Zinc ions cause, which has been found by us in previous investigations. (DOBREVA *et al*., 2008).

Unlike our results, the investigations of FERNANDES *et al*., (2007) show changes in the gills of the leaping grey mullet (*Liza saliens*) that are mostly of the circulation – aneurysms, hyperplasia, lifting and dilatation of the vessels but degenerative and hyperplastic changes are missing in the secondary lamellas.

Changes of gills histology of different fish species under the influence of Zinc are reported in the works of CERQUEIRA & FERNANDES (2002), TKATCHEVA *et al*., (2004), FERNANDES & PERNA–MARTINS (2001) as well but they do not track the relation between the metal content and the degree of the changes found.

However, according to our results, such a relation is present.

By the lower concentrations (0.1 and 0.5 mg.l-1) were detected mainly destructive changes (Fig. 1b).

By increasing the Zinc concentration (1.0, 1.5 $mg.l^{-1}$) were observed mainly hyperplastic changes that were reaching the final phase of adhesion of the gill plates (2.0 $mg.l^{-1}$). We consider that this is due to a compensatory reaction towards the Oxygen deficit related to formation of new epithelia cells in the gills aiming to improve the gas exchange.

We can come to the conclusion that the Zinc influence on the tissue structure of Gibelio carp gills is expressed by the causing of degenerative, circulation and hyperplastic changes. By increasing the Zinc concentration the hyperplastic processes predominate over the degenerative and circulation ones. The probable reason for this is the capillaries compensation of the enlarged epithelia tissue by the higher Zinc concentrations.

Acknowledgements

The present investigation is supported by the "Scientific Research" fund of the Applied Science Activities at the University of Plovdiv "Paisii Hilendarski".

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Received: 17.07.2010
Accepted: 18.08.2010

Union of Scientists in Bulgaria – Plovdiv
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ECOLOGIA BALKANICA

2010, Vol. 2 December 2010 pp. 25-38

Spatial Distribution and a Retrospective Analysis of the Herpetofauna in the City of Plovdiv, Bulgaria

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Abstract. The current study presents the contemporary status and distribution of the amphibians and reptiles in the city of Plovdiv. To track changes in the status of amphibians and reptiles in the research area through time, a retrospective analysis was made based on available literary data. During the past 100 years four species of amphibians (*Triturus karelinii, Lissotriton vulgaris, Bombina bombina, B. variegata*) and four species of reptiles (*Coronella austriaca, Zamenis longissimus, Elaphe sauromates, Vipera ammodytes*) probably have disappeared from the study area; one amphibian species (*Bufo bufo*) and one reptile (*Natrix natrix*) have not changed their status, one amphibian (*Pelobates syriacus*) and two reptile species (*Ablepharus kitaibelii, Podarcis muralis*) have reduced their localities and four amphibian species (*Epidalea viridis, Rana dalmatina, Pelophylax ridibundus, Hyla arborea*) and seven species of reptiles (*Mediodactylus kotschyi*, *Lacerta trilineata, Lacerta viridis, Podarcis tauricus, Emys orbicularis, Dolichophis caspius, Natrix tessellata*) have increased their localities and frequency of occurrence. The records of the two tortoise species (*Testudo hermanni* and *T. graeca*) in the city, and the presence of the Red-eared slider (*Trachemys scripta elegans*) should be considered as accidental. Important Herpetological Areas (IHA) in the study region are also identified.

Key words: amphibians, reptiles, spatial distribution, retrospective analysis, Plovdiv, Bulgaria

Introduction

The changes in the landscapes and their impact on distribution and abundance of animals are becoming more important issue for the ecologists (LUBCHENKO *et al.*, 1991). Studies of the biodiversity in urban landscapes are of particular importance because they are still poorly studied. A better understanding of the ecological processes governing the species composition and distribution of animals in an urban environment is necessary for adequate management and conservation (HUSTÉ, 2005). Under the conditions of urbanization some species undergo a process synanthropization, while other species can not adapt to new conditions and are isolated in separate fragmented populations, or pushed outside the city (VERSHININ, 1996). Adjustment and adaptation of amphibians and reptiles to urban development are probably the least studied of all classes of vertebrates in urban landscapes (GERMAINE & WAKELING, 2001).

So far there are few studies on species composition and distribution of amphibians and reptiles from various cities in Europe (HAMMER & MCDONNELL, 2008; MCKINNEY, 2008), while in Bulgaria they are extremely scarce (MILCHEV, 1985; UNDZHIYAN, 2000; DELOV *et al.*, 2005; MOLLOV, 2005a,b; MOLLOV & VALKANOVA, 2009; MOLLOV *et al.,* 2009; VALKANOVA *et al.,* 2009). The purpose of this study is to present the contemporary species composition and spatial distribution of the amphibians and reptiles in the city of Plovdiv and to trace the dynamics of their localities and frequency of occurrence in time and determine the Important Herpetological Areas in the city.

Material and methods

For the purposes of the current study a series of observations were conducted in the period from March to October 2007-2009 in the city of Plovdiv (South Bulgaria) and its surroundings. Study area covers 127 km^2, calculated from the UTM map of Bulgaria (10x10 km). The borders of the research area are identified on the basis of a 1-kilometer UTM grid (10x10 km standard quadrants are divided into 100 smaller quadrants of size 1x1 km) (LERER & DELCHEV, 1978). Thus, the study area includes the administrative boundaries of the city (53 km^2) and the surrounding areas, excluding other urban areas (Fig. 1).

The distribution of the established species of amphibians and reptiles is presented with UTM codes of the 1-kilometer grid. The logic of the codes is the same as in the formation of the 10-kilometer quadrants, and the number of each 1x1 km quadrant follows the number of the main 10x10 km quadrant.

Amphibians and reptiles were determined visually using the field guide of ARNOLD & OVENDEN (2002) and BISERKOV *et al.* (2007). For each species are given a valid Latin and common name after BISERKOV *et al.* (2007) and SPEYBROECK & CROCHET (2007). Each observed amphibian and reptile was identified at the species level and its exact position was marked using a hand-held GPS unit (Garmin Etrex Vista HCx, manufacturer specified accuracy ± 3 m). In some cases observed amphibians and reptiles were captured by hand or using a net, loops, etc. for the more precise identification and released at the same place. Some specimens were identified by the sounds they make, their eggs or larvae and skin sheds.

The Index of distribution of the species was calculated using the formula:

$$A = \frac{N}{S}.100\,,$$

where A is the Index of distribution, N is the number of squares in which the species was encountered, and S is the total number of squares in the study territory (DYAKOV, 1970; PETROV & MICHEV, 1986).

We identified Important Herpetological Areas (IHA) in the study area, based on number of localities and number of species detected in a given territory. Polygons were generated using the Kernel Index from Hawth's analysis tools extension for ArcGIS 9.3 (BEYER, 2004).

Results and Discussion

In the current study we identified a total of 6 species of amphibians in the study area, which represents 31.58% of the Bulgarian batrahofauna and 9 species of reptiles, which is 24.32% of the Bulgarian herpetofauna. In the literary data for the period 1905-2005, for the research area were reported a total of 10 amphibian and 17 reptile species (Table 1). For comparison, in Sofia city eight species of amphibians are recorded (MILCHEV, 1985), in Varna - 8 species of amphibians and 14 reptiles (DELOV *et al.*, 2005), for Ruse 7 species of amphibians and 13 reptile species were reported (UNDZHIYAN, 2000).

Species composition and distribution

Classis Amphibia (Amphibians)

Triturus karelinii (Strauch, 1870) - Southern Crested Newt

This species is reported for the first time by KOVACHEV (1912) for the Maritsa River in Plovdiv, and later by BURESH & TSONKOV (1941) for "Swamps along Maritsa River in Plovdiv (22.II.1930)". In December 1957 the species was discovered near Komatevo Village

near Plovdiv by ANGELOV & KALCHEV (1961). Since then, the Southern Crested Newt is not detected again in the city (it was not registered in this study as well) and in our view has disappeared from the study area.

Lissotriton vulgaris (L., 1758) - Common (Small) Newt

The common newt is reported for the first time for Plovdiv by KOVACHEV (1905, 1905b, 1912) from swamps along the Maritsa River in the city. Then this species is mentioned only in the fifties of last century by ANGELOV (1960a) and ANGELOV & KALCHEV (1961) in the area "Ostrova" (now Park "Otdih i kultura") in the outskirts of the city. After these reports the common newt was not found again in the city (it was not registered in this study either) and in our view has disappeared from the research area.

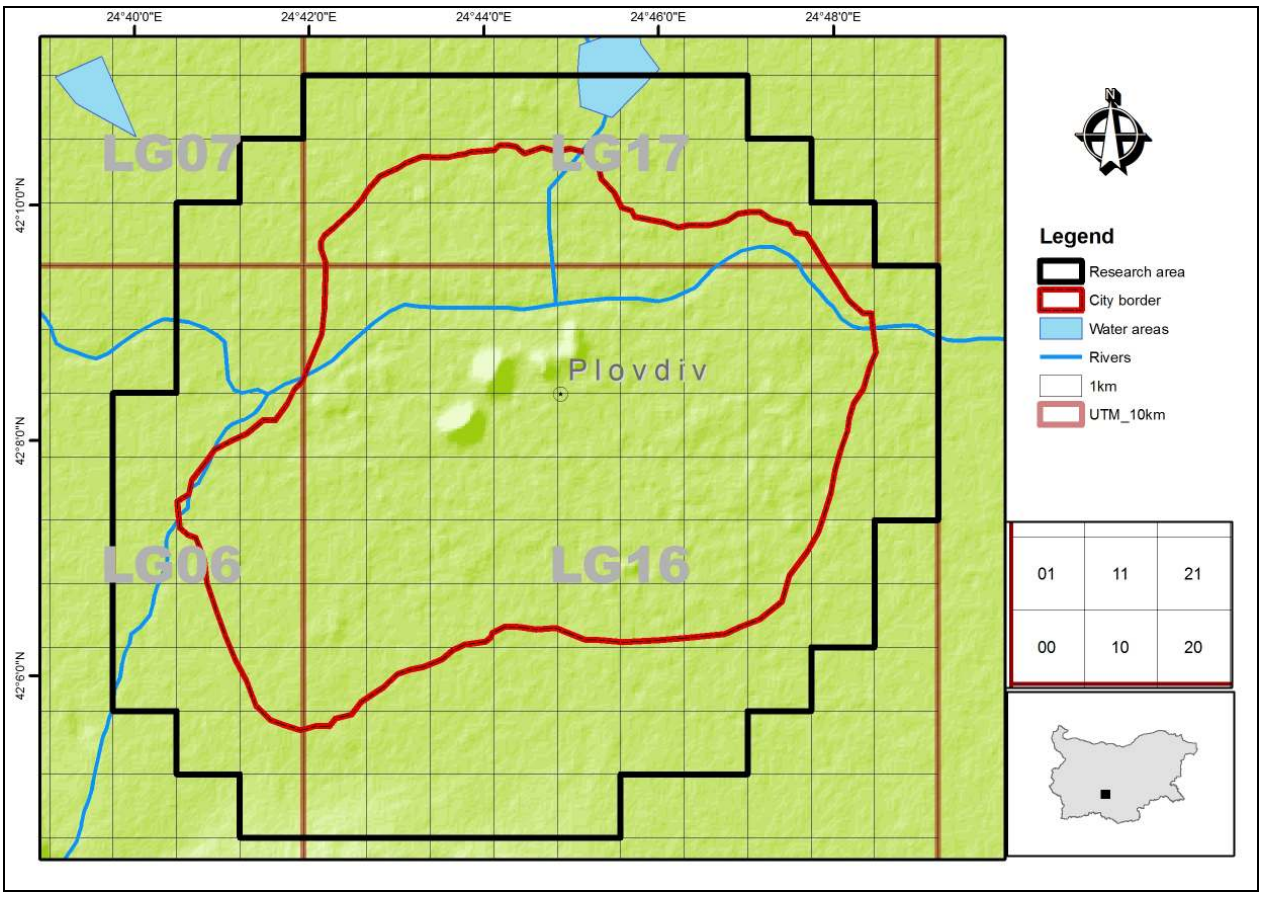

Fig. 1. UTM grid of the studied region – the city of Plovdiv and its surroundings. Scale of the UTM quadrants – 1x1 km.

Bombina bombina (Linnaeus, 1761) – Fire-bellied Toad

The species is reported for the city by KOVACHEV (1912) and later by BURESH & TSONKOV (1942) for Komatevo Village near Plovdiv (18.IV.1932). In May and October 1960 the species was found "near the village Komatevo" and "near the "Ostrova" area in Plovdiv" by ANGELOV & KALCHEV (1961). Since then the Fire-bellied Toad is not registered in the city again (and it was not found in this study). The nearest locality present to the study area is Radinovo Village - LG0754 02.VIII.2005 (Georgiev, Plovdiv, pers. comm., 2005).

Bombina variegata (Linnaeus, 1758) - Yellow-bellied Toad

The species is reported from the "Ostrova" area in Plovdiv by ANGELOV (1960a) and DONEV (1984c) and the southern suburbs of the city of ANGELOV (1960b) and ANGELOV &

Table 1. Species composition of the recorded from literary data and during the current study amphibians and reptiles in the study area.

№	Species	Species, reported by literary data (1905-2005)			Species, recorded in the current study (2007-2009)		
		N	U	A	N	U	A
Amphibia							
1.	*Triturus karelinii* (Strauch, 1870)	3	3	2.36	0	0	0
2.	*Lissotriton vulgaris* (L., 1758)	5	4	3,15	0	0	0
3.	*Bombina bombina* (L., 1761)	4	3	2.36	0	0	0
4.	*Bombina variegata* (l., 1758)	4	3	2.36	0	0	0
5.	*Bufo bufo* (L., 1758)	4	3	2.36	3	3	2.36
6.	*Epidalea viridis* (Laurenti, 1768)	12	9	7.09	150	11	8.66
7.	*Pelobates syriacus* Boettger, 1889	2	2	1.57	1	1	0.79
8.	*Rana dalmatina* Bonaparte, 1840	4	3	2.36	5	4	3,15
9.	*Pelophylax ridibundus* (Pallas, 1771)	6	4	3,15	426	54	42.52
10.	*Hyla arborea* (L., 1758)	5	4	3,15	21	11	8.66
Reptilia							
1.	*Mediodactylus kotschyi (Steindachner, 1870)*	10	10	7.87	132	16	12.60
2.	*Ablepharus kitaibellii* Bibron et Bory, 1833	1	1	0.79	0	0	0
3.	*Lacerta trilineata* Bedriaga, 1886	7	5	3.94	7	7	5.51
4.	*Lacerta viridis* (Laur., 1768)	6	4	3,15	61	31	24.41
5.	*Podarcis muralis* (Laur., 1768)	1	1	0.79	0	0	0
6.	*Podarcis tauricus* (Pallas, 1814)	7	7	5.51	38	10	7.87
7.	*Emys orbicularis* (L., 1758)	3	3	2.36	11	9	7.09
8.	*Trachemys scripta elegans* (Wied-Neuwied, 1839)	0	0	0	2	2	1.57
9.	*Testudo graeca* L., 1758	2	2	1.57	0	0	0
10.	*Testudo hermanni* (Gmelin, 1789)	2	2	1.57	0	0	0
11.	*Dolichophis caspius* Gmelin, 1789	1	1	0.79	12	11	8.66
12.	*Coronella austriaca* Laur., 1768	1	1	0.79	0	0	0
13.	*Zamenis longissimus* (Laurenti, 1768)	2	2	1.57	0	0	0
14.	*Elaphe sauromates* (Pallas, 1814)	2	2	1.57	0	0	0
15.	*Natrix natrix* (L., 1758)	5	5	3.94	5	5	3.94
16.	*Natrix tessellata* (Laur., 1768)	3	3	2.36	9	9	7.09
17.	*Vipera ammodytes* (L., 1758)	1	1	0.79	0	0	0

Legend: N – number of localities, U – number of UTM quadrants (1x1 km), A – distribution index.

KALCHEV (1961). In this study the Yellow-bellied Toad was not registered in the study area, but was found north of Parvenets Village - LG0661 and LG0662, 02.VIII.2005 (Georgiev, Plovdiv, pers. comm., 2005).

Bufo bufo (Linnaeus, 1758) – Common Toad

The species is rare for Plovdiv. In the literature is reported by KOVACHEV (1912), ANGELOV (1960), ANGELOV & KALCHEV (1961) and MOLLOV (2005b). In the present study was found in 3 localities in 3 UTM quadrants (Appendix 1).

Epidalea viridis (Laurenti, 1768) - Green Toad

The Green Toad is one of the most common amphibian species in the city. In literature is reported by CYREN (1941), BURESH & TSONKOV (1941) ANGELOV (1960a, b), ANGELOV & KALCHEV (1961), EUZET *et al.*

(1974), MOLLOV (2005a,b) and VALKANOVA *et al.* (2009). In this study the species was recorded in 150 localities in 11 UTM quadrants (Appendix 1).

Pelobates syriacus Boettger, 1889 - Syrian Spadefoot Toad

From all identified amphibian species the Syrian Spadefoot Toad is the rarest. From the city it has been reported so far only by ANGELOV & KALCHEV (1961) for the "Ostrova" area and DŽUKIĆ *et al.* (2008) for The State Fishery Pond. Detailed examination of the distribution of the species in the city and the country has been made in our previous study (MOLLOV *et al.* 2007), which includes the only locality found in this study period - LG1629.

Pelophylax ridibundus (Pallas, 1771) – Marsh Frog

This is the most common amphibian in the city. So far, from literary data it has been reported only from the outskirts of Plovdiv by ANGELOV (1960a, b), BACHVAROV (1968), POPOV (1973, 1975), DONEV (1984, 1986). In the present study was recorded throughout the studied area with a total of 426 localities in 54 UTM quadrants (Appendix 1).

Rana dalmatina Bonaparte, 1839 – Agile Frog

The Agile Frog is a relatively rare species in the study area and currently in the literature is reported only from around the city (near the "Ostrova" are and Komatevo) by ANGELOV (1960), ANGELOV & KALCHEV (1961) and BACHVAROV (1980). In this study the species is also found only in the outskirts of the city with a total of 5 localities in 4 UTM quadrants (Appendix 1). In the Bulgarian herpetological literature there is a report for the Common Frog (*Rana temporaria*) for the city of Plovdiv from the KOVACHEV (1905b). In our opinion, this report concerns the Agile Frog or it is a mistake. Due to the low altitude of the city, finding a Common Frog in it is quite improbable.

Hyla arborea (Linnaeus, 1758) - Tree Frog

The tree frog has been reported as "common" species from the outskirts of Plovdiv by ANGELOV (1960a, b) and ANGELOV & KALCHEV (1961) and the hills of Plovdiv by MOLLOV (2005a). In this study the species was found only in the outskirts of the city and in Mladezhki Halm Hill in the city center with a total of 21 localities in 11 UTM quadrants (Appendix 1).

Classis Reptilia (Reptiles)

Testudo graeca Linnaeus, 1758 – Spur-thighed Tortoise

The Spur-thighed Tortoise has been reported for the "outskirts of Plovdiv" by BURESH & TSONKOV (1933) and the "Ostrova" Area in Plovdiv by ANGELOV (1960). The species was recorded in the city on the Halm na osvoboditelite Hill (LG1628) on 13.IV.1999 (Nikolov, Plovdiv, pers. comm., 1999) and in a open meadow near arable land and farm buildings east of Trakia Quarter (LG1687) on 21.VI.2000, (Irikov, Plovdiv, pers. comm., 2000), but was not recorded in this study. According to BESHKOV & NANEV (2002) in many places in the Thracian Valley tortoises are destroyed by the human activity. Finding tortoises in the research area in our opinion should be considered accidental because most likely these are cases of specimens collected from other places in Bulgaria, kept alive as souvenirs and then released in various places in the city.

Testudo hermanni (Gmelin, 1789) - Hermann's Tortoise

The Hermann's tortoise has been reported by SHKORPIL (1897) for Plovdiv and one indeterminate species (*Testudo sp.*) by DRENSKI (1955). The species was registered in the city of Plovdiv on 12.V.2000 in the area of The Rowing Channel – LG0697 (Kirov, Ruse, pers. comm., 2000), but not in this study. According to BESHKOV & NANEV (2002) this species is absent in many places in the lowlands of northern Bulgaria and Thrace, which was destroyed by modern agriculture and the general modification of the landscape. We assume that the recorded specimens in Plovdiv are probably released tortoises, which had previously been kept alive as souvenirs collected from other parts of Bulgaria.

Emys orbicularis (L., 1758) - European Pond Turtle

The European Pond turtle has been reported for "Maritsa River in Plovdiv" by KOVACHEV (1910), "near the "Ostrova" area in Plovdiv" by ANGELOV (1960) and State Fisheries – Plovdiv by KIRIN (2001). In the current study it was recorded with a total of 11 localities in 9 UTM quadrants (Appendix 2).

Trachemys scripta elegans (Wied-Neuwied, 1839) – Red-eared Slider

The Red-eared Slider is a decorative freshwater turtle species, sold in pet shops as a pet. Unfortunately, when the turtles become too large to be kept in captivity, owners release them in different places in the country. Because of the high ecological plasticity of the species it successfully survives in natural conditions and that is why it has become a potentially dangerous invasive species in Europe (CADI & JOLY, 2004). So far there are two records of Red-eared sliders in Plovdiv. The first one is from 2004 when a specimen was spotted on the banks of Maritsa River near the bridge of the International fair - LG1649 (Dulev, Plovdiv, pers. comm., 2004) and the second was registered in this study - 14.07.2008 – a couple (male and female), released in a fountain in the garden behind the Natural History Museum (LG1648).

Mediodactylus kotschyi (Steindachner, 1870) – Kotschyi's Gecko

This is an extremely common and widespread species in Plovdiv (SHKORPIL, 1897; KOVACHEV, 1905b; 1910; 1912; BURESH & TSONKOV, 1933; MÜLLER, 1940; MOLLOV, 2005a). In Plovdiv is spread the endemic for Bulgaria subspecies "*rumelicus*" (BESHKOV & NANEV, 2002). In the current study the species was registered with 132 localities in 16 UTM quadrants (Appendix 2).

Ablepharus kitaibellii Bibron et Bory, 1833 - European Copper Skink

The European Copper Skink was found for the first time at Mladezhki Halm Hill in Plovdiv in a previous study (MOLLOV, 2005a) with two localities of the southern part of the hill on 31.III.2003 and in eastern on 02.V.2003. Unfortunately, the species was not re-recorded in this study.

Lacerta trilineata Bedriaga, 1886 – Balkan Green Lizard

This lizard was reported in and around the city center by KOVACHEV (1907, 1912), BURESH & TSONKOV (1933), ANGELOV *et al.* (1966) and MOLLOV (2005a). In the current study it was found only in the outskirts of the city with total 7 localities in 7 UTM quadrants (Appendix 2).

Lacerta viridis (Laurenti, 1768) - Green Lizard

This is the most common lizard species in the research area. It was reported by BURESH & TSONKOV (1933), ANGELOV (1960), ANGELOV *et al.* (1966), DONEV (1984d) and MOLLOV (2005a). In this study it was recorded with total of 61 localities in 31 UTM quadrants (Appendix 2).

Podarcis muralis (Laurenti, 1768) – Common Wall Lizard

This species is a typical petrophile – it inhabits only rocky and stony terrains. Therefore in the research area it was recorded only at two of the Plovdiv hills (KOVACHEV, 1905b; MOLLOV, 2005a). It seems that this species in Plovdiv is isolated from the other populations, but it exists due to the presence of near-natural conditions at the hills. The species was not recorded in the current study.

Podarcis tauricus Pallas, 1811 – Balkan Wall Lizard

The Balkan Wall Lizard is a relatively common species both in the urban and rural parts of Plovdiv (KOVACHEV, 1912; CYRÉN, 1933; BURESH & TSONKOV, 1933; DONEV, 1984b; MOLLOV, 2005a; MOLLOV & VALKANOVA, 2009). In the present study was established with a total of 38 localities in 10 UTM quadrants (Appendix 2).

Dolichophis caspius (Linnaeus, 1758) - The Caspian Whipsnake

This is the most common snake species in the research area. In this study it was recorded with 12 localities in 11 UTM quadrants (Appendix 2).

Coronella austriaca Laurenti, 1768 – Smooth Snake

So far, the species is reported for Plovdiv only by KOVACHEV (1905b, 1912). MOLLOV (2005a) reported finding a skin-shed of a young smooth snake at Mladezhki Halm Hill, but later when re-defining the material is found that the skin-shed belongs to a Caspian Whipsnake (*D. caspius*). In our opinion the Smooth snake has disappeared from the city limits.

Zamenis longissimus (Laurenti, 1768) - Aesculapian Snake

So far, the Aesculapian snake is reported for Plovdiv only by KOVACHEV (1912) and BURESH & TSONKOV (1934). Since then, the species has not been found again. It wasn't recorded in the current study.

Elaphe sauromates (Pallas, 1814) - Blotched Snake

Up to this moment the species is reported to the outskirts of Plovdiv only KOVACHEV (1912) and BURESH & TSONKOV (1934). Since then, not been found again and was not recorded in this study.

Natrix natrix (Linnaeus, 1758) - Grass Snake

The Grass snake has limited distribution in the city. So far the species is reported for the outskirts of Plovdiv by KOVACHEV (1912), BURESH & TSONKOV (1934), ANGELOV (1960), BACHVAROV (1969) and KIRIN (1994b). In the present study it was found in a total of 5 localities in 5 UTM quadrants (Appendix 2).

Natrix tessellata (Laurenti, 1768) - Dice Snake

This species occurs more frequently than the Grass snake. So far for Plovdiv it has been reported by KOVACHEV (1912), ANGELOV (1960) and KIRIN (1994a). In the present study it was found in a total of 9 localities in 9 UTM quadrants (Appendix 2).

Vipera ammodytes (Linnaeus, 1758) – Long-nosed Viper

The Long-nosed viper has been reported for Plovdiv only by KOVACHEV (1905b, 1912). It was not recorded in this study.

Retrospective analysis of the batraho- and the herpetofauna in the study area

The results of our study on the species composition and distribution of amphibians and reptiles in Plovdiv show a significant change in the last 100 years when comparing them with existing literary data.

From a total of 10 species of amphibians reported for the city of Plovdiv, in this study were found only 6. For the reptiles of 17 species reported for the city in this study were found only 9. In both groups, occurred around 50% reduction in the number of species. To trace the changes in the frequency of occurrence of amphibians and reptiles in the study area in time, we conducted an analysis of available data from the available literature sources (Table 2), using the calculated index of distribution. This enables us to gain even a vague idea about the state of the species of amphibians and reptiles in the past and compare it with their present condition. From the analysis of these data we could make some predictions for the future existence of the amphibians and reptiles in urban environment.

Of all recorded amphibian species four are increasing their frequency of occurance (Table 2), but only two were distributed almost throughout the whole study region and are relatively numerous - *Pelophylax ridibundus* and *Epidalea viridis*. One of the positive circumstances, which encouraged the widespread of the Marsh Frog, is the species' preference to the slow flowing waters. The presence of the Maritsa River and Parvenetska River and a dense network of irrigation canals in the outskirts of the city have played a peculiar role of corridors for the distribution of species in the urban area. Moreover, the high ecological plasticity of the species is well known and unlike most species of amphibians *Pelophylax ridibundus* can be found even in highly polluted water (LEONTEVA & SEMENOV, 1999). The Green Toad on the other hand is relatively drought-loving species and it uses for breeding small temporary ponds, which also abundant in the city. These circumstances and the fact that it is "explosive" breeding species and unlike the Common Toad (*Bufo bufo*) is not attached to the place of the laying of the eggs (KÜHNEL & KRONE, 2003), further

contributes to the prevalence of this species in the city.

Aside from both newt species and yellow- and fire-bellied toads both considered missing from the study area, the state of the remaining amphibian species remained relatively unchanged.

Table 2. Retrospective analysis of the batracho- and herpetofauna in the studied area

Species	Distribution index (A) - past state (1905-2005)	Dynamics	Distribution index (A) - current state (2007-2009)
Amphibia			
Triturus karelinii	2.36	disappearing	0
Lissotriton vulgaris	3,15	disappearing	0
Bombina bombina	2.36	disappearing?	0
Bombina variegata	2.36	disappearing?	0
Bufo bufo	2.36	no change	2.36
Epidalea viridis	7.09	increasing	8.66
Pelobates syriacus	1.57	decreasing	0.79
Rana dalmatina	2.36	increasing	3,15
Pelophylax ridibundus	3,15	increasing	42.52
Hyla arborea	3,15	increasing	8.66
Reptilia			
Mediodactylus kotschyi	7.87	increasing	12.60
Ablepharus kitaibelii	0.79	decreasing?	0
Lacerta trilineata	3.94	increasing	5.51
Lacerta viridis	3,15	increasing	24.41
Podarcis muralis	0.79	decreasing?	0
Podarcis tauricus	5.51	increasing	7.87
Emys orbicularis	2.36	increasing	7.09
Trachemys scripta	0	accidental	1.57
Testudo graeca	1.57	accidental	0
Testudo hermanni	1.57	accidental	0
Dolichophis caspius	0.79	increasing?	8.66
Coronella austriaca	0.79	disappearing	0
Zamenis longissimus	1.57	disappearing	0
Elaphe sauromates	1.57	disappearing	0
Natrix natrix	3.94	no change	3.94
Natrix tessellata	2.36	increasing	7.09
Vipera ammodytes	0.79	disappearing	0

Seven species of reptiles (*Mediodactylus kotschyi, Lacerta viridis, Lacerta trilineata, Podarcis tauricus, Emys orbicularis, Dolichophis caspius* and *Natrix tessellata*) are widely available in town and are in stable condition (Table 2). It should be noted that the distribution of the Balkan gecko in the city of Plovdiv is probably much wider than the recorded in this study, because the species occupied buildings, which are not accessible and it is difficult to record its presence.

The situation with the Grass snake (*Natrix natrix*) and the Dice snake (*Natrix tessellata*) in the city is interesting. In the current study, we record no change of the localities and frequency of occurrence of the

Grass snake and increase in the Dice snake. This may be due to the fact that *Natrix natrix* requires relatively large portions of unfragmented habitats and stable food base - mainly fish and frogs (LEONTEVA & SEMENOV, 1999), which are available mainly along Maritsa River and some parts in the outskirts of the city. This limits the speacie's distribution within the city, while the Dice snake occurs in irrigation canals and other more unsuited water bodies and it seems it has a higher ecological plasticity than the Grass snake.

The larger and easier to spot, thus more frequently becoming victims of humans, representatives of the snakes like *Coronella austriaca, Zamenis longissimus, Elaphe sauromates* and *Vipera ammodytes,* which have more specific habitat requirements are missing from the territory of the city.

The occurance of the two tortoise species and the Red Eared Slider in the city should be considered as accidental.

Important herpetological areas in the study region

Based on the number of localities and number of species detected, using the Kernel Index we identified the Important Herpetological Areas in the study region (Fig. 2).

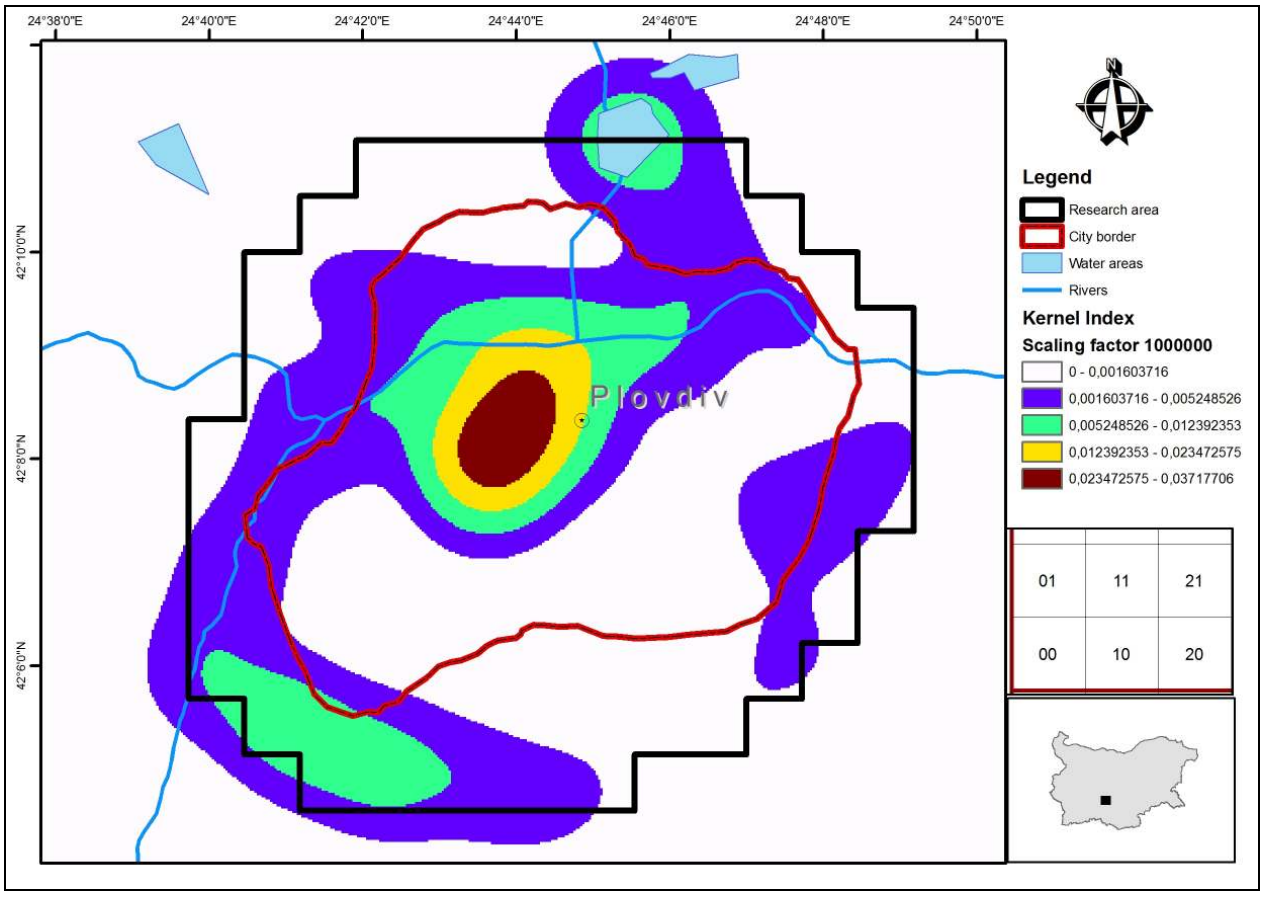

Fig. 2. Important Herpetological Areas in the study region.

Contrary with most urban studies on amphibian and reptile fauna, where the species richness declines form the rural parts of the city to its center (HAMMER & MCDONNELL, 2008; MCKINNEY, 2008), we identified the most number of species with the largest number of localities in the center of the city of Plovdiv. This is due to the position of the Hills of Plovdiv – areas which resemble natural conditions more closely which are in fact in the center of the city (MOLLOV, 2005). Along with that the center section of Maritsa River and the lower section of Parvenetska River offer good conditions for the spread of

amphibians and some reptiles. The irrigation cannals in the southern and eastern part of the city along with the State Fishery Ponds in the north are also playing significant role in the distribution of the amphibians and reptiles in the urbanized territory.

Most of the identified IHAs are with the boundaries of the three protected territories in the center of the city and with NATURA2000 sites. Nevertheless conservation measures concerning the amphibians and reptiles within the city of Plovdiv should be applied with priority for these areas.

Conclusions

In summary, from the data from retrospective analysis of the batraho- and the herpetofauna in the research area can be drawn the following conclusions:

- 4 species of amphibians (*Triturus karelinii, Lissotriton vulgaris, Bombina bombina, B. variegata*) and 4 reptiles (*Coronella austriaca, Zamenis longissimus, Elaphe sauromates, Vipera ammodytes*) probably have disappeared from the research area for the past 100 years;

- 1 species of amphibians (*Bufo bufo*) and 1 reptile species (*Natrix natrix*) have not changed their status over the past 100 years;

- 1 amphibian species (*Pelobates syriacus*) and 2 reptile species (*Ablepharus kitaibelii, Podarcis muralis*) have reduced their frequency of occurrence and the last 100 years;

- 4 amphibian species (*Epidalea viridis, Rana dalmatina, Pelophylax ridibundus, Hyla arborea*) and 7 reptile species (*Mediodactylus kotschyi, Lacerta trilineata, Lacerta viridis, Podarcis tauricus, Emys orbicularis, Dolichophios caspius, Natrix tessellata*) have increased their localities and frequency of occurrence for the past 100 years.

- The finding of the two tortoises in the city, and the presence of Red-eared Slider should be considered as accidental.

In our opinion, monitoring of the status and distribution of the batraho- and the herpetofauna in Plovdiv, and in other cities in the country is an important aspect of environmental studies as the results of such studies could help to take appropriate conservation measures for these species in urban environments.

Acknowledgements

This study is part of the project № MU-1/2008 – "Faunistic and ecological characteristics of amphibians and reptiles in the city of Plovdiv", funded by the NAP at the University of Plovdiv. The authors would like to express his sincerest gratitude to Dr. Georgi Popgeorgiev for his help on the maps and GIS analysis and his notes on the manuscript.

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Appendix 1. Amphibian distribution in the city of Plovdiv and its surroundings, registered in the current study based on UTM grid 1x1 km.

Bombina bombina - LG0754; *Bombina variegata* - LG0661, LG0662; *Bufo bufo* - LG1732, LG1608, LG1676; *Bufo viridis* - LG1730, LG1624, LG1626, LG1627, LG1628, LG1636, LG1637, LG1638, LG1648, LG1674, LG1675; *Pelobates syriacus* - LG1629; *Pelophylax ridibundus* - LG0790, LG1700, LG1710, LG1720, LG1750, LG1760, LG1770, LG1741, LG1751, LG1761, LG1752, LG1762, LG0693, LG0697, LG0698, LG0683, LG0684, LG0685, LG0686, LG0687, LG0688, LG0689, LG0674, LG0675, LG0676, LG1602, LG1603, LG1608, LG1609, LG1612, LG1613, LG1617, LG1618, LG1619, LG1622, LG1623, LG1629, LG1632, LG1639, LG1642, LG1649, LG1659, LG1663, LG1664, LG1669, LG1674, LG1675, LG1676, LG1679, LG1686, LG1687, LG1689, LG1697, LG1698; *Rana dalmatina* - LG0674, LG0675, LG1685, LG1686; *Hyla arborea* - LG0697, LG0674, LG0675, LG1621, LG1627, LG1686, LG1697, LG1700, LG1733, LG1743, LG1751.

Appendix 2. Reptilian distribution in the city of Plovdiv and its surroundings, registered in the current study based on UTM grid 1x1 km.

Testudo graeca - LG1628, LG1687; *Testudo hermanni* - LG0697; *Emys orbicularis* - LG1710,

LG1720, LG1762, LG1783, LG1607, LG1608, LG1619, LG1629, LG1649; *Trachemys scripta elegans* - LG1649, LG1648; *Mediodactylus kotschyi* - LG1616, LG1617, LG1626, LG1627, LG1628, LG1629, LG1636, LG1637, LG1638, LG1639, LG1649, LG1658, LG1659, LG1665, LG1676, LG1678; *Lacerta trilineata* - LG0674, LG0675, LG0687, LG1607, LG1608, LG1676, LG1686; *Lacerta viridis* - LG0692, LG0693, LG0697, LG0698, LG0683, LG0684, LG0687, LG0673, LG0674, LG0675, LG0676, LG1602, LG1603, LG1607, LG1612, LG1613, LG1622, LG1627, LG1629, LG1638, LG1639, LG1643, LG1649, LG1667, LG1675, LG1686, LG1697, LG1700, LG1741, LG1751, LG1760; *Podarcis tauricus* - LG0687, LG1616, LG1617, LG1625, LG1626, LG1627, LG1628, LG1667, LG1675, LG1678; *Dolichophis caspius* - LG0693, LG0694, LG0695, LG0696, LG1608, LG1612, LG1617, LG1627, LG1656, LG1657, LG1659; *Natrix natrix* - LG0689, LG0674, LG1629, LG1686, LG1752; *Natrix tessellata* - LG0675, LG0689, LG1602, LG1608, LG1611, LG1619, LG1629, LG1639, LG1752.

Received: 15.08.2010
Accepted: 25.10.2010

Union of Scientists in Bulgaria – Plovdiv
University of Plovdiv Publishing House

ECOLOGIA BALKANICA

2010, Vol. 2 December 2010 pp. 39-56

The Effects of Gap Disturbance on Soil Chemical and Biochemical Properties in a Mixed Beech – Hornbeam Forest of Iran

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Abstract. The present study aimed to examine the impacts of small (85.12 m²), medium (325.21 m²), large (512.11 m²) and very large (723.85 m²) gaps on soil properties in a mixed beech – hornbeam stand of northern Iran. As well as the value of pH, organic matter and carbon, total nitrogen, cation exchange capacity (CEC), phosphorus, potassium, calcium, nitrogen mineralization, microbial respiration, earthworms density and biomass of soil samples (0 – 15, 15 – 30 and 30 - 45 cm depths from gap center, edge and closed canopy positions) were measured in laboratory. Significantly effects of gaps size were found on soil properties as the highest values of CEC, density and biomass of earthworms observed in small gaps. The highest values of carbon to nitrogen ratio, phosphorus, potassium and calcium were detected in medium gaps. The most amounts of organic matter and carbon, nitrogen mineralization devoted in very big gaps. Greater amounts of pH, total nitrogen and microbial respiration were detected in big and very big gaps. It was found that gap position had a profound effect on soil characters as the highest values of pH, organic matter and carbon, total nitrogen, phosphorus, potassium, calcium, nitrogen mineralization, microbial respiration observed in gap center. The greatest amounts of carbon to nitrogen ratio, CEC, density and biomass of earthworms were detected in closed canopy. According to the results, soil upper layers showed the highest values organic matter and carbon, soil nutrition elements and biochemical activities, whereas the highest amounts pH, carbon to nitrogen ratio, CEC and density and biomass of earthworms were observed in deeper soil. It is concluded that opening areas of canopy cover should be less than 400 m² in beech forests of northern Iran with considering of soil properties.

Keywords: gap size, gap position, nutrient availability, earthworm, soil.

Introduction

Beech (*Fagus orientalis* Lipsky) is one of the most important forest species in the temperate broad - leaf forest biome and represents an outstanding example of the re - colonization and development of terrestrial ecosystems and communities after the last ice age, a process which is still ongoing (MOSADEGH, 2000; MARVIE MOHADJER, 2007). In the north of Iran, pure and mixed oriental beech forests cover 17.6 per cent of the surface land area and represent 30 per cent of the standing biomass. Beech is the most valuable wood – producing species in the Caspian forests (RESANEH *et al.*, 2001). The beech trees are found in small groups up to 500 m a.s.l. while individuals have been reported from 110 m up to 2650 m. At low altitudes, they occur mixed with hornbeam (*Carpinus betulus* L.) (MARVIE MOHADJER, 2007).

Disturbance is ubiquitous in forest ecosystems. Defined as "any relatively discrete event in time that disrupts ecosystems, community or population structure and changes resources, substrate availability, or the physical environment", disturbance determines forest species composition, structure, and process. Furthermore, disturbances exert their influence over a wide range of temporal and spatial scales. This disturbance, in turn, promotes changes in resource fluxes, changes that lead to some form of reorganization of the disturbed patch or gap at structural and functional levels that may be similar or dissimilar to pre - disturbance levels. Resource levels and inputs are changed and species respond accordingly (MCCARTHY, 2001; SAMONIL *et al*., 2009; JONASOVA *et al*., 2010; KATHKE & BRUELHEIDE, 2010).

In Iran beech forests, formation of gaps by wind throw is a characteristic natural disturbance event. Gap size varies greatly from the size of only a single crown to vast open fields with diameters of many tree lengths. However, changes in abiotic and biotic conditions depend both on gap size and within - gap position (HOLEKSA, 2003; KWIT & PLATT, 2003). Consequently, it is not easy to predict how soil properties react to gap formation. Disturbances caused by canopy gaps received much attention in the last decades and they are regarded as important factors in forest dynamics. Canopy openings as a result of tree falls create an environment different from the adjacent forest, which influences plant regeneration. In addition, gap processes partly determine forest structure and play an important role to maintain plant species richness. Thus, the creation of gaps in forests is an opportunity for the system to change in both species dynamics and ecological processes (MUSCOLO *et al*., 2007).

Although it is recognized that gaps modify soil resources, few studies have focused on below - ground processes (GRAY *et al*., 2002). Soil processes are controlled by a set of relatively independent state factors (climate, organisms, relief, parent material and time) and by a group of interactive controls (e.g., disturbance regime and human activities) (JENNY, 1994). Forest gaps are examples of natural interactive controls with direct impacts on state factors (e.g., climate and organisms). Forest gaps represent dramatic top - down trophic interactions between vegetation and the soil microbial - mediated processes (SCHARENBROCH & BOCKHEIM, 2007).

Most studies of gaps have addressed vegetation dynamics, regeneration through seedling establishment, effects of microclimate variables on the regeneration and, in general have concentrated on aboveground processes (RITTER *et al*., 2005; MUSCOLO *et al*., 2007). Relatively few studies have addressed belowground effects of canopy gaps on soil chemical and biochemical properties. Thus, the objectives of this study were to examine the impacts of small, medium, large and very large gaps on soil properties, a "sink" and "source" of plant nutrients in beech - hornbeam stands in northern Iran. The present paper assesses experimentally the effects of gap creation on soil nutrient supply. Specifically, the following hypotheses were tested: is gap size an important factor in controlling nitrogen mineralization, microbial respiration and consequently organic matter breakdown and nutrient amounts? The study included investigation of soil properties and biological activities about 4 years after gaps creation.

Material and methods

Site characteristics. This research was conducted in Tarbiat Modares University Experimental Forest Station located in a temperate forest of Mazandaran province in the north of Iran, between 36˚ 31ó 56˝ N and 36˚ 32´ 11˝ N latitudes and 51˚ 47´ 49˝ E and 51˚ 47´ 56˝ E longitudes. The maximum elevation is 1700 m and the minimum is 100m. Minimum temperature in December (6.6˚C) and the highest temperature in June (25˚C) are recorded, respectively. Mean annual precipitation of the study area were from 280.4 to 37.4 mm at the Noushahr city metrological station, which is 10 km far from the study area. For performing this research, 20 ha areas of reserve parcel (relatively undisturbed) considered that was covered by *Fagus orientalis* and *Carpinus betulus* dominant stands. This limitation had an inclination 60 - 70 percent with northeast exposure at 546 - 648 m a.s.l.

Bedrock is limestone - dolomite with sandy - clay - loam soil texture. Presence of logged and bare roots of trees is indicating rooting restrictions and soil heavy texture (ANONYMOUS, 2002). The current study is based on several wind throw events in the experimental forest station in during 2005 to 2006.

Gap selection. In the summer of 2009, twenty hectare areas of Tarbiat Modares University Experimental Forest Station were considered. Geographical position and all of canopy gaps were recorded by Geographical Position System (GPS). Gaps required a minimum canopy opening of 30 m^2 and trees growing in the gap to be less than two thirds the height of the closed adjacent forest (RUNKLE, 1992). Canopy gaps areas were measured in the field according to RUNKLE (1992). Sampling protocol was built up by locating and measuring two perpendicular lines in each gap: one along the longest line visible and one perpendicular to it at the widest section of the gap.

Soil sampling and analysis. For this purpose, three positions were distinguished including gap center, gap edge and closed canopy. Soil samples were taken at 0 - 15, 15 - 30 and 30 - 45cm depths from all positions using core soil sampler with 81cm^2 cross section (RAHMANI & ZARE MAIVAN, 2004). Roots, shoots and pebbles in each sample were separated by hand and discarded. The air - dried soil samples were sieved (aggregates were crushed to pass through a 2 mm sieve) to remove roots prior to chemical analysis. Soil pH was determined using an Orion Ionalyzer Model 901 pH meter in a 1:2.5, soil: water solution. Soil organic carbon was determined using the Walkey - Black technique (ALLISON, 1975). The total nitrogen was measured using a semi Micro - Kjeldhal technique (BREMNER & MULVANEY, 1982). The available P was determined with spectrophotometer by using Olsen method (HOMER & PRATT, 1961). The available K and Ca (by ammonium acetate extraction at pH 9) were determined with Atomic absorption spectrophotometer (AAS) and Cation Exchange Capacity (CEC) with flame photometer (BOWER *et al.*, 1952). Soil microbial respiration was determined by measuring the CO_2 evolved in 3 days incubation experiment at 25°C, in which 50 g of each soil samples (remoistened to 55% its water holding capacity) were placed in a glass jar. Glass vial holding 10 ml of 0.5 M NaOH was placed in the glass jar to trap the evolved CO_2. The excess alkali, after precipitating the CO_3^{2-} with 0.5 M $BaCl_2$ solution was titrated with standard 0.5 M dequate HCl to a phenolphthalein end point (ALEF, 1995).

Kinetic of nitrogen mineralization was measured using a laboratory incubation procedure under controlled conditions by 100 g of each soil samples. Soil samples were with moisture up to 55% of its water holding capacity. The containers were closed tightly and kept in the dark in a temperature - controlled chamber at 25°C. The samples were re-aerated weekly for adequate oxygen supply. Nitrogen mineralization was estimated from the increase KCl extractable inorganic N after incubating soil samples for 56 days. Initial inorganic N (NO_3-N and NH_4-N) was analyzed before incubation using the steam distillation method (BREMER, 1965) after extraction with 1 M KCl for 2 h (soil: extracting ratio of 1:5). Final inorganic N (NO_3-N and NH_4-N) concentrations were measured at the end of incubation on day 56. Net N-mineralization was calculated by subtracting initial mineral N from final mineral N for each sample (ROBERTSON *et al.*, 1999). The earthworms were collected simultaneously with the soil sampling by hand sorting, washed in water and weighed with milligram precision. Biomass was defined as the weight of the worms after drying for 48 hours on filter paper at oven (60˚C) (EDWARDS & BOHLEN, 1996).

Statistical analysis. Normality of the variables was checked by Kolmogrov - Smirnov test and Levene test was used to examine the equality of the variances. Differences between gap different areas, gap positions and soil depths in soil characteristics were tested with three - way analysis (ANOVA) using the GLM procedure, with areas (small, medium, large and very large), positions (gap center, gap edge and closed canopy) and depth (0 - 15, 15 - 30 and 30 - 45 cm) as independent factor. Interactions between independent factors were tested also. Duncan test was used to separate the averages of the dependent variables which

were significantly affected by treatment. Significant differences among treatment averages for different parameters were tested at P≤ 0.05. SPSS v.11.5 software was used for all the statistical analysis.

Results

Canopy gap characteristics. Twenty one canopy gaps with different areas were detected in study site (Table 1). Gaps classified in four classes: four gaps in 30 - 200 m^2 area class (small gap with area mean of 85.12 m^2), five gaps in 200 - 400 m^2 area class (medium class with area mean of 325.21 m^2), eight gaps in 400 - 600 m^2 area class (large class with area mean of 512.11 m^2) and four gaps in more than 600 m^2 area class (very large class with area mean 723.85 m^2). Result is indicating the most present gaps in study area have 300 - 500 m^2 area.

Table 1. Characteristics of canopy gaps in study area

Gap class (m^2)	Gap number	Gap area mean (m^2)	Minimum and maximum of gap area (m^2)
30 – 200	4	85.12	40.11 – 130.13
200 – 400	5	325.21	260.12 – 390.30
400 – 600	8	512.11	435.22 – 589
> 600	4	723.85	626.12 – 821.58

Soil properties. Soil pH was significantly ($P < 0.01$) higher in very large and large gaps in comparison to small and medium gaps (Table 2 and Fig. 1). The highest value of this character was detected in gap center position and deeper layers of soil (Table 2 and Fig. 1). Organic matter and carbon significantly ($P < 0.01$) increased with increasing size of the gaps, decreased with soil depth and from gap center to closed canopy (Table 2; Fig. 2 and 3). Greater amounts of carbon to nitrogen ratio were found in medium gap, closed canopy position and deeper layers of soil, significantly ($P < 0.01$) (Table 2 and Fig. 4). The greatest value of cation exchange capacity (CEC) resulted in small gap, closed canopy and gap edge position and beneath layers of soil. Significantly statistical differences ($P < 0.01$) were considered for this character (Table 2 and Fig. 5).

Compare mean of total nitrogen in the gap size indicated that large and very large gaps had the higher amounts ($P < 0.01$) than in the small and medium gaps. Gap center position and upper layer of soil had the greatest value of this character in comparison to the other positions and depth (Table 2 and Fig. 6). As can be seen in Table 2 and Fig. 7, the available P was significantly ($P < 0.01$) greater in medium gaps, gap center position and the 0 – 15 cm depth than in the other treatments. Medium gaps, gap center with gap edge position and the first soil depth devoted in the highest amounts ($P < 0.01$) of available K (Table 2 and Fig. 8), whereas the maximum available Ca ($P < 0.01$) was detected in medium gap, gap center position and soil upper layers (Table 2 and Fig. 9).

Gap sizes, position and soil depths were significantly ($P < 0.01$) different in terms of nitrogen mineralization. As Table 2 and Fig. 10 shows, its maximum values were detected in very large gaps, gap center position and soil upper layers. The similar results was observed in soil microbial respiration as the greatest values ($P < 0.01$) devoted in very large and large gaps, gap center position and upper layers of soil (Table 2 and Fig. 11). Earthworm density showed descending trend from small gap to very large and significantly statistical differences ($P < 0.01$) were detected (Table 2 and Fig. 12). The assemblage of earthworms was more in closed canopy ($P < 0.01$) and 30 – 45 cm depth ($P < 0.05$) than in the other position and depths (Table 2 and Fig. 12). Earthworm different biomass was found in gap area classes, positions and soil layers depending on earthworm density changes. Biomass decreased ($P < 0.01$) towards the greater areas of canopy openings (Table 2 and Fig. 13). Closed canopy position and soil deeper layers devoted in the greatest value ($P < 0.01$) of earthworm biomass (Table 2 and Fig. 13).

Table 2. Three - way analysis of soil properties in gap different areas, positions and soil depths.

Soil chara-cter	Variables source	F - Value
pH	Gap area	199.67**
	Gap position	964.59**
	Soil depth	3.39*
	Gap area × Gap position	54.63**
	Gap area × Soil depth	0.05 ns
	Gap position × Soil depth	0.20 ns
	Gap area × Gap position × Soil depth	0.05 ns
Organic matter (%)	Gap area	211.19**
	Gap position	1064.95**
	Soil depth	94.09**
	Gap area × Gap position	50.88 **
	Gap area × Soil depth	0.72 ns
	Gap position × Soil depth	0.42 ns
	Gap area × Gap position × Soil depth	0.45 ns
Organic carbon (%)	Gap area	209.45**
	Gap position	1063.77**
	Soil depth	92.65**
	Gap area × Gap position	50.50**
	Gap area × Soil depth	0.74 ns
	Gap position × Soil depth	0.43 ns
	Gap area × Gap position × Soil depth	0.43 ns
Carbon to nitrogen ratio	Gap area	13.35**
	Gap position	176.26**
	Soil depth	130.45**
	Gap area × Gap position	5.69**
	Gap area × Soil depth	0.58 ns
	Gap position × Soil depth	2.30 ns
	Gap area × Gap position × Soil depth	0.44 ns
CEC ($cmol_{(+)}\ kg^{-1}$)	Gap area	8.27**
	Gap position	9.54**
	Soil depth	16.92**
	Gap area × Gap position	5.32**
	Gap area × Soil depth	1.00 ns
	Gap position × Soil depth	20.78**
	Gap area × Gap position × Soil depth	1.16 ns
Total nitrogen (%)	Gap area	68.29**
	Gap position	526.92**
	Soil depth	211.74**
	Gap area × Gap position	24.40**
	Gap area × Soil depth	0.48 ns
	Gap position × Soil depth	1.89 ns
	Gap area × Gap position × Soil depth	0.50 ns
Available P (mg/kg)	Gap area	32.69**
	Gap position	20.36**
	Soil depth	5.54**
	Gap area × Gap position	3.67**
	Gap area × Soil depth	0.06 ns
	Gap position × Soil depth	0.14 ns
	Gap area × Gap position × Soil depth	0.20 ns
Available K (mg/kg)	Gap area	14.88**
	Gap position	5.42**
	Soil depth	4.80**
	Gap area × Gap position	1.54 ns
	Gap area × Soil depth	0.05 ns
	Gap position × Soil depth	0.07 ns
	Gap area × Gap position × Soil depth	0.03 ns
Available Ca (mg/kg)	Gap area	12.26**
	Gap position	34.63**
	Soil depth	24.92**
	Gap area × Gap position	11.48**
	Gap area × Soil depth	0.17 ns
	Gap position × Soil depth	0.07 ns
	Gap area × Gap position × Soil depth	0.17 ns
Nitrogen mineralization (mg N kg^{-1} soil)	Gap area	20.56**
	Gap position	181.27**
	Soil depth	49.41**
	Gap area × Gap position	9.02**
	Gap area × Soil depth	0.92 ns
	Gap position × Soil depth	0.90 ns
	Gap area × Gap position × Soil depth	1.85*
Soil microbial respiration (mg co_2-c/gr	Gap area	52.04**
	Gap position	453.32**
	Soil depth	180.16**
	Gap area × Gap position	23.23**
	Gap area × Soil depth	0.43 ns
	Gap position × Soil depth	1.38 ns
	Gap area × Gap position × Soil depth	0.58 ns
Earthworm density (number/m^2)	Gap area	28.70**
	Gap position	66.72**
	Soil depth	4.31**
	Gap area × Gap position	2.89**
	Gap area × Soil depth	1.10 ns
	Gap position × Soil depth	1.16 ns
	Gap area × Gap position × Soil depth	1.79 ns
Eartworm biomass (mg/m^2)	Gap area	26.78**
	Gap position	69.85**
	Soil depth	5.23**
	Gap area × Gap position	2.86*
	Gap area × Soil depth	0.77 ns
	Gap position × Soil depth	1.81 ns
	Gap area × Gap position × Soil depth	1.60 ns

** Different is significant at the 0.01 level. *Different is significant at the 0.05 level. (ns): Non significant differences ($P > 0.05$).

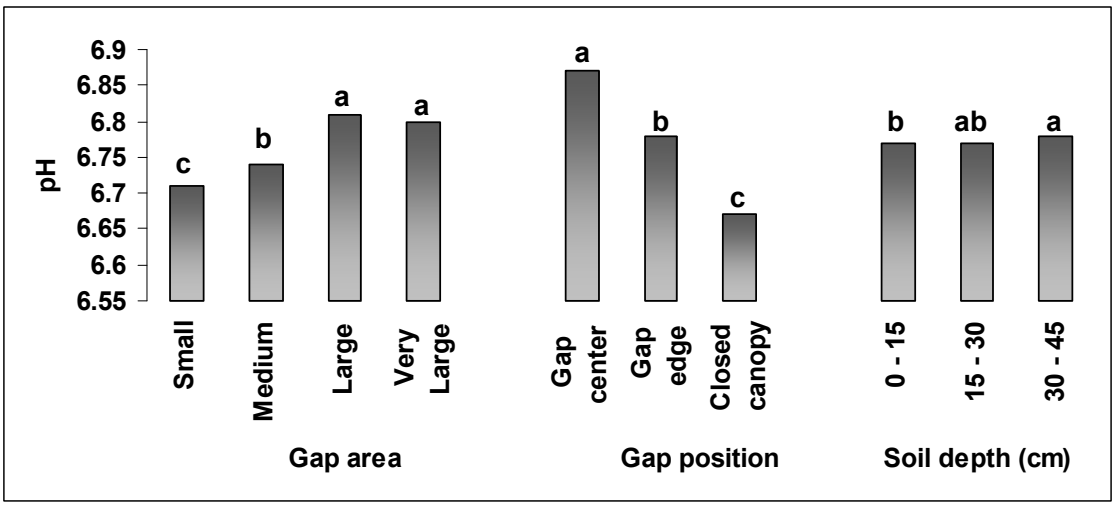

Fig. 1. Mean of soil pH in gap different areas, gap positions and soil depth

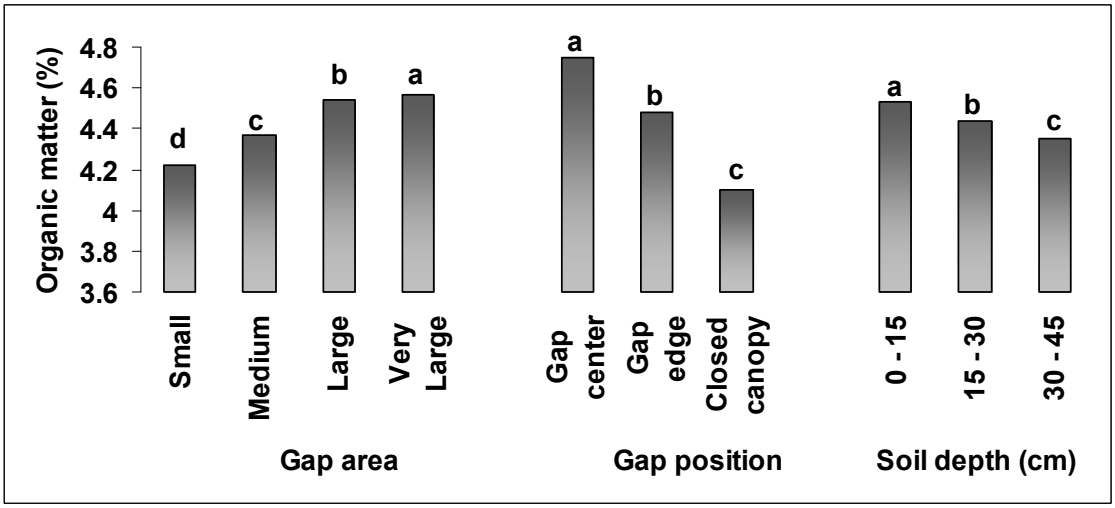

Fig. 2. Mean of soil organic matter in gap different areas, gap positions and soil depth

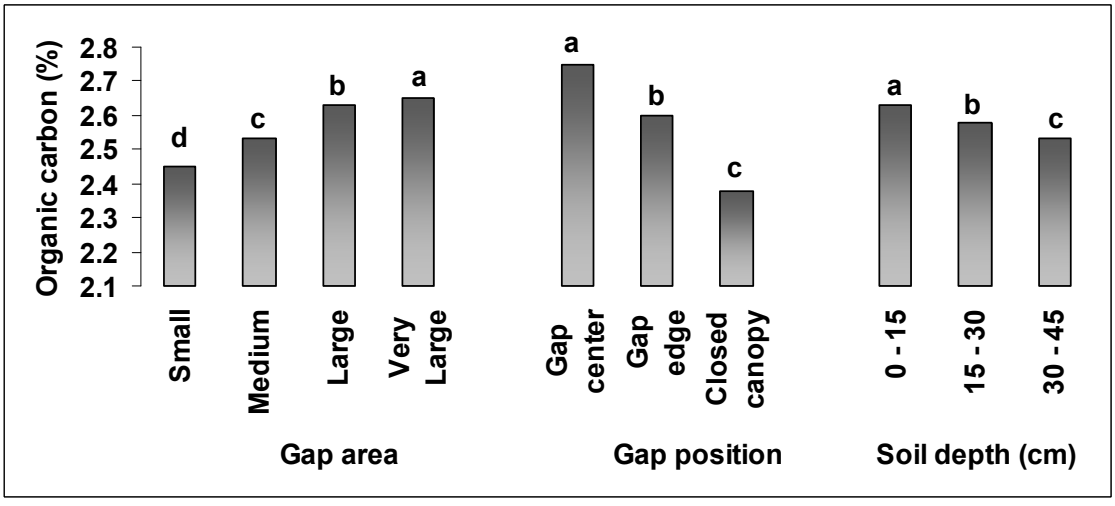

Fig. 3. Mean of soil organic carbon in gap different areas, gap positions and soil depth

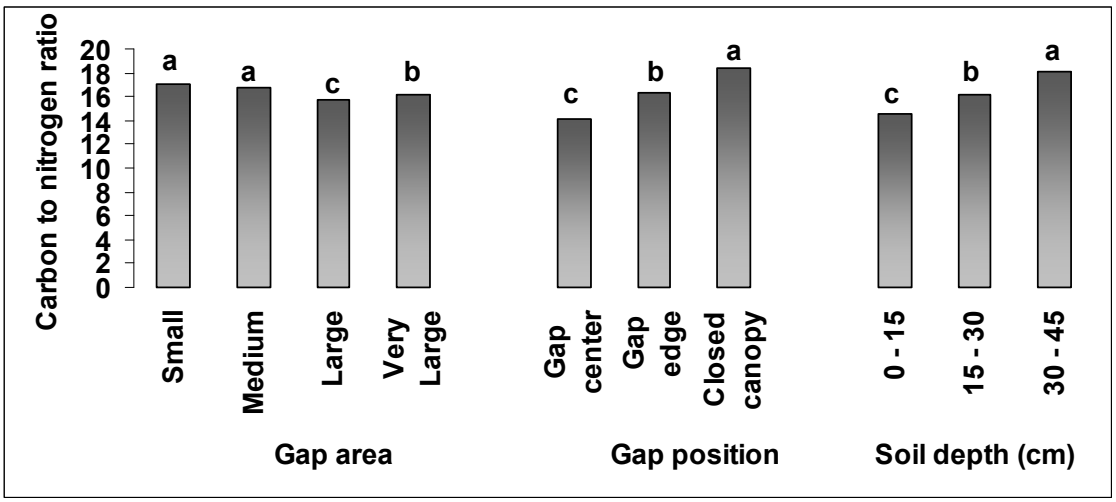

Fig. 4. Mean of soil C/N ratio in gap different areas, gap positions and soil depth

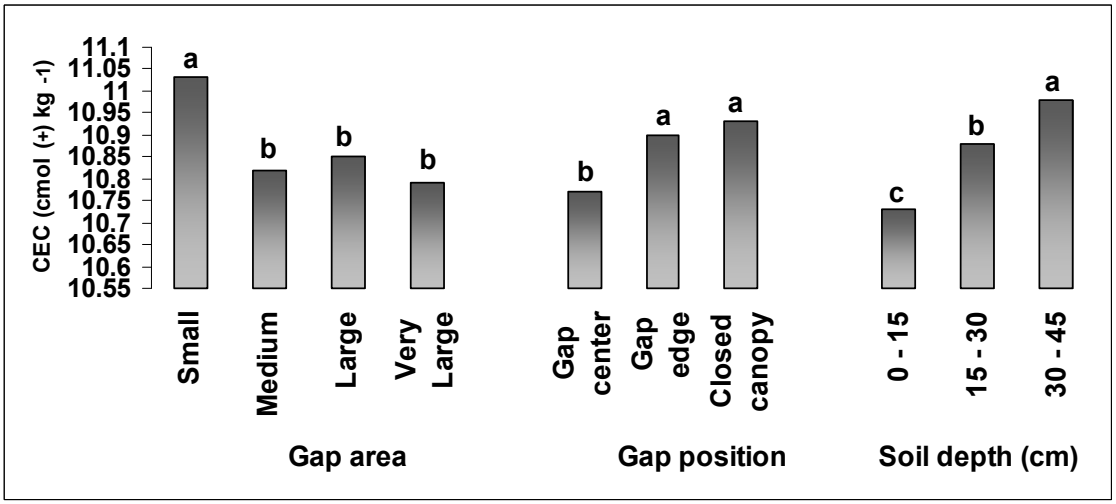

Fig. 5. Mean of soil CEC in gap different areas, gap positions and soil depth

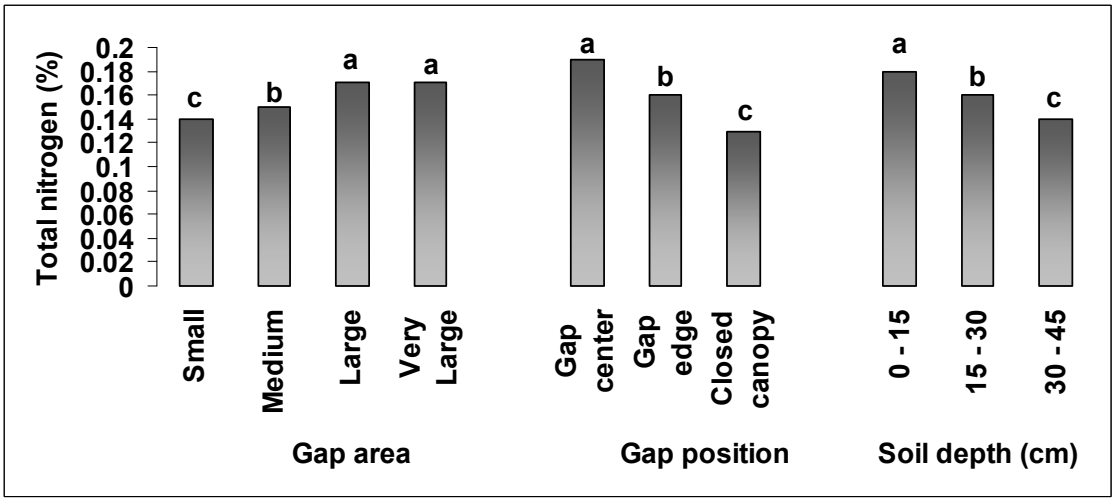

Fig. 6. Mean of soil total nitrogen in gap different areas, gap positions and soil depth

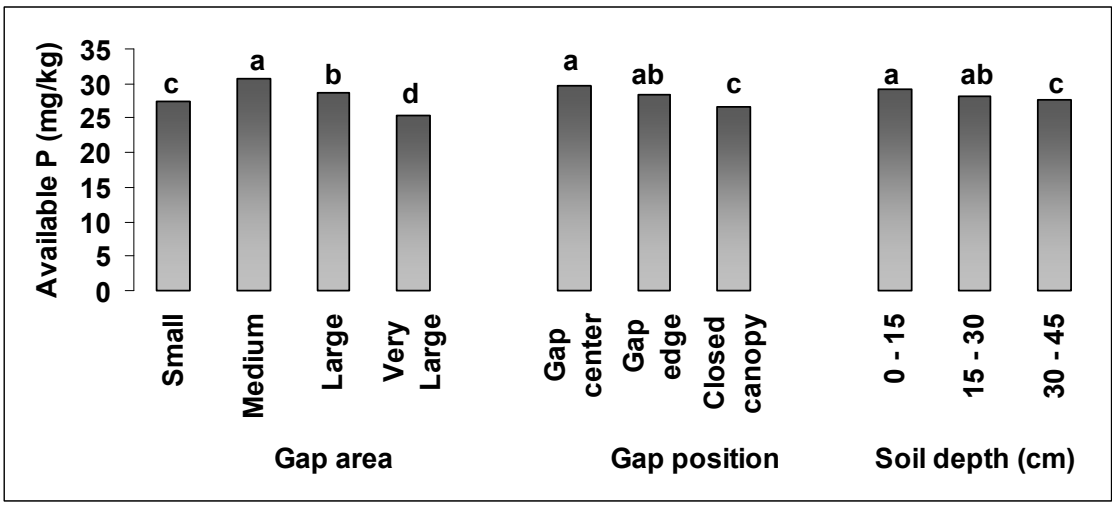

Fig. 7. Mean of soil available P in gap different areas, gap positions and soil depth

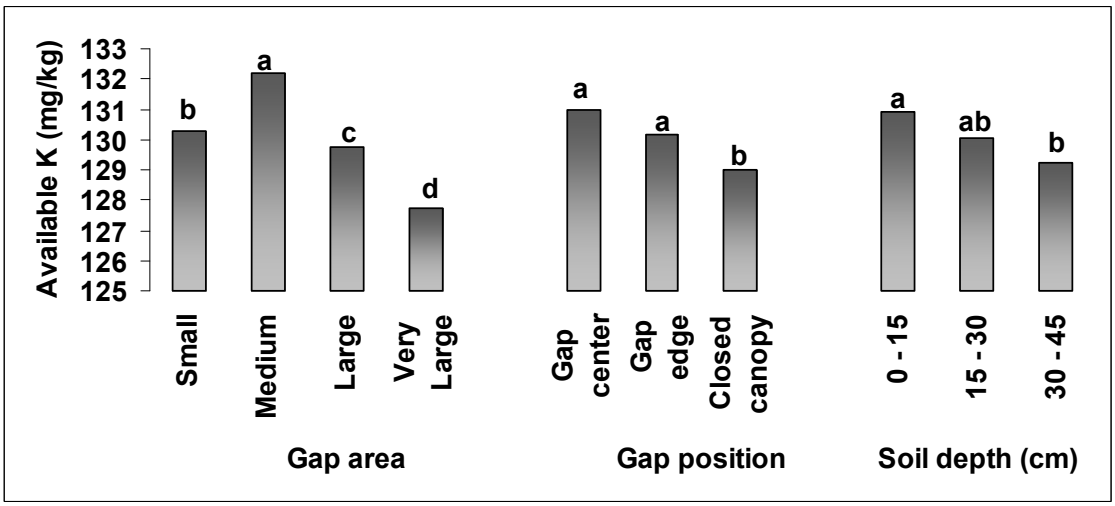

Fig. 8. Mean of soil available K in gap different areas, gap positions and soil depth

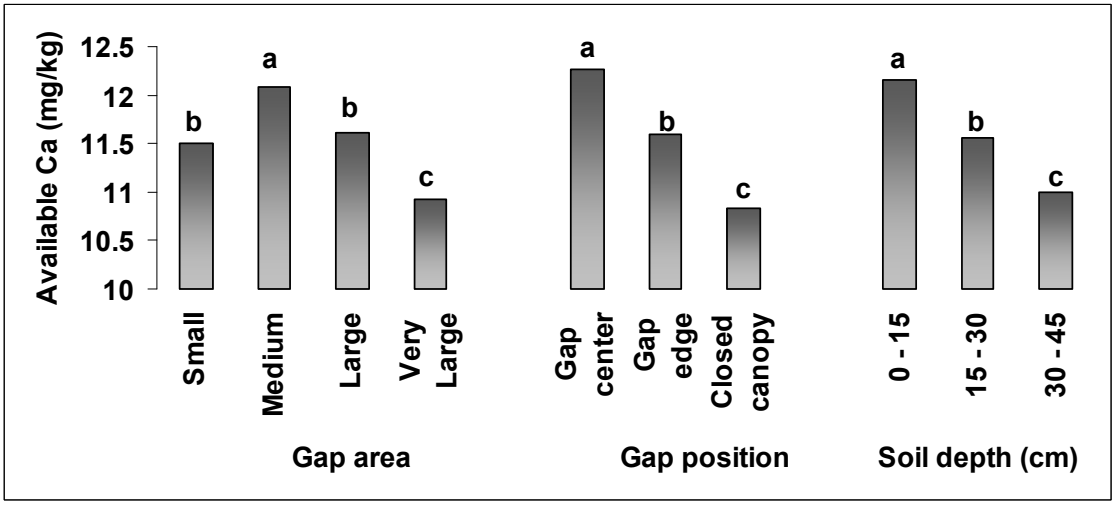

Fig. 9. Mean of soil available Ca in gap different areas, gap positions and soil depth

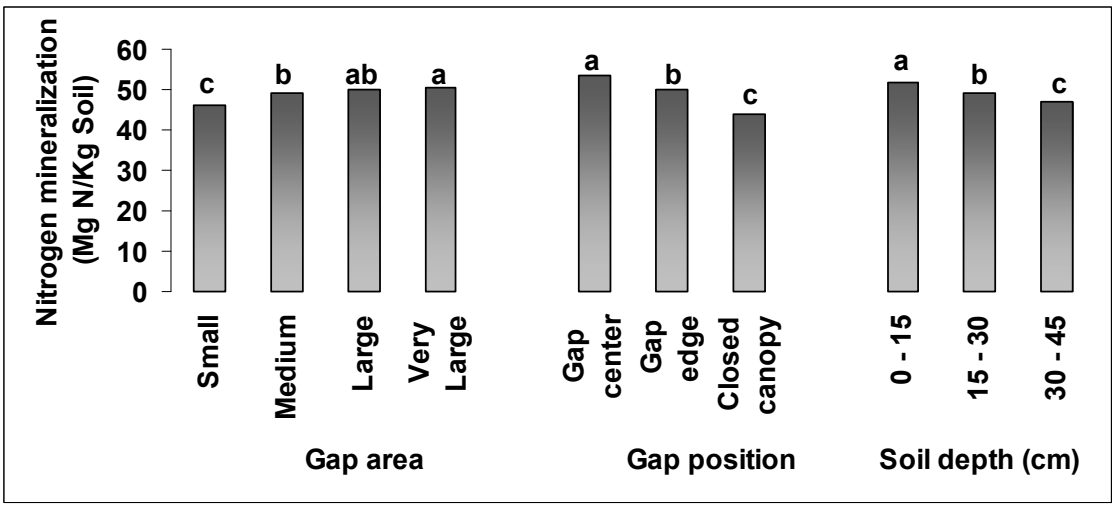

Fig. 10. Mean of nitrogen mineralization in gap different areas, gap positions and soil depth

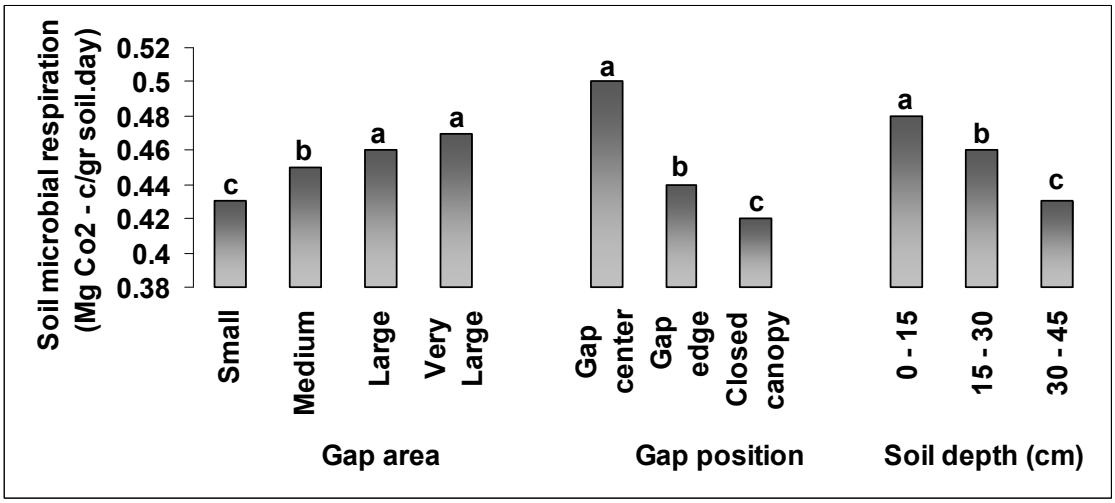

Fig. 11. Mean of soil microbial respiration in gap different areas, gap positions and soil depth

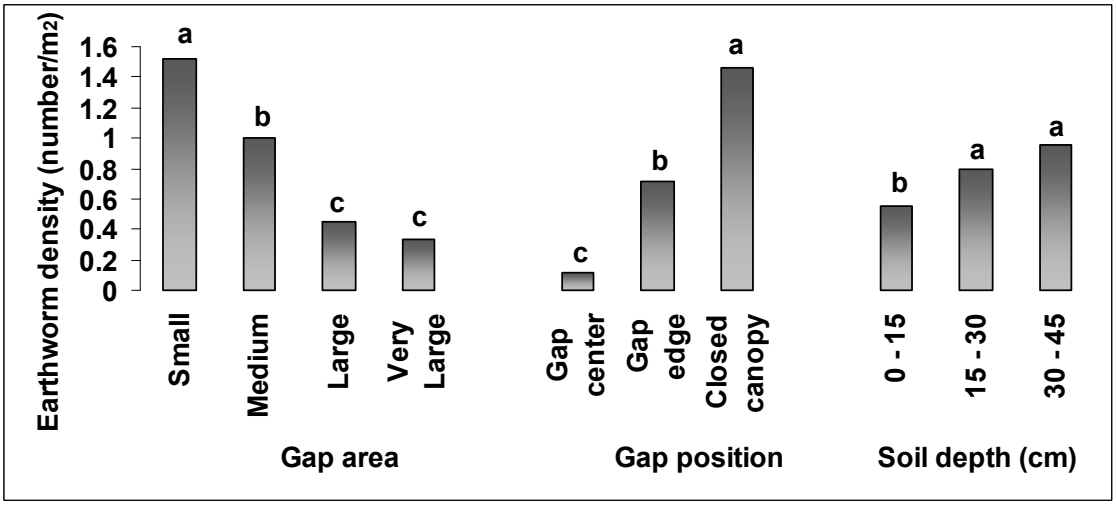

Fig. 12. Mean of earthworm density in gap different areas, gap positions and soil depth

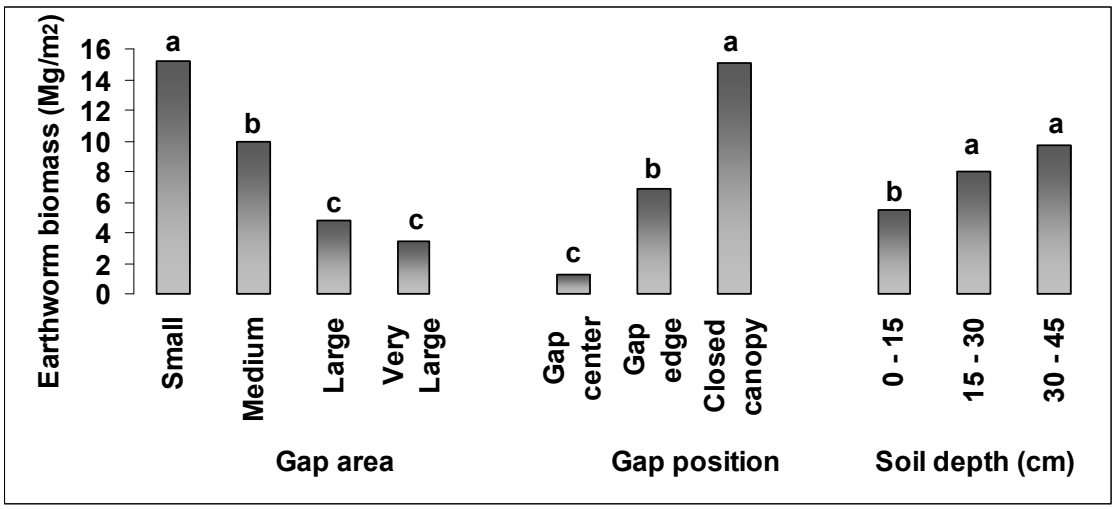

Fig. 13. Mean of earthworm biomass in gap different areas, gap positions and soil depth

Discussion

Soil pH. The result is indicating that large and very large gaps, gap center and 30 - 45 cm depth had greater pH than in the other gap size, position and depths. Soil acidification often occurs with NO^{3-} leaching and nitrification (GUNDERSEN & RASMUSSEN, 1990); thus, it is unlikely gap disturbance have a role in acidification of these forest soils through NO3- leaching or increased nitrification (GUNDERSEN & RASMUSSEN, 1990). Small gaps tended to have lower amounts of soil pH that can be related to presence complexes of sustain organic acids as in gaps with more openings these complexes are leaching from soil upper layers. Thus, gap larger areas tended to have higher pH. Similar status can be considered in different positions of gaps as in gap center leaching of acid complexes more occurred and soil pH is increased. SCHARENBROCH & BOCKHEIM (2007) detected no significant differences in soil pH character for gap different areas. SCHARENBROCH & BOCKHEIM (2008) studied the soil pH amounts in different positions of gaps. Theirs research result is indicating soil pH had no significant differences among gap center, gap edge and closed canopy. Soil pH higher amounts in soil deeper layers can be related to lower values of organic matter in soil beneath depths as inversely relation found between these characters (NOURBAKHSH *et al.,* 2003; YASREBI *et al.,* 2003).

Organic matter and carbon. The highest values of these characters found in very large gaps, gap center and soil upper layers and significant differences were considered. Density and particle size separations isolate district soil organic matter pools for relating stabilization and turnover of carbon in soil (ELLIOT & CAMBARDELLA, 1991; GREGORICH & ELLERT, 1993; SIX *et al.*, 2002). Soil organic matter associated with silt and clay particles are considerably more recalcitrant, with turnover times ranging from 400 to 1000 years (JENKINSON & RAYNER, 1977; PARTON *et al.*, 1988; BUYANOVSKY *et al.*, 1994). Soil microbial biomass is a mediator of carbon turnover (PAUL & JUMA, 1981) and is essential as both a flow and source of plant nutrients (SINGH *et al.*, 1989). The microbial biomass reflects long term quantitative and qualitative carbon inputs in soil (MCGILL *et al.*, 1986; INSAM & DOMSCH, 1988). In this research by reason presence of clay higher amounts in soil texture (result is unpublished) and microbes assemblage and density (with considering microbial respiration values), the organic matter amounts were more considerable in large gaps. Soil upper layers had more organic matter contents regarding near to litter sources and plant residuals. However, gap dynamics may be important in explaining discrepancies in theories suggesting old growth forests are inconsequential carbon sinks (for example, ODUM, 1969, 1985) or are actively accumulating carbon (BUCHMANN &

SCHULZE, 1999; ZHOU *et al.,* 2006). SCHARENBROCH & BOCKHEIM (2007) reported the canopy gaps effects on soil organic matter character was non significant.

Total nitrogen. The most values of total nitrogen found in gap large, gap center and soil upper layers. RITTER *et al.* (2005) also found that soil solution nitrate concentrations and nitrate losses were increased as a result of forest gaps. In Rocky Mountain coniferous forests, PARSONS *et al.* (1994a, b) found that the removal 15 - 30 trees together represented a threshold above which significant losses of available nitrogen to groundwater may be incurred. Of course, in this research the similar status can be occurred with formation of more opening areas. Many researchers (PRESCOTT *et al.,* 2003; RITTER *et al.*, 2005) observed the non significant effects gap different areas on soil nitrogen character. SCHARENBROCH & BOCKHEIM (2008) claimed that canopy gaps are susceptible to nitrogen leaching less, thus the amounts of this character is less in opening areas soil than in closed canopy. Changes in nitrogen uptake, microclimate (e. g., increased radiation, temperature and moisture), and substrate associated with gaps effect significant influence on forest nitrogen dynamics. In general, increased organic matter decomposition and nitrogen mineralization and reduced root nitrogen uptake tends to favour leaching of inorganic nitrogen in gaps relative to the undisturbed closed forest (e. g., MLADENOFF, 1987; PARSONS *et al.*, 1994a; 1994b; BRUMME, 1995; ZHANG & ZAK, 1995; DENSLOW *et al.,* 1998). The indicators that have been used to indicate N -saturation include increased N deposition (JOHNSON & LINDBERG, 1992), increased nitrogen mineralization and nitrification (FOSTER *et al.,* 1989), high soil nitrogen accumulation with decreased soil C/N ratios (MCNULTY *et al.,* 1991; ABER, 1992), and increased NO^{3-}/NH^{4+} ratios in drainage waters (HEDIN *et al.,* 1995). Clear - cut studies suggest the peak nitrogen loss period to be 2 to 3 after the cutting with a return to pre - cut levels after 5 years (GUNDERSEN *et al.,* 2006). Although minimal, SCHARENBROCH & BOCKHEIM (2008) results show that nitrogen loss in these gaps, 6 - 9 years old, is significant relative to the closed forest. Likewise, PRESCOTT *et al.* (2003) found gaps in Canadian spruce - fir forests still had significant nitrogen loss 7 years after disturbance. RITTER & VESTERDAL (2006) claimed that nitrogen concentration in growth season is more in within gaps than to closed canopy. In present study similar results detected pay attention to this research carried out in summer season. But, it is assumed that nitrogen losses will occurred after removal of the forest cover on large areas. Plant diversity and regeneration in the gap was vigorously growing and the nitrogen demand of young trees is relatively high until canopy closure. Thus, reduction of nitrogen concentration at this site can be strongly effected in use by plants as a significant part of ecosystem nitrogen pool must be accumulated in the living biomass (MILLER, 1981). RITTER & VESTERDAL (2006) mentioned that it takes a long time for a reduction in solution nitrogen concentration to take place when regeneration in the gap develops slowly. A similar conclusion was also reached by BARTSCH (2000) for gaps in a German beech forest. In RITTER & VESTERDAL (2006) research, an increase in nitrogen concentration in the gaps in ALS Nqreeskov and Rude forest in the second year after gap formation indicates a delay in response to the disturbance. Increased nitrogen concentration in the gaps may partly be attributed to a lack of nitrogen uptake by regeneration or ground vegetation in the early years after gap formation, as also found by KNIGHT *et al.* (1991). Therefore, in our study after 3 - 4 years of gaps formation wasn't enough time for nitrogen uptake by regeneration and vegetation. RITTER & VESTERDAL (2006) also pointed in the advanced regeneration in the seventh year after gap formation was still not enough to reduce nitrogen concentrations significantly. We suspect that a long time is need to reduction of nitrogen concentrations in within gaps, significantly.

Carbon to nitrogen ratio. The highest value of this character detected in small and

medium gaps, closed canopy position and the third depth. With considering the greatest amounts nitrogen observed in large, very large gaps and soil upper layers, thus greater C/N ratio found in small gap and soil deeper layer depending to carbon and nitrogen amounts.

Cation exchange capacity (CEC). This character was significantly greater in small gap, closed canopy and the third depth. Clay percent content in soil texture can be effective on cation exchange capacity amounts. The highest value of clay was detected in small gap, closed canopy position and deeper layers of soil (result is unpublished). Thus, cation exchange capacity increased in these treatments following more clay presence. SCHARENBROCH & BOCKHEIM (2007) resulted that gap positions (gap center, gap edge and closed canopy) had non significant effects on cation exchange capacity value. But, fewer amounts were observed in gaps than to closed canopy.

Available P, K and Ca. Medium gaps, gap center and upper soil had the greatest amounts of base cations. In general, solar radiation will increased with increasing of canopy opening areas that is due to accelerating decomposition of litters. But if the opening be very large, decrease in base cations in gaps is likely a result of leaching losses. SCHARENBROCH & BOCKHEIM (2007) reported the leaching is the most important reason for decrease of base cations in within gaps. Their results suggest an increased nutrient leaching potential as a result of relatively large (300 - 2000 m^2) gaps in old growth northern hardwood - hemlock forests. The results of current research is indicating that base cations leaching potential increased with expanding of canopy opening areas from medium to large; thus soil is poor of nutrient elements in large canopy gaps. This important should be considered in forest management and trees marking for utilization to prevent of gaps formation with large opening areas. Furthermore, plant diversity will increased with increasing of opening areas in canopy gaps (SHURE et al., 2006) that is observed in study area, also. On the other hand, nutrient retention is dependent upon the balance between inputs (atmospheric deposition, gas adsorption/fixation), transformations (mineral weathering, mineralization of soil organic matter), and outputs (soil leaching and volatilization) (BRUIJNZEEL, 1991; LESACK & MELACK, 1996). Canopies tend to enhance nutrient concentrations of incident precipitation (PARKER, 1983; LINDBERG & OWNES, 1993). There is a strong negative relation in nutrient elements with the amounts of precipitation. Total nutrient deposition, on the other hand, is positively related to precipitation amounts (PARKER, 1983). Therefore, leaching potential of soil nutrient will increased with expanding canopy gaps. Removal of canopy cover is generally known to increase water drainage and stream flow. This is reported from thinning, clear -fallings and gap formation (e.g. KNIGHT *et al.*, 1991; LESCH & SCOTT, 1997) and is also supported by the present study. In a study in a heterogeneous forest with mixed tree species, ZIRLEWAGEN & VON WILPERT (2001) emphasised the role of small - scale structural variation. They found crown interception to be a main factor reducing water fluxes, while crown gaps increased water fluxes. These effects were enhanced by variable root densities and thus water uptake. An influence of the forest structure (canopy, roots), tree sizes, species composition, soil properties and soil solution chemistry was reported in other studies (KOCH & MATZNER, 1993; BEIER, 1998), and hyrcanian forests of Iran are characterized by high variability in most of these parameters.

Nitrogen mineralization. The maximum values of nitrogen mineralization observed in very large gaps, gap center and 0 - 15 cm depth. Decomposition and mineralization tend to increase where smaller, non-occluded substrates that are low in resistant compounds (e.g., lignin), and high in available nitrogen are present (i.e., C/N ratio) (WAGNER & WOLF, 1998). Through the loss of canopy trees and subsequent alteration of the existing vegetation dynamics (e.g., BUSING & WHITE, 1997;

WEBSTER & LORIMER, 2002), gaps likely also impact the microbial substrate. Through top - down trophic interactions, gaps alter the soil environment and substrate for microbial - mediated processes. In current study, carbon and nitrogen amounts created a condition that is due to increasing of nitrogen mineralization in mentioned treatments. However, organic matter decomposition and nutrient mineralization may be greater in gaps than in the closed forest (MLADENOFF, 1987; PARSONS *et al*., 1994a, b; ZHANG & ZAK, 1995; BRUMME, 1995; DENSLOW *et al*., 1998) that is according to results of this research. SCHARENBROCH & BOCKHEIM (2007) resulted that nitrogen mineralization was significantly greater in gap center and edge positions in compare to closed canopy. BAUHUS (1996) found that nitrogen mineralization decreased with time in gaps relative to the forests, and hypothesized that mineralizable substrate had been depleted under gap conditions. Of course, the decrease of nitrogen mineralization is predicted in this research also. But, this decrease isn't logical by reason of substrate depleting. Because of with considering presence of deciduous broad leaved trees (leaf litter fall) in studied ecosystem, very much litters gathered in forest floor every year that are as pool of nutrition elements for mineralization. But, reduction of nitrogen mineralization is related to decreasing intensity solar radiation within gaps with gradual closing of opening areas in along time. RITTER & VESTERDAL (2006) pointed that gap disturbance is due to increasing of nitrogen mineralization in forest ecosystems that is according to this research. It was found the impacts on nitrogen mineralization decreased from the forest floor to the upper mineral soil by reason decreasing of substrate content to soil deeper layers.

Microbial respiration. This character was significantly greater in very large gaps, gap center and 0 -15 cm depth. Microbial processes are regulated by a variety of substrate and environmental conditions. Microbial activity generally increases with adequate soil moisture and aeration, warmer soil temperatures (optimum of 30 – 45^{0}C), and a near - neutral soil pH to allow for diverse active microbial populations (WAGNER & WOLF, 1998). It is imagined that these conditions are more appropriate in very large gaps, gap center position and soil upper layers in site area that is due to gathering of different microbes and increasing of soil microbial respiration amounts. The effects gaps have on microclimate are important as solar radiation, soil moisture, and soil temperature have direct impacts on soil microbial processes (WAGNER & WOLF, 1998). SCHARENBROCH & BOCKHEIM (2007) recognized that gaps modify soil resources and create an appropriate condition for microbes different activity that is confirming results of current study. In contrast, gaps can also adversely affect microbial activity and biomass through dramatic temperature increases (ZHANG & ZAK, 1995; ARUNCHALAM *et al*., 1996). Of course, greater microbial respiration in gap location in compare to closed canopy is indicating appropriate condition for activity of microbes. BAUHUS & BARTSCH (1995) and also SCHARENBROCH & BOCKHEIM (2007) observed that microbial activity increased in gaps, likely due to microclimate changes. In addition, gap microbial activity is negatively impacted as a result of decreased substrate availability. They believed that gap edges may represent regions of optimal microclimate and substrate availability for microbial - mediated processes. But, in current study pay attention to presence of broad leaved trees, the shortage of substrate isn't visible and microclimate condition is appropriate for microbe's activity within gaps. Thus, we suspect that within gap (especially gap center) has more appropriate conditions for different microbes activities as microbial respiration was increased in this position. BAUHUS & BARTSCH (1995) found leaf litter fall in gaps to be 75% of the adjacent closed beech forest. Although, litters mass is fewer in within gap than in closed canopy, but intensity solar radiation is greater in within gap that is due to more accelerate decomposition of litters. Thus,

greater available nutrient elements are gathered in within gap that can be used by microbes. Disturbances influence the microbial community locally through microclimate change (MCGILL *et al.*, 1986; INSAM *et al.*, 1989), and temperature and moisture are positively correlated with microbial activity (BUNNELL et al., 1977). The soil microbial biomass responds more quickly to disturbance than does the amount of organic matter in the soil (INSAM & DOMSCH, 1988) thus, it can be more sensitive index in disturbances of forest ecosystems (ANDERSON & DOMSCH, 1989). BAUHUS (1996) found a substantial decrease in microbial biomass in the gap center compared to gap edge and found equal microbial biomass in the forest and gap edge. The other research showed that with gap disturbance it is expected that soil bacteria will increase and fungi decrease (BRADY & WEIL, 2002; COLEMAN *et al.*, 2004).

Earthworm density and biomass. These characters were significantly greater in small gaps, closed canopy position and soil lower layers. The most important effective factors can be soil higher moisture and lower temperature in small gaps (SALEH RASTIN, 1978). Almost, 80 to 90 % of earthworms live weight is water, thus soil moisture is essential for their life and will kill them (SALEH RASTIN, 1978). Furthermore, soil moisture amounts have descending trend with increasing of canopy gaps but soil temperature has ascending trend (SCHARENBROCH & BOCKHEIM, 2007). Thus, earthworm density and biomass is decreased with increasing of canopy cover opening areas by reason of soil moisture reduction and increase of temperature. As similar, closed canopy position has greater soil moisture and less temperature in compare to the other positions. Therefore, this position created more appropriate condition for gathering of earthworms. Gap edge has medium condition for assemblage of earthworms than to gap center and closed canopy. In general, low moisture and high temperature created fatal conditions for earthworms (NACHTERGALE *et al.*, 2002) in gap center. Earthworms (especially endogeic) are able to migration more beneath layers and avoid of soil drought, especially in summer season (HALE & HOST, 2005). Thus, earthworms were gathered in soil lower layers in this research with considering weather heating in growth season (summer).

Conclusion

Forest gaps irregularly affect the availability degree of materials and micro region resources, soil and the site. The existence of the above - mentioned factors are changeable in time and place. The purpose of the present study was recognizing the appropriate way in forest management that prevents the wasting of materials and sources in forest ecosystems. So, it is clear that using the gaps in medium area is an appropriate guideline to maintain the balance in cycle of food materials and the climatic factors of temperature, moisture and transmittance light especially in temperate ecosystems. In conclusion, within the range of gap sizes included in this study, results have shown that gap size is effective on soil chemical and biochemical. However, on the basis of the results, we believe that the creation of medium gaps (200 - 400 m^2) may be important from an ecosystem perspective representing the appropriate management procedures for an adequate conservation of ecological functions, capable to preserve soil properties and favour beech natural regeneration. Since this study was not replicated across a range of site types, we cannot generalize our conclusion. We hope that these results will be tested in a replicated study to determine whether they are general. We believe that such a study in different natural forest could be conducted using the set of measurements and the analytical tools we have presented.

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Received: 17.07.2010
Accepted: 18.10.2010

ECOLOGIA BALKANICA

2010, Vol. 2 December 2010 pp. 57-60

Habitat selection of the Shelduck *Tadorna tadorna* (Aves: Anseriformes) in Evros Delta, Greece

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Abstract. Evros Delta is the most important wetland habitat in Greece. Thousands of aquatic birds use to breed and winter in the area but the reduction of the habitat is still continuing. We used road transects and Shelduck *Tadorna tadorna* habitat selection was recorded. Based on Ivlev's electivity index, Shelducks have a preference for places with sweet and salted/brackish waters. Food availability and abundance are the key factors that influence the Shelduck movements in Evros Delta. Actions are required to maintain these habitats in order to protect the Shelduck population in the area.

Key words: aquatic birds, habitat, Shelduck, Evros Delta, Greece.

Introduction

The dramatic reduction of wetlands extend is a major subject of discussion for many years. Deltas are very important ornithological sites since birds are attracted by the estuaries taking advantage of their high food availability and the variety of their dwellings. Many rare species used Evros Delta for breeding, wintering and as a stopover site during migration, but thoughtless human activities caused a significant shrinkage of the wetland with negative impacts in the status of the populations (PARASKEVOPOULOS *et al.*, 1993). The major threats of the Evros Delta habitats were reported from SCHEPERS *et al.* (1990) and these are drainage, extensive cultivations, canalization, pumping ground-water etc. which have already affected bird population and distribution. Habitat destruction and fragmentation caused many problems worldwide, reducing bird populations to a lower level (NEWTON, 1998), and this emphasizes the meaning of understanding the ecological requirements of the wildlife species. The protection and the management of the key habitats for aquatic birds, requires the knowledge of the habitat selection during foraging activities.

Shelduck (*Tadorna tadorna*) is evaluated as Least Concern species and its European breeding population is relatively small (<65.000 pairs) but the population size is stable or increasing (BIRDLIFE INTERNATIONAL, 2009). It is a common wintering and breeding bird in Evros Delta (STUART *et al*. 1990) but data on the population status in Greece, remains unknown. However, local studies provide some useful information about the

populations of the Shelduck (HAILEY & GOUTNER, 2002) in Greek wetlands.

The aim of our study was to evaluate the most important habitats for the Shelduck in Evros Delta during the breeding season.

Material and methods

The study was carried out in Evros Delta wetland (40° 47'N, 26° 05'E) which covers a surface of 20.000 ha and 9.500 of them are under protection from the RAMSAR convention. The climate is of Mediterranean type, the mean annual precipitation is about 560 mm, the mean annual temperature is 15°C and the warmer period of the year is during August (PARASKEVOPOULOS *et al.*, 1993).

Evros Delta is a natural wetland of international importance, used by many rare and threatened bird species e.g. Lesser White-fronted Goose (*Anser erythropus*), Red-breasted Goose (*Branta ruficollis*), Collared Pranticole (*Glareola pratincola*) and Slender-billed Curlew (*Numenius tenuirostris*) in the previous years (GOUTNER & KAZANTZIDIS, 1989; HANDRINOS & GOUTNER, 1990; GOUTNER, 1997). The most common mammals in the study area that were observed accidentally form the first author during surveys were Golden jackal (*Canis aureus*), Badger (*Meles meles*), Red fox (*Vulpes vulpes*) and Wild cat (*Felis sylvestris*). Moreover, the area is gazed by numerous cows.

Surveys were conducted during the period March – June 2008 and only adult birds were recorded. Road transects, 47 km in length each, were carried out ten times (470 km in total) using a car, with an average speed of 25 km/h and all data were collected one hour after dawn until noon. The main route was along the main bund of the Delta, which is served as the main entrance for the area and passed through the six different habitats. Its height was about 3m and bird observations were made as far as possible using 10x42 binoculars. Observations from raised points are a commonly used method in wetlands and birds can be viewed without disturbance from a long distance (BIBBY *et al.*, 2000). Moreover road transects are practical in large open areas for conspicuous species (MEUNIER *et al.*, 2000).

Breeding pairs and non-breeding flocks were all included in the analysis. When a flock of birds was seen, habitat type that was used at the specific time was recorded. Habitat surveyed was categorized into six types: salted/brackish water, grasslands, tamarisks, reeds, inland sweet waters and cultivated fields (BABALONAS, 1979; GOUTNER, 1983; PARASKEVOPOULOS *et al.*, 1993). Other habitats of the area were coastal islets and riverside forests but they were not surveyed due to inaccessibility.

We used Ivlev's electivity index (JACOBS, 1974) to indicate habitat selection. Electivity varies from -1.0 to +1.0, where -1.0 indicates avoidance and +1.0 preferences for a particular habitat. The formula is:

$$Ei = \frac{r_i - p_i}{r_i - p_i - 2r_i p_i},$$

where r_i is the proportion of shelducks observed over habitat i and p_i is the proportion of habitat i in the study area. The proportions of available habitats were obtained from FOREST SERVICE OF ALEXANDROUPOLIS (2009).

Results and Discussion

We counted 1453 individuals of Shelducks at 6 habitats during ten surveys within the breeding season in Evros Delta. During observations it became apparent that Shelducks were using areas with salted/brackish and inland water all over the breeding season and Ivlev's electivity index was 0.492 and 0.334 respectively. Highest bird abundance was recorded on these habitats, which accounted for >50% of all birds counted and feeding was the most common behavior. Moreover, areas with *Tamarix* sp. and reeds were avoided (Table 1). Grasslands were used only from breeding pairs or colonies during nest prospecting, laying and incubation period. All birds were recorded in a distance of >50 meters from the bund. Problems of wetland degradation and their impact on wildlife were reported many years ago for Greece (HOFFMAN, 1989).

Several ornithological studies were conducted in Evros Delta many years ago (e.g. GOUTNER & ECONOMIDIS, 1986; GOUTNER, 1990) but comparative data for the habitat shrinkage and the effect on the aquatic birds populations is missing.

Table 1. *Habitat selection based on the Ivlev's electivity index of the Shelduck in Evros Delta, Greece.*

Habitat category	Birds observed	Proportion of the habitat %	Ivlev's electivity index
Cultivated areas	156	37.3	-0.664
Tamarix sp.	10	8.0	-0.852
Grasslands	535	28.6	0.185
Reeds	2	0.9	-0.723
Inland waters	357	14.0	0.334
Salted/brackish waters	393	11.2	0.492

This study emphasizes the importance of some habitats in the most important Greek wetland. Places with water (salted or sweet) seem to attract the Shelducks and most of the waterfowl species, in all stages of the breeding season but these areas are highly irrigated (GERAKIS & KALBURTJI, 1998) and available foraging habitats during summer are absent. Moreover, the most important species in the diet of the Shelduck is the small, hard-shelled mollusc *Hydrobia* (BUXTON & YOUNG, 1981; PATTERSON, 1982) and it can be found in large quantities in the Evros Delta especially in salted/brackish waters (KEVREKIDIS *et al.,* 1996). This can explain the Shelduck preference, for this type of habitat. Tamarisks and reeds are highly avoided, proving their typical desire for coastal places (PATTERSON, 1982). Therefore, Shelduck habitat selection is strongly depended on mollusk occurrence and abundance.

The continuing decrease of the available foraging habitat will have negative effects on the Shelduck population within following years. Local authorities should pay attention on preventing the damage of this international importance wetland.

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Received: 07.03.2010
Accepted: 24.03.2010

ECOLOGIA BALKANICA

2010, Vol. 2 December 2010 pp. 61-63

Colour Variation of an Individual of Hart's Rivulus *(Rivulus hartii)* found in a Habitat Rich in Polycyclic Aromatic Hydrocarbons in the Pitch Lake of Trinidad

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Abstract. Typical coloured *Rivulus hartii* have been documented in South America and the twin island state of Trinidad and Tobago in habitats ranging from rock pools to slow moving streams. After repeated sampling at the Pitch Lake, an area rich in polycyclic aromatic hydrocarbons in Trinidad, a single individual was found within a pool of the lake with abnormal colouration. The fish exhibited normal behavior and showed no signs of pathology.

Key words: *Rivulus hartii,* Pitch Lake, colour variation.

Hart's rivulus (*Rivulus hartii*) is one of the larger members of the Killifish family (Cyprinodontidae family) in the ornamental trade. They have a natural distribution in Trinidad and Tobago and drainages along the northern coast of Venezuela, where they inhabit streams, swamps, ponds, and rock pools at the bases and above waterfalls (AXELROD & SCHULTZ, 1983). Adult males are light brown with blue-green hints. Longitudinal rows (approximately eight rows) of red spots from the operculum to the caudal peduncle are prominent. The caudal fin has yellow stripes to the top and bottom (Fig. 1). Females also have similar, but less intense coloration. The adult fish show clear sexual dimorphism when the fish reaches about 30mm total length (TL). Males develop a white fringe on the caudal fin, both dorsally and ventrally, whereas females have a black fringe at the periphery of the caudal fin (AXELROD & SCHULTZ, 1983). Females above 50mm TL and about 50-80% of all males have a dark pigmented spot on the dorsal part of the caudal peduncle which is also visible in juveniles. There is hardly any size difference between sexes of this species (AXELROD & SCHULTZ, 1983).

In Trinidad and Tobago Hart's rivulus has a wide distribution across both islands (KENNY, 1995; PHILLIP, 1998). PHILLIP (1998) showed their existence in south west Tobago, and we have observed specimen from both central (Gilpin Trail, UTM 20P: 759065.37 E, 1249820.56 N) and northern regions (UTM 20P: 767634.95 E, 1253366.84 N and 766369.16E, 1253366.84N) of the island in forested areas with seasonal streams. They show a greater diversity in habitat types in Trinidad, however, deviating from the typical freshwater habitat mentioned above. We have recorded them in small streams within less than 10.0 meters

from sea water at salinities between 3‰ to 5‰, at both the west (Iros Bay, UTM 20P: 640103.04 E, 1123072.10N) and east coastlines (Mayaro, UTM 20P 719148.75 E, 1137293.55 N) in Trinidad. The most interesting site they have been recorded at, however, is the Pitch Lake (UTM 20P: 650341.45 E, 1131668.93 N) of Trinidad.

Fig 1. Normal coloured *Rivulus hartii.*

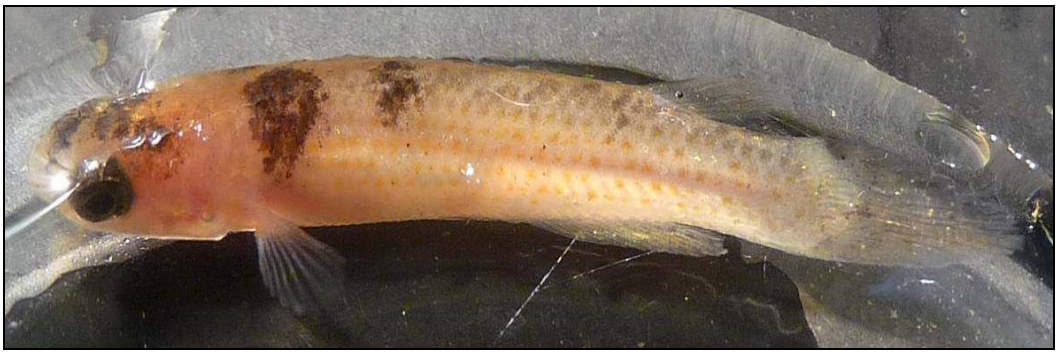

Fig. 2. Unique coloured *Rivulus hartii* from the Pitch Lake, Trinidad.

The Pitch Lake can be described as relatively flat crater that closely resembles a large asphalt car park with pitch folds creating several freshwater pools, furrows and connecting drains. The lake is approximately 0.8km^2. The perimeter is fringed with marshes and the pools surrounding the lake have several aquatic plants such as lilies (*Nymphaea* sp.) and algae (*Nitella* sp.). Thus far, Kenny (1995) has documented three species of fish for the lake; namely, *Poecilia reticulata* (guppy), *Polycentrus schomburgkii* (Guyana leaf fish) and *Rivulus hartii* (Hart's rivulus). *Rivulus hartii* has the highest density amongst the three species within the lake (personal observations by authors). We have documented pools with fish having mean temperatures of 31.26°C, pH of 4.94, conductivity of 1328 µs and salinity of 0.6‰. During June 2009 a specimen of Hart's rivulus was collected from the Pitch Lake showing coloration unlike the description above (Fig. 2). After reviewing several pieces of literature on *Rivulus* and Killifish coloration it was determined that this natural color morph is unique. The individual collected is 41mm TL, however, the sex cannot be determined due to the unusual color of the fish. The individual lacks the peduncle spot and seems to have a pink and white hue. Two mottled darker patches are also visible on the dorsal posterior area. The markings along the body are dull orange, only a faint orange band is visible on the caudal fin. With the exception of color, the morphology and attributes of our specimen is in keeping with what can still be taxonomically called a *Rivulus hartii*. When viewed at 4x magnification, minute dots of dark pigment are seen within the scales. No signs of pathology have been

observed. The fish's health, behavior and feeding are the same compared to other *Rivulus hartii* collected at the same time in the lake. During captivity, the specimen was kept in both illuminated and un-illuminated conditions to observe changes in the intensity of coloration. This yielded no change in the fishes' hue or pattern.

Harrington and RIVAS (1958) noted color variation of *Rivulus marmoratus* (collected in Cuba), reflective of the habitat and substrate; light coloration of fish collected in areas of light colored sediments and darker colored fish collected in environments with dark leaf litter substrates. This contradicts our findings for this specimen considering the substrate at the Pitch Lake is mostly black or dark colored. Other *Rivulus hartii* collected within the lake do conform to this adaptation, however (personal observation by authors), but this is temporary with individuals changing color intensity when the substrate is altered.

In Trinidad only the catfish *Rhamdia quelen* has ever shown a color morph in extreme habitat. ROMERO *et al*. (2004) and KENNY (1995) documented several pale catfish in cave systems and their associated drainages in Northern Range of Trinidad. The catfish documented had variable eye diameters and barbell lengths. This reduction in pigmentation and other changes of morphology was attributed to an adaptation to reduced exposure to sunlight; in dark conditions pigmentation is not important. This situation does not occur at the Pitch Lake, as this habitat is exposed to sunlight with little cover by vegetation. Changes in faunal community and individuals within oil impacted habitat have previously been noted (AGARD *et al*. 1993) in marine assemblages. Rather, we hypothesize that due to large natural oil seeps, this habitat is rich in polycyclic aromatic hydrocarbons (PAHs) which are mutagenic and carcinogenic compounds of crude oil. Consequently, one or more loss-of-function mutations may have occurred in the melanin pathway, resulting in the abnormal colour morph. Further investigations into the mutational load of *Rivulus* and guppies are warranted to examine the impact of PAHs on the genetics of wild fish populations. Following this we are now seeking explanations our observations. This is the first of a series of our ongoing investigations into the dynamics of the ichtyological fauna of the Pitch Lake.

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Received: 17.10.2010
Accepted: 02.11.2010

Union of Scientists in Bulgaria – Plovdiv
University of Plovdiv Publishing House

ECOLOGIA BALKANICA

2010, Vol. 2 December 2010 pp. 65-74

Studies on Terrestrial Herbaceous Plants Tolerance to Excess Heavy Metals: Methodological Approach

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Abstract. Plant tolerance to heavy metals is a scientific issue attracting significant attention due to the possible use of tolerant plants for phytoremediation purposes as well as due to the fact that the molecular mechanisms of this phenomenon are not clear enough. Despite of the increasing volume of research on the problem, the available information in many cases is incomplete and/or difficult to compare with other studies because of the significant differences in the experimental designs, range of used metal concentrations, exposure time, etc. In this review-paper both the advantages and limitations of the used experimental designs as well as the methods for evaluation of heavy metal tolerance are briefly discussed.

Key words: heavy metals, plant tolerance, experimental design, methods

Introduction

The contamination of the environment by heavy metals (HM) represents a real ecological problem. In Bulgaria the soils contaminated by HM cover an area of about 200 000 da (GRANCHAROV & POPOVA, 2003). The sustainable use of these soils can be achieved by developing various remediation phytotechnologies as well as adaptive agriculture practices (VASSILEV *et al*., 2005a). For this purpose, it is necessary to have a detailed understanding of the interaction between the HM and the plants, which includes the mechanisms of 1) the uptake and the distribution of HM by the plants, 2) the metal phytotoxicity, and (3) the plant's tolerance towards the excess of metal ions in the environment.

In general, the plant's tolerance towards HM represents the ability of particular plants or populations to thrive under conditions that are characterized by having excess of metal ions, which have toxic effect for other plants (MACNAIR *et al*., 2000). The first research on the plants' tolerance towards HM ever made dates back to the beginning of the 20th century, when it was established that two populations of the species *Silene dioica* have different tolerance towards the excess of Cu (ERNST *et al*., 1992). Thanks to the development of experimental biology, our understanding of the plant tolerance towards HM is gradually enriched. At present day, the assumption is that the tolerance is based on two different strategies: 1) to avoid the entry of excess HM

into the plants, and 2) to achieve effective intra-cellular detoxification. The main tolerance-related mechanisms are already well-known, the most important of which include 1) the reduced uptake and/or accelerated excretion of HM by the cells, 2) the metal detoxification and compartmentalization, 3) the control of the metal-induced oxidative stress, etc. (VASSILEV & NIKOLOVA, 2010).

The scientific interest with respect to the plant tolerance towards HM has become considerably larger in recent years. On one hand, this is due to possible usage of tolerant plants for phytoremediation of soils contaminated by HM (KULAKOV *et al.*, 2009), and on the other, the interest is a result of the possible wider usage of the plants as model objects for eco-toxicological studies (HOCK & ELSTNER, 2005). The number of research papers related to the identification of plants that have high tolerance and hyper-accumulative abilities towards HM are constantly increasing (SCHULZE *et al.*, 2005).

At the same time the gathered information is not always accurate, objective or comparable to other results. The main reasons for this include the considerable differences in the utilized experimental designs, the metal concentrations, the exposure time, etc. For the reasons mentioned above, there is a need for critical analysis of the available information, which would identify the strengths and weaknesses of the different types of tests, and would also describe the established methods that are used to determine the plant tolerance towards HM.

Types of research

The research on the plant tolerance towards HM is carried out on the basis of different experimental designs, which can be generally classified as either *in vitro* or *in vivo*. The *in vivo* research is carried out through hydroponic, pot, or field tests, where every type of test has its advantages and disadvantages.

When employing the *in vitro* framework, the cellular organelles, groups of cells and leaf blades, are being incubated into solutions of different concentrations of HM. Under these conditions, the expositions are shorter, the applied concentrations of HM are larger, and the observed effects are more direct. The *in vitro* framework is being used to draw the characteristics of the potential effects of HM on the plant cells.

Under the *in vivo* framework the plants are being cultivated in conditions marked by excess of the necessary HM (Cu, Zn, Mn), or by the presence of other HM (Cd, Pb, As), where the latter have been biologically inactive. This framework is closer to the natural growing conditions; however, when employing the latter, it is difficult to differentiate the direct effects of HM from the indirect ones, as far as the separate physiological processes are concerned. In recent years, research has predominantly been based on the *in vivo* framework.

The applied effects of the *in vivo* research can have chronic or acute characteristics. In the former case the plants suffer from the negative effects of the HM, but remain alive, whereas in the latter, they die. The tests involving concentrations that cause acute phytotoxicity are still predominant, however, this approach is subject to growing levels of criticism (MILONE *et al.*, 2003) since the used concentrations are often unrealistically high and do not let the plants' defensive mechanisms to manifest themselves.

Types of experimental designs

Hydroponic experiments

In the hydroponic experiments the plants are grown on nutritious solutions, while controlling the environment's parameters (light, temperature, relative air humidity, photoperiod, mineral nutrition). The main advantage of these tests is that they are accurately reproducible (Fig. 1A). One of the disadvantages is the inability for a mycorrhiza to develop, which in natural conditions is important for the absorption of HM and for the plants' tolerance towards the latter.

The absorption and phytotoxicity of HM are most accurately measured in hydroponic conditions. In these conditions it is of particular importance to maintain the predefined concentration of the studied HM. This is achieved to a degree by changing often the solutions, and by controlling the levels of pH. This way, one limits the possibility of having a part of the studied HM transformed into a form that is impossible to be absorbed by the plants.

In the traditional hydroponic solutions, the element Fe is being introduced as a chelate of ethylene-diamine-tetraacetic acid (Fe-EDTA) in order to prevent its being transformed into a non-absorbable form. When introduced into the root cells, the complex becomes dissociated and the free Fe penetrates into the protoplasm (CHANEY *et al.,* 1972). The presence of EDTA in the hydroponic solutions can, however, create problems for some tests involving HM (CHANEY, 1993). For instance, the presence of EDTA in the solution hinders the sampling of frameworks involving Zn phytotoxicity. Compared to the Fe, the Zn has a higher constant for connecting to the EDTA, as a result of which it can replace it from the complex. This in turn reduces the number of free Zn ions in the solution, and furthermore, it causes a Fe deficit, since the replaced Fe has been transformed into a non-absorbable form. The sample frameworks that are characterized by an excess of Pb or Cd are also problematic for research. In hydroponic conditions, the Pb practically becomes entirely precipitated. In order to ensure the absorption of Pb by the plants, some researchers mix the Pb with EDTA beforehand (GEEBELEN *et al.,* 2002). In this case, about 70% of the Pb is being absorbed and transported into the plants in complex state (SARRET *et al.,* 2001), which does not reflect the real situation in the contaminated soils. As far as sample frameworks involving Cd are concerned, when used in realistic concentrations (1-2 μM) they create the possibility for the reduction of the number of free Cd ions in the solution.

In order to avoid the above-mentioned shortcomings of the traditional hydroponic solutions, it is recommended to use the so-called "chelate-buffered solution". The main way in which they differ from the traditional solutions, is that they create sample frameworks, which are based on "ion activities", and not on ion concentrations. The concentrations of the ions in the soil solution are generally much lower than those in the hydroponic solutions. However, because of the dynamic ion balance between the solid and the liquid phase in the soil, the ion activities in the soil solution are very well buffered. The situation is different in the hydroponic solutions, especially in relation to the macro elements. The selection of the chelating compound in the chelate-buffered solutions depends on the studied HM, on the type of stress (toxicity, deficit), and on the type of plant. The selected chelating compound is usually added in excess, generally in the range of 20 to 100 μM more than the sum of the macro elements in the solutions. The ion activities of the HM could be determined with the help of specialized computer software, such as for instance GEOCHEM-PC (PARKER *et al.,* 1995).

Pot experiments

In these experiments the plants are being grown in soil or other inert substrata such as sand, perlite, vermiculite, etc. in greenhouses. As a rule of thumb, the duration of these tests is longer than that of the hydroponic ones, and could encompass the entire vegetation.

The main advantage of the soil tests is their proximity to the natural conditions and the possibility for the development of mycorrhiza. Their main disadvantage, on the other hand, is the strong dependency of the observed effects upon the properties of the used soil (Fig. 1B). Sample designs are often being used with artificially contaminated soils (metal-spiking studies), in which case their properties have a considerable effect on the mobility,

absorption and, consequently, the effects of the HM on the plants.

A critical juncture when working with soils is the way in which they are treated with HM. Usually, the contamination is being carried out with salts of the examined HM, which are easily soluble. From a technical point of view, the HM can be introduced into the soil in a relatively homogenous way by means of fine sprinkles of the water solutions of the compounds onto a thin layer of dry soil. After treating the soil, it needs to be left aside for several months, in order to reach a balance between the separate pools of HM in it (VASSILEV *et al.*, 1998). When inducing complex contamination with several HM, it is necessary to monitor the level of the salts, in order to avoid any side effects, related to possible soil salinity.

When testing with inert substrata (sand, perlite, etc.) the HM is being changed in regular intervals, while ensuring that the excess volume of the solution is leaked from the containers. When changing the solutions, the substrata first needs to be washed with distilled water, in order to prevent a possible overdose of HM.

When working with sand cultures, the used sands needs to soak for 24 hrs in a solution with 20% of hydrochloric acid, in order to have all the salts dissolved. The sand then needs to be well washed with distilled water. The advantage of the tests involving substrata over those involving soils is the inherent possibility to separate and measure the mass of roots. Furthermore, the sand cultures can also host the development of mycorrhiza, unless they have been sterilized.

The container soil tests involve the application of mineral fertilization and maintenance of particular water regime. It is recommended to introduce the necessary mineral elements in the soil in the beginning of the tests, because if they are introduced in parts, this could lead to changes in the reaction (pH), and consequently to the mobility and absorption of the HM by the plants during the vegetation period. The water regime has to be maintained by pouring water into the pots until they become a certain weight, the latter being calculated in relation to pre-determined soil humidity.

A B

Fig. 1. General view of the experimental design involving heavy metals in the case of (A) hydroponic and (B) soil cultivation of the plants (Koleva and Vassilev, original)

Those opposed to the use of salts in sample soil tests involving HM, base their arguments on two main facts – "salt" effect and the effect of the "plateau", both of which exert strong influence on the derived results (BASTA *et al.*, 2005). The salt effect reflects the higher mobility and consequently the plants' access to the HM when the latter have been added as salts into the soil, in comparison to their being accessible in soils contaminated by industrial activity. The

effect of the plateau is characterized by a lower absorption of HM by the plants when the latter are grown on soils contaminated by industrial activity, as opposed to being contaminated by salts of HM. Usually, the absorption of HM in the soils contaminated by salts has a direct dependency upon the common concentration, whereas in the industrially contaminated soils the dependency is expressed by a curve, which reaches saturation, i.e. the absorption decreases as the concentrations of HM in the soil increase.

The mentioned facts favor the avoidance of the experimental frameworks that introduce salts of HM, whenever this is possible. It is better if the different levels of HM are derived on the basis of mixing non-contaminated soils with soils that are contaminated by industrial activity, or with biological sediments containing HM. It is necessary, however, that the physico-chemical characteristics of the mixed components to be as similar as possible, and that they lead initially to the same reaction (pH).

In certain cases, in order to avoid the dependency of the biological effects of HM on the properties of the specific soil, the so-called 'artificial' soils are being used (a mix of quartz sand, clay and calcium carbonate) which are prepared according to established methodologies (VASSILEV *et al.*, 2005b).

Field experiments

The main advantage of the field trials is the fact that they provide information about the interaction between the plants and the HM in the soil, in the context of specific environmental factors. There are, however, some shortcomings.

The plants that grow on soils contaminated by HM can, to a certain degree, absorb HM from the air as a result of closely situated industrial sources. In such conditions, in addition to HM in the soil, air pollutants can also be phytotoxic. The compared variations in these tests are usually determined by the distance from the industrial source, as well as by the direction of the winds (IANKOV *et al.*, 2000). Despite the fact that the variations are not big, some soil and climate-related differences do nevertheless exist. It is also known that the industrially contaminated soils are characterized by heterogeneity with respect to the content of HM, both, in horizontal and vertical directions. The roots of plants generally avoid areas covered by soils contaminated by HM, as a result of which there are substantial differences between the productivity under homogenous and heterogeneous distribution of HM in the soil (PODAR *et al.*, 2004). The mentioned peculiarities of the field trials with regards soils contaminated by HM need to be taken into account when interpreting the results.

The combined use of the different experimental frameworks in research work compensates to a certain degree for their individual shortcomings and leads to objective results.

Approaches and methods for determining the tolerance of plant genotypes towards heavy metals

The tolerance towards HM is determined through various laboratory and vegetative tests (KÖHL & LÖSCH, 1999). The tests include easy-to-determine parameters such as root length, biomass of the roots or of the entire plants, fertility of the seeds, pollen development, as well as physiological parameters such as leaf gas exchange, chlorophyll fluorescence, the ability of cells to plasmolyze and others.

The tolerance of the genotypes is determined either on the basis of the changes of the parameters relating to a single concentration of HM, or to a concentration interval. In the former case the tolerance index (TI) is being measured, which represents the ratio between the magnitudes of the parameter measured under conditions of higher concentration of HM vis-à-vis its relative value under controlled conditions, expressed in percentage. Table 1 contains data regarding the TI of various plant types with respect to Cd.

When determining the TI, two approaches are being used – sequential determination, and parallel determination. The sequential determination is used when it is necessary to determine the tolerance of every member of the population, mainly for genetic research and for

selection purposes. The used parameter in this case is measured in a non-destructive way, by firstly, having the individual plants cultivated in a controlled environment, and then in environment characterized by higher concentration of HM.

Table 1. Values of TI (%) measured on the basis of lengthening the roots in the nutritional solution of Rorison, containing 10 μM Cd (as per BAKER & WALKER, 1989)

Plant species	TI (%)
Festuca rubra L.	101 ± 21
Agrostis capillaries L.	83 ± 20
Holcus lanatus L.	65 ± 12
Poa annua L.	47 ±13
Lolium perenne L.	24 ± 6

The tolerance index, as mentioned above, is calculated as a fraction of the value of the parameter of the environment that has increased metal concentration, and the value in the controlled environment, multiplied by 100. The disadvantage in this approach is the dependency of the effect on changes in the growth rate of the ontogenesis, as well as the necessity to maintain constant conditions during the experimentation. In the latter case, the plants of a particular genotype are being grown in a parallel fashion – in controlled environment and in environment having an increased concentration of HM. Under this framework the dependency on ontogenetic effects is smaller, however the observed differences can be the result of not only the concentration of the HM, but also of differences in the tolerance of the separate individual plants within the population, unless cloned material is being used.

The curve "dose-response" is being described on the basis of the parallel cultivation of plants in controlled environment and in environment contaminated by HM. The latter serves as the basis for determining the so-called effective concentrations (EC) of HM. The most frequently determined concentrations are the following: (no-observed-adverse effect-concentration), which represents the highest external concentration of HM, where there isn't effect on the studied parameter; EC_{25} и EC_5 – external concentrations of HM, which lead to changes in the parameter by 25 and 50%, respectively (Table 2). The change usually leads to inhibition, but when determining enzyme activities or other dynamic variables, this could be a matter of temporary stimulation.

Table 2. NOAEC и EC_{50} values with respect to the growth of the roots and leafs of four plant types under conditions of soil contamination with Cd (mg kg^{-1} DW) (AN, 2004)

Plant species	NOAEC (roots)	NOAEC (leaves)	EC_{50} (roots)	EC_{50} (leaves)
Sorghum bicolor (L.) Moench	20	20	61	39
Cucumis sativus L.	40	40	88	102
Triticum aestivum L.	< 40	40	113	98
Zea mays L.	160	160	268	208

Root lengthening

The root test is the most popular method in the experimental research, used for determining the tolerance towards HM. It is based on the fact, that when in toxic concentrations, the HM inhibits the linear growth of the roots (WOOLHOUSE, 1983). It is

established that the length of the roots is a very sensitive indicator, because the HM have influence on, both, the cell division in the meristem zone, and on the cell elongation.

The root lengthening is a typical study of relatively short tests. In most cases, its duration does not last for more than 7-8 days. It is suitable for determining the tolerance of plant species, whose root growth is relatively quick. On the other hand, it is more suitable for determining the tolerance towards heavy metals that have a relatively quick effect on the root growth. In this aspect, Cu is more suitable than Zn or Cd. Usually, the longer the test becomes the lower the established effective concentrations, which could be a consequence of the plants' acclimation towards the specific heavy metal.

When a heavy metal is necessary for the plant growth and development (for instance Cu or Zn) it is also present in the solution of the controlled version. It is necessary that its concentration in the controlled version is optimal for the root growth, because in cases when the concentration is lower, their growth can be stimulated (WILKINS, 1978). The concentration of HM that is used for screening of the tolerance of the genotypes needs to be selected in such a way, as to ensure that the TI in the most sensitive genotypes is not higher than 0, and in the most tolerant – not lower than 100. This can be achieved by conducting preliminary experiments.

In most cases, the growth of the longest root is measured, unlike cases involving dicotyledonous plants, where the length of the main root is measured (VASSILEV *et al.*, 2005c). Certain authors, however, measure the growth of all roots (ŠTEFANOVIČOVÁ *et al.*, 2000). In order to determine the growth, in the beginning of the test the tips of the roots are marked with a permanent marker or are being submerged into a suspension of active carbon. Since the roots grow apically, the growth (usually in mm) is measured in the end of the test as the difference between the mark and the new length.

Plant biomass increase

The duration of this test is longer (several weeks or months) and is always carried out using the parallel method, due to the unavoidable influence of ontogenetic and ecological factors. Both, hydroponic and substrata-container tests can be used for the test. The fresh or dry mass of the surface roots or of the entire plants is being used as criteria for the determination of the TI in the end of the examination period, both, in the controlled environment and in the environment having an increased concentration of (VASSILEV *et al.*, 2007). Some authors use the relative growth rate of the entire plants as a parameter (ERNST *et al.*, 1992). The latter is being determined according to the formula described below, on the basis of the plant's dry mass in the beginning (DW_1) and in the end (DW_2) of the examination period (ΔT in days):

$$RGR = (ln\ DW_2 - ln\ DW_1) / \Delta T$$

The main advantage of using the weight of the biomass as an indicator is its integral character and easy measurement. On the other hand, its precise determination a long period of time, and its accuracy is not always high enough.

Plasmolytic test

The tolerance criterion in this test is the ability of the cells of different genotypes to plasmolyze after a period of 24 to 48 hours of being in solutions that have different concentrations of HM. Usually, bits of epidermis or tissue are being put in the solutions, where the latter would have 3-4 layers of cells, and the plasmolysis is being induced by 1M of sucrose. The cell membrane's integrity, and respectively, the vitality of the cells, is being calculated as a percentage of plasmolyzed cells. This method can be used for comparative research on the phytotoxicity of different HM.

Other methods

The test of seed germination can also be added in addition to the above-mentioned methods. In general, this method is not sensitive enough towards excess of HM (BAKER & WALKER, 1989), but continues to be used for different types of toxic tests (AN, 2004). The HM concentrations, which inhibit the seed

germination, are usually much higher than those inhibiting the growth of the sprouts or of the young plants.

Recently, in order to increase the sensitivity of the tolerance tests, a number of functional parameters have also been included (Table 3). In this respect, the non-destructive physiological analyses are of most interest, such as the leaf gas exchange and the chlorophyll fluorescence (VASSILEV, 2002), as well as the activity of antioxidant enzymes (CLIJSTERS *et al.*, 1999; VASSILEV, 2003). The combination of growth and functional indicators best characterizes the tolerance of the plant genotype towards HM (VANGRONSVELD & CLIJSTERS, 1992).

Table 3. Ecotoxicological values characterizing the tolerance of barley (*Hordeum vulgare* L.) plants towards the toxic influence of Cd (as per VASSILEV, 2003). A – net photosynthetic rate; E – transpiration rate; stomatal conductivity; Chl.a – chlorophyll "a"; GPOD – root peroxidase activity; RGR – relative growth rate; DW and FW, dry and fresh weight.

Parameter	Regression equation	EC_{25}	R^2
A (µmol CO_2 m^{-2} s^{-1})	Y = -0.0009*X+0.245	72	0.75
E (mmol H_2O m^{-2} s^{-1})	Y = -0.005*X+1.98	99	0.87
q_s (mol m^{-2} s^{-1})	Y = -0.0008*X+0.122	38	0.90
Chl.a (mg g^{-1} DW)	Y = -0.03*X+7.83	64	0.86
GPOD (U g FW)	Y = 1.46*X+188.83	40	0.86
RGR (mg g^{-1} day^{-1})	Y = 0-0.53*X+32.17	13	0.95

Conclusion

The problem relating to plant tolerance towards HM has a number of theoretical and practical aspects. The accurate determination of the species and genotype tolerance towards particular HM is an important issue, which concerns the phytotechnologies used for the sustainable use of contaminated soils, as well as the ecotoxicology. There is increasing research on the topic, however, due to considerable differences in the experimental designs, the results are not straight forward or objective enough. On the other hand, the main methods used for determining the plant tolerance towards HM are based primarily on biometric measurements, which are labor-intensive and moderately accurate. To increase the reliability of the results it is necessary that the research includes sensitive functional indicators - enzymatic activities, non-destructive photosynthetic parameters, and the different experimental designs need to be combined.

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Received: 01.05.2010
Accepted: 12.07.2010

Union of Scientists in Bulgaria – Plovdiv
University of Plovdiv Publishing House

ECOLOGIA BALKANICA

2010, Vol. 2 December 2010 pp. 75-96

Revision of River & Lake Typology in Bulgaria within Ecoregion 12 (Pontic Province) and Ecoregion 7 (Eastern Balkans) According to the Water Framework Directive

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Abstract. A revision of river and lake typology in Bulgaria has been done within the Ecoregion No. 12 Pontic province and Ecoregion No. 7 Eastern Balkans according to the Water Framework Directive 2000/60/EC. Certain geographic changes between the Ecoregion No. 12 and Ecoregion No. 7 have been proposed based on existing biogeographic data, at the beginning. The whole part of the Danube and Black Sea River Basin district have been associated to the Pontic Province (ER No. 12) as well as the rest part of the Southern Bulgaria (Maritsa river basin, Mesta RB and Struma RB) has been allied to the Eastern Balkan (ER No. 7). A serious reduction of the total number of river types (from 33 to 16 types) was justified using clear hydromorphological and biological criteria, e.g. ecoregion, vertical factors (4 altitude zones, main substratum characteristics, slope, and other supplementary factors such as fish and vegetation zonation, climate maps) and horizontal factors (calcareous geology, salinity, size category). Similar reduction of the number of lake types (from 33 to 17 types) was proposed in parallel with a significant modification of the lake typology method. Lake typology in Bulgaria was based on the obligatory factors (4 altitude zones, size typology based on surface area, depth, salinity and geology) and optional factors (residence time, mixing characteristics, e.g. monomictic, dimictic and polymictic, presence of profundal zone, reference trophic status). Seven lake types were identified as "reservoir types", which were only presented by heavily modified and artificial water bodies without any possibility for natural lake equivalent within the country or region. Four coastal lake types with various salinity (from freshwater <0.5 ‰ to hypersaline >40‰) have been reviewed as belonging to the category "transitional waters".

Keywords: ecoregion, typology, rivers, lakes, Water Framework Directive, Bulgaria

Abbreviations:
BEQ – Biological Quality Element
CPOM - Coarse Particular Organic Matter
ER – Ecoregion
FPOM – Fine Particular Organic Matter
RB – River Basin
WB – Water Body
WFD – Water Framework Directive 2000/60/EC

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Introduction

The territory of Bulgaria is divided into 4 river basin districts for water management, govern by 4 river basin directorates (competent water authorities) in frame of Ministry of Environment and Water: Danube river basin (covering about half of the Bulgarian territory), Black Sea river basin district (comprising small river basins of all direct tributaries of Black Sea), East Aegean Sea river basin (river basin of transboundary Maritsa / Merich / Evros River) and West Aegean Sea river basin district (transboundary river basins of Struma/Strymonas and Mesta/Nestos). The analysis of the existing information show considerable unconformities in the approach and number of types at four river basin districts in Bulgaria, although applied in principle common algorithm at the initial typologization according System B of the Water Framework Directive (WFD) 2000/60/EC (EUROPEAN COMMISSION, 2000). The different level of river types' validation is one of the problems. For example East Aegean Sea River Basin Directorate has been reduced the total river types number to 6, while the number of types varies between 12 and 14 at the other Directorates.

There is a variety of possibilities towards unifying of types and thus reduction of the total river types number from 33 to 16, based on hydromorphological system of ROSGEN & SILVEY (1996) and classical altitude hydrobiological zonation (trout, barbell-chub and carp zones) (River continuum concept and its derivatives).

Similar opportunity exists on reduction of lake types from 33 to 17. The lake typology according System B is slightly developed in comparison with river one due to insufficient biological data and heavily modified water bodies domination. Dams (heavily modified and artificial water bodies) represent more than 90% from total lake water bodies. Therefore determination of ecological potential of separate types artificial lakes is essential for Bulgaria, rather than determination of ecological status of small number natural lakes (high mountain glacial lakes, a few landslide lakes and Black Sea and Danube riparian lakes and swamps, usually under strong anthropogenic influence).

There are 4 types coastal lakes in Bulgaria depend on the salinity degree (from freshwater to hypersaline). They have to be referred to the category «transitional waters». A set of specific phenomena is insufficiently studied: seasonal and annual salinity variations (so called mixohaline conditions), heterogenic character of salinity in the range of one lake (vertical – halocline, horizontal variability), as well as unstable salinity (so called poikilohaline water bodies). Various salinity degrees determine unique ecosystems which in many cases have no analogue among neither freshwater nor coastal waters.

The border between two ecoregions № 12 Pontic province and № 7 Eastern Balkans is represented in Appendix № XI, map A of WFD 2000/60/EC (EUROPEAN COMMISSION, 2000) close to the Black Sea and does not respond to Bulgarian biogeography and thus is the basis of serious problems in determining typology, reference conditions, water bodies (WB) boundaries and hence in the accomplishment of additional requirements of the WFD (monitoring programmes, measurement programmes, etc.).

The existing river separation in Black Sea river basin district into two ecoregions (№ 12 for northern and № 7 for southern rivers) is unjustified from biogeographic point of view and will embarrass and even prevent developing of classification systems for fish fauna due to merging of diverse fish species inhabiting Black Sea rivers and lacking in Aegean Sea region and vice versa. Similar perplexities will emerge with metrics for benthic macroinvertebrates (macrozoobenthos).

Material and methods

Study on all surface freshwaters (rivers and lakes) on the Bulgarian territory, as well as identification of "transitional waters" category close to the Black Sea was accomplished. GIS layers were applied at the research, available at the government

authorities (Basin Directorates), as well as specialized biological and hydromorphological approaches and data.

The applied approach was based on the following main pillars:

(i) Critical analysis and alteration of borders of the two ecoregions in Bulgaria № 12 Pontic province and № 7 Eastern Balkans: Appendix № XI, map A of WFD 2000/60/EC (EUROPEAN COMMISSION, 2000);

(ii) Holistic concepts for river continuum;

(iii) Hydromorphological basis of river and lake types.

Holistic concepts

Type-specific approach was applied, covering 16 river types and 17 lake types, determined according hydromorphological and biological criteria (biogeographic data, altitude zonation, connected with "river continuum" theories, „interrupted river continuum", etc.). The above approach gravitates around so called holistic concept (VANNOTE *et al.*, 1980), which was extended by the following concepts:

(i) Interrupted river continuum, i.e. Serial Discontinuity Concept (STANFORD & WARD, 1983) – includes geomorphology and detailed hydromorphology, barriers, tributaries, accumulation → discharging;

(ii) Hyporheic Corridor Concept (STANFORD & WARD, 1993) – revealing connections between rivers and ground waters;

(iii) Flood Pulse Concept (TOCKNER *et al.*, 2000) – for lowland river types and link between nutrients, organic matter and seasonal periodical floods;

(iv) Integrated conceptual models for standing waters (lakes and dams) – lakes are examined as more than trophic status (classical concept of lake ecosystems).

In the above connection the existing typology of rivers and lakes in Bulgaria made by competent water authorities (River Basin Directorates) without any field validation (hydromorphology and biology) was reconsidered. Actualization of types was proposed, based on existing science foundation, principles and concepts, as well as on the gained practical experience in Bulgaria and other countries.

"Top – down" approach was applied. The validation process of the types' boundaries, identification of potential subtypes or special cases, and detail additional validation (especially for lake types) should be started with the first River Basins Management Plans 2010 – 2015.

The holistic concept defined several basic zones along every river system, connected with particular type ecosystems:

- **High mountain zone** (crenon) with little organic matter (leaves and twigs), on which base to be developed more complex and rich ecosystems. Fish fauna is often lacking and the species composition and abundance of the rest Biological Quality Elements (BEQ) (benthic macroinvertebrates, macrophyte flora, phytobenthos) are poor and limited by the environmental conditions. Generally the basic primary producer is periphyton (phytobenthos) from microalgae (diatoms, cyanobacteria, green algae, etc.), lower fungi, etc. overgrowth on rocks,
- **Mountain zone of** CPOM forming (Coarse Particular Organic Matter) (epirhithron, metarhithron). There are created complex benthos ecosystems with main participation of shredders and collectors. Fundamental role have communities developing on the fallen leaves and twigs from coniferous and deciduous trees. Trout zone with low fish diversity (2-3 species). Some macrophytes with scarce abundance appeared. Periphyton still plays major role.
- **Semi - mountain zone** (hyporhithron). Transitional zone between mountain rivers and streams and lowland rivers with low flow velocity. The rivers could reach middle size. Complex benthic communities are formed. Species scraping substrate (grazers & scrapers) dominate. Filter feeders species has serious presence. Periphyton (phytobenthos) has important meaning for the ecosystem. Typical barbell-chub zone in Bulgaria**.** More sizable macrophyte overgrowth is formed in

sections with slower velocity and adequate substrate.

- **Lowland zone** (potamon). The zone gains river mouth and is characterized by slow velocity. FPOM (Fine Particular Organic Matter) is formed in the zone. The rivers could reach very large size with typical slow velocity and sections of accumulated organic sediments. Specific benthic communities are characteristic on the basis of collectors (especially so called detritus feeders (detritivores) and sediment feeders). Phytoplankton and zooplankton become of significance for the first time. Aquatic macrophytes are abundant and determinant as primary producers. Periphyton' (phytobenthos) role tends to diminish. Fish communities are extremely rich (over 15 species) and include economically important fish resources (large biomass). Typical carp zone in Bulgaria.

Reviewing rivers as hyporheic corridor - Hyporheic Corridor Concept (STANFORD & WARD, 1993) defines particular river sections (whole rivers in some cases), which are strongly influenced by groundwaters into both directions (discharging, infiltrating). Generally such rivers have high values of hardness (electrical conductivity) and specific hyporheic fauna. Typical illustration is Dobrudja's losing rivers.

The Flood Pulse Concept (TOCKNER *et al.*, 2000) defines some specific lowland river types as so called flood plain rivers. For example small and middle sized lowland rivers (marsh or sandy rivers) in Upper Thracian Valley and the lowland sections of large rivers Danube, Maritsa, Tundja, etc.

Integrated conceptual approaches for standing waters (lakes and dams) determine basic criteria of lake typology:

✓ Trophic level of the WB – classical conception defining main types according nutrient status and eutrophication degree:

- **Oligotrophic** „lakes" (high mountain and mountain zone);

Current type lakes are naturally poor in nutrients (P, N) and lacking eutrophication (phytoplankton growth).

- **Mesotrophic** „lakes" (semi mountain and part of the lowland zone);

„Lakes" with average nutrient level and slight eutrophication as phytoplankton and macrophytes growth.

- **Eutrophic** „lakes" (shallow lowland lakes)

Lakes significantly loaded with organic material and nutrients in lowland areas, usually shallow (mean depth <6 m). High levels of eutrophication are characteristic (phytoplankton blooms) and generally rapid macrophyte growth.

✓ Depth zonation and micticity – shallow zone is determined (littoral) and deep zone (profundal, >15 m depth), presence and absence of stratification, thermocline and different residence time (water exchange);

- **Shallow lake ecosystems** (mean depth <15 m)

Shallow lake ecosystem determined by permanent water circulation (polymictic), absence of summer or winter stratification and in general species richness (phytoplankton, zooplankton, benthic invertebrates, macrophytes and fish fauna). The conditions are proper especially for marcophytes which sometimes overgrowth large part of the lake area.

- **Deep lake ecosystems** (mean depth >15 m)

There is clear delimiting of shallow littoral zone (often similar characteristics with shallow lakes) and well differentiated deeper zone (profundal) with completely different characteristics. Profundal is defined as a WB, separated form littoral in the range of a single lake in some countries. Well expressed dimictic conditions (spring and autumn circulation), presence of stratification and thermocline (in summer). Both water layers above and under the thermocline usually have different physico-chemical and biological features. Phytoplankton is concentrated in the upper layer (pelagial, epipelagial), called also compensatory zone, concurrent with euphotic layer. Macrophytes are located at the littoral zone. Benthic macroinvertebrates communities are completely different in the littoral (insects' larvae, molluscs, etc. with high extent of diversity) and profundal

(almost 100% detritus feeders' speices, representatives of Oligochaeta and Chironomidae, low biodiversity). Deeper water layer (called batipelagial) has also very low degree of diversity (mainly bacterial communities and some zooplankton species). Heavy quantities of organic sediments conglomerate frequently in profundal and lead to anaerobic bacterial decomposition (ammonification, sulphate reduction and methanogenesis) and hypoxia/anoxia at bottom layer. Fish fauna also is concentrated basically in littoral zone and epipelagial. Thus profundal is defined as a "biological desert" (poor in species composition and very low productivity) in comparison to littoral. Both zones are in active interaction during spring and autumn water circulations.

Similar to rivers, determination of "lakes" types depends also on connections with ground waters (infiltration or discharging of aquifers) and water level variance. Some Black Sea and riparian lakes and swamps are completely dependent on groundwater supplies (Shabla Lake, Durankulak Lake, Velyov Vir Lake, Alepu swamp, Arkutino, etc.). Water level fluctuations could influence very strongly on the lake ecosystem, destroying completely macrophytes and macroinvertebrates in eulittoral zone.

Hydromorphological basis of river typology

The following hierarchical scheme is proposed towards river typology in Bulgaria (Fig. 1):

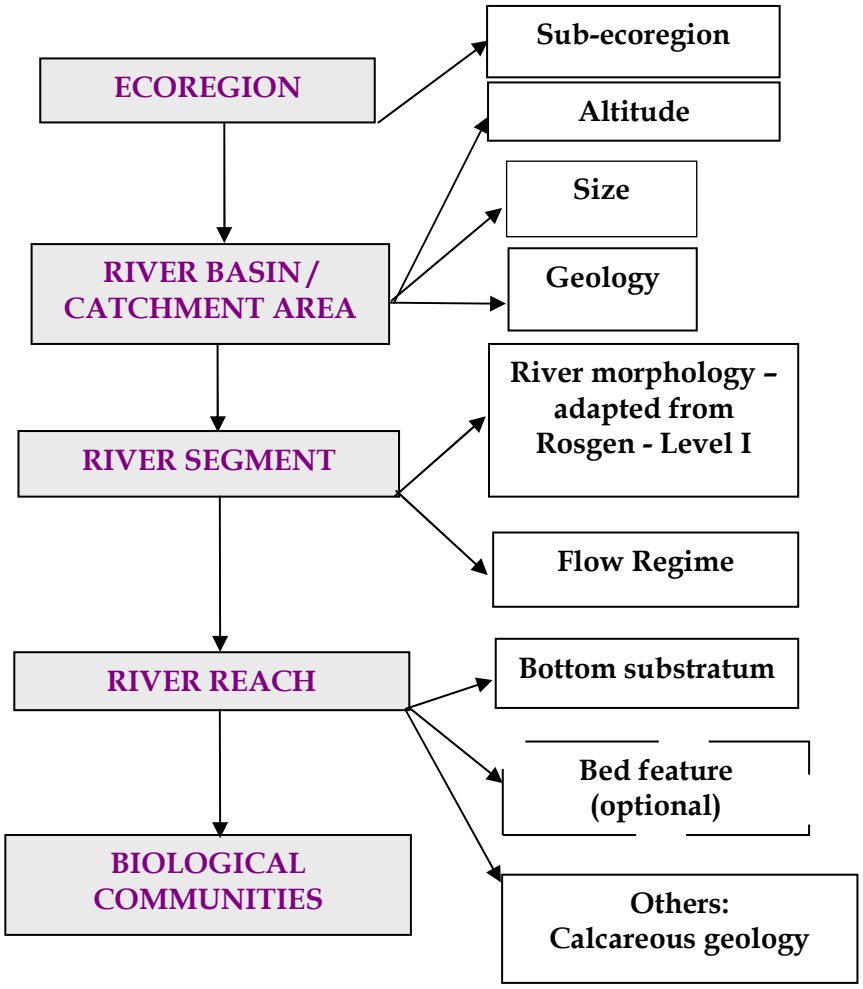

Fig. 1. Hydromorphological basis of river typology.

Altitude

The altitude defines 6 principal river types (Fig. 2 and 3) according classification system of ROSGEN & SILVEY (1996).

Morphological characteristics – level 1

1. High mountain rivers with very steep slope – **type** (**Aa+**), **slope J >10%** and **type** (**A**), **slope J = 4 – 10%**;

2. Mountain rivers with moderate slope, low channel sinuosity- **type** (**B**), **slope J = 2 – 4%**;

3. Semi-mountain or lowland rivers with gentle slope, meandering - **type (C, E)**, **slope J < 2%**;

4. Typical lowland rivers with braided channel or anastomosing (multiple channels) in depositional sections or at broad river valleys - **type (D)**, **slope J < 4%**) and **type** (**DA**), **slope J < 0.5%**;

5. Rivers with classic "entrench-ed, meandering" channels, gentle slope - **type (F)**, **slope J <2%**;

6. "Gully" stream type is an entrenched, narrow, and deep, step/pool channel with a low to moderate sinuosity. - **type (G), slope J = 2 – 4%.**

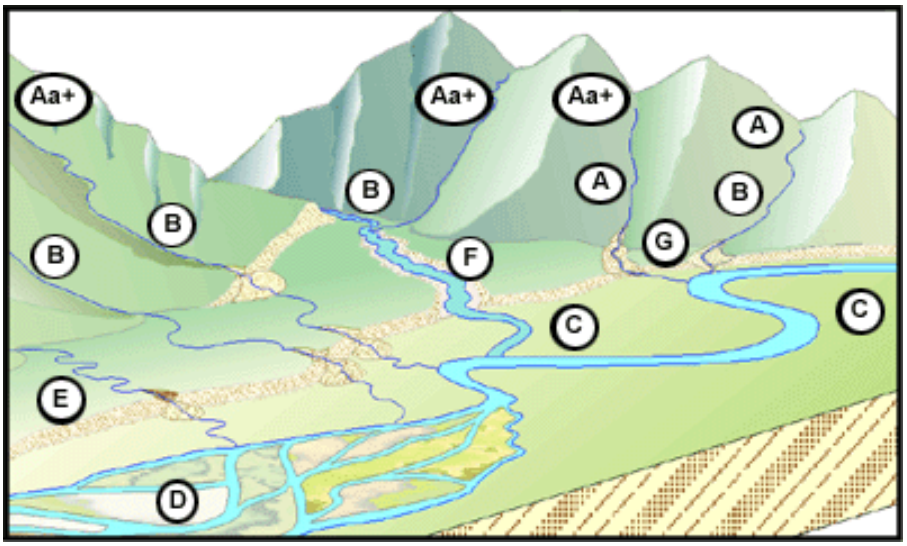

Fig. 2. Hydromorphological classification of rivers according altitude (after ROSGEN & SILVEY, 1996).

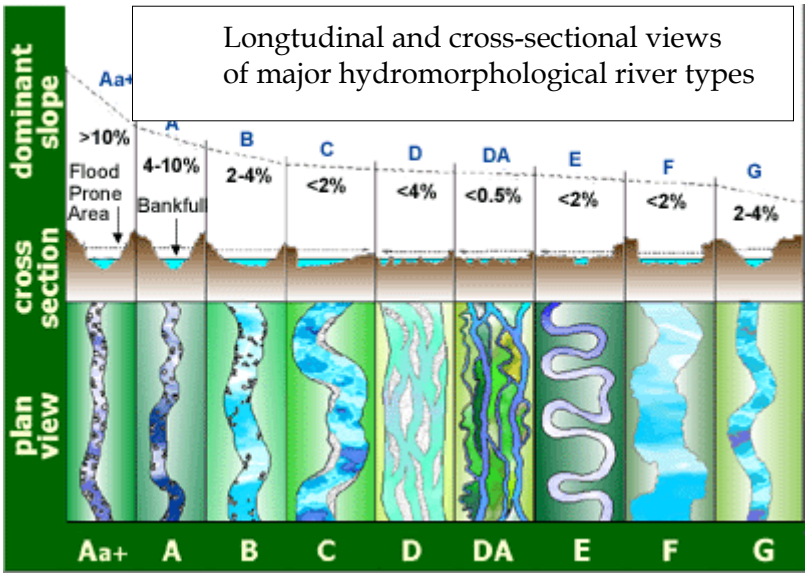

Fig. 3. Longitudinal and cross-sectional views of major hydromorphological river types (after ROSGEN & SILVEY, 1996).

Size (catchment area and river size)

The catchment area is one of the most easily and reliably measurable parameters for river classification. According to the WFD 2000/60/EC (EUROPEAN COMMISSION, 2000) four dimension groups of catchment area are determined in applying System A towards river typologization: small size (<100 км^2), medium (100 – 1000 км^2), large (1000 – 10000 км^2) and very large (10000 – 25000 км^2).

After dimension classification following catchment area of WFD 2000/60/EC (EUROPEAN COMMISSION, 2000) there are 15 large rivers in Bulgaria (Danube Region – 6; Black Sea Region – 2; East Aegean Sea Region – 4; West Aegean Sea Region - 2). Special instance are Dobrudja's temporary rivers with large catchment areas (Suha, Tsaratsar, Kanagyol) which could not be adopted as large rivers due to their temporary character. There are many other tributaries of main rivers with catchment area >1000 km^2 (Sazliika, Vacha, etc.), which have also to be reviewed as large ones.

The following dimension criteria were defined in the current study in connection with the adopted approach for typologization after System B consistent with Bulgarian conditions (Table 1).

Table 1. *Size criteria after System B consistent with Bulgarian conditions. * - Catchment areas between 2500 km^2 (Large Danube tributaries) and 1300 km^2 (Medium and small rivers) are not registered in Bulgarian part of Danube RB. ** - There are some exceptions, e.g. rivers Iskar, Struma, Mesta, etc.*

River type according size	Catchment area	Examples
Very large rivers	> 800 000 km^2	only Danube River
Large Danube tributaries *	> 2 500 km^2	Ogosta, Iskar, Vit, Osam, Yantra, Rusenski Lom (6 rivers)
Large lowland rivers in Ecoregion 7	> 7 000 km^2	Maritsa, Tundja
Large Black Sea rivers	> 1 000 km^2	Kamchiya, Veleka, (Rezovska)
Medium and small rivers in Danube Region and Ecoregion 7 *	< 1 300 km^2	Archar, Lom, Tsibritsa, Gostilya, Pyasachnik, Mochuritsa
Medium and small Black Sea rivers	< 1 000 km^2	Ropotamo, Batova, Dyavolska
Semi mountain rivers (up to medium size) **	< 1 300 km^2	Semi mountain zone of all rivers in Bulgaria
Mountain rivers	< 500 km^2	Beli Iskar, Chepelarska, etc.
Small alpine rivers and springs	< 100 km^2	Malyovishka, Banderitsa, Damyanitsa, etc.

Geology

Current study adopted the approach assumed by RB Directorates separating rivers in Bulgaria into 2 major groups:

☑ Siliceous or mixed geology (silicate, calcareous);

☑ Strongly karst rivers (high values of hardness/electrical conductivity)

Third type defined in WFD – organic peat basis, is represented with insignificant cases (some small mountain peat marshes and lakes) and could be neglected in the view of general river typologization in Bulgaria.

River segment (morphological characteristics after ROSGEN & SILVEY, 1996)

River segment is a part of river restricted for points of tributaries' inflows. It is a factor of continuity in attitude to river morphology.

River segment (flow regime)

Hydrological regime is suggested to be generally classified for the needs of river typology in the following manner:

1. Ephemeral stream – river streams, forming ephemeral flow under direct influence of local precipitations.
2. Intermittent stream – river streams, forming seasonal flow in different year periods – usually in the period of spring rainfall and thawing of snow, as well as separate WB in the period of dryness.
3. Perennial stream – permanent river streams, feeding in the dryness periods form groundwaters.
4. Headwater (river sections close to source) – could be perennial, intermittent or ephemeral.
5. Transitional river streams. River flows under influence of periodical inflows of sea water.

Rivers reach (substrate, river bed characteristic, slope)

„River reach" is a part form the river segment. It is also a factor of continuity, connected to a great extent with the river bed morphology (Fig. 4).

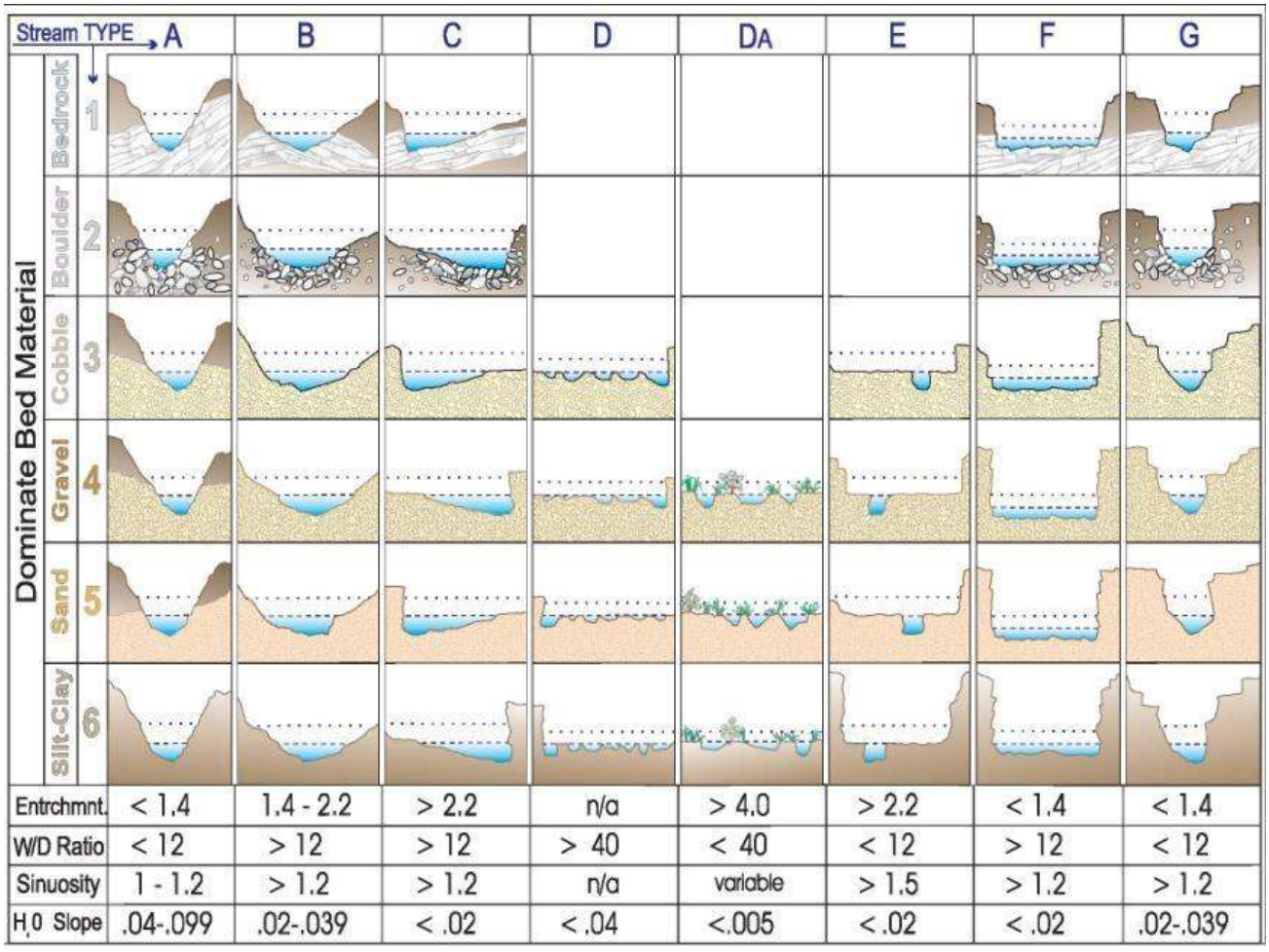

Fig. 4. Connection between hydromorphological river type, dominant substrate, river bed form, slope and other characteristics (after ROSGEN & SILVEY, 1996).

The slope is important characteristic of different river types and has been classified as follows: >10% (very steep), 4-10% (steep), 2-4% (moderate steep), <2% (slight sleep).

According to the river classification of ROSGEN & SILVEY (1996), 6 general river types are determined towards dominant bottom substrate (bedrock, boulders, cobble, gravel, sand and silt/clay) (Fig. 5).

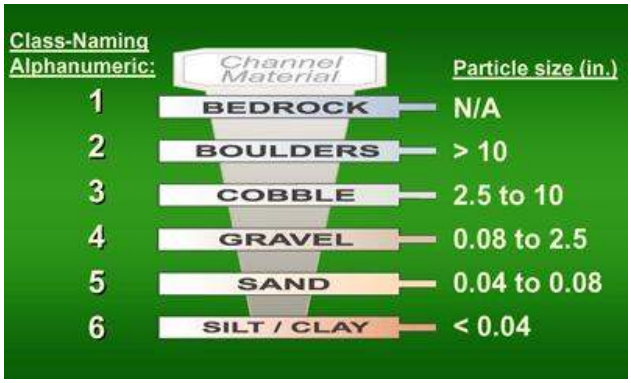

Fig. 5. Classification according dominant substrate (after ROSGEN & SILVEY, 1996). Substrate size is given in inches.

Ecological and hydromorphological basis of lake typology

The general criteria towards defining lake types after System B are as follows:

(i) "Lake" types which have natural equivalent; and "lake" types having no natural equivalent and represented only by heavily modified and artificial WBs;

(ii) Altitude differentiation of 3 basic trophic groups:

- High mountain and mountain types – correspond to ultra-oligtrophic and oligotrophic conditions (altitude parameters correspond to alpine and mountain river types);
- Semi mountain types – respond to mesotrophic conditions (altitude parameters correspond to semi mountain river types);
- Lowland types – mesotrophic to eutrophic (for shallow „lakes") conditions;

(iii)Size characteristics;

- Large „lakes" > 10 km^2 surface area;
- Middle and small < 10 km^2 surface area;

(iv) Bathymetric characteristics:

- Deep „lakes" (Fig. 6). Availability of well developed and significant in size depth zone (profundal and bathyal (bathypelagic) zone), >15 m mean depth, which exists together with shallow littoral zone (<15 m) and epipelagial (euphotic zone);
- Shallow „lakes" (<15 m mean depth), lack of marked and large scale depth zone (profundal).

(v) Geology – absence of specific division of siliceous, calcareous and organic in Bulgaria. Common heterogeneous geology is in use at the moment.

(vi) Mixing characteristics

- Polymictic (generally shallow „lakes") – lack of marked summer stratification and thermocline;
- Monomictic (one annual circulation) – rare type, probably only deep alpine glacial lakes;
- Dimictic (deep „lakes" with developed profundal) – spring and autumn circulation, typical stratification and thermocline;

(vii) Salinity – important characteristic only for transitional waters (Black Sea coastal lakes):

- Freshwater <0.5 ‰ salinity;
- Oligohaline (low salinity), 0.5 – 5 ‰ salinity;
- Meso- to polyhaline (medium to high salinity), 5 – 30 ‰ salinity;
- Hyperhaline (extreme salinity), >40 ‰ salinity.

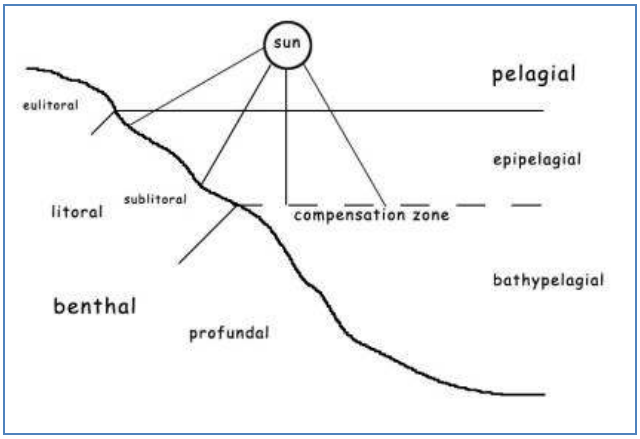

Fig. 6. Biological zonation of deep „lakes" (after http://spoiwzp.sggw.pl/).

Results and Discussion

Alteration of boundary between Ecoregion № 12 (Pontic Province) and Ecoregion № 7 (Eastern Balkans)

Suggested alterations

On the basis of the received results the

boundary between Ecoregion № 12 (Pontic Province) and Ecoregion № 7 (Eastern Balkans) to be specified and altered is suggested (Fig. 7) on the ground of the available biogeographic data (especially for fish and macroinvertebrate fauna) verified by the followed projects:

- "Assessment of reference conditions and maximum ecological potential of surface water types (rivers and lakes) on Bulgarian territory" (Consortium for Biomonitoring, 2009-2010);
- "Developing classification system towards ecological status and potential assessment of the defined surface water types (rivers and lakes) on Bulgarian territory (in compliance with System B (Consortium for Biomonitoring, 2009-2011).

Fig. 7. Altered border between Ecoregion № 12 (Pontic Province) and Ecoregion № 7 (Eastern Balkans) in Bulgaria.

Thus both river basin districts (Danube and Black Sea) which naturally belong to the Black Sea catchment area are united (Ecoregion № 12 Pontic Province). In a similar manner both Mediterranean river basins (East Aegean and West Aegean) follow borders of the particular catchment areas, flowing towards Aegean Sea (Mediterranean Sea). Suggested new boundaries of Ecoregion 12 will cover the total area of Danube RB and respectively Black Sea RB district. Parts of Romania, Moldova, Ukraine and Belarus are included in the same Ecoregion. An intercalibration process has been launched including Bulgarian participation (Ministry of Environment and Water, Executive Environmental Agency, River Basin Directorates) in the frame of so called East Europe Geographical Intercalibration Group (EE GIG).

The boundary between two ecoregions is presented at Appendix № XI, map A of WFD 2000/60/EC (EUROPEAN COMMISSION, 2000) sharing the Black Sea RB district and does not respond to the biogeographic data of the region as well as raises serious problems in defining typology, reference conditions, WBs' boundaries and thus the accomplishment of the additional requirements of WFD (monitoring programmes, programmes of measures, etc.).

The existed separation of the rivers from Black Sea RB district into two ecoregions (Pontic Province for northern and Eastern Balkans for southern rivers) is ungrounded by biogeographical point of view and will perplex

and even could prevented the development of multimetric fish index since unification of species common for Black Sea rivers and not typical for Aegean ones, and conversely. Similar difficulties will be faced with metrics of benthic invertebrate fauna characterized by substantial biogeographic distinctions (Table 2).

Table 2. *Typical species/taxa for Black Sea, East and West Aegean RB districts.*

Black Sea Region	**East Aegean Sea and West Aegean Sea Regions**
Ecoregion № 12 Pontic Province	**Ecoregion № 7 Eastern Balkans**
Fish fauna	
Chalcalburnus chalcoides *Vimba vimba tenella* *Barbus bergi* *Rutilus frisii* *Leucaspius delineates* *Pungitius platygaster* *Clupeonella cultriventris* *Neogobius gymnotrachelus* *Mesogobius batrachocephalus* *Neogobius constructor* *Neogobius syrman* *Neogobius melanostomus* *Neogobius fluviatilis* *Salmo labrax*) *Cobitis pontica* *Syngnathus abaster* Including recently registered species *Phoxinus stranjae*, *Gobio kowatchevi* (, *Alburnus mandrensis*, *Alburnus shishcovi* and salt-tolerant (eurohalinic) species entering from the sea.	*Vimba melanops* *Chondrostoma vardarense* *Squalius orpheus* *Barbus cyclolepis* *Barbatula bureshi*, etc.
Benthic invertebrate fauna	
Trichoptera *Helicopsychidae* *Odonata* - some subspecies and species Ephemeroptera и Plecoptera - separate species Gastropoda Turbellaria/Tricladida Plus many other typical Pontic and Ponto-Euxinic species	*Odonata* - some subspecies and species Ephemeroptera и Plecoptera - separate species Gastropoda Turbellaria/Tricladida
Aquatic macrophytes	
The prevailing part of aquatic species is cosmopolitan and has no clear geographical specificity in the region. An exeption are riparian species (helophytes) but they have minimal meaning.	
Phytobenthos (Diatoms)	
There are not enough data on biogeographical distribution of species, nevertheless geographical defferences are expected.	

About 30% of the Black Sea rivers' fish species is not registered at the Aegean catchment area. At the same time none of the Aegean fish species is found at the prevailing part of small and middle Black Sea rivers. On the other hand all Bulgarian Black Sea tributaries show presence of Danube ihtiofauna with pontic taxa such as gobies (*Gobiidae*) – character for Ecoregion № 12 Pontic Province. It could be summarized that species form Pontic fauna and flora, biogeographically defining river basins characteristics are typical for the whole Black Sea region.

New concept towards river and lake types in Bulgaria

River and lake types in Bulgaria are substantially reduced from 33 to 16 river types, and from 33 to 17 lake types. Fundamental factors defining river and lake typology (in compliance with System B of WFD) (EUROPEAN COMMISSION, 2000) and a general view of new concept are presented at Tables 3 and 4.

Conclusions

Some of the major and significant conclusions after analysis of the results from the current study could be summarized as follows:

(i) Alteration of boundary between Ecoregion № 12 (Pontic Province) and Ecoregion № 7 (Eastern Balkans) was implemented and it would be a basis of concrete typologization at Black Sea Region for Basin Management in Bulgaria;

(ii) New concept was created for river and lake typology with the purpose of unifying the approach and existing types between separate basin regions;

(iii) Reduction of the number of river types in Bulgaria from 33 to 15 river types for freshwaters was accomplished;

(iv) Reduction of the number of lake types in Bulgaria from 33 to 13 lake freshwater types, among them 4 dam types (heavily modified and artificial water bodies) without natural equivalent in the region was established;

(v) For the first time in Bulgaria the category „transitional waters" was identificated, represented by 1 river type and 4 lake types.

Acknowledgments

This work was supported by EU- funded projects under Operational Programme "Environment" 2007 - 2013, Priority Axis I: "Determination of reference conditions and maximum ecological potential for the types of surface waters (rivers and lakes) on the territory of the Republic of Bulgaria" and "Development of a classification system for assessing the ecological status and ecological potential of the determined types of surface waters (rivers and lakes) on the territory of the Republic of Bulgaria (on the basis of a system "B" typology)".

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Received: 07.07.2010
Accepted: 12.08.2010

Table 3. *Ecological factors that determine the river typology in Bulgaria according to System B of the WFD 2000/60/EC.*

Type code	River type	Obligatory factors				Optional factors					Comments
		Ecoregion / Sub-ER[1]	Altitude[2]	Geology	Size	Distance from river source	Mean water slope/ Energy of flow	Valley shape	Mean substratum composition	Sali-nity[3]	
R1	**High mountain (Alpine) rivers**	12-1; 7	> 1800 m vary to some extent	Mixed, siliceous, calcareous	<100 km², streams	<15 km	> 10%; Very steep; High E of flow	V, narrow U	Bedrock, rocks, boulders	Fresh water; <0.5 ‰	High sediment supply systems, erode rivers
R2	**Mountainous rivers (Uplands)**	12-1,2	Mountain zone – sometimes get down (validation)	Mixed, siliceous, calcareous	<100 km², streams (small rivers)	<40 km	4 – 10 % steep or 2-4% moderately steep; High E of flow	V, narrow U	Boulders (>256 mm), cobles (64 – 256 mm)	Fresh water; <0.5 ‰	Small riverbank terraces or non; sediment supply systems, erode rivers
R3	**Mountainous rivers (Uplands)**	7	> (600) 800 m, vary to some extent (validati	Mixed, siliceous, calcareous	<150 km², streams (rarely medium rivers <500 km²)	<80 km	4 – 10 % steep or 2-4% moderately steep;	V, narrow U	Boulders (>256 mm), cobles (64 – 256 mm)	Fresh water; <0.5 ‰	Small riverbank terraces or non; sediment supply

[1] Ecoregions (ER): 12 – Pontic province, 7-Eastern Balkans; Sub-ecoregions (sub-ER) only for ER Pontic province: 12-1 Danube sub-ER, 12-2 Black Sea sub-ER
[2] The altitude usually varies to great extent and it requires a validation during First River Basin Management Plan (RBMP) 2010-2015
[3] Salinity is an obligatory factor for transitional waters represented by R16 Black Sea river firths

Type code	River type	Obligatory factors				Optional factors					Comments
		Ecoregion / Sub-ER[1]	Altitude[2]	Geology	Size	Distance from river source	Mean water slope/ Energy of flow	Valley shape	Mean substratum composition	Sali-nity[3]	
			on)				High E of flow				systems, erode rivers
R4	**Semi mountain rivers (gravel rivers)**	12-1,2	Big variation, semi-mountain zone	Mixed, siliceous, calcareous	<1300 km², small and medium (rarely large)	Usually significant distance	<2% slight steep; Moderate E of flow	U with broader valley	Coarse gravels (16 – 64 mm), fine and medium gravels (2-16)	Fresh water; <0.5 ‰	Serious transport ability
R5	**Semi mountain rivers (gravel rivers)**	7	Big variation, semi-mountain zone	Mixed, siliceous, calcareous	<1300 km², small and medium (rarely large)	Usually significant distance	<2% slight steep; Moderate E of flow	U with broader valley	Coarse gravels (16 – 64 mm), fine and medium gravels (2-16)	Fresh water; <0.5 ‰	Serious transport ability
R6	**Lower Danube**	12-1	<30 m	Mixed	>800,000 km², very large	> 1500 km	<0.1% (practically lack of slope)	Broad river valley	Sand (0.064 - 2), silt (<0.064), clay, loess	Fresh water; <0.5 ‰	Sediment deposit area; Possible gravel supplements
R7	**Large Danube**	12-1	<80 m (certain	Mixed	>2500 km², large	> 70 km	<0.5%; insignificant	Broad river	Sand (0.064 - 2), silt	Fresh	Sediment deposit

Type code	River type	Obligatory factors				Optional factors					Comments
		Ecoregion / Sub-ER[1]	Altitude[2]	Geology	Size	Distance from river source	Mean water slope/ Energy of flow	Valley shape	Mean substratum composition	Sali-nity[3]	
	tributaries		variation)				slope; Low E of flow	valley	(<0.064), clay, loess	water; <0.5 ‰	area; Possible gravel supplements
R8	**Medium and small Danube rivers**	12-1	<100 m (certain variation)	Calcareous, mixed, siliceous	<1300 km², medium and small (rarely large)	Variable significantly	<0.5% insignificant slope; Low E of flow	Broad river valley (often entrenched or deeply incised)	Sand (0.064 - 2), silt (<0.064), clay, loess	Fresh water; <0.5 ‰	Sediment deposit area; Possible gravel supplements
R9	**Losing karst rivers in Dobrudja area**	12-1	<300 m	Calcareous	<4000 km², very small and temporary rivers (as Q)	Variable	Variable	Often entrenched or deeply incised	Variable	Fresh water; <0.5 ‰	Rapid seasonal variation of water level; Temporary rivers in many cases
R10	**Large Black Sea rivers**	12-2	<90 m (variable	Mixed, siliceous	>1000 km², large	> 50 km	<0.5% insignificant	Broad river	Sand (0.064 - 2), silt	Fresh	Sediment deposit

Type code	River type	Obligatory factors				Optional factors					Comments
		Ecoregion / Sub-ER[1]	Altitude[2]	Geology	Size	Distance from river source	Mean water slope/ Energy of flow	Valley shape	Mean substratum composition	Sali-nity[3]	
			)				slope; Low E of flow	valley	(<0.064), clay	water; <0.5 ‰	area; Possible gravel supplements
R11	**Small and medium Black Sea rivers**	12-2	<70 m (variable)	Mixed, siliceous	<900 km², small and medium	Variable	<0.5% insignificant slope; Low E of flow	Broad river valley (often incised)	Sand (0.064 - 2), silt (<0.064), clay	Fresh water; <0.5 ‰	Sediment deposit area; Possible gravel supplements
R12	**Large floodplain rivers**	7	<150 (200) m variable	Mixed, siliceous	>7000 km², large and very large	> 60 km	<1% gentle slope;	Broad river valley	Sand (0.064 - 2)	Fresh water; <0.5 ‰	Sediment deposit area; Often gravel supplements
R13	**Small and medium floodplain rivers**	7	<150 (350) m variable	Mixed, siliceous	<1300 km², Medium and small	Variable	<1% gentle slope;	Broad river valley	Sand (0.064 - 2), silt (<0.064), gravel	Fresh water; <0.5 ‰	Sediment deposit area
R14	**Sub-Mediterran**	7	<500 (650) m	Mixed, siliceous,	<1100 km², Medium	Variable	Variable	Variable,	Variable	Fresh	Typical temporary

Type code	River type	Obligatory factors				Optional factors					Comments
		Ecoregion / Sub-ER[1]	Altitude[2]	Geology	Size	Distance from river source	Mean water slope/ Energy of flow	Valley shape	Mean substratum composition	Sali-nity[3]	
	ean rivers (temporary)		variable	calcareous	and small			all shapes		water; <0.5 ‰	rivers ; Often torrential rivers with huge variation in water flow
R15	**Karst springs**	12, 7	Variable	Calcareous	<10 km², small streams near to the source	< 5 km	N.A.	N.A.	Variable	Fresh water; <0.5 ‰	Often high hardness (conductivity.) and calcareous substratum
R16	**Black Sea river firths**	12-2	<5 (12) m	Mixed, siliceous	Variable	> 15 km	Non or very gentle slope	Broad river valley	Sands (0.064 - 2), silt (<0.064), clay	Mixo-oligohaline, often with seasonal salinity;	Sediment deposit area; Standing waters during low water level season

Table 4. *Ecological factors that determine the lake typology in Bulgaria according to System B of the WFD 2000/60/EC.*

Lake type code	Lake type	Obligatory factors					Optional factors				Comments
		ER/ Sub-ER[4]	Altitude[5]	Mean[6] depth	Geology	Size/ surface area	Depth (max.)	Residence time	Mixing characteristics	Salinity[7]	
L1	**High mountain glacial lakes**	12-1; 7	> 2000 m	< 3 до 15 m (rarely >20 m)	siliceous, mixed	<0.15 km², very small	< 50 m	N.A.	monomictic	<0.5 ‰;fresh water	ultra-oligotrophic lakes
L2	**Mountain lakes in ER 12**	12-1,2	mountainous zone – getting down sometimes (validation)	Unstudied (<15 m or very shallow)	mixed, siliceous, calcareous	<0.1 km², very small	<15 m <80 m reservoirs	N.A.	polymictic	<0.5 ‰;fresh water	oligotrophic conditions
L3	**Mountain lakes in ER 7**	7	> (600) 800 m, variable (validation)	< 3 m (peaty lakes); <15 m (for reservoirs) - variable	organic (peat), mixed, siliceous, calcareous	<0.1 km², very small (natural lakes); < 5 km² for HMWB	<6 m lakes <80 m reservoirs	N.A.	polymictic	<0.5 ‰;fresh water	natural dystrophic (peaty) lakes + mountain reservoirs (HMWB)
L4	**Lowland or semi-mountain natural lakes & swamps in**	12-1,2	variable, semi-mountain and lowland zones	< 3 m (rare more, small natural lakes and	mixed, siliceous, calcareous	<0.5 km² dominated < 5 km² (rarely, res.	<15 m	N.A.	polymictic	<0.5 ‰;fresh water	mesotrophic conditions

[4] Ecoregions (ER): 12 – Pontic province, 7-Eastern Balkans; Sub-ecoregions (sub-ER) only for ER Pontic province: 12-1 Danube sub-ER, 12-2 Black Sea sub-ER
[5] The altitude usually varies to great extent and it requires a validation during First River Basin Management Plan (RBMP) 2010-2015
[6] There is lack of data for mean water depth of most of lakes and reservoirs in Bulgaria
[7] Salinity is an obligatory factor for all transitional waters, e.g. for all Black Sea coastal lakes (types L7, L8, L9 и L10).

Lake type code	Lake type	Obligatory factors					Optional factors				Comments
		ER/ Sub-ER[4]	Altitude[5]	Mean[6] depth	Geology	Size/ surface area	Depth (max.)	Residence time	Mixing characteristics	Salinity[7]	
	ER 12		(validation)	swamps)		Rabisha)					
L5	**Riverine marshes in ER 12**	12-1,2	<80 m (variable)	< 3 m (rare more – for some excavation lakes)	mixed, siliceous	<5 km², very small to medium	<10 m deeper for artificial excavation lakes	N.A.	polymictic	<0.5 ‰;fresh water	mesotrophic to eutrophic conditions
L6	**Riverine wetlands in ER 7**	7	<300 m (variable) (validation)	< 3 m (rare more – for same excavation lakes)	mixed, siliceous, calcareous	<0.5 km², very small	<6 m Deeper for artificial excavation lakes	N.A.	polymictic	<0.5 ‰;fresh water	mesotrophic to eutrophic conditions
L7	**Black Sea freshwater coastal lakes**	12-2	<12 m	< 3 m (rare deeper)	mixed, siliceous, calcareous	<3.5 km², medium; >10 km² for HMWB	<15 m Deeper for HMWB	N.A.	polymictic	<0.5 ‰;fresh water	mesotrophic to eutrophic conditions; Transitional waters
L8	**Black Sea oligohaline coastal lakes**	12-2	<10 m	< 3 m	mixed, siliceous	Variable up to >10 km² (Burgas Lake)	<10 m	N.A.	polymictic	0.5-5‰ oligo-haline	mesotrophic to eutrophic conditions; Transitional waters
L9	**Black Sea**	12-2	< 5 m	< 3 m	mixed,	Variable	<15 m	N.A.	polymi	5-	salinity equal to

Lake type code	Lake type	Obligatory factors					Optional factors				Comments
		ER/ Sub-ER[4]	Altitude[5]	Mean[6] depth	Geology	Size/ surface area	Depth (max.)	Residence time	Mixing characteristics	Salinity[7]	
	mesohaline or polyhaline coastal lakes			(natural) < 15 m (HMWB)	siliceous, calcareous	up to >15 km² (Varna Lake)			ctic	30‰ meso-haline & poly-haline	the Black Sea; Transitional waters
L10	**Black Sea euhaline & hyperhaline coastal lakes**	12-2	< 5 m	< 1.5 m	mixed, siliceous	<20 km², large	<3 m	N.A.	polymictic	>40‰ super-haline	eutrophic to polytrophic conditions; Transitional waters
L11	**Large deep reservoirs / artificial lakes**	12-1,2; 7	variable	>15 m	mixed, siliceous, calcareous	>10 km², large	<120 m profundal zone	multi-annual	dimictic	<0.5 ‰;fresh water	Well-presented deep zone (profundal), with different ecological conditions than littoral zone; presence of thermocline in summer/autumn
L12	**Small and medium-size semi-mountain reservoirs / artificial lakes in ER**	12	>150 (200) m (up to mountain zone) variable (validation)	variable	mixed, siliceous, calcareous	1-10 km², medium; or 0.5 – 1 km² small	<80 m	annual, monthly or shorter period	dimictic; polymictic	<0.5 ‰;fresh water	Usually oligotrophic to mesotrophic conditions

Lake type code	Lake type	Obligatory factors					Optional factors				Comments
		ER/ Sub-ER[4]	Altitude[5]	Mean[6] depth	Geology	Size/ surface area	Depth (max.)	Residence time	Mixing characteristics	Salinity[7]	
	12										
L13	**Small and medium-size semi-mountain reservoirs / artificial lakes in ER 7**	7	150 (350) – 600 (800) m variable	variable	mixed, siliceous, calcareous	1-10 km², medium; or 0.5 – 1 km² small	<80 m	annual, monthly or shorter period	dimictic; polymictic	<0.5 ‰;fresh water	usually oligotrophic to mesotrophic conditions
L14	**Large lowland reservoirs with medium depth in ER 12**	12	usually <120 m variable	< 15 m	mixed, siliceous, calcareous	>10 km², large	<50 m profundal lacks or slightly developed	multi-annual; annual	polymictic	<0.5 ‰;fresh water	mesotrophic conditions; Lack of profundal zone and lack of summer stratification
L15	**Large lowland reservoir with medium depth in ER 7**	7	usually <200 m variable	<15 m	mixed, siliceous, calcareous	>10 km², large	<50 m profundal lacks or slightly developed	multi-annual; annual	polymictic	<0.5 ‰;fresh water	mesotrophic conditions; Lack of profundal zone and lack of summer stratification
L16	**Small and medium-size lowland reservoirs / artificial lakes in ER**	12-1,2	<120 m (validation)	<15 m (often <6m)	mixed, siliceous, calcareous	1-10 km², medium; or 0.5 – 1 km² small	<50 m variable	annual, monthly or shorter period	polymictic	<0.5 ‰;fresh water	mesotrophic conditions

Lake type code	Lake type	Obligatory factors					Optional factors				Comments
		ER/ Sub-ER[4]	Altitude[5]	Mean[6] depth	Geology	Size/ surface area	Depth (max.)	Residence time	Mixing characteristics	Salinity[7]	
	12										
L17	**Small and medium-size lowland reservoirs / artificial lakes in ER 7**	7	<200 m (validation)	<15 m (often <6m)	mixed, siliceous, calcareous	1-10 km², medium; or 0.5 – 1 km² small	<50 m variable	annual, monthly or shorter period	polymictic	<0.5 ‰;fresh water	mesotrophic conditions

BLAGOVEST KIRILOV TEMELKOV (1956 – 2010)

On October 12th 2010 the Department of Ecology and Environmental Conservation said the final goodbyes with our beloved and respected colleague - Blagovest Kirilov Temelkov who passed away after short illness.

He was born on June 18th 1956 in Plovdiv. In the period 1978 - 1989 he works as a laboratory technician at Higher Medical Institute - Plovdiv. Graduates University of Plovdiv "Paisii Hilendarski", specialty "Biology" (MSc) in 1992. In 1994 is employed as assistant professor at the Department of Ecology and Environmental Conservation, Faculty of Biology, University of Plovdiv. For years he develops and reads lectures of "Geology and Petrography", "Fundamentals of Palaeontology", "Historical geology" and "Palaecology" to the students from BSc "Ecology", "Biology" and "Biology and Chemistry" specialties.

His scientific interests consisted historical geology and palaeontology, taphonomy and ecology of fossil fauna, taxonomy and ecology of recent and fossil Foraminifera from the Black Sea Coast, Marine and aquatic ecology. He discovered and described one new species - G*uanduella podensis* and one new subspecies - *Porosononion subgranosus bulgaricus* of recent Foraminifera from the Bulgarian Black Sea Coast. Successfully defends his PhD thesis in 2002. He is author of 21 scientific publications and 3 books. He worked in the Department of Ecology and Environmental Conservation till 2010.

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IVELIN A. MOLLOV

GEORGI KANCHEV BACHVAROV
(1934-2010)

After long illness our respected colleague prof. Georgi Bachvarov passed away on November 2nd 2010.

Born in 1934 in Plovdiv. Graduated Sofia University "Kliment Ohridski" in 1957. He started working as assistant professor in Plovdiv University in 1962. He defended his PhD dissertation in 1970, specialized in the USSR in 1968 and in France in 1973. From 1971 is Associate Professor. Since 1973, he was deputy dean of Chemical-Biological Faculty of The University of Plovdiv and Head of the Department of Biology in 1974. Doctor of biological sciences since 1986. Professor since 1988. He is the creator and head of the Department of Ecology and Environmental Conservation since 1988 until his retirement in 2002.

Member of Society of Parasitologists in Bulgaria, the French Society of Parasitology and Balkan Federation of Ecology. Under his leadership are successfully defended two doctoral dissertations and over 100 theses.

His main research interests are in the field of environmental parasitology and aquatic toxicology. Were studied helminths of amphibians and reptiles from Bulgaria, France, Serbia, Guadeloupe, Togo and others. Specialist in the family Polistomatidae of international renown. He described 12 new species new to the science. After his name was named a parasite from West Africa (*Polistoma bachvarovi*). He has won several international competitions, the latter being for "high-level scientists and international renown". He worked together with experts from France, Togo, Russia, Poland, Yugoslavia, Turkey and Greece.

He has read lectures at the University of Thessaloniki and University of Perpignan (France). Lecture courses read by prof. Bachvarov include: Hydrobiology, Parasitology, Ecology of Animal General Ecology, Ecology and Environmental Protection, protected natural sites, Environmental parasitology, environmental protection.

He has participated in congresses of the Bulgarian Parasitological Society, Congress of the French Parasitological Society, International Congress of Parasitology in Montpellier, IKOPA - Warsaw, Kongpec in diseases of wildlife - the Netherlands, First Balkan Conference on Ecology – Thessaloniki and others.

Prof. Bachvarov's Parasitological collection of scientific materials and invertebrates collected in a period of more than 50 years and scientific literature were donated to the Zoological Institute and the National Museum of Natural History at the Bulgarian Academy of Sciences in Sofia, Bulgaria.

IVELIN A. MOLLOV

ECOLOGIA BALKANICA - INSTRUCTIONS TO AUTHORS

General information

Submissions to "Ecologia Balkanica" can be original studies dealing with all fields of ecology, including ecology and conservation of microorganisms, plants, aquatic and terrestrial animals, physiological ecology, behavioral ecology, population ecology, population genetics, community ecology, plant-animal interactions, ecosystem ecology, parasitology, animal evolution, ecological monitoring and bioindication, landscape and urban ecology, conservation ecology, as well as new methodical contributions in ecology. Studies conducted on the Balkans are a priority, but studies conducted in Europe or anywhere else in the World is accepted as well. All submission must be accompanied with filled out letter of submission, which can be downloaded from our site - http://eb.bio.uni-plovdiv.bg/about/instructions-to-authors/ and sent to the e-mail of the journal – ecologia_balikanica@abv.bg.

Manuscript submission

The following types of manuscripts are accepted: *short research notes* (up to 4 pages), *research articles* (4 to 10 pages) and *review papers* (10 to 20 pages). *Short research notes* are shorter submissions of a preliminary nature or those including new records or observed phenomenon etc. *Research articles* should present significant original research in the various fields of ecology, mentioned above. *Review papers* should deal with topics of general interest or of contemporary importance, being synthetic rather than comprehensive in emphasis. Authors of Review papers should consult with the Editor before submission. The Editor may also invite review articles concerning recent developments in particular areas of interest. The Editor reserves the right to decide if a manuscript should be treated as a Short note or Research article. In general, studies that are purely descriptive, mathematical, documentary, and/or natural history will not be considered.

Manuscripts must conform strictly with the instructions to authors and sent to the Editor. Incoming manuscripts are initially judged by the Editor. Manuscripts may be rejected without peer review if they do not comply with the instructions to authors or are beyond the scope of the journal. If the manuscript is acceptable in principle, it will be forwarded to referees for evaluation. All manuscripts are peer-reviewed by 2 or 3 independent reviewers. After final edition and approval by the editorial board, the manuscript will be accepted for publication. The Editor reserves the right to make editorial changes. Authors agree, after the manuscript's acceptance, with the transfer of copyright to the publisher.

Legal requirements

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract, or as part of a published lecture, or thesis); that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities -

tacitly or explicitly - at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Manuscript preparation

Language
The manuscripts must be prepared in English. Contributors who are not native English speakers are strongly advised to ensure that a colleague fluent in the English language, if none of the authors is so, has reviewed their manuscript. Spelling should be British or American English and should be consistent throughout. All abbreviations and acronyms should be defined at first mention. To facilitate reader comprehension, abbreviations should be used sparingly.

Technical information
Manuscripts must be submitted in **electronic version only**, as well as the original figures and tables. The manuscript text should be **MS-Word** processed, justified, font size 12, "Book Antiqua" or "Times New Roman", without footnotes, column or page breaks, single spaced (about 60 lines per page), on A4 (210 x 297 mm) paper, with margins of exactly 2.5 cm on each side. Pages should not be numbered.

The manuscripts should conform to the following format:
Title: Provide a title that is concise but also an informative synthesis of the study. Where appropriate, include mention of the family or higher taxon.
Author(s): Full first name(s), middle initials and surname(s) in bold italic.
Address(es): As complete as possible, including e-mail address(es).
Abstract: Maximum of 300 words and should summarize the essential results and conclusions with no description of methods, discussions, references and abbreviations.
Key words: Normally 3–10 words suitable for information-retrieval system.

The standard order of sections should be: Abstract, Key words, Introduction, Material and Methods, Results, Discussion (or Results and Discussion), Conclusions (optional), Acknowledgements and References.
The *Introduction* has to explain the actuality of the researched problem and give the aim of the study.
Materials and methods have to provide sufficient information to permit repetition of the experiment and/or fieldwork. The technical description of study methods should be given only if such methods are new; otherwise a short presentation is enough.
The *Results* section must be a concise presentation of the finding of the study. Avoid the presentation of same information as text and/or figure and/or table.
Discussion section should be separate from the results section at full-length papers and should deal with the significance of the results and their relationship to the aims of the paper. Also include how the findings of the paper will change, influence the state of our knowledge about model organism. In separate cases a joint section "Results and Discussion" is allowed but not preferable.

Conclusions should shortly describe the main contributions and recommendations of the study without including citations and statistics.
In the *Acknowledgements* section all persons and organizations that helped during the study in various ways, as well as the organization that financed the study must be listed.

Short Notes (generally less than four-five manuscript pages) should be produced as continuous text, preceded by an abstract of no more than 150 words.

Tables: The tables must not repeat information already presented in the figures or in the text. Each table must be self-explanatory and as simple as possible. No fold-outs are accepted. Tables must be numbered consecutively. They should be placed within the text at the desired position by the author(s). An explanatory caption, located on the top of the table, should be provided.

Example:

Table 1. *Shannon-Wiener indexes in the burned ($\boldsymbol{H}_{burned}$) and control ($\boldsymbol{H}_{control}$) territory for the total duration of the study (2004–2006).*

Illustrations: They must not repeat information already presented in the tables or in the text. Lines and letters in figures must be able to be enlarged or reduced without reduction in quality. They should conform to the size of the type area (16 × 24 cm) which is the limit for all illustrations. Magnification should be shown by scale bars. Colour illustrations are accepted, but will appear only in the electronic version of the journal (PDF). The illustrations in the hardcopy printed version will be greyscale. All illustrations must be sharp, of high quality with at least 300 dpi. The following formats are acceptable: JPEG, GIF, TIFF, EPS. Figures must be numbered consecutively and should be provided with an explanatory legend below them. They must be placed within the text at the desired position by the author(s).

Example:

Fig. 1. Indicative map of the study area.

All tables and figures must be referred to in the text.

Citations and references
Literature citations in the text should indicate the author's surname in SMALL CAPITALS with the year of publication in parentheses, e.g. CARLIN (1992); BROOKS & CARLIN (1992); SHAPIRO *et al.* (1968). Citations in brackets should be divided with semicolons and the author's name and the year of publication with comma (*example:* CARLIN, 1992; BROOKS & CARLIN, 1992; SHAPIRO *et al.*, 1968). If there are more than two authors, only the first should be named, followed by "*et al.*" in *italic*. References at the end of the paper should be listed in alphabetical order by the first author's family name and chronologically. If there is more than one work by the same author or team of authors in the same year, a, b, etc. is added to the year both in the text and

in the list of references. Each citation in the text must be accompanied by a full reference in the list of references and vice versa.

Examples:

A journal article:
AUTHOR A. 1990. Title of the article. - *Full title of the journal*, 56(3): 35-105.
AUTHOR A., B. AUTHOR. 1990. Title of the article. - *Full title of the journal*, 56(2): 35-105.
AUTHOR A., B. AUTHOR. C. AUTHOR. 1990. Title of the article. - *Full title of the journal*, 56(1): 35-105.

A book:
AUTHOR A. 2000. *Title of the book*. Vol. I. Place of publication. Publishing house. 220 p.

Proceedings or book chapter:
AUTHOR A., B. AUTHOR 1990. Title of the contribution. - In: Author A. (Ed.): *Title of the book or proceedings*. Place of publication. Publishing house, pp. 235-265.

Software:
STATSOFT INC. 2004. STATISTICA (Data analysis software system), Vers. 7. Computer software. [http://www.statsoft.com].
GARMIN LTD. 2007. MapSource, Vers. 6.12. Computer software. [http://www.garmin.com]

Website:
FAUNA EUROPAEA. 2007. Invertebrates. Fauna Europaea. Vers. 1.1. Available at: [http://www.faunaeur.org]. Accessed: 12.10.2009.

In case of papers written in other than Latin letters, if there is an English (or German, or French) title in the summary, it may be used. If there is not such a summary, the author's must be transcribed and the title of the paper must be translated into English and put in square brackets. If the name of the journal is also not in Latin letters it also should be transcribed. This should be noted in round brackets at the end of the paragraph, for instance: (In Bulgarian, English summary).

Example:

ANGELOV P. 1960. Communications entomologiques. I. Recherches sur la nourriture de certaines espèces de grenouilles. – *Godishnik na muzeite v grad Plovdiv*, 3: 333-337. (In Bulgarian, Russian and French summary).

KOROVIN V. 2004. [Golden Eagle (*Aquila heliaca*). Birds in agricultural landscapes of the Ural]. Ekaterinburg, Published by Ural University, 57 p. (In Russian).

Names of persons who provided unpublished information should be cited as follows: "(ANDERSSON, 2005, Stockholm, pers. comm.)".

Additional requirements

For special symbols (Greek letters, symbols for male and female etc.) use the Symbol list on the Insert menu in Microsoft Word with the following preferable fonts: Symbol, Webdings, Wingdings, Wingdings 2 and Wingdings 3. Degree symbols (°) must be used (from the Symbol list) and not superscript letter "o" or number "0". Multiplication symbols must be used (×) and not small "x" letters. Spaces must be inserted between numbers and units (e.g., 3 kg) and between numbers and mathematical symbols (+, –, ×, =, <, >), but not between numbers and percent symbols (e.g., 45%).

Small capitals and italic letters. The Latin genus and species names must be cited completely once in the text and should be typed in *italic*. Family names of authors of taxa and for publications listed in reference must be in SMALL CAPITALS, but never for collectors, preparators, acknowledgements, etc.

Word 2007. Unfortunately, the journal **cannot accept** Microsoft Word 2007 documents. Please use Word's 'Save As' option therefore to save your document as an older (2003 or earlier) ***.doc file** type.

Statistics

Mean values should always be accompanied by some measure of variation. If the goal is to describe variation among individuals that contribute to the mean standard deviation (SD) must be used. When the aim is to illustrate the precision of the mean standard errors (SE) should be given. The last paragraph of Materials and Methods section should briefly present the significance test used. Quote when possible the used software. Real p values must be quoted both at significance or non-significance. The use of the sign is acceptable only at low values of p (e.g. $p<0.0001$).

Ethics

The authors of articles that are based on experiments that caused injuries or death of animals should explain and justify the grounds of the study and state that the scientific results of the study is at least in trade-off with the sufferings caused. In the Materials and Methods of the manuscript, authors should detail as precisely the conditions of maintenance, transport, anaesthesia, and marking of animals. When available, references should be added to justify that the techniques used were not invasive. When alternative non-harming techniques exist, but were not used, the manuscripts may not be considered for publication.

Proofs and Reprints:

Proof will be sent to the **first (or corresponding) author** for checking (a PDF file) only once and it should be returned without delay. Corrections should be limited to typographical errors. No additional changes of the manuscript are allowed. Following publication, the first (or corresponding) author will be provided with both electronic copy (PDF) and **10 reprints** of the article.