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# Genome Instability of *Chironomus riparius* Mg. (Diptera, Chironomidae) from Polluted Water Basins in Bulgaria

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**Abstract.** Larvae of *Chironomus riparius* Mg. (Chironomidae, Diptera) collected from two polluted water basins in Bulgaria, the Maritsa and Chaya Rivers (adjacent to Plovdiv and Asenovgrad respectively), a small pool (near Plovdiv) plus controls reared in the laboratory were studied. High concentrations of the heavy metals Pb, Cu and Cd were recorded in the sediments of the polluted stations. Marked somatic structural chromosome aberrations were found in *C. riparius* salivary polytene chromosomes from the field stations and their frequency was significantly higher ($p<0.01$) compared to the control. The observed somatic chromosome changes are discussed as a response of the chironomid genome to aquatic pollution. A new cytogenetic index based on the number of aberrations found in larvae from polluted regions in comparison with the control was applied to the data to more easily evaluate the degree of heavy metal pollution in aquatic ecosystems. Our study of a polluted site near the River Chaya showed that the somatic index was very high at 3.35 for 2010 and 11.66 for 2013 compared to 0.5 in the control. The cytogenetic index was effective in showing that all studied sites were highly polluted in comparison with the control. To determine the mechanism involved in the concentration of aberration breakpoints within specific regions of the chironomid polytene chromosome the FISH method was applied. The localization of a transposable element TFB1 along the polytene chromosomes of *C. riparius* was analyzed and the sites of localization were compared with breakpoints of chromosome aberrations. A significant correlation ($p<0.05$) was found which shows that most of the aberrations do not appear randomly but are concentrated in sites rich in transposable elements.

**Key words:** Chironomidae, polytene chromosomes, somatic and cytogenetic index, heavy metals.

## Introduction

Pollution of aquatic ecosystems is a major ecological problem globally, coinciding with the rapid industrialization and urbanization that began in the early nineteenth century. In Bulgaria, numerous rivers have been classified as very polluted and shown to be contaminated with heavy metals by the National Biomonitoring Program (PEEV & GERASIMOV, 1999). The Maritsa and Chaya Rivers are polluted rivers in Bulgaria, with sediments highly contaminated with metals such as Cr, Cu, Mn, Pb and Cd (MICHAILOVA *et al*., 2012a). The biological effect of heavy metal exposure is of growing concern due to the increasing release of such metals into the environment via industrial and agricultural processes, exacerbated by their tendency to accumulate in biotic systems (WHITESIDE *et al*., 2010).

Biological indicators are useful in assessing the overall effect of environmental contaminations by virtue of their important role aquatic ecosystem structure and function (MENG *et al*., 2009). The larvae of

non-biting aquatic midge *Chironomus riparius* are dominant in many aquatic ecosystems, especially those with moderate to high levels of pollution (AL-SHAMI *et al*., 2010). *C. riparius* is considered as an ideal organism for ecotoxicological monitoring as the larvae spend most of their life cycle at the sediment-water interface where they therefore are exposed to toxicants both in the water and sediment. They have a short life cycle (MEBANE *et al*., 2008; AL-SHAMI *et al*., 2010; MICHAILOVA *et al*., 2012b) and are ideal organisms for comparative bioassays in the laboratory because they are relatively easy to culture (WARWICK, 1988). Moreover, our previous studies have showed that the polytene chromosomes of this species can be used as a bioassay of heavy-metal-induced genome instability (MICHAILOVA *et al*., 2012b).

In order to evaluate the impact of heavy metals on biota of the aquatic basins we used structural and functional alterations of the salivary gland chromosomes of Chironomids which provide early warning indicators of contaminants in the environment.

In this paper we outline a new, sensitive and a reliable method to assess the impact of toxicants, specifically heavy metals, on the genome of the larva stage of *C. riparius*. On the basis of different somatic chromosome alterations we propose a cytogenetic index as a sublethal method to assess the degree of pollution in freshwaters. For this purpose cytogenetic damage was investigated by analyzing the somatic alterations in individual *C. riparius* collected from the polluted Maritsa and Chaya Rivers and compare with mean somatic rearrangements found in laboratory reared control populations of the same species. The index, a ratio of somatic rearrangements in a control to potentially polluted environment is simple to apply and, by definition, provides as sublethal measure of pollution damage. We also performed in situ hybridization with selected transposable element in order to assess whether the somatic chromosome rearrangements are randomly distributed.

## Material and Methods

### Material

*Chironomus riparius* larvae were collected from the three stations in Maritsa and Chaya Rivers (Kemera, Asenovgrad and Katuniza) (in 2009, 2010, and 2013) and a farm near Plovdiv (in 2010). On the basis of data from the Bulgarian National Biomonitoring Program (1999), the Maritsa and Chaya Rivers are subject to industrial pollution, including the heavy metals Pb and Zn, and also $S0_2$, $N0_2$ (MICHAILOVA *et al*., 2012a). The pollution originates from producing metal products. A further site near Plovdiv is subject to domestic sewage and animal wastes.

The control (lab. stock) *C. riparius* larvae were reared from an existing laboratory population in pure mash filter paper under standard conditions (temperature 18 - 20°C, photoperiod 16h light & 8h dark; feeding 2 times a week and constant aeration).

### Methods

*Cytogenetic analysis.* For cytogenetic analysis, the larvae of *C. riparius,* collected in the field and those from the lab. stock were fixed in alcohol: acetic acid (3:1). Polytene chromosome preparations were prepared using conventional cytogenetic method (MICHAILOVA, 1989). Chromosome maps of HäGELE (1970) and KIKNADZE *et al*. (1991) were used as a standard and applied to localize the somatic chromosome rearrangements.

*In situ hybridization.* A probe of fold-back transposable element was used. The TFB1 326 bp probe was obtained by using the following primers:

TFB1311-F (5'-GCAACGACTATTCCTACCTTGCC-3') and TFB1636-R (5'-TCACACCGTTTTCACGTGTGAATCT-3') and labeled with Digoxigenin. The hybridization signals were detected by the anti-digoxigenin antibody (Roche).

The locations of the TE copies along the salivary gland chromosomes were determined by FISH, following the methods of SCHMIDT (1992) and HANKELN *et al*.

(1993). The signals found in all studied cells and individuals were considered as fixed signals; variable signals appeared in all or almost all cells but not in all studied individuals (MICHAILOVA *et al*., 2009).

*Water and sediment metal analysis.* Two samples of water and sediment were collected from each site. The water samples were acidified on site using trace metal grade nitric acid. Acidification ensured that there was no adsorption on to the walls of the container and that the metals remained in their dissolved state for later analysis. Sediment samples were stored on plastic containers and frozen until analyzed.

Sediment samples were placed in an oven until dry, passed through a 250 micron sieve and a known amount in the range of 0.08-0.15g from each site transferred to glass vials for immediate analysis. Five ml of trace metal grade nitric acid was added to each sediment sample and digested on a hotplate. A marble was placed on top of each glass vial to facilitate reflux and the samples boiled gently until fumes of $NO_2$ were no longer produced. Once the digest had been completed the solutions were transferred to 10ml volumetric flasks and made up to volume using deionized water. Any remaining particulate in the samples was then removed by filtering through a 45 micron Whatman glass fiber filter paper.

After appropriate dilution in deionised water, metal (Cu, Cd, Pb, Cr and Mn) analysis of both water samples and sediment digests was carried out by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using a Perkin-Elmer Optima 5300. The spectroscope was calibrated using an internal standard solution, which was a matrix matched serial dilution of Specpure multi element plasma standard solution 4 (Alfa Aesar).

*Data analysis.* We compared the somatic alterations in the polytene chromosomes of *C. riparius* from polluted stations with those from the laboratory stock used as a control. The types and localization of chromosome alterations of the polytene chromosomes of *C. riparius* from Maritsa and Chaya Rivers collected in 2009, 2010 and the Plovdiv farm collected in 2010 were analyzed as reported previously (MICHAILOVA *et al*., 2012a). In this paper we analyzed somatic chromosome rearrangements, their types and localization in *C. riparius* from Chaya River (2013) and the control.

Somatic aberrations affect only a few cells of the salivary glands of *C. riparius* and these were used for analyzing the somatic index (SELLA *et al*., 2004) by dividing the number of aberrations to the number of analyzed individuals at each site.

Here we present a new cytogenetic index of the polytene chromosomes of *C. riparius* collected from the Chaya River in 2013 and the control and further statistical analysis of somatic chromosome rearrangements found in this species from the Maritsa and Chaya Rivers collected in 2009, 2010 and from the Plovdiv farm in 2010. We applied the cytogenetic index as the ratio of the percentage aberrations in control to percentage aberrations in polytene chromosome of *C. riparius* from each polluted site.

We statistically analyzed somatic aberrations to make a comparative analysis between polluted and unpolluted material (control), using contingency G test (SOKAL & ROHLF, 1995). The values $p<0.05$ was considered as significant.

## Results and Discussion

*1. Water and sediment analysis*

Concentrations of Cu, Cd, Pb, Cr and Mn in the water collected in 2013 were not elevated when compared to water quality standards (data not shown). However, in the four sites the concentrations of trace metal in the sediments were higher than those of reference data (FÖRSTNER & SALOMONS, 1980) and, as previously shown (MICHAILOVA *et al*., 2012a) in 2009 and 2010 (Table 1). The concentrations of Cu in the Maritsa River sediment collected in 2009 and 2010 were 5.8 and 4.8 times higher than the reference data; in Chaya River the concentration was between 3 to 12 times higher in 2009 and 2010; the Farm - 2.6 times. Lead in the Maritsa River sediments in 2009 and 2010 were 24 and 35 times

higher respectively; in the Chaya River (2009, 2010 and 2013) between 24-36 times higher and 7.8 times greater at the farm site. Cadmium concentrations in the Maritsa River - 84-94 times higher in 2009 and 2010; concentrations in the Chaya River (2009, 2010, 2013) were between 14-105 times higher; the farm site contained 14 times more metal than the reference data reported in FÖRSTNER & SALOMONS (1980). The concentrations of Pb, Cu and Cd in the sediments of the field stations were all higher than the control sediment (Table 1).

*2. Cytogenetic characteristics and somatic chromosome alterations*

*Chironomus riparius* has a chromosome set 2n = 8, belonging to the thummi cytocomplex (KEYL, 1962) with chromosome arm combination AB, CD, EF, G. Chromosomes AB, CD are metacentric, chromosome EF – submetacentric and chromosome G acrocentric (Fig. 1). Three Balbiani rings (BRa, BRb, BRc) and a Nucleoli Organizer (NOR) are localized in chromosome G. BRa is expressed in only a few cells of the salivary glands.

**Table 1.** Concentration (μg/g) of trace metals in the sediments of the each station

| **Stations** | **Cr** | **Cu** | **Mn** | **Pb** | **Cd** |
|---|---|---|---|---|---|
| Fossil sediment | 59 | 25 | 406 | 16 | 0.2 |
| Maritsa River, 2009 | 45.19 | 120 | 1885.4 | 388.2 | 16.83 |
| Maritsa River, 2010 | 0.173 | 144 | 63.8 | 559.78 | 18.81 |
| Chaya River, 2009 | 70.4 | 314.3 | 434.7 | 585.9 | 7.3 |
| Chaya River, 2010 | 0.114 | 213.8 | 60.21 | 536.75 | 2.86 |
| Chaya River, 2013 | 18.4 | 75.23 | 765.87 | 383.41 | 21.03 |
| Plovdiv-farm, 2010 | 0.127 | 66.44 | 74.05 | 293.07 | 2.84 |
| Control, 2013 | 45.76 | 134.97 | 720.35 | 125.94 | 1.99 |

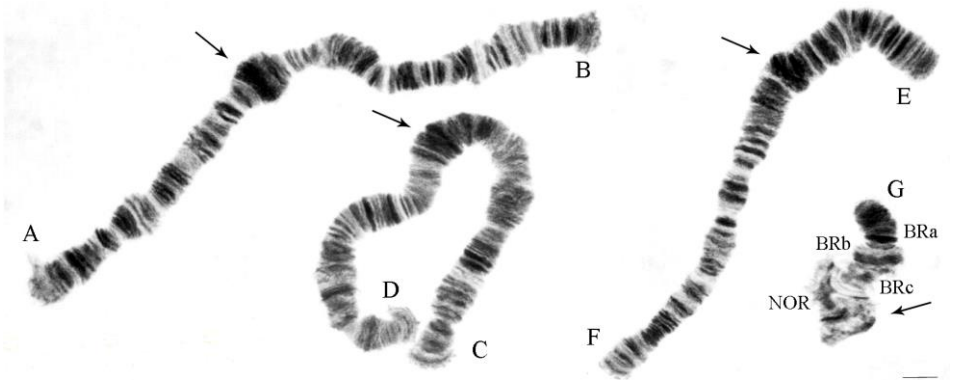


**Fig 1.** Standard karyotype of *C. riparius;* Balbiani Ring (BR); Nucleolar Organizer (NOR). The arrow indicates the centromere region in each chromosome. Bar - 10μm

A number of different somatic chromosome rearrangements (para- and pericentric heterozygous inversions, amplifications, deletions and deficiencies) were observed in the polytene chromosomes of *C. riparius* from all sites on the Chaya and Maritsa Rivers (Fig.2). The highest somatic index was calculated in *C. riparius* from the Chaya River (2013, 2010 – 11.66 and 3.25) (Table 2) where the concentrations of trace metals were highest.

As shown in Fig. 3, a significantly higher frequency of somatic alterations was found in larvae collected from all polluted sites when compared with larvae reared under laboratory conditions and hence not exposed to toxicants (Maritsa River, 2009 – $G = 66.631$, $df = 1$, $p<0.001$; Maritsa River,

2010 – G = 36. 889, df = 1, $p< 0.001$; Chaya River, 2009 - G = 73.766, df = 1, $p<0.001$; Chaya River, 2010 - G = 40.802, df = 1, $p< 0.001$; Chaya River, 2013 – G = 97.496, df = 1, $p<0.001$; Plovdiv, farm 2010 – G = 39.447, df = 1, $p<0.001$). Our comparative study of this biomarker revealed marked differences in the natural populations of *C. riparius* larvae collected from different polluted habitats. However, it is important to bear in mind that, under field conditions, it is difficult to attribute observed changes to a single metal because synergistic and antagonistic interactions may occur. Therefore, on the basis of applying, somatic and cytogenetic index we are not able to ascertain which trace metal specifically affects the genome structure, but we can show the degree of pollution at the different sites.

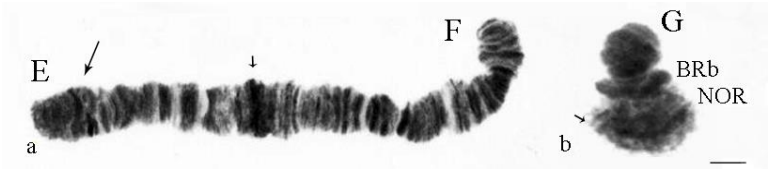


**Fig. 2.** Somatic aberrations in *C. riparius* polytene chromosomes; Balbiani Ring (BR); Nucleolar Organizer (NOR). The large arrow indicates the somatic aberration; the small arrow shows the localization of the centromere region. Bar - 10μm; a) Somatic heterozygous inversion in arm E; b) deletion of BRc

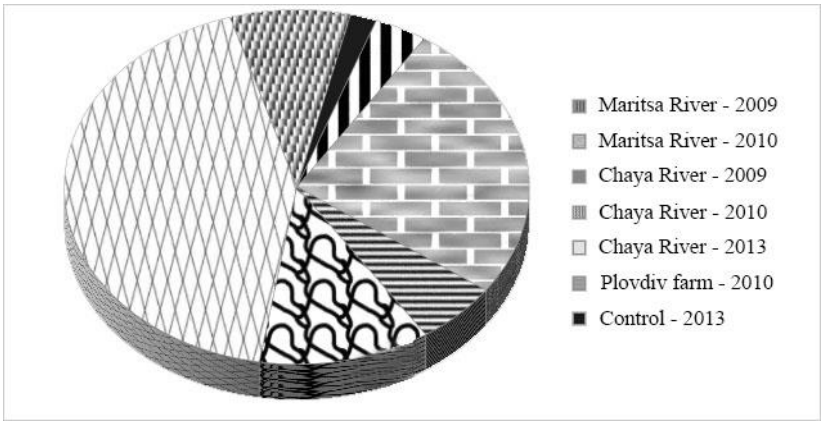


**Fig. 3.** Somatic index at each site studied

For the first time we suggest to use a cytogenetic index as a biomarker for evaluating the degree of environmental pollution (Table 2). The laboratory reared stock, which we used as a control therefore has a cytogenetic index of 1. On the basis of this index we proposed three levels of pollutants: slight pollution with a cytogenetic index near to the control (0.9-0.8), moderate polluted (0.7-0.5) and highly polluted (<0.5). Comparing our data using this cytogenetic index we can conclude that the all stations of both rivers are highly polluted. The Maritsa River (2010) and the Chaya River (2010, 2013) are the most heavy metal polluted, followed by the farm near Plovdiv and the Maritsa and Chaya Rivers in 2009 (Table 3).

The present results indicate that heavy metal pollutants at sublethal concentration influence the genome of the ubiquitous aquatic insect *C. riparius*. Our study suggests a relationship between concentrations of trace metals and a cytogenetic index of somatic chromosome rearrangements in *C. riparius* and this approach therefore merits further investigation. This could include examining the response to other stressors such as pesticides. We further suggest that our cytogenetic index using this model chironomid species would be a useful approach to be included in the suite of bioassessment methods.

*3. In situ hybridization*

The localization of TFB1 copies along polytene chromosomes of *C. riparius* from

the Chaya River (2010) and the farm adjacent to Plovdiv was studied and compared with observed breakpoints of somatic aberrations. The number of observed TFB1 signals is shown in Table 3. The fixed signals of the TE were concentrated in the proximal parts of the chromosomes (centromere regions) and variable signals were localized along the chromosome arms.

**Table 2.** Somatic chromosome rearrangements in the polytene chromosome of *C.riparius*, collected from polluted stations in Bulgaria and control plus the cytogenetic index for each site studied

| Site /year | No indiv-iduals | No cells | No /% indv. with aberr. | No / % cells with aberr. | No somatic aberr. | S. index | Average No aberr. per indv. ($\bar{x}$ ±SD) | Cyto-genetic index | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Maritsa River (2009) | 13 | **394** | 12/92.31 | 90/22.84 | 13 | 1 | 22.30±10.61 | **0.0968** | MICHAILOVA *et al*., 2012a; this study |
| Maritsa River (2010) | 1 | 38 | 1/100 | 14/36.84 | 7 | 7 | 37±15.10 | **0.0584** | MICHAILOVA *et al*., 2012a; this study |
| Chaya River (2009) | 14 | 347 | 14/100 | 87/25.07 | 22 | 1.57 | 23.88±9.63 | **0.0904** | MICHAILOVA *et al*., 2012a; this study |
| Chaya River (2010) | 4 | 44 | 4/100 | 16/36.36 | 13 | 3.25 | 33.33±29.16 | **0.0648** | MICHAILOVA *et al*., 2012a; this study |
| Chaya River (2013) | 3 | 106 | 3/100 | 46 / 43.4 | 35 | 11.66 | 34±7.72 | **0.0635** | This study |
| Plovdiv farm (2010) | 7 | 111 | 7/100 | 26/23.42 | 16 | 2.28 | 28.71±16.67 | **0.0742** | MICHAILOVA *et al*., 2012a; this study |
| Control (2013) | 14 | 282 | 5 | 7/ 2.48 | 7 | 0.5 | 2.16±3.22 | **1** | This study |

**Table 3.** Number of TFB1 signals and common breakpoints of chromosome aberrations in *C. riparius* polytene chromosomes collected from two polluted stations

| Station | Number of breakpoints | Common breakpoints of chromosome aberrations | Number of signals | |
|---|---|---|---|---|
| | | | Fixed signals | Variable signals |
| **Plovdiv farm, 2010** | 65 | 46 | 17 | 135 |
| **Chaya River, 2010** | 85 | 60 | 13 | 94 |

In the larvae from the farm site, 22 of common breakpoints coincided with sites of TFB1. The correlation between frequency of common breakpoints and frequency of insertion sites of the TE was significant (rs = 0.53; df = 10; $p$<0.05). In the individuals from the other polluted sites on the Chaya River (Asenovgrad) we found 18 common breakpoints which significantly coincided with the TFB sites (rs = 0.85, df = 10; $p$<0.01) (ILKOVA *et al*., 2013).

These data support the hypothesis that aberration breakpoints do not appear randomly in the *C. riparius* genome and some sites on the chromosomes, called by us "hot spots", are more sensitive to various

environmental stress factors. BOVERO *et al*. (2002), MICHAILOVA *et al*. (2007), ILKOVA *et al*. (2007) found at these sites different mobile elements which are involved in producing the somatic chromosome rearrangements. The same effect have been established for other transposable elements (NLRCth1, CTRT) in the *C. riparius* genome (ILKOVA *et al*., 2013), in *C. piger* genome (ILKOVA *et al*., 2007; MICHAILOVA *et al*., 2009), plus in the fruit fly *Drosophila* (ZELENTSOVA *et al*., 1999, CASALS *et al*., 2005). These results support the hypothesis that stress agents in the environment activate transposable element mobility in the genome and influence genome instability, resulting in numerous somatic chromosome rearrangements (GUERREIRO, 2012; KALE *et al*., 2005).

In summary, our study has shown that the *C. riparius* genome is very sensitive to heavy metals stress and a number of different chromosome changes can be observed. On the basis of these changes and using somatic and cytogenetic index we are able to estimate the degree of pollution in river basins. Further work is required to establish a clearer relationship between individual heavy metal contaminants and the cytogenetic index over a range of metal concentrations and conditions, plus an examination of other anthropogenic stressors such as anoxia arising from pesticide release, organic pollution and eutrophication. The cytogenetic index could then prove to be a reliable and effective biomarker tool to assess the degree of genotoxicity of different toxicants.

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# *Ex Situ and In Vitro Conservation of Glicyrrhiza glabra L. – Crop Wild Relative from Fabaceae*

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**Abstract.** *Glycyrrhiza glabra* L. (Licorice), belongs to the Fabaceae family. The species is included in the Crop Wild relatives list for Bulgaria and is close to beans and peas. Its origin is Southeast Europe, the Mediterranean and Asia, where Bulgaria falls. The roots of the plant contain glycyrrhizin, 30 to 50 times sweetener than the sucrose. As a CWR, as well as due to the high level of glycyrrhizin in the roots, the conservation and maintenance of the species is of interest, although it is not included in the list of threatened plant species yet. In the Institute of Plant Genetic Resources - Sadovo *Glycyrrhiza glabra* is maintained in situ in the Botanical garden. Besides the in situ conservation of the species, *in vitro* techniques are a reliable means of reproduction and long-term storage. After introduction of the raw cuttings from plant species in culture *in vitro*, the process of micropropagation is accomplished by single bud microcuttings in nutrient medium fitted with growth regulators, enabling the development of single-rooted stems with options of repeatedly subcultivating. Along with that the possibility for long term *in vitro* propagation by reduction of the composition of the nutrient medium was tested, where the period for conservation of the cultivated explants reaches 8 months.

**Key words:** *Glycyrrhiza glabra, ex situ* conservation, *in vitro* conservation, Fabaceae

## Introduction

*Glycyrrhiza glabra* L. belongs to *Fabaceae.* The species is included in the Red Book of protected plants according to the Convention for biodiversity (Secretariat of the Convention on Biological Diversity, 2005.). Part of the population is in the protected area Dolni Vit. Othert part is in protected areas from NATURA 2000 (PEEV, 2011). The genera is CWR of beans and peas. For further protection of *G. glabra* are necessary additional investigations to show it`s potential to grow in different soil and climatic conditions, particularly in terms of Sadovo, which are quite different from the conditions in Danube plain.

The area of origin of *G. glabra* is the Mediterranean, where Bulgaria is situated (ZEVEN *et al*., 1982)

Other option for preservation of *G. glabra* is the *in vitro* techniques for long-term storage with opportunities for precultivation. Besides low temperatures, growth retardants, osmotic stress reduction of the growth potential *in vitro* can be easier obtained by reduction of the chemical compound of the nutrient substance (REED, 1999; PAULA, 2000; LAMBARDI *et al.*, 2006; PREVIATI *et al*., 2008; OZUDOGRU *et al*., 2010). Similar studies have been made for many plant species – potatoes, roses, humulus, ornamental plants etc. The reduction of

mucro- and micro salts in the nutrient substance (MURASHIGE & SKOOG, 1962) up to 25, 50 and 75% the *in vitro* growth of tomatoes and carnations is suppressed. For *in vitro* preservation of vitis SHIBLI *et al.* (2006) makes investigation by reduction only of the amonium nitrate up to 6 and 25% of it's total quantity in the nutrient substance. The cultivated nodal segments (0.5mm) are kept 262-290 days with preserved vitality up to 70-80%. There are no investigations in that area for *G. glabra* up to now.

The purpose of the current study is to establish the possibilities for *ex situ* and *in vitro* preservation and propagation of *G. glabra* with the aim it`s preservation and practical use.

**Material and Methods**

*G. glabra* is kept *ex situ* in the Botanical garden of the IPGR – Sadovo. The species is collected during expeditions in Danube plain. In order to establish if the plant can succesfuly develop in the condition of Sadovo, different from the natuaral habitat, 3-years biomentrical investigations have been done as follows: lengh of the stem (cm), lengh and width of leaf (cm), dimensions of the blossom parts (cm) – corrola, flag and wings, lengh and width of the pod (cm) and diameter of seed (mm).

For the *in vitro* experimental work 10 mm microcuttings with one stem nod are used as starting material. The nutrient medium with mineral solution after Knop, modified after Mur, microelements after Berthelot and vitamins after Morel (GRENAN, 1979) is a relative good composition for root development and single leading shoot for a period of 25-35 days. To reduce the growth force of cultured explants by reducing the composition of the nutrient medium, the experimental work was displayed in the following variants:

1. Reduction of the Nitrogen compounds up to ¼ of their total quantity (KNO3 - 237 mg, NH4NO3 -90mg) (¼ PAC).

2. Reduction of macro- and micro elements up to ¼ of their composition (¼ PMM).

3. Reduction of the overall chemical composition of the medium to ¼ of the total intensity (¼ POXC).

Plants were grown under lights 1000 lx, photoperiod 16/8 hours day/night and temperature 24°C.

For each option were included 20 explants with 4-4-fold repetition of the experiments.

The survival rate (%) and the duration of maintaining in *in vitro* conditions (days) of the explants was recorded. After the storage period of the regeneration capacity of the stored material was determined by re-culturing in a standard culture medium, expressed in time (days) required for the formation of the leading shoot stem with 5 knots. For control were used unstored microcutings from *G. glabra.*

**Results and Discussion**

According to JORDANOV (1976) *G. glabra* in it1s natural habitats is perrenial shrub with thickq woody roodstock with shoots. Stems are erectedm 50-100 cm heigh, ribbed, reddish, highly branched, fibrous, rarely acute rough. Leaves longer than internodes, petals elliptical, curved on the top, (Fig. 1).

**Fig. 1.** *Glycyrrhiza glabra* L.

The inflorescence spherical to cylindrical, loose. The flowers sessile. Calyx shorter than the corolla. The corolla 8-10 mm, lilac. The flag eleptical, curved on the top, in the bottom concave in triangular claw. Wings equal to the flag or little shorter. Keel equal to the flag and wings, acute on the top, petiole twice longetr than the claw. Pods are

1-3 cm long and 4-5 mm wide, smooth. Seeds greenish, spherical. Blossoms from July to August. Grows in dry, grassy areas.

To study the growth behavior of *G. glabra* in nonspecific conditions for the plant species, namely, *ex situ* maintenance in the Botanical garden IPGR - Sadovo three-year biometric analyzes were carried out, summary results of which are shown in Table. 1.

**Table 1.** Summary results from three-year biometric analyzes of *G glabra* L.

| Index \ Value | | x±Sx | min | max | CV% |
|---|---|---|---|---|---|
| Lengh of the stem (cm) | | 65.06±2.99 | 53.0 | 85.0 | 89.19 |
| Leaf | length (cm) | 10.9±0.57 | 9.0 | 14.0 | 3.21 |
| | width (cm) | 3.71±0.26 | 2.5 | 4.6 | 0.7 |
| Blossom | corrola length (cm) | 9.24±0.37 | 7.5 | 11.0 | 1.33 |
| | flag length (cm) | 10.88±0.17 | 10.0 | 11.9 | 0.29 |
| | wings length (cm) | 9.43±0.14 | 8.9 | 10.1 | 0.2 |
| Pod | length (cm) | 2.7±0.15 | 1.5 | 2.9 | 0.22 |
| | width (cm) | 3.43±0.18 | 2.8 | 4.5 | 0.33 |
| Seed diameter, mm. | | 3.06±0.06 | 2.9 | 3.5 | 0.033 |

The lengh of the stem is 65.06 cm ±2,99. The minimum is 53 cm and the maximum is 85 cm. The degree of variation of this indicator is very high (89.19%). Leaf size vary from 9-15 cm in length (10.9±0.57) and 2.5 – 4.6 cm width (3.71±0.26). The variation of that indexes is insignificant. The variation of the size of the flower is also very low. The corolla length is 7.5 – 11 cm (9.24±0.37), th flag length is 10.0-11.9 cm (10.88±0.17) and length of the wings is 8.9 – 10.1 cm (9.43±0.14). The pod is comparatively short, 1.5 – 2.9 cm (2.7±0.15) long and 2.8 – 4.5 cm (3.43±0.18) wide. The seed is small, spherical in shape with diameter 2.9 – 3.5 mm (3.06±0.06). These results confirm the data of other authors (IVANOV *et al.,* 1977) and shows that *G. glabra* can successfully grow in different climatic conditions.

The level of survival of the *in vitro* explants as well as the duration of their conservation are the twoo basic indexes, having equal importance for *in vitro* preservation. The results fro that study in Table 2 show some regularities and dependencies between both indexes under the influence of investigated factors. The main tendency, that comes out from the data from the three repetitions is elongation of the storage period under lower survival and the explants as well as the opposite, the more explants survived, the period for storage id shorter. That tendency is a result from the interaction between the compounds of the nutrient media, which is cleared by detailed analysis of the results for the different variants. In the variant of reduction of the Nitrogen compounds up to 1/4 was recorded comparatively high percent of vitality of the explants (76.5%), but the duration of their preservation in *in vitro* conditions was very short – 102.2 days.

In reduction of the macro- and micro elements up to 1/4 from the compound od the nutrient media the level of survival of the explants is reduced (61.6%) and the recorded period of storage is 137.7 days.

In the case of total reduction of the chemical compounds in the nutrient media up to 1/4, 59.9% from the cultivated microcuttings preserve their vitality, but the period of storage is elongated up to 239.7 days. That is due to the lower concentration of growth regulators and thus the growth process is suppressed and the time of preservation is elongated, (Fig. 2&3).

After the storage period, in recultivation of the explants to standard nutrient media (with full compound) was recorded that there is no difference in time, nececary

**Table 2.** Development of microcuttings from *G. glabra* in in vitro storage and after that. Legend: RNC - Reduction of Nitrogen Compounds, RMM - Reduction of Macro- and Micro elements, RNC - Reduction of the Nutrient Compound.

| | **Number of microcuttings** | **Vitality of the explants** | **Duration of the storage** | **Time for regeneration** | **Control** |
|---|---|---|---|---|---|
| Variant 1 1/2 RNC | number | /%/ | days | days | days |
| | 80 | 76.5 | 102.2 | 33.4 | 32.7 |
| Variant 2 1/2 RMM | number | /%/ | days | days | days |
| | 80 | 61.6 | 137.7 | 30.5 | 35.2 |
| Variant 3 1/2 RNC | number | % | days | days | days |
| | 80 | 59.9 | 239.7 | 34.6 | 31.9 |

**Fig. 2.** In vitro storage of *G. glabra*

**Fig. 3.** Control

for formation of the shoots up to 5- stem nod between the stored in ¼ PAC, ¼ RMM, ¼ RNC (30.5 – 34.6 days), as well as between them and unstored explants (control – 31.9 – 35.2 days). So in *in vitro* storage of microcuttings of *G. glabra* by reducing of the chemical compound af the nutrient media the explants do not change their regeneration and growing potential, which is realised in equal level with th eunstored explants.

**Conclusions**

1. *Glycyrrhiza glabra* can be succesfully grown ex sity in the botanical garden of the IPGR – Sadovo, which ensures the presrvation of the species as valuable plant genetic resources in different conditions.

2. The optimalway for *in vitro* preservation of explants from *Glycyrrhiza glabra* is the variant with 1/4 reduction of the whole chemical compound of the

nutrient media (RNC), where 8 month period for conservation in 60% preserved vitality is ensured, without any changes in their regeneration potential.

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# *Determination of Heavy Metals in Roach (Rutilus rutilus) and Bleak (Alburnus alburnus) in Zhrebchevo Dam Lake*

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**Abstract.** The aim of this study was to examine the concentration of iron (Fe), nickel (Ni), lead (Pb), manganese (Mn), copper (Cu), chromium (Cr), cadmium (Cd) and zinc (Zn) in roach (*Rutilus rutilus*) and bleak (*Alburnus alburnus*) as species for human consummation. Two fresh water fish species, roach and bleak were caught from Zhrebchevo Dam Lake in Bulgaria. Determination of heavy metals (Fe, Cu, Ni, Pb, Zn, Mn, Cr and Cd) in muscle samples were performed with electro thermal atomic absorption spectrometry (ETAAS). The heavy metal content in the meat of roach and bleak were found to be 0.59±0.032 - 0.69±0.128 mg $kg^{-1}$ for Cu, 6.59±0.224 - 7.34±0.142 mg $kg^{-1}$ for Fe, 0.03±0.025 - 0.04±0.012 mg $kg^{-1}$ for Ni, 0.06±0.044 - 0.07±0.031 mg $kg^{-1}$ for Pb, 4.05±0.263 - 5.46±0.388 mg $kg^{-1}$ for Zn, 0.49±0.060 - 0.72±0.080 mg $kg^{-1}$ for Mn, 0.09±0.036 - 0.1±0.045 mg $kg^{-1}$ for Cr, 0.01±0.002 - 0.01±0.003 mg $kg^{-1}$ for Cd. The data show that the differences between the content of Fe, Cu, Mn, Zn, Cr and Ni in roach and bleak are significant, while these ones of Pb and Cd are not significant. The significant differences in the content of heavy metals in muscles of roach and bleak are as a result of multiple factors, including season, food, chemical properties of water or sediment**.** The data established during the investigation show that the edible part of fish do not carry heavy metals loads and concentrations are below the legal value for fish and fish products established by the Food and Agriculture Organization and national legislation. This paper is helpful to consumers and academics concerning the mineral of body composition of roach (*Rutilus rutilus*), and bleak (*Alburnus alburnus*).

**Key words:** Fish, roach, bleak, *Rutilus rutilus*, *Alburnus alburnus*, heavy metals, ETAAS.

## Introduction

The roach widely distributed in Europe fresh water is species mostly caught together with bleak (OKGERMAN *et al*., 2009). The fish inhabit nearly every type of water bodies, from large lakes, coastal brackish lagoons and rivers, to small oxbow lakes and clay-pits (RACZYŃSKI *et al*., 2008). The roach and bleak represent one of the most commonly found and most widely distributed fishes in Bulgaria – river Danube and many dam lakes as Dospat, Batak, Koprinka, Zhrebchevo wetlands along the Bulgarian Black Sea coast (Durankulak and Shabla Lake). The species also represent and important component of recreational catches, carried out by amateur anglers (BRYLIŃSKA, 2000; PETROVA & STOYKOV, 2002; 2009).

The content of minerals in fish tissue has an important role in human metabolism (ÖZDEN, 2010). The levels of contaminants,

especially toxic trace minerals in fish tissue are of particular interest because of potential risk to human. These minerals can be classified as potentially toxic (aluminum, arsenic, cadmium, lead, mercury etc.), probably essential (nickel, vanadium, cobalt) and essential (cooper, zinc, selenium) (CANLI & ATLI, 2003). The effects of toxic metals, presented in aquatic food products upon human health and environment are of great interest today (ÖZDEN *et al.*, 2013; YORDANOVA *et al.*, 2008, STANCHEVA *et al.*, 2013a, b). The accurate determination of mineral elements in fish meat is therefore important in nutrition studies, particularly because meat, as a biological material, exhibits natural variations in the amounts of nutrients contained (GREENFIELD & SOUTHGATE, 2003).

Overall, the most widely used trace elements determination techniques in fish meat are UV-Vis spectrophotometry (BLAND *et al.*, 1999), ETAAS (ANGELOVA *et al.*, 2006; DOSPATLIEV *et al.*, 2008), X-ray fluorescence, ICP-OES (DOSPATLIEV *et al.*, 2010; DOSPATLIEV *et al.*, 2011) and ICP-MS (FORRER *et al.*, 2001; MATSUURA *et al.*, 2001).

The aim of this study was to examine the concentration of iron (Fe), nickel (Ni), lead (Pb), manganese (Mn), copper (Cu), chromium (Cr), cadmium (Cd) and zinc (Zn) in roach (*Rutilus rutilus*) and bleak (*Alburnus alburnus*) as species for human consummation.

## Materials and Methods

*Study area*. The Zhrebchevo Dam Lake is located in Southeastern Bulgaria, geographic coordinates (42° 36′ 56″ N, 25° 51′ 33″). Surface area 25 km², maximum depth is 50.50 m. The dam water is used as a source of irrigation water and as a recreational area. There are more than 15 freshwater fish species, including roach and bleak.

*Sample preparation*. The study was carried out in February-March 2013. Roach and bleak were caught by recreational anglers' using fishing rod. A freshly caught fish (27 fish per group) should be anaesthetized (el stunning) and stored on ice in an insulated box at a temperature 0-4° C and transported to the Central laboratory of Trakia University, Stara Zagora on the next day. The mean weight and length of the roach were 127.15±43.73 g and 206.90±26.20 mm, of bleak were 32.63±4.73 g and 156.60±7.00 mm respectively. The meat samples (35 g), without skin from all fish specimens were taken and examined. They were prepared for the experiment after dried at 105° C in a fan oven and stored in dark plastic bottles.

*Reagents*. Reagents were qualified as pure (Merck® and Fluka®). The standard solutions for ETAAS determination of Cu, Fe, Ni, Pb, Zn, Mn, Cr and Cd, with concentration of 1000 mg.l$^{-1}$ were supplied by Merck (Darmstadt, Germany). Double-distilled water was used for all procedures. To determine the pH of $H_2O$ was used pH-meter (Consort C932, Belgium).

*Mineralization of samples*. For the determination of Cu, Fe, Ni, Pb, Zn, Mn, Cr and Cd, 3.0 g (weighted to the nearest 0.01 g) of each air-dried muscle sample was put in a round-bottomed 100 ml flask 22.5 ml of HCI and 7.5 ml of $HNO_3$ were added. After that the flask was connected to a reflux condenser and let it stand for no less than 16 hours at room temperature, then heated gently to boiling for 2 hours. After cooling and flushing the condenser with 25 ml of 12.5 % nitric acid, the sample was filtered and 100 ml of 12.5 % nitric acid was added to the part of it in liquid phase. For analyzed of the muscle samples were used Perkin-Elmer AAnalyst 800 atomic absorption spectrometer (Norwalk, CT).

*Statistical analysis*. Statistical analyses were performed using STATISTICA 6 (StatSoft Inc., 2002). The accuracy of the measurements was assessed by standard deviation (SD) for n=10.

## Results and Discussion

The iron content of these two species was very low compared to that of mammals (WATANABE *et al.*, 1997) and another fish like salmon (ATANASOFF *et al.*, 2013). Mean concentrations of iron in the samples of roach were 6.59±0.224 mg kg$^{-1}$ and bleak 7.34±0.142 mg kg$^{-1}$ respectively (Table 1). A

joint effort of the FAO/WHO (1999) has set a limit for iron intake based on human body weight. For an adult weighting (60 kg), the provisional tolerable daily intake for iron is 48 mg (MITEV *et al.*, 2013). The statistical analysis of the iron data showed that significant differences ($p \leq 0.001$) were found between two studied fish species.

Copper is essential trace element for fish metabolism and important micronutrients in the human diet (AMES, 1998). This element is a cofactor in a wide range of enzymes, including cytochrome oxidase, superoxide dismutase and lysyl oxidase (WATANABE *et al.*, 1997). Copper is essential for good health, but very high intake can cause adverse health problems, such as liver and kidney damage (IKEM & EGEIBOR, 2005; VELCHEVA *et al.*, 2006). Muscle copper levels in roach were 0.69±0.128 mg $kg^{-1}$ and bleak 0.59±0.032 mg $kg^{-1}$ respectively. The statistical analysis of the cooper data showed that significant differences ($p \leq 0.001$) were found between two fish species (Table 1). The established maximum level for copper in fish tissue according to Bulgarian legislation, above which fish consumption is not permitted, is 10 mg $kg^{-1}$ (REGLAMENT 31/2004).

Zinc is another important essential element and it presents active site of many enzymes of fish. Its deficiency can lead to loss appetite, growth retardation and skin changes in marine species (ÖZDEN, 2013). The muscle concentration of zinc in roach was found to be 5.46±0.388 mg $kg^{-1}$ and in bleak 4.05±0.263 mg $kg^{-1}$ respectively (Table 1). The FAO (1983) set a limit daily human intake for Zn 30 mg $kg^{-1}$. The established maximum level for zinc in Bulgarian legislation above which fish consumption is not permitted is 50 mg $kg^{-1}$ (REGLAMENT 31/2004). The statistical analysis of the zinc data showed that significant differences ($p \leq 0.001$) were found between two species.

Lead exists in water mostly in particulate form. The WHO suggests a maximum tolerable weekly intake 25 µg $kg^{-1}$ body weight for adult per week for lead (MITEV *et al.*, 2013). Lead content of catching fish were determined in roach as 0.07±0.031 mg $kg^{-1}$ and bleak 0.06±0.044 mg $kg^{-1}$ (Table 1). The established maximum level for lead in Bulgaria legislation, above which fish consumption is not permitted, is 0.20 mg $kg^{-1}$ (REGLAMENT 31/2004).

Cadmium is a non-essential element in food and in natural waters it can cause kidney and liver damages at any concentration. The EU has set the upper limit of cadmium concentration in fish for human consummation at 1.0 mg $kg^{-1}$ (ÖZDEN *et al.*, 2009). In our study were determined in roach 0.01±0.002 mg $kg^{-1}$ and in bleak 0.01±0.003 mg $kg^{-1}$ (Table 1). The established maximum level for cadmium, according Bulgaria legislation above which fish consumption is not permitted, is 0.05 mg $kg^{-1}$ (REGLAMENT 31/2004).

Nickel concentration in roach was found as 0.04±0.012 mg $kg^{-1}$ and in bleak as 0.03±0.025 mg $kg^{-1}$. Many trace amounts, could be beneficial to activate some human enzymes systems. The WHO (1984) recommends 100-300 µg $kg^{-1}$ body weight nickel for daily intake. However, nickel toxicity in humans is not very common occurrence because the absorption of nickel is very low (ÖZDEN, 2008). The statistical analysis of the nickel data showed that significant differences ($p \leq 0.01$) were found between two studied species. The established maximum level for nickel, in Bulgaria legislation above which fish consumption is not permitted is 0.05 mg $kg^{-1}$ (Reglament 31/2004).

Manganese is recognized as essential trace element for human, but the human requirements or levels of absorption from the diet have not been clearly determined (ÖZDEN *et al.*, 2010). The manganese content was 0.49±0.060 mg $kg^{-1}$ in roach and in bleak was 0.72±0.080 mg $kg^{-1}$ (Table 1). The statistical analysis of the manganese data showed that significant differences ($p \leq 0.001$) were found between these two species.

Chromium concentrations in selected biological materials are elevated in the vicinity of industrial operations and municipal waste treatment facilities (ATANASOFF, 2014). Chromium content was also determined in this study (Table 1). The fish samples that had the higher concentration of chromium were from roach

muscles 0.10±0.045 mg kg$^{-1}$ and they were 10 % more than in these ones of bleak ($p<0.05$). The established maximum level for chromium, in Bulgaria legislation above which fish consumption is not permitted is 0.3 mg kg$^{-1}$ (REGLAMENT 31/2004).

**Table 1.** Heavy metals content (mg kg$^{-1}$) in roach and bleak meat.

| Elemental concentration (mg.kg$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|
| Element | n | Roach | | Bleak | | Reference (REGLAMENT 31/2004) |
| | | Mean | SD | Mean | SD | |
| Fe | 10 | 6.59 | ±0.224 | 7.34*** | 0.142 | - |
| Pb | 10 | 0.07 | ±0.031 | 0.06$^{ns}$ | 0.044 | 0.20 |
| Mn | 10 | 0.49 | ±0.060 | 0.72*** | 0.080 | - |
| Cd | 10 | 0.01 | ±0.002 | 0.01 $^{ns}$ | 0.003 | 0.05 |
| Zn | 10 | 5.46 | ±0.388 | 4.05*** | 0.263 | 50 |
| Cu | 10 | 0.69 | ±0.128 | 0.59*** | 0.032 | 10 |
| Ni | 10 | 0.04 | ±0.012 | 0.03* | 0.025 | 0.05 |
| Cr | 10 | 0.10 | ±0.045 | 0.09** | 0.036 | 0.3 |

*ns* - no significant correlation; *correlation is significant ($p<0.01$); **correlation is significant ($p<0.05$); *** correlation is significant ($p<0.001$)

It is know that the variation in the mineral composition of fish meat is closely related to seasonal and biological differences (species, size, dark/white muscle, age and sex), food source and environmental conditions. As can be seen from the results of both two fish, legal limitations for toxic metals were content not exceeded.

**Conclusion**

Fish are often at the top of aquatic food chain and may concentrate large amounts of metals from the water. Multiple factors, including season, food, chemical properties of water or sediment can play a significant role in toxic metal accumulation.

This investigation provides practical and useful information on the heavy metal content of roach and bleak for first time in Zhrebchevo Dam Lake. Based on the samples analyzed, the edible parts of fishes do not carry heavy metals loads and concentrations are below the legal value for fish and fish products established by the Food and Agriculture Organization (1983).

It can be concluded that this study contributes to a description of the investigated elements of fish meat which could be use to extend existing information. These results will be important for the nutritionists and researchers for improving processing of fish. It is also helpful for similar academic studies and to prepare tables of compositions of food.

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# *Biochemical Changes of the Organism of Apodemus flavicollis (Rodentia: Muridae) Under Conditions of Environmental Anthropogenic Pollution by Heavy Metals in Northern Areas of Ukraine*

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**Abstract.** The present research dedicates the integral assessment of biochemistry indexes of nature populations of rodents under conditions of environment pollution by heavy metals. The raised content in soils of mobile forms Pb, Cd, Cr, Ni and Co was revealed on distance of 500 m to the South-West from Tripillya Thermal Power Plant (Kyiv region, Ukraine). That's considerably (3–5 times) exceeds levels for territory of Kaniv Nature Reserve (Cherkassy region, Ukraine). Territory of National Nature Park "Holosiivsky" (Kyiv, Ukraine) characterized by rather increased content of active form of researched heavy metals especially Pb. Increase of the concentration of diene conjugates (3–7 times) and malonic dialdehyde (2–4 times) in yellow-necked mouse liver (*Apodemus flavicollis*) of under pollution by heavy metals has been discovered. Insignificant increasing of content of Schiff basis in liver cells of rodents in region of impact of Tripillya TPP (in 2 times in spring and in summer, in autumn – in 2.5 times) was detected. Seasonal dynamics of the maintenance of lipid peroxidation has been revealed. The registered changes of biochemical indicators testify about presence ecological-biochemical stress in an organism of the yellow-necked mouse in the district of influence of Tripillya TPP.

**Key words:** heavy metals, *Apodemus flavicollis*, lipid peroxidation, pollution.

## Introduction

Man-caused chemical pollution of ecosystems is an actual problem at the present. Concentration of industrial enterprises in big cities close by small cities and villages makes difficult to monitor its negative impact on environment (AIJUAN *et al.*, 2011; AL-SHAYEB & SEAWARD, 2001; BAKER & CHESNIN, 1975). Intensive production results in releasing of considerable amounts of heavy metals that extend in atmosphere and involve in circulation of elements. Their ranges in many times exceed the natural amount of metals in different levels of biosphere. Lead, mercury, cadmium, copper and zinc widely distributed in biosphere among man-caused metals. They are releasing mainly on burning of mineral fuel. The main products that are forming on burning of coal are ashes, nitric oxides, sulfur anhydride, vanadium pentoxide, carbon oxide and benzapilene. In coal ashes concentrated by heavy metals the maintenance of Cu is 35 mg/kg, Zn – 85 mg/kg, Pb – 20 mg/kg, Cr – 123 mg/kg, Ni – 62 mg/kg, Cd – 4 mg/kg,

Co – 114 mg/kg (KABATA-PENDIAS & PENDIAS, 1984). Anthropogenic emission is so strong that on burning of bituminous coal much more metals are dispersing in atmosphere than human extracts them from deposits (LEYGONIE, 1993). In snow cover nearby big thermoelectric power stations, the regions of pollutions form in radius 10–20 km. Winds contribute to formation of dispersion halo (MAATOUG *et al.*, 2007). Kyiv region is one of the most contaminated in Ukraine. 443 industrial enterprises are exposing as sources of air pollution in Kyiv region. Among areas of region the most pollutants are in Obuchiv district, amount of its industrial discharges arranges from 83000 ton per year. Among them the strongest pollutant is Tripillya TPP – over 21000 ton per year. It forms 84% of all discharges in atmosphere by industrial enterprises in Kyiv region (BESKOROVAYNIY, 2011).

Because of it the clarification of mechanisms and parameters of impact on environment by large plant had placed in densely populated region (e.g. thermoelectric power station) is actual. Small mammals are the most convenient objects for such investigations as long as they are one of some animals that inhabit transformed ecosystems directly next to human. Thanks to short life circle forests rodents have time to reflect the impact of environment on their organism (SALDIVA & BOHM, 2002). It is known that the activation of lipid peroxidation processes of cellular membranes is one of display of toxic heavy metals' impact (BRIGANTI & PICARDO, 2003). Liver is most important organ of detoxication. The present research dedicates the integral assessment of biochemistry indexes of nature populations of rodents under conditions of environment pollution by heavy metals.

## Material and Methods

*Study area*

Researches were conducted on nature population of yellow-necked mouse (*Apodemus flavicollis* Melchior, 1834), which is living on territories with different level of anthropogenic pollution. Environment of this species closely concerned with soil bedding. Therefore, *A. flavicollis* may come as a biomonitor of man-caused pollution of environment. Three areas with different level of anthropogenic loading were chosen for comparative analysis. The territory of Kaniv Nature Reserve (Cherkassy region, Ukraine) that is the nature reserve of highest protection status was chosen as the least disturbed landscape. Territory of National Nature Park "Holosiivsky" (Kyiv, Ukraine) is similar on phytocenosis structure (hornbeam forest) to Kaniv Nature Reserve. It feels direct and indirect impact of human activity starting with disturbing factor and ending with air pollution by discharges. The zone of impact of Tripillya TPP (Obuchiv district, Kyiv region) where are the small hornbeam plantation adjusts very close to the South-West edge of industrial area of thermoelectric power plant (about 500 meters). Industrial area rather works on bituminous coal getting under the torch of dispersion (PHATEEV & PASHCHENKO, 2003).

*Methods*

The results of control catches of *A. flavicollis* on chosen areas were used for material. The control catches were conducted according to generally accepted methods in spring, in summer and in autumn 2012 (CRISTALD & MASCARZON, 1990; ZADYRA & LUKASHEV, 2012). General amount of individuals were analyzed is 89 specimens. Among them 36 specimens were from Kaniv Nature Reserve, 23 specimens – from National Nature Park "Holosiivsky", 30 specimens – from Tripillya TPP. The collection of material was conducted on registered spots by square (3025 $m^2$) which are chosen for assessment of density of individuals' distribution.

The heavy metals content in upper soil stratum of 5 cm and in liver were determined by flame atomic-absorption spectrophotometer C115-M1 (SELMI-CHROM, Ukraine) with heavy hydrogen corrector of the background and computer complex CAS-120. The content of acid-soluble metals and their exchange fraction in soil have been analyzed by extraction by

acetate-ammonium buffer (pH 4.8) according to standard methods (ALLEN, 1989). The content of metals in recoveries was estimated in mg per kg of masses of air dried recoveries (HANNA, 1964).

The portion of products of lipid peroxidation was estimated on maintenance of primary (diene conjugates – DC), second (malonic dialdehyde – MDD) and terminal (Schiff bases – SB) metabolites. During the lipid peroxidation on stage of formation of free radicals in molecules of polyunsaturated higher fatty acids the system of conjured binary connections is forming. As a result conjugated dienes are forming. It is accompanying by appearance of new maximum in spectrum in absorption on wave of 223 nm (BRIGANTI & PICARDO. 2003).

Since conjugated dienes differ by intensive absorption in UV specters spectrophotometric methods was used for identification of DC. Identification of contents of MDD was conducted by spectrophotometric methods (SHIMADZU, UV mini-1240 model, Japan). MDD was detected in reaction with 2-thiobarbituric acid (TBA) (ORECHOVICH, 1977). Calculation of concentration of TBA-active compounds was conducted taking into account the value of coefficient of molecular extinction of malonic dialdehyde on 552 nm. Content of DC and MDD were calculated in nmol per mg of sample. Content of Schiff basic was calculated in conventional units per 1 ml of sample that was detected by spectrofluoremetric method (the length of excitation wave is 360 nm; the length of emission wave is 420 nm; SHIMADZU, RF-1501 model, Japan) (BRIGANTI & PICARDO, 2003).

The samples for definition of products of lipid peroxidation were obtained by preparation of 10% liver homogenate counting on 1 gm of liver tissue per 10 ml of 0.9% solution of sodium chloride (physiological solution).

Because of misfit of normal distribution of some sampling variative ranks of investigated indexes the average size of products of lipid peroxidation and content of metals were presented as median (*Me*). Standard declination of median ($SD_{Me}$) was used as index of variation. Man-Whitney's U-Index was used for comparative characteristic of sampling parameters. For all statistical analysis the STATISTICA 6.0 statistical package was used (StatSoft, 2001).

**Results and Discussion**

Soil is a specific component of biosphere as long as it not only accumulates contaminants but appears as natural buffer that control the transfer processes of chemical elements and compounds from lands to atmosphere, hydrosphere and living organisms (PHATEEV & PASHCHENKO, 2003). The lifetime of contaminants' being in soil is too much longer than in other components of biosphere. Therefore, contamination of soil particularly by heavy metals is practically perpetual. Compounds of heavy metals that are falling on ground surface together with atmospheric precipitates are accumulating in soil thickness especially in upper humus levels and slowly are removing under leaching, erosion, deflation and extraction by plants. The period of semi-extraction for Pb from soil is a few thousand years, for Cd is to 1.1 thousand years, for Zn – to 0.5 thousand years (ALLOWAY, 1990). It may suppose that under permanent source of air contamination gradual increase of heavy metals' content in upper level of soil will occur. It may serve as index of contamination of researched territory (GROMOW & EMELINA, 1994). Examination of content of heavy metals (Pb, Cd, Cr, Ni, Co) in soil samples displays the significant differences of selected regions under exchange fraction (Table 1).

Differences of soil in researched regions under content of acid-soluble fraction of heavy metals were founded statistical insignificant and were corresponded to value of regional clarke typical for forest-steppe zone of Ukraine (PHATEEV & PASHCHENKO, 2003). Excess of normative indexes of boundary permissible concentrations for arable soil have not founded. However, the analysis of exchange fraction of heavy metals has showed what

**Table 1.** Content of exchange fraction of heavy metals in samples of upper soil level in researched territories

| Content of metals, mg/kg / Territory of investigation | Pb | | Cd | | Cr | | Ni | | Co | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Me** | **$SD_{Me}$** | **Me** | **$SD_{Me}$** | **Me** | **$SD_{Me}$** | **Me** | **$SD_{Me}$** | **Me** | **$SD_{Me}$** |
| Kaniv Nature Reserve (Cherkassy region, Ukraine) | < 0.19 | – | < 0.002 | – | 0.07 | 0.03 | 0.08 | 0.07 | 0.05 | 0.04 |
| National Nature Park "Holosiivsky" (Kyiv, Ukraine) | 0.27 | 0.09 | < 0.003 | – | 0.12 | 0.02 | 0.10 | 0.06 | 0.11 | 0.06 |
| Region of impact of Tripillya TPP (Kyiv region, Ukraine) | 0.34 | 0.05 | 0.03 | 0.00 | 0.17 | 0.14 | 0.30 | 0.05 | 0.25 | 0.01 |

its content in Kaniv Nature Reserve is the least. Soil at National Nature Park "Holosiivsky" characterized by increased content of Pb. In region of impact of Tripillya TPP the content of active forms of all researched heavy metals in soil are greatly (in 3–5 ones, $p<0.05$) exceeding the levels are typical for nature reserve territory. Thus it may affirm that the consistent increase in soil the part of biological available fraction of researched heavy metals was observed: Kaniv Nature Reserve < National Nature Park "Holosiivsky" < region of impact of Tripillya TPP. Increase of content of exchange fraction of heavy metals in soils at last two regions probably was conditioned by processes of atmospheric contaminants transportation and falling out (the presence of big city nearby with National Nature Park "Holosiivsky" and powerful Tripillya TPP). It will be showed that in every case the exceeding of normative indexes of boundary permissible concentrations of heavy metals for arable soils was not determined. It may understand by existing environmental standards as a satisfactory ecological situation in all researched territories.

The same results of pollution of soils by heavy metals at territories were adjacent to Tripillya TPP were obtained by other researchers (KRASOVSKIY *et al.*, 2005). Estimation is showing that thanks to irregular dispersion of smoke fumes discharges of station 26.3–36.0 ton per $km^2$ of man-caused dust are falling out in South-East direction annually. Because of it soils are enriching by compounds of Cd, Pb, Cr. Thus it may be contend that region around Tripillya TPP under content of heavy metals (Pb, Cd, Cr, Ni, Co) is a man-caused contaminated territory. Territory of National Nature Park "Holosiivsky" characterized by rather increased content of moveable form of researched heavy metals especially Pb.

In National Nature Park "Holosiivsky" significant increase of density of yellow-necked mouse's population during different seasons of year: in spring – 5 individuals per hectare, in summer – 6 individuals per hectare, in autumn – 8 individuals per hectare. In Kaniv Nature Reserve and in region of impact of Tripillya TPP the highest density was observed in summer (17 individuals per hectare and 16 individuals per hectare accordingly). The value of density was minimal in spring and in autumn: 5 individuals per hectare 8 individuals per hectare accordingly in Kaniv Nature Reserve; 6 individuals per hectare and 7 individuals per hectare accordingly on territory next to Tripillya TPP. Thus maximum density on most contaminated territory next to powerful TPP was observed in spring while minimum density is observed in autumn.

Analyzing the content of heavy metals in yellow-necked mouse's liver on researched territories the insignificant increase of content of Cu, Zn, Cr, Mn in region of impact of Tripillya TPP was detected. Thus the content of Cu and Cr in liver on 17% greater on contaminated

territory than on reserved territory, the content of Zn and Mn – on 22%. So the heavy metals are accumulating in yellow-necked mouse's liver from population in region of impact of Tripillya TPP. It probably may cause physiological disturbance in organism.

Biochemical changes in organism are one of most accurate indexes of direct impact of heavy metals (CHRISTINA & CHANG, 2005). The products of lipid peroxidation in homogenate of yellow-necked mouse's liver in natural populations were analyzed for confirmation of presence of biochemical indexes' changes. Analysis of content of products of lipid peroxidation in animals' organism from researched territories showed considerable differences ($p<0.05$) (Table 2).

**Table 2.** Content of products of lipid peroxidation in liver homogenate of natural populations of animals from researched territories

| Territory of investigation | Season 2012 | Diene conjugates, nmol/mg | | TBA-active compounds, nmol/mg | | Schiff bases, conventional units | |
|---|---|---|---|---|---|---|---|
| | | Me | $SD_{Me}$ | Me | $SD_{Me}$ | Me | $SD_{Me}$ |
| Kaniv Nature Reserve | spring | 0.10 | 0.08 | 0.13 | 0.01 | 0.0017 | 0.0003 |
| | summer | 0.26 | 0.05 | 0.21 | 0.06 | 0.0019 | 0.0003 |
| | autumn | 0.25 | 0.04 | 0.21 | 0.03 | 0.0017 | 0.0002 |
| National Nature Park "Holosiivsky" | spring | 0.27 | 0.12 | 0.16 | 0.03 | 0.0017 | 0.0002 |
| | summer | 0.54* | 0.03 | 0.25 | 0.03 | 0.0028 | 0.0002 |
| | autumn | 0.49* | 0.05 | 0.27 | 0.05 | 0.0026* | 0.0003 |
| Region of impact of Tripillya TPP | spring | 0.72* | 0.07 | 0.42* | 0.02 | 0.0030* | 0.0004 |
| | summer | 0.73* | 0.04 | 0.81* | 0.16 | 0.0035* | 0.0001 |
| | autumn | 0.72* | 0.07 | 0.43* | 0.09 | 0.0042* | 0.0004 |

* – statistically significant differences ($p<0.05$)

As a result of investigation of diene conjugates' content in liver homogenate the increasing of these products in yellow-necked mouse on contaminated territory during the different seasons 2012 was determined. In spring it was in 7 times greater in region of impact of Tripillya TPP as against the nature reserve territory, in summer and in autumn it was greater in 3 times accordingly.

As deserve to point out in National Nature Park "Holosiivsky" the maximum concentration of DC was detected in summer while the content of primary metabolites had descended. As it is well known diene conjugates concern to toxic metabolites that has damage impact on enzymes, ferments and nucleic acids (KAPPUS, 1985). Therefore it may assume that content of DC depends on intensity of metabolism in organism. In our investigations the increasing of quantity of toxic product in individuals of yellow-necked mouse was detected during the year on territory next to Tripillya TPP.

The study of content of MDD had showed the increasing of its concentration near territory of Tripillya Thermal Power Plant during 2012 in comparison with reserved territory: increasing on 3 times had been recorded in spring, in summer – on 4 times, in autumn – on 2 times. Such increasing of MDD possibly closely concerned with entry of heavy metals with food. It's possible leads to affection of liver cells' membranes.

Important role in synthesis of prostaglandins, progesterone and other steroids belongs to MDD (BRIGANTI & PICARDO, 2003). The negative role of MDD is lying in lacing of lipids' molecules and deterioration of penetration of membranes. As a result the membrane becomes less labile. The processes concerned with change

of membrane surface (e.g. phagocytosis, pinocytosis, cell migration) were broken (ORECHOVICH, 1977).

Formation of Schiff bases is final product of lipid peroxidation as a result of interaction of secondary products of lipid peroxidation with proteins and phospholipids that contains amides (KAPPUS, 1985). The analysis of content of Schiff basis insignificant but statistically considerable exceeding of its concentration in liver of mouse was detected for region of impact of Tripillya TPP. The clear gradual growth of its content could to note during the vegetative season from 0.0030 conventional units in spring to 0.0042 conventional units in autumn. On reserved territories the content of Schiff basis in liver of animals was practically constant during the vegetative season. Such difference in dynamics of Schiff basis on territories with different level contamination of top-soil it is possible to explain by compliance of accumulation that product to more deep changes of oxide homeostasis (CHRISTINA & CHANG, 2005). Considerable increasing of that index mostly is the consequence of serious tissue affection.

It is well known that the entrance of toxic substances to organism of warm-blooded animals stimulates the generation of active forms of oxygen (BRIGANTI & PICARDO, 2003). Under disturbance or overcharge of molecular mechanisms of inactivation of these radicals intensification of processes of free-radical oxidation and accumulation of products of lipid peroxidation are possible.

Prohibition of these processes is carried out due to endogenous antioxidants – vitamin A, E and others (CRISTALD & MASCARZON, 1990). Accumulation of products of lipid peroxidation under conditions of toxic pollution is concerned with exhaustion of resources of endogenous protectors (DIXON & PAIVA, 1995). Disturbance of cell membranes' structure and ferment system of organism's metabolism that is appearance of intoxication characteristic is a consequence of these.

**Conclusions**

The results of this study show that content of heavy metals in soil and liver in population of yellow-necked mouse is the highest in region of impact of Tripillya TPP (most contaminated among researched territories) as against Kaniv Nature Reserve what is background territory. Considerable accumulation of products of lipid peroxidation in liver cells of rodents from Tripillya TPP area as against natural reserve territory in spring (diene conjugates are in 7 times; MDD is in 3 times), in summer and in autumn (diene conjugates are in 3 times; MDD is in 4 times and in 2 times) was stated. Insignificant increasing of content of Schiff basis in liver cells of rodents in region of impact of Tripillya TPP (in 2 times in spring and in summer, in autumn – in 2.5 times) was detected.

Thus exceeding of levels of maximum permissible concentrations for soil of researched territories was not detected but biochemical features of disturbance in organism of yellow-necked mouse from the natural populations were observed. Conformity of chemical composition of soils to the maximum permissible concentrations was not conforms to condition of prosperity of animal organism existence of which was concerned with paedosphere. Therefore it may conclude about absolute content of heavy metals in soil is not a marker of ecological conditions of environment. Recorded changes of biochemical parameters are sensible indexes of presence of ecological-biochemical stress for an organism under condition of pollution.

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# *Some Chemical Characteristics of Sediments from Carp Fishponds Treated With Different Fertilizers*

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**Abstract.** The bottom sediments of earthen carp fishponds treated with mineral or organic fertilizers and areas ranging from 0.38 to 7.0 hectares were analyzed. The analyses included determination of basic chemical characteristics in the 0-0.15 m sediment layer like organic matter and organic nitrogen in percentages of dried substance, ammonium and nitrate nitrogen in $mg.kg^{-1}$ dried substance too. The sediments of the fishpond fertilized with ammonium nitrate showed highest concentrations of ammonium and nitrate nitrogen. There was an increased level of organic matter in sediments of organically manured and of other fishponds with area of 2.4 and 7.0 hectares correspondingly. The control fishpond showed higher level of organic nitrogen than the others did. The degree of macrophyte coverage determined the content of organic matter and organic nitrogen while the kind of applied fertilizer influenced the concentrations of inorganic nitrogen forms in sediments.

**Keywords**: fishponds, bottom sediment, organic and mineral fertilization.

## Introduction

The manuring of fish ponds with mineral and organic fertilizers is one of most frequently applied intensification measure in practice of fish farming. The controlled addition of mineral fertilizers can increase the natural productivity of waters, expressed by quantity of plankton and benthos, which are playing an important role in supplementing the fish food shortage (GAS & JANA, 2003). The development of these food resources is directly connected with availability of the basic nutrients nitrogen and phosphorus. The necessary balance between nitrogen and phosphorus and their ratio in the water column depends also on the processes in sediment, which are controlling the nutrient cycle, and distribution in the aquatic ecosystems.

In that sense the bottom sediments, absorbing mineral fertilizers are playing an important role in the fertilization. Colloidal bound nutrients are released thus gradually increasing pond productivity. The bottom depositions serving the exchange between organic substances and nutrients might act as source or depot of organic and mineral resources.

The understanding of properties as well as reactions and processes of the soils might be very useful for pond's aquaculture (BOYD, 1995b). GARD & BHATNAGAR (2002)

showed an increase of organic nitrogen and ammonium ion levels after manuring. In that sense the goal of the study is to reveal the effect of mineral and organic manuring on some indicative characteristics of bottom sediments from fishponds.

**Materials and Methods**

The investigation was carried out on earthen carp fishponds with area size varying from 0.38 to 7.0 ha belonging to the institute of fishery and aquaculture in town of Plovdiv. The traditional technology for breeding of warm water fish species in polyculture was applied.

During the vegetation period two of the ponds were treated either with mineral (pond No 8) or organic (pond No 24) fertilizer, while the third pond No 29 was not manured. The Table 1 presents a scheme of pond fertilization and composition of bred fish polyculture.

The monthly sampling from a 0-0.15 m sediment layer was carried out in May-September interval of year 2009 by means of modification of Kachinski core sampler.

**Table 1.** Scheme of studied ponds with applied fertilizers and farmed fish species

| Pond No | Area, ha | Applied fertilizer kg.ha$^{-1}$ | Farmed fish species |
|---|---|---|---|
| 8 | 0.38 | 466 ammonium nitrate 20 superphosphate | Common Carp, Silver Carp |
| 28 | 2.4 | 2000 manure | Common Carp, Silver Carp, Grass Carp |
| 29 | 7.0 | without manure | Common Carp, Grass Carp, Silver Carp, Northern Pike, Wels |

The sediment analysis includes determination of organic substances in percents of dried matter (%), after Bosworthetal (www.geo.arizona.edu/nyanza/pdf/BharmalLaurent); of organic nitrogen (%) by Kjeldahl method after mineralization with selenium by means of semiautomatic system for analysis of DK-6 type for decomposition and UDK-132 for distillation of VELP-Scientifica firm; of mobile nitrogen forms determined spectrophotometrically after its preliminary extraction from soil by solution of KCl (after TOMOV *et al.*, 1999); determination of inorganic forms of phosphorus by double lactate method of Egner - Riem (after TOMOV *et al.*, 1999)

By means of the "Statistica 7.0" package Wilcoxon paired t-test for significance testing of the differences between ponds and treatments was applied.

**Results and Discussion**

*Organic substances.* The average seasonal percentage of organic substances in the surface slit layer varied from 5.16% to 7.29% with higher values recoded in pond No 29 (Fig. 1). Probably this is related to the regular overgrowing of this pond with soft and hard aquatic plants vegetation during the vegetation season (up to 50% coverage of water surface) and impossibility to remove it due to large size of the pond (7.0 ha). The application of organic fertilizer is the reason for accumulation of organic substances in the sediments of pond No 28 (2.4 ha), where relatively high percentage is recorded (6.9%) The percentage of organic substances in two mentioned ponds is significantly higher than in pond No 8 ($8 < 28$ $P = 0.0012$; $8 < 29$ $P = 0.011$) where the average seasonal level of organic matter in the surface bottom layer is about 5.2%. Therefore, we can conclude that the mineral fertilization does not increase the organic matter in the surface sediment layer. The recorded average values are from 30 to 82% higher than the reported by BOYD (1995a) average content of organic matter in ponds (4%).

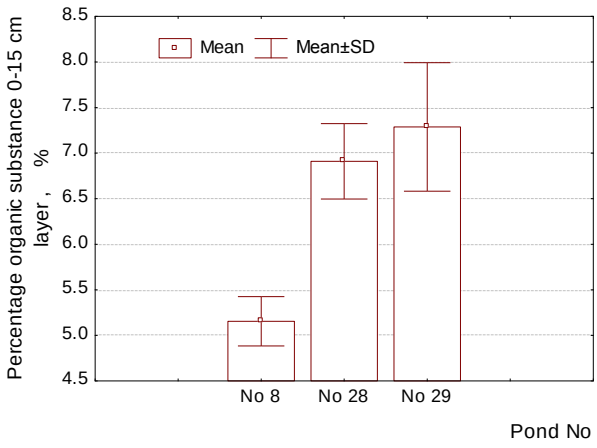


**Fig. 1.** Average seasonal percentage of organic matter (OM, %) in 0-0.15 m sediment layer of three investigated ponds.

*Organic nitrogen.* The average seasonal values of percentage of organic nitrogen in sediment layer 0-0.15 m vary from 0.29% (pond No 28) to 0.37% (pond No 29) (Fig. 2). The percentage of organic nitrogen in pond No 29 is significantly higher than in pond No 8 (29>8, P = 0.008), while the differences between ponds No 8 and 28 (P = 0.65) and ponds No 28 and 29 (P = 0.11) we are not statistically significant.

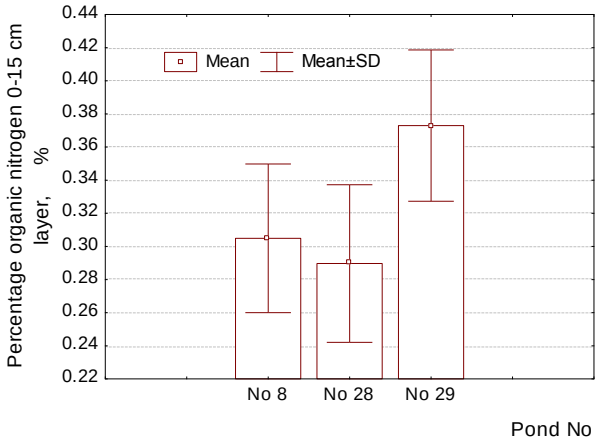


**Fig. 2.** Average seasonal percentage of organic nitrogen (ON, %) in 0-0.15 m sediment layer of three investigated ponds.

The obtained values for organic nitrogen are close to those reported in previous investigations (HADJINIKOLOVA *et al.*, 2007). Bearing in mind that the total content of organic nitrogen in soil surface layers is varying between 0.1% - 0.85% (ATANASOV *et al.*, 1979), the stock of organic nitrogen in bottom sediments of fish ponds might be estimated as "average" one. In freshly formed depositions like the bottom sediments of fish ponds a big part of organic nitrogen is mineralized by nitrification or lost by denitrification (BHARMAL & LAURENT, 2004**).**

*Inorganic nitrogen forms.* The average seasonal concentration of ammonium nitrogen in the sediment layer 0-0.15 m varies between 23.91 and 67.19 mg.dm$^{-3}$. The concentration in pond No 8 is significantly higher than in pond No 28 (P = 0.025) and in pond No 29 (P = 0.043) (Fig. 3). The differences of ammonium nitrogen concentration between ponds No 28 and No 29 are not significant (P = 0.11). This leads to the conclusion that application of mineral fertilization influences ammonium nitrogen directly, what is in accordance with investigations of GARD & BHATNAGAR (2002).

The average seasonal concentration of nitrate nitrogen (Fig. 4) is varying from 4.79 (pond No 29) to 11.05 mg.dm$^{-3}$ (pond No 8). The highest stock of inorganic nitrate is encountered in sediments of pond No 8, where mineral fertilization was applied. The concentration of nitrate ions in this pond is 2.3 times higher than in the control pond and by 18.8% higher than in pond No 28, where organic fertilizer was applied. Independent from the mentioned large differences for nitrate concentrations there were no statistically significant differences detected between investigated ponds No 8, 28 and 29.

*Inorganic phosphorus.* The average seasonal values of phosphate ions (Fig. 5) are varying in the range 42 (pond No 8) – 63.55 mg.dm$^{-3}$ (pond No 29). The highest stock of inorganic phosphorus compounds is observed in sediments of pond No 29, which is by 33.9% higher than in pond No 8 (with applied mineral fertilizer) and by 8.9% higher than in pond No 28 (with applied organic manure).

Independent of recorded variations of average seasonal values for phosphate ions no statistically significant differences between investigated ponds No 8, 28 and 29 were found. In our case the treatment with

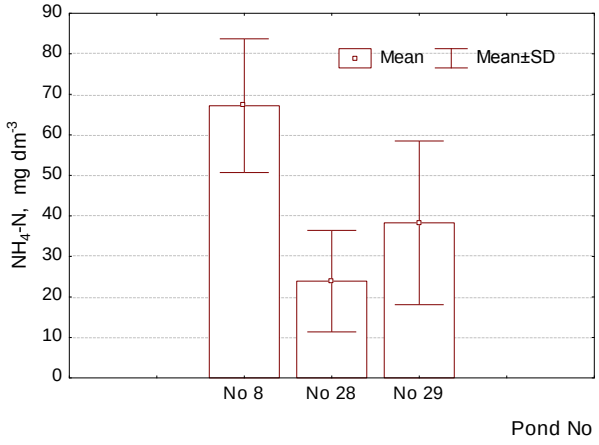


**Fig. 3.** Average seasonal values of concentrations of ammonium nitrogen ($NH_4$ - N, mg.dm$^{-3}$) in 0-0.15 m sediment layer of three investigated ponds

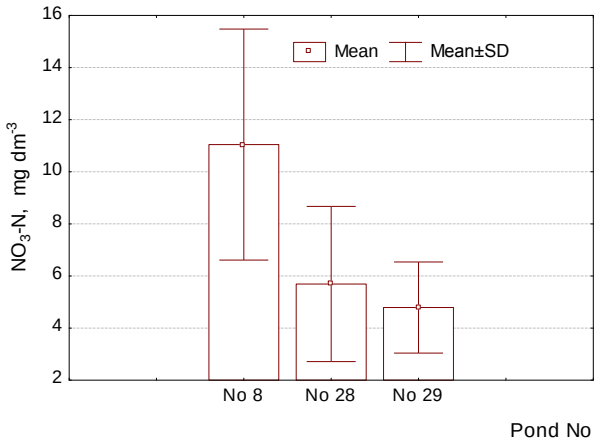


**Fig. 4.** Average seasonal values of concentrations of nitrate nitrogen ($NO_3$ - N, mg.dm$^{-3}$) in 0-0.15 m sediment layer of three investigated ponds

mineral phosphorus fertilizer does not directly influence the phosphate concentration in surface sediment layer, as observed for ammonium ions. One possible reason for this might be the binding of incoming with water phosphorus with calcium and iron ions in insoluble complexes. On the other hand, in principle the effect of fertilization depends on preliminary pond preparation and factors of aquatic environment like water temperature, oxygen concentration and pH (HADJINIKOLOVA, 2013).

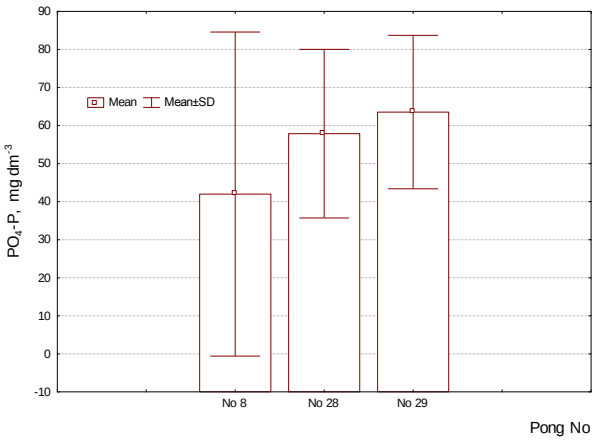


**Fig. 5.** Average seasonal values of phosphate phosphorus ($PO_4$ – P, mg.dm$^{-3}$) in sediment layer 0-0.15 m

**Conclusions**

The average seasonal content of organic substance in the surface silt layer of carp fish ponds varies from 5.16% to 7.29%, whose values are more strongly and significantly affected by organic in comparison with mineral manuring (pond No 8 < pond No 28 for P = 0.0012)

The organic nitrogen stock of sediment layer is in the range 0.29% - 0.37% and could be estimated as average compared to the reported total content of organic nitrogen in soil surface layers (0.1% - 0.85%).

Reported are variations of concentrations of ammonium ions from 23.91 mg.dm$^{-3}$ (pond No 28 with applied organic fertilization) to 67.19 mg.dm$^{-3}$ (pond No 29 control, without fertilization). Significantly higher ammonium concentrations are reported in pond No 8 than in pond No2 8 (P = 0.025) and then in pond No 29 (P = 0.043), which is indication for direct influence of mineral nitrogen fertilization on concentration of ammonium nitrogen in sediments.

The investigations do not show significant differences between studied ponds for their concentrations of nitrate nitrogen and phosphate phosphorus.

More clear differences between average seasonal concentrations of nitrate nitrogen of pond No 29 (4.79 mg.dm$^{-3}$) and of pond No 8 (11.05 mg.dm$^{-3}$) were observed, which indicated the tendency for higher stocks of

nitrate in pond No 8, where mineral nitrogen fertilization was applied. The average seasonal phosphate ion concentrations are varying in the range 42 $mg.dm^{-3}$ (pond No 8) – 63.55 $mg.dm^{-3}$ (pond No 29) indicating that the mineral phosphorus fertilization does not directly affect the concentration level of phosphates in the surface sediment layer.

**Acknowledgements**

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# *Biological Treatment of Wastewater by Sequencing Batch Reactors*

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**Abstract.** In the present paper the operation of wastewater treatment plant (WWTP) in the town of Hisarya which includes a biological stage with aeration basins of cyclic type (SBR-method) was studied. The values of the standard indicators of input and output water from the wastewater treatment plant were evaluated. Moreover, the reached effects due to the biological treatment of the wastewater in terms of the COD (95.7%), $BOD_5$ (96.6%), total nitrogen (81.3%), total phosphorus (53.7%) and suspended solids (95.7%) were established. It was concluded that the indexes of the treated water were significantly below the emission limits specified in the discharge permit.

**Key words:** wastwater, biological, treatment, SBR-technology, performance

## Introduction

Nature conservation and improvement of the environment is one of the most considerable problems of the modern world. An important component of the environment is the water whose quality is the basis of the balance of ecosystems. Recent development of urbanization, tourism and industry leads to increasing of worldwide water consumption. On the other hand the volume of wastewater effluents into water intakes containing a variety of pollutants is continuously growing. Wastewater treatment before its discharging into water bodies is an important assignment of any civilized society, central and local government. Domestic and industrial wastewaters incoming into the urban treatment plants are characterized by irregularity in the amount and type of the pollutants. Therefore, the facilities for the treatment of this type of water are combined and typically include a mechanical, biological and in some cases, chemical step. The biotransformation of organic pollutants is carried out in the aeration tanks, where under the action of the existing biocenosis and in the presence of the required amount of dissolved oxygen in the water, the pollutants are converted into environmentally safe substances (TSACEV, 2001; TCHOBANOGLOUS *et al*., 2002; RAITCHKOV *et al*., 2004; DAVIS, 2010).

For the first time, sequencing batch reactors (SBRs)-technology has been used in 1914. Later in 20th century, it is becoming more and more popular due to the excellent opportunities for adaptation to seasonal changes without limitation of the required optimal treatment capacity at each load. This technology offers great flexibility in terms of the implementation and control of different phases of the biological treatment process,

such as biological phosphorus removal, aerobic oxidation of nitrogen (nitrification) and anoxic elimination of nitrate (denitrification). Several studies demonstrated the effectiveness of SBR-technology and its application as an alternative to conventional flow system with respect to the treatment of municipal and industrial wastewater, especially for smaller flow (JANCZUKOWICZ *et al*., 2001; MACE & MATA-ALVAREZ, 2002).

Bulgarian experience in wastewater treatment with a total biomass for complete removal of $BOD_5$, nitrogen and phosphorus is relatively new and limited. Recent projects for new wastewater treatment plants (WWTP) are developed by mathematical models and programs. These plants are not susceptible to mathematical verification. For examination of plant's design and efficiency of operation can only be used the results from the wastewater analysis at the inlet and the outlet of the already constructed plant during its exploatation (KUZMANOVA, 2011).

In the town of Hisarya which is one of the famous Bulgarian resorts with its mineral springs and SPA-centers attracting thousands of tourists especially in summer is located one of the newly WWTP.

The purpose of this paper is to evaluate the performance of the WWTP-Hisarya which includes biological stage in aeration basins of cyclic type (SBR-method).

**Materials and Methods**

The object of this study was WWTP-Hisarya. The results presented are for the period of January to December 2012. The plant was put into operation in 2011. Design values of the performance of the plant are: load 10000-25000 PE, wastewater dry weather flow 7250 $m^3/d$, wastewater wet weather flow up to 2000 $m^3/h$, daily treatment volume in wet weather 1080 $m^3/h$, organic load as $BOD_5$ up to 1500 kg/d, total nitrogen load 275 kg/d, total phosphorus load 45 kg/d, three aeration basins SBRs, aerobic stabilization of sludge, dewatering machine (centrifuge) and conditioning with lime, installed capacity of about 430 kW, daily consumption of electricity at full load about 2000 kWh/d, specific consumption of electric energy per unit volume of wastewater 0.27 $kWh/m^3$ and specific electricity consumption equivalent per capita per year 29 kWh/PE.

The WWTP operation was evaluated by the values of the following standard indicators: chemical oxygen demand (COD), five-day biological oxygen demand ($BOD_5$), total nitrogen, total phosphorus and suspended solids. Determination of all indicators was performed by standard methods with triplicates.

The processes in the plant and the sequence are presented in the schematic flow diagram shown in Fig. 1.

For monitoring of the WWTP operation and to determine the effects of wastewater treatment an appropriate data were taken from the laboratory records which reflect the characteristics of the wastewater influent and effluent. Monthly and average wastewater treatment effects are defined by the following equations:

Required wastewater treatment effect:

$$L = \frac{C_{inlet} - C_{outlet}^{limit}}{C_{inlet}}.100\ ,\ \%\quad (1)$$

Reached wastewater treatment effect:

$$R = \frac{C_{inlet} - C_{outlet}}{C_{inlet}}.100\ ,\ \%\quad (2)$$

where:

$C_{inlet}$ – concentration of the respective pollutant at the inlet of the WWTP, $mg/dm^3$;

$C_{outlet}$ – concentration of the respective pollutant at the outlet of the WWTP, $mg/dm^3$;

$C_{outlet}^{limit}$ - individual emission limit for the respective pollutant, $mg/dm^3$.

Individual emission limits are specified in the permit for the use of water body "Blue River" - Hisarya for discharge of the wastewater.

**Results and Discussion**

Table 1 shows the characteristic of wastewater influent in the aeration basins.

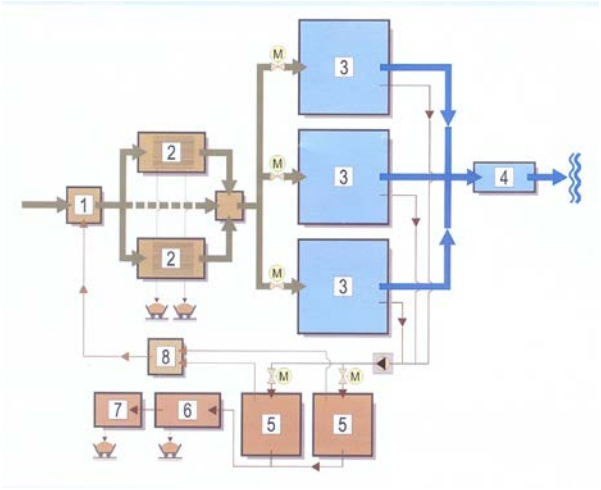


**Fig. 1.** Schematic flow diagram of the WWTP-Hisarya:

1 - input shaft, 2 - mechanical pre-treatment units, 3 - SBRs, 4 - UV-disinfection, 5 - excess sludge stabilisation tanks, 6 - excess sludge dewatering machine, 7 - conditioning of dewatered excess sludge, 8 - pumping station.

The values of standard indicators of treated water for the investigated period are presented in Tables 2 and 3.

The results in Table 1 show that the actual load of the WWTP is less than the design, which allows for the treatment of additional volumes of wastewater in any expansion of the business in the city and to increase the number of tourists.

Data in Tables 2, 2ª, 3 and 3ª shows that the average values of indicators COD, $BOD_5$, total nitrogen, total phosphorus and suspended solids in treated wastewater are significantly below emission limits specified in the discharge permit. For the COD reduction is 9.8 times, for $BOD_5$ is about 6.1 times, and for total nitrogen, total phosphorus and suspended solids decrease is 2.9, 1.1 and 7.8 times respectively. This indicates that the treated water has a significantly better perfomance compared to the requirements for the category of water intake.

**Table 1.** Characteristic of wastewater influent in the aeration basins (SBRs)

| Month-2012 | Influent $Q_1$, l/s | | Wastewater flow $Q_2$, $m^3/d$ | | $BOD_5$, mg/l | | COD, mg/l | | N-total, mg/l | | P-total, mg/l | | SS, mg/l | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* |
| January | 25.56 | *20.89* | 2208 | *1805* | 132 | *37* | 261 | *60* | 25.5 | *2.3* | 3.5 | *0.9* | 74 | *32* |
| February | 104.90 | *93.64* | 9067 | *8091* | 141 | *60* | 228 | *56* | 24.5 | *2.6* | 3.1 | *0.9* | 68 | *18* |
| March | 20.17 | *1.99* | 1744 | *172* | 90 | - | 247 | *55* | 25.0 | *3.4* | 3.6 | *0.7* | 71 | *14* |
| April | 26.73 | *28.30* | 2310 | *2445* | 106 | - | 238 | *31* | 26.3 | *2.6* | 3.6 | *0.3* | 69 | *18* |
| May | 40.24 | *45.21* | 3476 | *3906* | 134 | *46* | 236 | *70* | 23.6 | *4.3* | 3.3 | *0.5* | 72 | *23* |
| June | 21.32 | *8.46* | 1842 | *731* | 111 | *27* | 260 | *77* | 25.5 | *2.4* | 3.8 | *0.6* | 82 | *20* |
| July | 18.71 | *8.82* | 1616 | *762* | 124 | *20* | 293 | *29* | 24.9 | *2.6* | 4.1 | *0.7* | 93 | *24* |
| August | 21.86 | *10.79* | 1889 | *932* | 118 | *18* | 300 | *37* | 26.8 | *3.2* | 4.3 | *0.4* | 92 | *32* |
| September | 20.28 | *8.02* | 1753 | *693* | 143 | *18* | 309 | *25* | 27.9 | *3.0* | 4.2 | *0.2* | 91 | *17* |
| October | 19.78 | *5.69* | 1709 | *491* | 104 | *21* | 313 | *41* | 26.0 | *3.0* | 4.2 | *0.4* | 82 | *14* |
| November | 18.78 | *5.56* | 1622 | *480* | 111 | *16* | 297 | *37* | 29.3 | *5.1* | 4.2 | *0.6* | 80 | *14* |
| December | 23.33 | *12.80* | 2016 | *1106* | 113 | *22* | 303 | *43* | 23.6 | *1.5* | 3.6 | *0.4* | 78 | *15* |
| **Average** | **30.14** | ***24.27*** | **2604** | ***2098*** | **119** | ***16*** | **274** | ***32*** | **25.7** | ***1.7*** | **3.8** | ***0.4*** | **79** | ***9*** |

Av – average; STD – standard deviation; $BOD_5$ – five-day biological oxygen demand; COD – chemical oxygen demand; N-total – total nitrogen; P-total – total phosphorus; SS – suspended solids;

**Table 2.** Characteristic of wastewater effluent from aeration basin $SBR_1$

| Month-2012 | pH | | N-total, mg/l | | $NH_4$-N, mg/l | | $NO_3$-N, mg/l | | DO, mg/l | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* |
| January | 7.1 | *0.10* | 5.40 | *1.70* | 0.20 | *0.10* | 3.90 | *1.60* | 2.68 | *0.15* |
| February | 7.2 | *0.04* | 9.98 | *2.10* | 1.26 | *3.96* | 7.85 | *1.47* | 2.65 | *0.08* |
| March | 7.2 | *0.03* | 5.78 | *2.26* | 0.20 | *0.06* | 5.24 | *2.89* | 2.66 | *0.16* |
| April | 7.2 | *0.10* | 3.34 | *0.37* | 0.22 | *0.03* | 2.47 | *0.29* | 2.70 | *0.20* |
| May | 7.2 | *0.10* | 6.49 | *5.34* | 0.36 | *0.11* | 4.73 | *5.04* | 2.70 | *0.21* |
| June | 7.1 | *0.10* | 4.00 | *2.40* | 0.44 | *0.12* | 3.87 | *2.40* | 2.72 | *0.11* |
| July | 7.2 | *0.10* | 3.15 | *0.50* | 0.39 | *0.21* | 2.20 | *0.40* | 2.73 | *0.10* |
| August | 7.1 | *0* | 3.44 | *0.78* | 0.42 | *0.10* | 2.31 | *0.62* | 2.68 | *0.15* |
| September | 7.1 | *0.03* | 3.13 | *0.06* | 0.50 | *0.10* | 2.24 | *0.39* | 2.72 | *0.13* |
| October | 7.2 | *0.10* | 3.72 | *1.23* | 0.58 | *0.20* | 2.60 | *0.50* | 2.66 | *0.15* |
| November | 7.2 | *0.04* | 5.72 | *2.18* | 0.44 | *0.17* | 4.18 | *1.78* | 2.64 | *0.14* |
| December | 7.1 | *0.02* | 6.48 | *1.56* | 0.37 | *0.08* | 5.31 | *1.39* | 2.60 | *0.15* |
| **Average** | **7.2** | ***0.05*** | **5.10** | ***2.00*** | **0.45** | ***0.28*** | **3.91** | ***1.71*** | **2.68** | ***0.04*** |
| **Limit** | **6-9** | **-** | **15** | **-** | **-** | **-** | **-** | **-** | **-** | **-** |

Av – average; STD – standard deviation; N-total – total nitrogen; $NH_4$-N – ammonia-nitrogen; $NO_3$-N – nitrate-nitrogen; DO – dissolved oxygen.

**Table 2[a].** Characteristic of wastewater effluent from aeration basin $SBR_1$

| Month-2012 | COD, mg/l | | $BOD_5$, mg/l | | P-total, mg/l | | t, $^0C$ | | SS, mg/l | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* |
| January | 9.8 | *1.6* | 3.1 | *0.7* | 1.49 | *0.30* | 16.6 | *1.5* | 3.4 | *0.5* |
| February | 12.0 | *3.7* | 5.0 | *-* | 1.48 | *0.09* | 14.2 | *1.0* | 3.7 | *0.5* |
| March | 10.8 | *1.8* | 3.4 | *0.6* | 1.82 | *0.21* | 18.2 | *1.8* | 16.6 | *28.9* |
| April | 9.9 | *1.6* | 4.2 | *1.6* | 1.79 | *0.20* | 21.5 | *1.2* | 3.3 | *0.6* |
| May | 12.7 | *3.1* | 4.4 | *1.0* | 1.74 | *0.09* | 22.3 | *2.1* | 3.4 | *0.6* |
| June | 9.1 | *1.4* | 2.3 | *0.1* | 1.98 | *-* | 26.1 | *1.5* | 3.4 | *0.6* |
| July | 14.0 | *1.5* | 4.4 | *0.6* | 1.76 | *0.20* | 28.0 | *0.6* | 3.6 | *0.6* |
| August | 10.8 | *0.3* | 5.6 | *0.6* | 1.76 | *-* | 27.4 | *0.9* | 3.0 | *1.0* |
| September | 14.1 | *2.1* | 5.1 | *0.2* | 1.84 | *-* | 26.3 | *0.5* | 3.9 | *0.5* |
| October | 13.4 | *2.4* | 3.5 | *0.7* | 1.85 | *0.12* | 24.9 | *1.4* | 3.4 | *0.5* |
| November | 13.2 | *3.8* | 3.7 | *1.6* | 1.84 | *0.07* | 22.7 | *0.8* | 3.0 | *0.5* |
| December | 11.3 | *1.5* | 4.1 | *0.3* | 1.73 | *0.23* | 17.0 | *1.2* | 3.4 | *0.6* |
| **Average** | **11.8** | ***1.7*** | **4.1** | ***0.9*** | **1.76** | ***0.14*** | **22.1** | ***4.7*** | **4.5** | ***3.8*** |
| **Limit** | **125** | **-** | **25** | **-** | **2** | **-** | **-** | **-** | **35** | **-** |

Av – average; STD – standard deviation; COD – chemical oxygen demand; $BOD_5$ – five-day biological oxygen demand; P-total – total phosphorus; t – water temperature; SS – suspended solids.

**Table 3.** Characteristic of wastewater effluent from aeration basin $SBR_2$

| Month-2012 | pH | | N-total, mg/l | | $NH_4$-N, mg/l | | $NO_3$-N, mg/l | | DO, mg/l | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* |
| January | 7.1 | *0.04* | 5.20 | *1.60* | 0.17 | *0.03* | 4.24 | *1.59* | 2.70 | *0.10* |
| February | 7.1 | *0.1* | 8.70 | *2.00* | 0.17 | *0.11* | 7.34 | *2.32* | 2.65 | *0.23* |
| March | 7.2 | *0.04* | 6.40 | *3.60* | 0.20 | *0.04* | 5.11 | *2.87* | 2.69 | *0.14* |
| April | 7.2 | *0.04* | 3.14 | *0.46* | 0.23 | *0.05* | 2.54 | *0.40* | 2.70 | *0.18* |
| May | 7.2 | *0.1* | 6.22 | *7.40* | 0.39 | *0.15* | 3.98 | *4.55* | 2.67 | *0.16* |
| June | 7.1 | *0.04* | 4.89 | *2.98* | 0.42 | *0.10* | 4.08 | *2.46* | 2.72 | *0.11* |
| July | 7.1 | *0* | 3.12 | *1.03* | 0.33 | *0.16* | 2.11 | *0.30* | 2.68 | *0.13* |
| August | 7.1 | *0.04* | 2.94 | *1.10* | 0.42 | *0.09* | 2.36 | *0.54* | 2.65 | *0.10* |
| September | 7.1 | *0.02* | 3.10 | *0.90* | 0.50 | *0.10* | 2.16 | *0.52* | 2.69 | *0.09* |
| October | 7.2 | *0.08* | 3.89 | *0.81* | 0.63 | *0.22* | 2.60 | *0.49* | 2.70 | *0.13* |
| November | 7.2 | *0.05* | 5.04 | *1.75* | 0.43 | *0.18* | 4.24 | *1.68* | 2.72 | *0.13* |
| December | 7.1 | *0.04* | 5.00 | *1.00* | 0.35 | *0.06* | 5.83 | *2.53* | 2.66 | *0.14* |
| **Average** | **7.1** | ***0.05*** | **4.80** | ***1.73*** | **0.35** | ***0.14*** | **3.88** | ***1.64*** | **2.69** | ***0.02*** |
| **Limit** | **6-9** | **-** | **15** | **-** | **-** | **-** | **-** | **-** | **-** | **-** |

Av – average; STD – standard deviation; N-total – total nitrogen; $NH_4$-N – ammonia-nitrogen; $NO_3$-N – nitrate-nitrogen; DO – dissolved oxygen.

**Table 3[a].** Characteristic of wastewater effluent from aeration basin $SBR_2$

| Month-2012 | COD, mg/l | | $BOD_5$, mg/l | | P-total, mg/l | | t, $^0C$ | | SS, mg/l | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* |
| January | 9.6 | *2.1* | 3.0 | *0.1* | 1.50 | *0.30* | 17.0 | *1.1* | 3.5 | *0.6* |
| February | 12.3 | *1.9* | 5.1 | *1.3* | 1.49 | *0.19* | 14.4 | *0.8* | 3.2 | *0.7* |
| March | 11.0 | *5.3* | 4.5 | *2.0* | 1.60 | *0.20* | 18.2 | *1.6* | 3.2 | *0.4* |
| April | 10.6 | *2.1* | 3.4 | *2.3* | 1.68 | *0.21* | 21.5 | *1.2* | 3.4 | *0.5* |
| May | 13.6 | *5.6* | 3.3 | *1.0* | 1.95 | *0.04* | 22.5 | *2.1* | 3.3 | *0.9* |
| June | 15.8 | *8.1* | 3.6 | *0.8* | 1.97 | *0.01* | 26.1 | *1.5* | 3.7 | *0.6* |
| July | 12.0 | *0.7* | 3.0 | *-* | 1.98 | *-* | 27.9 | *0.7* | 3.9 | *0.6* |
| August | 12.0 | *1.0* | 5.1 | *1.4* | 1.84 | *0.09* | 27.3 | *0.8* | 3.5 | *0.5* |
| September | 13.4 | *2.0* | 4.5 | *1.1* | 1.90 | *-* | 26.4 | *0.7* | 3.6 | *0.5* |
| October | 13.0 | *2.9* | 3.4 | *-* | 1.80 | *0.20* | 25.1 | *1.2* | 3.3 | *0.4* |
| November | 15.7 | *4.0* | 5.7 | *0.4* | 1.82 | *0.07* | 22.3 | *0.8* | 3.3 | *0.4* |
| December | 13.1 | *1.1* | 5.0 | *1.2* | 1.74 | *0.20* | 17.0 | *1.1* | 3.3 | *0.8* |
| **Average** | **12.7** | ***1.9*** | **4.1** | ***1.0*** | **1.77** | ***0.17*** | **22.1** | ***4.6*** | **3.4** | ***0.2*** |
| **Limit** | **125** | **-** | **25** | **-** | **2** | **-** | **-** | **-** | **35** | **-** |

Av – average; STD – standard deviation; COD – chemical oxygen demand; $BOD_5$ – five-day biological oxygen demand; P-total – total phosphorus; t – water temperature; SS – suspended solids.

Values of the required and reached effects of the biological wastewater treatment are presented in Tables 4 and 5.

**Table 4.** Effects of biological wastewater treatment in aeration basin $SBR_1$

| Month-2012 | Wastewater treatment effect, % | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | by COD | | by $BOD_5$ | | by N-total | | by P-total | | by SS | |
| | L | R | L | R | L | R | L | R | L | R |
| January | 52.1 | 96.2 | 81.1 | 97.7 | 41.2 | 78.8 | 42.9 | 57.4 | 52.7 | 95.4 |
| February | 45.2 | 94.7 | 82.3 | 96.5 | 38.8 | 59.3 | 35.5 | 52.3 | 48.5 | 94.6 |
| March | 49.4 | 95.6 | 72.2 | 96.2 | 40.0 | 76.9 | 44.4 | 49.4 | 50.7 | 76.6 |
| April | 47.5 | 95.8 | 76.4 | 96.0 | 43.0 | 87.3 | 44.4 | 50.3 | 49.3 | 95.2 |
| May | 47.0 | 94.6 | 81.3 | 96.7 | 36.4 | 72.5 | 39.4 | 47.3 | 51.4 | 95.3 |
| June | 51.9 | 96.5 | 77.5 | 97.9 | 41.2 | 84.3 | 47.4 | 47.9 | 57.3 | 95.9 |
| July | 57.3 | 95.2 | 79.8 | 96.5 | 39.8 | 87.3 | 51.2 | 57.1 | 62.4 | 96.1 |
| August | 58.3 | 96.4 | 78.8 | 95.3 | 44.0 | 87.2 | 53.5 | 59.1 | 62.0 | 96.7 |
| September | 59.5 | 95.4 | 82.5 | 96.4 | 46.2 | 88.8 | 52.4 | 56.2 | 61.5 | 95.7 |
| October | 60.1 | 95.7 | 76.0 | 96.6 | 42.3 | 85.7 | 52.4 | 56.0 | 57.3 | 95.9 |
| November | 57.9 | 95.6 | 77.5 | 96.7 | 48.8 | 80.5 | 52.4 | 56.2 | 56.3 | 96.3 |
| December | 58.7 | 96.3 | 77.9 | 96.4 | 36.4 | 72.5 | 44.4 | 51.9 | 55.1 | 95.6 |
| **Average** | **54.4** | **95.7** | **79.0** | **96.6** | **41.6** | **80.2** | **47.4** | **53.7** | **55.7** | **94.3** |

L – required effect; R – reached effect.

**Table 5.** Effects of biological wastewater treatment in aeration basin $SBR_2$

| Month-2012 | Wastewater treatment effect, % | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | by COD | | by $BOD_5$ | | by N-total | | by P-total | | by SS | |
| | L | R | L | R | L | R | L | R | L | R |
| January | 52.1 | 96.3 | 81.1 | 97.7 | 41.2 | 79.6 | 42.9 | 57.1 | 52.7 | 95.3 |
| February | 45.2 | 94.6 | 82.3 | 96.4 | 38.8 | 64.5 | 35.5 | 51.9 | 48.5 | 95.3 |
| March | 49.4 | 95.5 | 72.2 | 95.0 | 40.0 | 74.4 | 44.4 | 55.6 | 50.7 | 95.5 |
| April | 47.5 | 95.5 | 76.4 | 96.8 | 43.0 | 88.1 | 44.4 | 53.3 | 49.3 | 95.1 |
| May | 47.0 | 94.2 | 81.3 | 97.5 | 36.4 | 73.6 | 39.4 | 40.9 | 51.4 | 95.4 |
| June | 51.9 | 93.9 | 77.5 | 96.8 | 41.2 | 80.8 | 47.4 | 48.2 | 57.3 | 95.5 |
| July | 57.3 | 95.9 | 79.8 | 97.6 | 39.8 | 87.5 | 51.2 | 51.7 | 62.4 | 95.8 |
| August | 58.3 | 96.0 | 78.8 | 95.7 | 44.0 | 89.0 | 53.5 | 57.2 | 62.0 | 96.2 |
| September | 59.5 | 95.7 | 82.5 | 96.9 | 46.2 | 88.9 | 52.4 | 54.8 | 61.5 | 96.0 |
| October | 60.1 | 95.8 | 76.0 | 96.7 | 42.3 | 85.0 | 52.4 | 57.1 | 57.3 | 96.0 |
| November | 57.9 | 94.7 | 77.5 | 94.9 | 48.8 | 82.8 | 52.4 | 56.7 | 56.3 | 95.9 |
| December | 58.7 | 95.7 | 77.9 | 95.6 | 36.4 | 78.8 | 44.4 | 51.7 | 55.1 | 95.8 |
| **Average** | **54.4** | **95.4** | **79.0** | **96.6** | **41.6** | **81.3** | **47.4** | **53.4** | **55.7** | **95.7** |

L – required effect; R – reached effect.

Reached biological effects of wastewater treatment are significantly above the calculated required effects. For example, difference between the reached and the required effect for COD index is on average 41%, for $BOD_5$ was 17.6%, and for suspended solids that difference is 38.6%.

**Conclusion**

The results obtained from this research and statistical analysis of the performance of the wastewater influent and effluent show that the WWTP-Hisarya operates under the regulations. Values of all standard indicators are significantly below established emission

limits. Reached biological effects of wastewater treatment significantly exceed the required.

Analysis of the results of WWTP-Hisarya operation shows that it is designed and built a future oriented, modern wastewater treatment plant with all conditions to work reliably for many years, which in terms of equipment, structure and economical effectiveness set new accents.

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# *Hydrobiological Investigation of the Activated Sludge from Sequencing Batch Reactors of WWTP –Hisarya*

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**Abstract.** The operations of the relatively new wastewater treatment plant of Hisarya, Bulgaria were evaluated in the present paper. The bacterial diversity of activated sludge from aeration basins of cyclic type (SBR-method) during the seasons was studied. The Cyclic Activated Sludge System is one of the most popular sequencing batch reactor (SBR) processes. The hydrobiological characteristic of the activated sludge was performed with regular observations by using light microscopic examinations, but also by evaluation of the flocculation ability and settleability. The presence of positive bioindicators - *Aspidisca* and *Epistyllis* species during the investigated period of time (2012 Year) was established, which confirmed the gut purification of the wastewater and the carrying out of the nitrification process. Moreover, the results obtained demonstrated that the performance of the SBR maintained high level, and the SBR system remained stable during this study.

**Keywords:** wastewater treatment, activated sludge, SBR-method

## Introduction

Biological treatment is an important and integral part of any wastewater treatment plant that treats wastewater from either municipality or industry having soluble organic impurities or a mix of the two types of wastewater sources. The obvious economic advantage, both in terms of capital investment and operating costs, of biological treatment over other treatment processes like chemical oxidation; thermal oxidation etc. has cemented its place in any integrated wastewater treatment plant.

Cyclic Activated Sludge System as the name suggests is one of the most popular sequencing batch reactor (SBR) processes employed to treat municipal wastewater and wastewater from a variety of industries including refineries and petrochemical plants. For the first time sequencing batch reactors-technology has been used in 1914. Later in 20th century it is becoming more and more popular due to the excellent opportunities for adaptation to seasonal changes without limitation of the required optimal treatment capacity at each load. The technology offers several operational and performance advantages - great flexibility in terms of the implementation and control of different phases of the biological treatment process, such as biological phosphorus removal, aerobic oxidation of nitrogen (nitrification) and anoxic elimination of nitrate (denitrification). Several studies

demonstrated the effectiveness of SBR-technology and its application as an alternative to conventional flow system with respect to the treatment of municipal and industrial wastewater, especially for smaller flow (JANCZUKOWICZ *et al*., 2001; MACE & MATA-ALVAREZ, 2002).

Communities of prokaryotic micro-organisms present in activated-sludge or biofilm reactors are responsible for most of the carbon and nutrient removal from sewage and thus represent the core component of every biological wastewater treatment plant (WWTP). By contrast, mass occurrence of certain bacterial species can also be detrimental for sewage treatment by negatively influencing the settling properties of activated-sludge in the secondary clarifiers, by contributing to the formation of foam or by out competing microorganisms required for nutrient removal. Consequently, a thorough knowledge of the ecology of the microbial communities is required to reveal factors influencing the efficiency and stability of biological WWTPs and to develop promising strategies for improved process performance.

The characteristic of the activated sludge flocks are important not only for researcher but also in the everyday operations of the wastewater treatment plants. Many different living beings live in sewage water. Bacteria constitute the major component of activated sludge flocks and they are responsible for the biological oxidation of organic substrates, nitrification of ammonia, denitrification of nitrate and accumulation of phosphorous (BITTON, 1994). Some bacteria form conglomerates or clusters, others float as individual cells or threads in the interconglomeratic fluid. Typically bacteria clusters include every group of protozoa (*Protozoa*): flagellates (*Zooflagellata*), rhizopods (*Rhizopoda*), heliozoans (*Heliozoa*), ciliates, sectarians (*Suctoria*). Metazoans are represented mostly by worms: nematodes (*Nematoidea*), rotifers (*Rotatoria*), and oligochaetes (*Oligochaeta*). *Protozoa* are predators of bacteria or consume dissolved substrate. So the composition of protozoa fauna of an activated sludge not only depends on environmental conditions (temperature, pH, dissolved oxygen) but the available dissolved substrate and the particular bacteria present in the sludge. That is why *Protozoa* can be used as indicator of plant performance and technological parameters (SANGJIN *et al*., 2004). Protozoa can be classified practically in three groups: ciliates, flagellates, rhizopoda. More than 200 ciliate species have already been determined in activated sludge samples, but only a few of them can be found in a particular activated sludge. Most of the ciliates graze on bacterial cells, but some of them feed on other ciliates. Generally, diverse and high abundance of ciliate population indicate good plant behavior and excellent effluent parameters (SEVIOUR & BLACKALL, 1999).

The crawling or creeping ciliates (*Aspidisca*, *Euplotes*) feed on bacteria on the surface of sludge flocks. *Aspidisca* sp., *Euplotes affinis* are well known bioindicators of nitrification (GULÝAS, 1990). Free ciliates (eg.: *Chilodonella*, *Paramecium*, *Lionotus*) graze on free-swimming bacteria, so they clean the liquid phase of the activated sludge (MARTIN-CRECEA *et al*., 1996; SANGJIN *et al*., 2004). Stalked ciliates (eg.:*Vorticella*, *Carhesium*, *Epistylis*) are attached to flocks by their stalks. An ideal activated sludge contains stalked and crawling ciliates in abundance, but some of them can indicate bad effluent quality (e.g.:*Vorticella microstoma*) (MADONI *et al*., 1993).

Flagellates can take up dissolved substrate by absorption, so they can live without bacterial cells. Flagellates are indicators of high substrate concentration and low dissolved oxygen concentration (GULÝAS, 1990). Rhizopoda (amobae) subdivided into amoeba (e.g., *Amoeba proteus*) and shell covered the camoeba. High number of the camoeba in the activated sludge indicates underloaded system and long sludge age. The generation time of small metazoa (eg.: *Rotifier* sp., *Tradigrada* sp., *Nematoidea* sp.) is long, they can be washed out in case of short solid residence time. They indicate stabile, old sludge with good flock structure (PÁSZTOR & SZENTGYÖRGYI, 2004).

The Bulgarian experience in wastewater treatment plants with a total biomass for complete removal of $BOD_5$, nitrogen and phosphorus is relatively new and limited. Recent projects for new wastewater treatment plants are designed by mathematical models and programs. (KUZMANOVA, 2011). One of the recently build WWTP is located in Hisarya, which is one of the famous resorts in Bulgaria with its famous mineral springs and spa, attracting thousands of tourists especially in summer. The installation was developed with the finance help of the EU Project by collaboration with Germany.

The purpose of the present paper was to evaluate the performance of the WWTP - Hisarya, which includes biological stage in aeration basins of cyclic type (SBR-method) in terms of presence of bioindicators, which evaluate the purification process itself.

**Materials and Methods**

Object of the present study was WWTP - Hisarya, Bulgaria. The presented results are for the period of one year (January to December, 2012). The station was put into operation in 2011 and is relatively new. Design values of the performance of the plant are: load 10000-25000 PE, wastewater dry weather flow 7250 $m^3/d$, wastewater wet weather flow up to 2000 $m^3/h$, daily treatment volume in wet weather 1080 $m^3/h$, organic load as $BOD_5$ up to 1500 kg/d, total nitrogen load 275 kg/d, total phosphorus load 45 kg/d, three aeration basins (SBRs, Fig.1), aerobic stabilization of sludge, dewatering machine (centrifuge) and conditioning with lime, installed capacity of about 430 kW, daily consumption of electricity at full load about 2000 kWh/d, specific consumption of electric energy per unit volume of wastewater 0.27 $kWh/m^3$ and specific electricity consumption equivalent per capita per year 29 kWh/PE.

The operation of the WWTP was evaluated by determination of the values of the following standard indicators: dissolved oxygen and temperature of the water. For characterization of the activated sludge the parameters settled sludge volume and sludge volume index were determined and the dry matter and the humidity of the waste activated sludge were also established. The evaluation of all parameters was performed by standard methods in triplicates and the characteristic of the activated sludge was performed with regular observations by using light microscopic examinations.

**Fig. 1.** SBR aeration basins

**Results and Discussion**

Since one of the critical elements in designing an aeration system are the estimation oxygen demand requirements by the microorganisms and the estimation the waste activated sludge production in the present study the values of those parameters were evaluated.

It is known that the concentration of oxygen must be enough in the aerobic zone for microorganisms that have accumulated phosphorus for complete or near complete

nitrification. The concentration of oxygen must be 2–6 mg/l, no less than 1 mg/l in the aerobic zone, because otherwise the phosphates will split from the micro-organisms to wastewater (HENZE *et al*., 1997; DROSTE, 1996). In our case, the oxygen concentrations are in the appropriate range during the whole 2012 year. The average of dissolved oxygen content in aeration basins $SBR_1$ and $SBR_3$ were 2.68 ± 0.04 and 2.69 ± 0.02 mg/l, respectively, which means sufficient aeration (Table 1). The temperatures were in range 22.1 ± 4.7 and 22.1 ± 4.6 °C, respectively.

An ideal activated sludge flock is dense, big, has spherical shape and contains only a few filamentous bacteria. An activated sludge with ideal flocks is easy to settle, filters the wastewater during the clarifying process, and produces a transparent effluent with little suspended solids content. In table 2 are represented the values of the activated sludge in the aeration basins in terms of settled sludge volume (SSV), dry matter (DM), and sludge volume index (SVI). In table 3 are represented the values for excess sludge.

**Table 1.** Characteristic of wastewater effluent from aeration basins $SBR_1$ and $SBR_3$

| Months 2012 | Aeration basin $SBR_1$ | | | | Aeration basin $SBR_3$ | | | |
|---|---|---|---|---|---|---|---|---|
| | $O_2$, mg/l | | t, °C | | $O_2$, mg/l | | t, °C | |
| | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* |
| January | 2.68 | *0.15* | 16.6 | *1.5* | 2.70 | *0.10* | 17.0 | *1.1* |
| February | 2.65 | *0.08* | 14.2 | *1.0* | 2.65 | *0.23* | 14.4 | *0.8* |
| March | 2.66 | *0.16* | 18.2 | *1.8* | 2.69 | *0.14* | 18.2 | *1.6* |
| April | 2.70 | *0.20* | 21.5 | *1.2* | 2.70 | *0.18* | 21.5 | *1.2* |
| May | 2.70 | *0.21* | 22.3 | *2.1* | 2.67 | *0.16* | 22.5 | *2.1* |
| June | 2.72 | *0.11* | 26.1 | *1.5* | 2.72 | *0.11* | 26.1 | *1.5* |
| July | 2.73 | *0.10* | 28.0 | *0.6* | 2.68 | *0.13* | 27.9 | *0.7* |
| August | 2.68 | *0.15* | 27.4 | *0.9* | 2.65 | *0.10* | 27.3 | *0.8* |
| September | 2.72 | *0.13* | 26.3 | *0.5* | 2.69 | *0.09* | 26.4 | *0.7* |
| October | 2.66 | *0.15* | 24.9 | *1.4* | 2.70 | *0.13* | 25.1 | *1.2* |
| November | 2.64 | *0.14* | 22.7 | *0.8* | 2.72 | *0.13* | 22.3 | *0.8* |
| December | 2.60 | *0.15* | 17.0 | *1.2* | 2.66 | *0.14* | 17.0 | *1.1* |
| **Average** | **2.68** | ***0.04*** | **22.1** | ***4.7*** | **2.69** | ***0.02*** | **22.1** | ***4.6*** |

Av – Average value; STD – Standard deviation

The results represented in both tables 2 and 3 confirmed the high quality of the technological process of water purification and the very good sedimentation properties of the sediment. All values of sludge index are in the recommended range.

The purity level of water, the current relevant properties of water quality can be determined in a fast, efficient, and cost effective way using bioindicators. Bioindicators indicate the presence and condition of the different stages of sewage treatment, also indicating the absence or excessive level of an entity. Observing the bioindicators, the quality of water, and the condition and operations of the treatment equipment can be continuously checked and controlled in a cost effective way. Thus, the study of bioindicators is justifiable. The optimal conditions in the aeration basins are indicated not only by the sufficient aeration but also by the presence of certain ciliate protozoan: *Aspidisca* species. They signal the process of nitrification, decreased ammonia level, and favorable aerobic (i.e. pertaining to the amount of oxygen) conditions. *Epistylis* ciliate protozoa are present in large numbers when the efficiency of sewage treatment is above 65%.

In table 4 are represented the presented indicator microorganisms found in the examined activated sludge.

**Table 2.** Characteristics of the activated sludge in the aeration basins $SBR_1$ and $SBR_3$

| Month-2012 | Aeration basin $SBR_1$ | | | | | | Aeration basin $SBR_3$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SSV, $cm^3/l$ | | DM, g/l | | SVI, $cm^3/g$ | | SSV, $cm^3/l$ | | DM, g/l | | SVI, $cm^3/g$ | |
| | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* | Av | *STD* |
| January | 484 | *61* | 5.5 | *0.3* | 88 | *6* | 468 | *42* | 5.6 | *0.3* | 84 | *4* |
| February | 552 | *105* | 5.4 | *0.5* | 103 | *13* | 543 | *63* | 5.3 | *0.5* | 102 | *7* |
| March | 451 | *88* | 4.6 | *0.5* | 97 | *11* | 383 | *46* | 4.4 | *0.3* | 87 | *15* |
| April | 453 | *79* | 4.6 | *0.5* | 99 | *11* | 453 | *36* | 4.5 | *0.4* | 101 | *9* |
| May | 489 | *89* | 5.1 | *0.6* | 95 | *8* | 395 | *79* | 4.3 | *0.4* | 88 | *13* |
| June | 365 | *84* | 4.5 | *0.7* | 84 | *5* | 388 | *64* | 4.3 | *0.8* | 91 | *8* |
| July | 486 | *77* | 5.2 | *0.6* | 93 | *7* | 389 | *81* | 4.4 | *0.9* | 89 | *10* |
| August | 569 | *72* | 5.5 | *0.6* | 102 | *7* | 321 | *37* | 3.4 | *0.7* | 97 | *12* |
| September | 379 | *49* | 4.0 | *0.3* | 94 | *8* | 438 | *67* | 4.7 | *0.6* | 94 | *3* |
| October | 417 | *90* | 4.4 | *0.5* | 94 | *10* | 349 | *41* | 4.0 | *0.2* | 86 | *6* |
| November | 516 | *53* | 5.0 | *0.5* | 104 | *3* | 389 | *83* | 4.3 | *0.6* | 90 | *8* |
| December | 448 | *139* | 4.6 | *0.9* | 95 | *12* | 607 | *55* | 5.6 | *0.3* | 108 | *5* |
| **Average** | **467** | ***62*** | **4.9** | ***0.5*** | **95** | ***6*** | **427** | ***81*** | **4.6** | ***0.6*** | **93** | ***7*** |

Av – Average value; STD – Standard deviation

**Table 3.** Characteristics of the waste activated sludge

| Month - 2012 | Humidity,% | | DM, g/l | |
|---|---|---|---|---|
| | Av | *STD* | Av | *STD* |
| January | 97.8 | *0.2* | 29.5 | *1.2* |
| September | 98.0 | *0.1* | 30.0 | *6.0* |
| **Average** | **97.9** | ***0.14*** | **29.75** | ***0.35*** |

Av – Average value; STD – Standard deviation

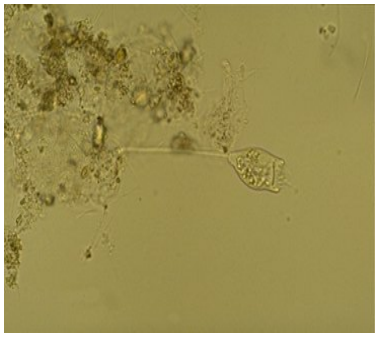
**Table 4.** Presence of bioindicators in the activated sludge

| Bioindicator | Month - 2012 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 | 09 | 10 | 11 | 12 |
| *Flagellatae sp.* | *2* | *2* | *2* | *4* | *3* | *2* | *3* | *2* | *4* | *1* | *2* | *2* |
| *Euglypha laevis* | *1* | *1* | *1* | *1* | *1* | *1* | *1* | *1* | *4* | *1* | *1* | *1* |
| *Arcella vulgaris* | *1* | *1* | *1* | *1* | *1* | *2* | *1* | *1* | *4* | *1* | *2* | *1* |
| *Epistyllis plicatilis* | *4* | *2* | *2* | *4* | *2* | *3* | *3* | *4* | *2* | *4* | *2* | *4* |
| *Turricolla similis* | *1* | *2* | *3* | *2* | *2* | *1* | *2* | *4* | *4* | *4* | *2* | *2* |
| *Arcella discoides* | *2* | *2* | *2* | *2* | *3* | *1* | *1* | *2* | *4* | *1* | *2* | *2* |
| *Vorticella microstoma* | *4* | *2* | *2* | *4* | *4* | *4* | *2* | *2* | *2* | *3* | *2* | *4* |
| *Rotatoria* | *3* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *3* | *3* |
| *Hyalodiscus linax* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* |
| *Aspidisca sp.* | *2/3* | *4* | *4* | *2* | *3* | *3/2* | *3* | *3* | *2* | *2* | *4* | *2* |
| *Vorticella convallaria* | *2* | *4* | *3* | *1* | *2* | *1* | *4* | *4* | *1* | *3* | *2* | *2* |
| *Opercularia coarctata* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *3* | *4* | *2* | *4* | *4* |
| *Hydracarina* sp. | *4* | *4* | *4* | *4* | *4* | *4* | *3* | *4* | *4* | *3* | *3* | *4* |
| *Aspidisca costata* | *4* | *4* | *3* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* |
| *Nematoda* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *4* | *3* |
| *Philodina roseola* | *4* | *4* | *4* | *4* | *4* | *4* | *3* | *4* | *4* | *4* | *4* | *4* |

*1- commonly represented*
*2- mid represented*
*3- poorly represented*
*4- not represented*

Many different living beings live in sewage water. Bacteria represent the most populous group. The protozoa communities of the activated sludge were considerably different. In the sludge of WWTP-Hisarya several genera of protozoa were found. The most abundant genera in sludge were the stalked ciliates *Vorticella*. The dominant group of protozoa was ciliates.


*Arcella vulgaris*



*Vorticella convallaria*

**Fig. 2.** Bioindicators presented in the activated sludge

The most important bioindicative processes could be observed e.g.: abundance of *Aspidisca* species indicated good nitrification and dominancy of *Ciliates* sowed excellent treatment performance. The presence of *Vorticella convallaria* and *Arcella vulgaris* (Fig. 2) was a positive indicator for the wastewater treatment process whereas the presence of *Vorticella microstoma* was undesirable and indicates bad effluent quality. The numerous flagellates in the activated sludge were unexpected, because they generally indicate low oxygen concentration or overloading. However, the appearance of activated sludge is good, is fast settling of dense flocks supernatant is clear.

**Conclusions**

Analysis of the results of WWTP-Hisarya operation shows that it is designed and built a future oriented, modern wastewater treatment plant with all conditions to work reliably for many years, which in terms of equipment, structure and economical effectiveness set new accents.

The massive presence of positive bioindicators and the rare presence or absence of undesirable bioindicators indicates that the aeration basins work well and microorganisms are able to purify to a sufficient degree the incoming wastewater.

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# *Comparative Study on the Effect of the Climatic Conditions on Biological, Economic and Chemical Characteristics of Large-Leaved Tobacco Samples of Burley and Virginia Groups*

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**Abstract.** Correlations between the climatic characteristics: average daily temperature and precipitation sum, on the one hand, and, the biological, economic and chemical characteristics of ten large-leaved Burley and Virginia tobacco samples, on the other, were studied and compared. Except for the vegetation period length, for which a positive correlation was reported, the effect of the climatic conditions on the biological characteristics was insignificant. In both tobacco types the amount of precipitation showed positive correlation with the yield and negative one with the nicotine content ($p<0.05$). Out of all the studied large-leaved tobacco samples, the climatic conditions had a stronger effect on the biological, economic and chemical characteristics of Burley tobacco, i.e. that tobacco type was influenced to a larger degree by the environmental conditions.

**Keywords:** Burley tobacco, Virginia tobacco, Biological, Economic and Chemical Characteristics biological indicators, Climatic Conditions, correlation analysis, dependencies.

## Introduction

Many researchers emphasized the importance of the genotype-environment interrelations. NGUYEN *et al.* (1980) considered that the relative performance of different genotypes often varied depending on the environment. That caused some difficulties in plant breeding activities (HOTAYLEVA & TARUTINA, 2002). According to GRAVOIS *et al*. (1991), breeding should be based on good knowledge of the genetic structure of the breeding material and the effect of genotype-environment interaction. Due to the great plasticity of tobacco and its dependence on the environment (climate, soils and agrotechniques), there should be a good knowledge about the constancy of the separate morphological traits and the limits of varietal variation. When making technological decisions, it is very important to know well the variation amplitude of the phenotypic expression of the major economic and technological characteristics (NAUMOVSKI, 1985; DIMANOV & ZAPRYANOVA, 2002; VELICHKOVA *et al*., 2012).

Temperature and precipitation are the major factors affecting the rate of growth and development of tobacco plants, the productivity and the chemical content of the raw material (DIMITROVA, 2005). It was established that the morphological traits of tobacco plants depend mainly on the variety and the climate and soils have less effect. Studies of MOHAMAD (1969) and TOMOV (1990) showed that climatic conditions affected significantly plant height and had less effect on the number of leaves.

The chemical composition of the leaves depends on the varietal and biological

characteristics of the plants but the effect of the climate and soil is the most significant. The content of nicotine and soluble carbohydrates is in a positive correlation with temperature and in a negative one with rainfalls (TOMOV, 1989).

The aim of the present study was to compare the response of local and introduced Burley and Virginia varieties and variety groups to the changes of temperature and rainfalls in five consequent years characterized by quite different climatic conditions and to evaluate their susceptibility to the environment.

**Material and Methods**

The experiment was carried out in the period 2006-2010, following the plot design. Ten varieties and lines of each of Burley (Burley 21, Burley 1322, Burley 1344, Line 1354, Line 1362, Line 1386, Line 1393, Line 1400, Line 1458, Line 1466) and Virginia (Virginia 0454, Virginia 0514, Virginia 330, Virginia 250, Line 543, Line 586, Line 607, Line 642, Line 842, Line 843) variety groups, both local and introduced, were the object of the study. Tobacco growing, harvesting and curing followed the adopted technology for those varietal groups. The interdependdencies between the climatic characteristics: average daily temperature and precipitation sum, on the one hand, and the most important biological, economic and chemical characteristics, on the other, were established. The climatic data were measured in the period from 1 June to 31 August (for each studied year, respectively), i.e. the period of tobacco growing in the fields. The following characteristics were studied: plant height; number of leaves; length and width of the 7$^{th}$, 14$^{th}$ and 21$^{st}$ leaf, representative for the lower, medium and top harvesting layer of leaves, respectively; vegetation period length; yield and percentage of the first-, second- and third-class of cured tobacco; percentage of nicotine content, sugars, total nitrogen and proteins.

The correlation analysis of data was made by SPSS statistical package. The linear correlation coefficient was used for measuring the strength of the relation between the studied characteristics.

**Results and Discussion**

The results of the correlation analysis between the climatic and biological characteristics of Burley tobacco were presented in Table 2. A significant positive correlation between plant height and the precipitation amount was established. There was no significant correlation between the plant height and the temperature sum.

**Table 1.** Meteorological data obtained at Plovdiv station, 2006 – 2010

| Meteorological index | 2006 | 2007 | 2008 | 2009 | 2010 |
|---|---|---|---|---|---|
| Daily average temperature $^{o}$C June - August | 23.1 | 24.6 | 22.3 | 23.5 | 23.9 |
| Sum of rainfalls, l m$^{-2}$ | 170.8 | 289 | 105.5 | 129.2 | 204.6 |

**Table 2.** Correlations coefficients of the relationships between climatic conditions and biological characteristics for Burley tobacco

| Meteorological index | Biological characteristics for Burley tobacco | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Length of plants | Number of leaves | Length of 7–th leaf | Width of 7-th leaf | Length of 14-th leaf | Width of 14-th leaf | Length of 21-st leaf | Width of 21-st leaf | Length of vegetativ e period |
| Sum of rainfalls | 0.368(*) | 0.189 | 0.195 | 0.148 | 0.158 | 0.254 | 0.335(*) | 0.251 | 0.678(**) |
| Sum of temperature | 0.048 | 0.122 | -0.133 | -0.121 | -0.041 | -0.085 | 0.256 | 0.204 | -0.442(*) |

Legend: **p<600; *p<300

Data from the correlation analysis showed that the precipitation sum had an insignificant effect on the leaf size of the bottom harvesting leaf layer in Burley tobacco. The correlation between precipitation and the leaf size of the middle layer had higher values, although statistically insignificant again. The correlation coefficient of the leaves of the top harvesting layer increased, reaching to +0.34 for the leaf length, i.e. the value was statistically significant.

The effect of the temperature sum on the leaf size of the three leaf layers in Burley tobacco changed from negative for the leaf width of the bottom and middle layers to slightly positive for the leaf width of the top harvesting layer.

The correlation between the climatic factors and the vegetation period length in Burley tobacco was considerably stronger compared to the biometric indices. An average positive correlation was established between the vegetation period length and the precipitation amount (+0.68, p<600), (Table 2). The correlation with temperature was slightly expressed and it was negative, the value being significant again (– 0.44, p<300).

The results of the correlation analysis between the climatic and biological characteristics of Virginia tobacco were presented in Table 3. As a whole, they were slightly different from those of Burley tobacco.

Out of all the reported biometrical characteristics, a significant correlation (+0.31, p<300) was found only between the amount of precipitation and the plant height. For all the other characteristics, no correlation was established, and the calculated coefficients were lower compared to the Burley tobacco ones.

A moderate positive correlation was found between the vegetation period length and the amount of precipitation (+0.58, p<600), (Table 3). The temperature sum had a negative effect on that characteristic (–0.34, p<300).

The results of the correlation analysis between the climatic and economic characteristics of Burley tobacco were presented in Table 4. Positive correlation was established between the yield and the amount of precipitation (+0.47, p<300). Lower, but also significant positive relation was found between the amount of precipitation and the percentage of the first-class tobacco leaves (+0.32, p<300). The amount of precipitation had a negative effect on the percentage of third-class leaves, but the correlation between them was insignificant.

The temperature sum did not exert an effect on the yield and the percentage of the different classes of leaves in the studied samples of Burley tobacco.

The results of the correlation analysis of the climatic and economic characteristics of Virginia tobacco were presented in Table 5. Values obtained were close to those for Burley tobacco. In that case, the correlation between the yield and the amount of precipitation was statistically significant and positive (+0.43, p<0.05) again), but the value was lower compared to Burley tobacco. The tendency towards a positive effect on the percentage of first- and second-class leaves and a negative effect on the percentage of third-class leaves was preserved but the reported correlation coefficients were statistically insignificant.

The temperature sum did not exert any effect on the yield and the percentage of the leaf classes of the studied samples of Virginia tobacco.

Table 6 shows the relationship between the climatic and chemical characteristics of Burley tobacco. The correlation between the nicotine content and the amount of precipitation was significantly negative (p<0.05). Concerning the content of soluble sugars, the correlation was lower expressed, negative and significant (p<0.05). The total nitrogen content correlated negatively with the amount of precipitation and positively with temperature, but it was significant only concerning the temperature sum. The climatic factors did not have an effect on the percentage of protein content.

The results of the correlation analysis of the climatic and chemical characteristics of Virginia tobacco were presented in Table 7. In that case, again the differences to Burley

tobacco were small. A negative significant correlation between the nicotine content and the amount of precipitation was found ($p<0.05$). Referring to the content of soluble sugars, total nitrogen and proteins, the correlation coefficients had negative values and no statistical significant relationships.

**Table 3.** Correlations coefficients of the relationships between climatic conditions and biological characteristics for Virginia tobacco

| Meteorological index | Biological Characteristics for Virginia tobacco | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Length of plants | Number of leaves | Length of 7-th leaf | Width of 7-th leaf | Length of 14-th leaf | Width of 14-th leaf | Length of 21-st leaf | Width of 21-st leaf | Length of vegetative period |
| Sum of rainfalls, | 0.317(*) | 0.125 | 0.124 | 0.112 | 0.140 | 0.188 | 0.273 | 0.206 | 0.583(**) |
| Sum of temperature | 0.026 | 0.074 | 0.108 | 0.067 | 0.011 | -0.019 | 0.121 | 0.115 | -0.344(*) |

Legend: **p<600; *p<300

**Table 4.** Correlations coefficients of the relationships between climatic conditions and economic characteristics for Burley tobacco ($p<0.05$)

| Meteorological index | Economic Characteristics for Burley tobacco | | | |
|---|---|---|---|---|
| | Yield | % of I class | % II class | % III class |
| Sum of rainfalls | 0.475(*) | 0.328 (*) | 0.146 | -0.220 |
| Sum of temperature | 0.101 | -0.059 | 0.082 | 0.129 |

Legend: **p<600; *p<300

**Table 5.** Correlations coefficients of the relationships between climatic conditions and economic characteristics for Virginia tobacco

| Meteorological index | Economic Characteristics for Virginia tobacco | | | |
|---|---|---|---|---|
| | Yield | % of I class | % II class | % III class |
| Sum of rainfalls | 0.432(*) | 0.247 | 0.111 | -0.132 |
| Sum of temperature | 0.033 | 0.024 | 0.009 | 0.049 |

Legend: **p<600; *p<300

**Table 6.** Correlations coefficients of the relationships between climatic conditions and chemical characteristics for Burley tobacco

| Meteorological index | Chemical characteristics for Burley tobacco | | | |
|---|---|---|---|---|
| | % nicotine | % sugar | % total nitrogen | % proteins |
| Sum of rainfalls | -0.424 (*) | -0.378(*) | -0.357 (*) | 0.163 |
| Sum of temperature | 0.265 | 0.262 | 0.232 | -0.107 |

Legend: **p<600; *p<300

**Table 7.** Correlations coefficients of the relationships between climatic conditions and chemical characteristics for Virginia tobacco

| Meteorological index | Chemical Characteristics for Virginia tobacco | | | |
|---|---|---|---|---|
| | % nicotine | % sugar | % total nitrogen | % proteins |
| Sum of rainfalls | -0.337(*) | -0.157 | -0.243 | - 0.192 |
| Sum of temperature | 0.186 | 0.184 | 0.150 | 0.248 |

While the effect of the temperature sum on the percentage of proteins was negative and the effect of precipitation was positive in Burley tobacco, in Virginia tobacco the tendency was just the opposite, but the values were statistically insignificant.

The temperature sum did not have a considerable effect on the chemical composition of the studied Virginia tobacco samples. In that case, again the correlation with the two studied climatic factors was also lower in Virginia tobacco than in Burley.

**Conclusions**

The effect of the climatic conditions on the biological characteristics was insignificant, except for the vegetation period length. The precipitation sum had a stronger effect compared to the temperature sum.

Moderate positive correlation was found between the yield and the amount of precipitation in both tobacco types. The temperature sum did not have an effect on the yield and the percentage of classes in the studied samples of Burley and Virginia tobacco.

The amount of precipitation had a positive effect on the yield and quality of both tobacco types, the effect being stronger in Burley tobacco.

A significant negative correlation was registered between the nicotine content and the amount of precipitation in Burley and Virginia tobaccos. Concerning the content of soluble sugars, the relation was lower, negative and significant only in Burley tobacco.

Out the studied samples of large-leaved tobaccos, the climatic conditions had a stronger effect on the biological, economic and chemical characteristics of Burley tobacco.

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# *Plant Succession in Post Fire Communities of Pinus nigra Arn.*

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**Abstract.** Fires around settlements are a logical consequence of climate drought and the increased human activity today. Plant communities with specific composition and structure are formed on burned areas. This study aims to analyze the floristic composition on three of the after- fire communities, occurring at different times in the Stara Zagora green shelter belt. Analysis of Raunkiaer life forms is made, and the degree of canopy cover is calculated on Braun Blanquet. The results allow predicting the development of secondary plant succession on burned areas.

**Key words:** post fire communities, higher plants, plant succession, Stara Zagora region.

## Introduction

Today fires are one of the most common disorders of forest ecosystems worldwide. Naturally or artificially caused, they inflict severe damage to plant communities. But if in the past they were described as a natural disaster, today it is good to know, that they have very positive consequences as well - they assist natural regeneration of vegetation cover and are used as a remedy for cleansing felling (HICKEY, 1994); initiate succession eliminating of all vegetation or separate floors of the plant community (ZWOLINSKI, 1988); determine the existence of a number of woody and herbaceous species (GILL, 1974; DE LUIS *et al.*, 2006). Natural fires leave more structural elements (DUNKAN, 1985; HICKEY *et al.*, 1998), their recurrence over a period of 90-100 years eliminates indigenous forest species (BAKER *et al.*, 2004; LINDERMAYER & FRANKLIN, 2002), but also supports the development of species of lower floors, enriches the soil with minerals, purifies the forest of old and diseased trees and the pathogenic microorganisms in them, clears weed plant remains in the understory. In after fire communities, we observe a gradual replacement of small, short-lived species with large, long-lived species, there begins a succession, described by Egler as "the initial floristic model" (EGLER, 1954). Usually, after the fire, patches of indigenous, vegetation remains (GARANDEL *et al.*, 2009; KEELEY, 2010)

First, the seed breeding species disappear while those with vegetative propagation develop (SMITH, 1970). More resistant to fires are oaks, giant sequoia, eucalypts, and other tree species with thick corky bark (SWAN, 1970). Of particular importance are the fires of the coniferous species - though they are highly vulnerable because of the flammable resin in their tissues; high temperature is required for the opening of the cone and seed germination (VERROIOS & GEORGIADIS, 2002; GANATSAS *et al.*, 2012).

Despite the obvious devastation that natural fires cause, they are defined as "the essence of health" for most ecosystems (KNEITEL, 2012).

The purpose of this study is to analyze the floristic composition of three after fire

communities, formed at different times, having established early and late succession species in terms of the transitional continental climate of Stara Zagora, South Bulgaria.

**Materials and Methods**

The burned areas are situated in the northern part of the green shelter belt of Stara Zagora, South Bulgaria, as the vegetation losing became at different time: № 1 - 2010, area - 1,2 ha, location – South East; № 2 -2007, area - 0,8 ha, location – North East; №3 - 1998, area - 0,6 ha, location South West. The route method is used; these sites are passed several times within the period from March to October. The altitudes 500 m above sea level, the soil is maroon, the climate - transient - continental with Mediterranean influence - cool summers and mild winters. Species diversity was determined based on the works of JORDANOV (1963-1989) and KOUZHUHAROV (1992).

The projective cover is calculated on the scale of Braun-Blanquet (POORE, 1955).

**Results and Discussion**

In the area, burnt in 2010 (№1), there lacks any vegetation except for shoots of *Quercus cerris* L. (Fig. 1, A - B).

B

**Fig.1.** A-B. Shoots of *Quercus cerris* L. in the burnt territory № 1.

In the burnt area №2 (2007) there are 32 recorded species belonging to 30 genera and 15 families of vascular plants (Table 1). Dominating are the representatives of *Asteraceae* family (18.7%), *Lamiaceae* family (15.6%) and *Poaceae* family (9.4%). The prevailing biological type is perennials (56%), all the other biological types (a,b, a - b, a - p, h, H) are with a little equity (3 - 9%). Raunkiers life forms follow this distribution, dominated by hemicryptophytes (69%) and a relatively good representation of phanerophytes (18.7%).

Burnt area №3 includes 40 higher plant species, belonging to 38 genera and 21 families. Among the biological types, perennials predominate (57.5%), others are under-represented. Among the life forms, hemicryptophytes prevail (50%), the phanerophytes group constitutes 20% of the studied flora, the therophytes and the cryptophytes are both represented by 15%. The prevailing family is *Rosaceae* (17.5%), *Liliaceae* family, *Lamiaceae* family and *Boraginaceae* family are represented by 10%.

The results can be interpreted as follows: The most recently burnt area (2010) lacks any species because the burnt plantation was from *Pinus nigra* Arn. and hardly allowed development of other species in the understorey. The recovery of the plant community starts from the understory (GARANDEL *et al*., 2009) - we observe this process with coppice regeneration of single survived specimens of *Carpinus orientalis* Mill. - native species, highly resistant to high temperatures. Due to the short period after the fire, plant diversity is still at its minimum, and then it will successively go through the phase of dominance of annuals and perennials.

**Table 1.** Floristic composition of burnt area.

| Family/Species | Biological type | Raunkiaer Life forms | Burnt area №2 | Burnt area №3 |
|---|---|---|---|---|
| **Anacardiaceae** | | | | |
| *Cotinus coggygria* Scop | ħ | Ph | | + |
| **Asteraceae** | | | | |
| *Achillea millefolium* L. | p | H | + | |
| *Coniza canadensis* (L.) Cronq. | a | Th | + | |
| *Cirsium vulgare* L. | b | H | + | |
| *Crepis foetida* L. | a-b | H | + | |
| *Lactuca serriola* L. | a-b | H | + | |
| *Sonchus arvensis* L. | p | H | + | |
| **Apiaceae** | | | | |
| *Eringium campestre* L. | p | H | + | |
| *Orlaya grandiflora* (L.) Greuter. | a | Th | | + |
| **Apocynaceae** | | | | |
| *Vinca herbacea* L. | p | H | | + |
| **Betulaceae** | | | | |
| *Carpinus orientalis* Mill. | Ħ | Ph | + | |
| **Boraginaceae** | | | | |
| *Nonea atra* L. | a | Th | | + |
| *Myosotis arvensis* L. | b | H | | + |
| *Buglossoides purpurocaerulea* (L.) Johnst. | p | H | | + |
| *Echium vulgare* L. | b-p | H | | + |
| **Brassicaceae** | | | | |
| *Sinapis arvensis* L. | a | Th | | |
| *Thlaspi perfoliatum* L. | p | H | + | + |
| **Caprifoliaceae** | | | | |
| *Sambucus nigra* L. | ħ | Ph | + | |
| **Caryophyllaceae** | | | | |
| *Cerastium vulgare* Hartm. | a-p | H | + | |
| **Dipsacaceae** | | | | |
| *Dipsacus lacinatus* L. | p | H | | + |
| **Euphorbiaceae** | | | | |
| *Euphorbia cyparissias* L | p | H | + | + |
| **Fabaceae** | | | | |
| *Chamaecytisus hirsutus* (L.) Link. | p | H | | + |
| *Bituminaria bituminosa* (L.) Stirt. | p | H | | + |
| *Lathyrus annus* L. | a | Th | | + |
| **Fagaceae** | | | | |
| *Quercus cerris* L. | Ħ | Ph | + | + |
| **Geraniaceae** | | | | |
| *Erodium cicutarium* (L.) L'Hér. | a -b | H | + | |
| *Geranium sanguineum* L. | p | H | | + |
| **Hypericaceae** | | | | |
| *Hypericum perforatum* L. | p | H | + | |
| **Iridaceae** | | | | |
| *Crocus biflorus* L. | p | Cr | | + |
| **Lamiaceae** | | | | |

| | | | | |
|---|---|---|---|---|
| *Ajuga chamaepitys* (L.) Schreb. | p | | + | |
| *Calamintha sylvatica* L. | p | H | + | |
| *Clinopodium vulgare* L. | p | H | + | |
| *Lamium amplexicaule* L. | a | Th | | + |
| *L. purpureum* L . | a | Th | | + |
| *Marrubium peregrinum* L. | p | H | | + |
| *Origanum vulgare* L. | p | H | + | |
| *Teucrium chamaedrys* L. | p | H | + | + |
| **Liliaceae** | | | | |
| *Muscari racemosum* Mill. | P | Cr | | + |
| *Leopoldia tenuiflora* (Tausch.) Heldr. | P | Cr | | + |
| *Scila bifolia* L. | P | Cr | | + |
| *Fritilaria pontica* Wahb. | p | Cr | | + |
| **Oleaceae** | | | | |
| *Fraxinus ornus* L. | Ħ | Ph | | + |
| **Papaveraceae** | | | | |
| *Fumaria officinalis* L. | a | Th | | + |
| **Poaceae** | | | | |
| *Brachypodium sylvaticum* (Huds.) Beauv. | p | H | + | |
| *Festuca pratensis* L. | p | H | + | + |
| *F. pseudovina* Wiesb. | p | H | + | + |
| *Poa pratensis* L. | p | H | | + |
| **Polygalaceae** | | | | |
| *Polygala major* L | p | H | | + |
| **Primulaceae** | | | | |
| *Anagalis arvensis* L. | a | Th | + | |
| **Ranunculaceae** | | | | |
| *Clematis vitalba* L. | ħ | Ch | + | |
| *Heleborus odorus* L. | p | Cr | | + |
| **Resedaceae** | | | | |
| *Reseda lutea* L. | a-p | H | | + |
| **Rosaceae** | | | | |
| *Amygdallus communis* L. | Ħ | Ph | | + |
| *Agrimonia eupatoria* L. | p | H | | + |
| *Crataegus monogyna* L. | ħ | Ph | + | + |
| *Fragaria vesca* L. | p | H | + | |
| *Potentilla argentea* L. | p | H | + | |
| *Prunus spinosa* L. | ħ | Ph | | + |
| *Rosa canina* L. | ħ | Ph | | + |
| *Rubus caesius* L. | ħ | Ph | + | + |
| *Sanguisorba officinalis* L. | p | H | + | + |
| **Violaceae** | | | | |
| *Viola odorata* L. | p | H | | + |

At this second phase is the plant community in the burned area № 2 (2007) in which already dominate perennial representatives of the largest, evolutionarily advanced and plastic environmentally families - Asteraceae, Lamiaceae, Rosaceae, Poaceae. The majority of taxa have well-developed vegetative propagation, the role of seed propagation is lowered. The bushes reopening goes from dormant buds, grass

species form clumps (RUSSEL - SMITH *at al.*, 2006). Species are still arranged in small groups at random - projective cover 30-50%.

As an example of an open fire forest community (SOARES *et al.*, 2006) is that in burnt territory № 3. Long modified soil composition determines the development of new plant community, built mainly of shrubs and perennial herbaceous with projective cover 80-90 %. For a period of over 10 years, the dominant position has been taken by dicotyledonous shrubs in the family *Rosaceae* - dog rose, blackthorn, bramble, hawthorn and perennial grass species from family *Lamiaceae* - *Ajuga chamaepitys* (L.) Schreb., *Marrubium peregrinum* L., *Origanum vulgare* L., *Teucrium chamaedrys* L**.** Therophytes and cryptophytes found their place in the community**.**

In conclusion, we can summarize, that in its development the analyzed after-fire communities do not differ in their genesis from the established by general laws. The regeneration of plant communities does not restate the output, i.e. we have not observed auto succession. In the newly formed communities, local deciduous trees prevail as well as perennial grass species.

Oaks are especially fire resistant under transient - continental climate with Mediterranean influence, which predominate in Stara Zagira region; they first started breeding with shoots.

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# *Challenges for the Conservation of the Norway Spruce Forests in Vitosha Nature Park after Large-scale Natural Disturbances*

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**Abstract.** In 1934 was established the first national park on the Balkan Peninsula, today Vitosha Nature Park. One of its management objectives is conservation of the subalpine Norway spruce (*Picea abies*) forests in the park. In the last 60 years, significant areas of these forests have been affected by a series of medium and large-scale natural disturbances such as windthrows, bark beetle outbreaks and fires. To study the influence of these natural disturbances on the development of Norway spruce forests in the park we made a literature review and GIS identification of the disturbed forests on the basis of actual forest maps and aerial photographs from 1966, 2005 and 2011. Our results show that in the last 60 years medium and large-scale natural disturbances affected a total of 28% of all spruce forests in the protected area. Most frequent have been the windthrows, while largest impacts have caused the bark beetle outbreaks. About 8% of all natural spruce forests have been replaced with plantations as result of the implementation of forest protection measures, which indicates worsening of the conservation status of the Norway spruce habitat in the park. In order to prevent further worsening of its conservation status, we recommend differential zoning of the park territory so that core zones are surrounded by buffer zones, as well as implementation of appropriate protection measures outside the non-intervention areas. Such measures include prompt treatment of bark-beetle infested trees, leaving sufficient amounts of deadwood in the cutting areas, tolerating natural regeneration and forming multi-species and uneven aged forest structures.

**Key words:** Norway spruce, *Picea abies*, natural disturbances, climate change, protected areas, Vitosha Nature Park.

## Introduction

Subalpine Norway spruce (*Picea abies* Karst.) forests in Vitosha Mountain cover a total area of about 1450 ha. In order to preserve their high conservation value, in 1934 was established the first national park on the Balkan Peninsula, today Vitosha Nature Park. Part of these forests are strictly protected in the reserves "Bistrishko branishte" and "Torfeno branishte". Presently, the natural Norway spruce forests in the park form part of habitat 9410 "Acidophilous *Picea* forests of the montane to alpine levels (*Vaccinio-Piceetea*)", which falls under the protection of the Habitats Directive 92/43/EEC. The conservation of this habitat requires measures which maintain or restore its favorable conservation status. This means that the natural range of the habitat is stable or increases, its specific structure and functions continue to exist and its typical species are preserved.

During the last 60 years, significant areas of the Norway spruce forests in Vitosha Nature Park have been affected by a

series of medium and large-scale natural disturbances such as windthrows, bark beetle outbreaks and fires (DOUNTCHEV, 2007; PANAYOTOV & GEORGIEV, 2012). After 2001, unprecedented forest damages in the park resulting from large windthrows followed by severe bark beetle outbreaks triggered expert discussions about the implications concerning the management and conservation of spruce forests influenced by natural disturbances. The discussions have been polarized by the opposing of economic and nature conservation interests with regard to the management of the spruce forests in the park. At the same time, similar discussions have been ongoing in Europe and Northern America, where the scale of recent natural disturbances in protected and managed coniferous forests exceeds by hundreds of times (table 1) the scale of the natural disturbances in Vitosha Nature Park (by FORSTER *et al.*, 2000; FREY *et al.*, 1995; HALTER, 2011; SCHELHAAS *et al.*, 2003). Back in the 1990s, the storms "Vivian" and "Wiebke" caused windthrows and subsequent bark beetle outbreaks affecting hundreds of thousands of hectares of Norway spruce forests in Western and Central Europe (FISHER *et al.*, 1998). In 1999, the hurricane "Lothar" felled another 400 000 hectares of forests in Western and Central Europe (FISHER *et al.*, 2002), followed again by bark beetle outbreaks in vast areas of spruce forests. On November 19, 2004, a storm devastated over 10 000 hectares of coniferous forests in the Slovak Tatra mountains, whereby mainly affected were spruce plantations originating from the beginning of the 20th century (WWF, 2004) and the *Picea abies* trees in mixed Picea-Larix forests which originated after stand-replacing windthrows about a century ago (ZIELONKA *et al.*, 2009). On January 8, 2005, the storm "Gudrun" felled over 200 000 ha of Scots pine and Norway spruce forests in southern Sweden (SKOGSSTYRELSEN, 2005). On January 14, 2007, a hurricane damaged another 40,000 hectares of forests in Sweden. Even more alarming is the rate at which have been developing the bark beetle outbreaks in North America, where in recent decades were killed more than 28 million hectares of coniferous forests (HALTER, 2011; BERG *et al.*, 2006; WESTFALL, 2006).

**Table 1.** Area of coniferous forests affected by bark beetle outbreaks in Europe and North America in the last two decades

| Vitosha Nature Park, Bulgaria | Bayerische Wald National Park, Germany | Western Europe | North America |
|---|---|---|---|
| 300 ha | 4 000 ha | 1 000 000 ha | 28 000 000 ha |

In the past, the natural disturbances were perceived as "natural disasters". Latest scientific knowledge about the ecology of the forest ecosystems, however, proves that the natural disturbances play an integral role in forest dynamics by enhancing forest structural and biological diversity (FRELICH, 2002; PICKETT & WHITE, 1985). This is particularly valid for subalpine and boreal spruce forests, which are highly susceptible to natural disturbances such as windthrows or bark beetle outbreaks (HEURICH, 2001; SCHMIDT-VOGT, 1991). In the same time, natural disturbances create favourable conditions for the regeneration of spruce forests and contribute to the development of heterogeneous structures. Especially under the harsh climatic conditions of the high mountains and in the boreal zone, the regeneration of *Picea abies* occurs primarily on decaying wood that provides optimal microclimatic conditions for the growth of spruce saplings (EICHRODT, 1970; SCHÖNENBERGER *et al.*, 1992).

Recent studies on the dynamics of spruce forests in "Bistrishko Branishte" reserve indicate that the effective management and conservation of these spruce ecosystems in Vitosha Nature Park require extended knowledge about the

ecology of these ecosystems and better understanding of the role that natural disturbances and human activities play in their development (DOUNTCHEV, 2007; PANAYOTOV & GEORGIEV, 2012; TSVETANOV & PANAYOTOV, 2013).

In this regard, the aim of this study was to survey the changes in the development and distribution of Norway spruce forests in Vitosha Nature Park as result from natural disturbances during the last 60-70 years and to identify and address the main challenges for the management of those forests in the light of the conservation objectives of the protected area.

**Materials and Methods**

The natural Norway spruce (*Picea abies* Karst.) forests in Vitosha Nature Park (42°35'N; 23° 17'E) are situated in the water catchment areas of the rivers Bistrishka, Yanchovska, Vladayska and partially along the currents of the rivers Struma, Boyanska and Dragalevska at an altitude between 1400 and 2000 m (Fig. 1). The climate is typical for high mountain location with average annual temperature of 3.3 °C, ranging from -5.6 °C in January to 12.4 in July (data for Aleko hut climate station, 1800 m). Annual precipitation amounts to 1228 mm with maximum in the April-June and minimum in the August-September periods.

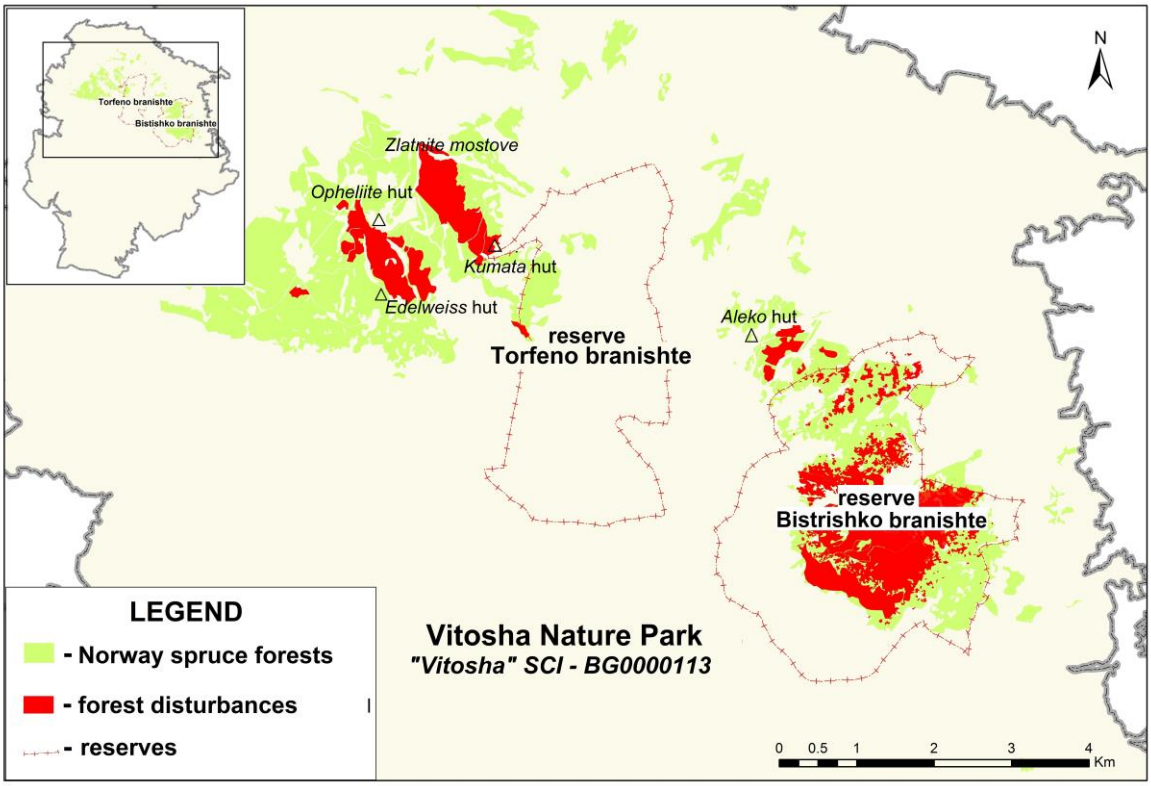


**Fig. 1.** Map of Norway spruce forests in Vitosha Nature Park.

Historical information about the natural disturbances in the Norway spruce forests in Vitosha Nature Park was collected on the basis of a review of available literature. Data for the distribution and development of spruce forests was acquired from the forest management plans of Vitosha Nature Park. Detailed identification and mapping of all polygons with disturbed Norway spruce forests was performed on the basis of actual forest maps and orthorectified digital aerial photographs from 1966, 2005 and 2011. The statistical data and the aerial photographs were processed and analyzed in Geographic Information Systems (GIS) using specialized software ArcGIS 10 (ESRI Inc.).

**Results**

According to the available scientific data, the Norway spruce forests in Vitosha Nature Park are most often affected by windthrows (Table 2.). On November 14-15

1941 a storm caused many small-scale windthrow gaps in spruce forests in the area between the "Zlatnite mostove" site and "Edelweiss" hut, whereby more than 2000 $m^3$ of spruce wood was sanitary logged (ZHEKOV, 1943). On June 14, 1956, a storm felled 110 hectares of spruce forests in the region of "Kumata" hut and in the tree line zone of Bistrishko branishte reserve (TASHEV, 1978). In both areas local people performed salvage logging and transported the logs by oxes. The windthrow area in the region of "Kumata" hut was planted over the years with Norway spruce and Macedonian pine (*Pinus peuce* Gries.). In this way, nearly 64 ha of the natural spruce habitats affected by the natural disturbance were converted into coniferous plantations. In "Bistrishko branishte" reserve, the windthrow area was estimated at 38 ha. It was left to natural regeneration. In 1986, a windthrow on an area of 3,5 ha occurred in spruce forests nearby "Aleko" hut (PANAYOTOV, 2006). There, the wind felled wood was removed, but because of its small size the windthrow area was also left to natural regeneration.

**Table 2.** List of the large-scale natural disturbances in the spruce forests in Vitosha Nature Park, 1940-2012

| Year of distur-bance | Type of natural disturbance | Management measures | Site | Dis-turbed area |
|---|---|---|---|---|
| 1956 | Windthrow | cleared and planted | "Vetrovala" site - "Kumata" hut | 64 ha |
| 1956 | Windthrow | cleared | "Bistrishko branishte" reserve | 38 ha |
| 1986 | Windthrow | cleared | near "Aleko" hut | 3,5 ha |
| 2001 | Windthrow | cleared and planted | near "Opheliite" hut | 12 ha |
| 2001 | Windthrow | uncleared | "Torfeno branishte" reserve | 2 ha |
| 2001 | Windthrow | uncleared | "Bistrishko branishte" reserve | 60 ha |
| 2002-2007 | Bark beetle outbreak | non-intervention | "Bistrishko branishte" reserve | 181 ha |
| 2002-2007 | Bark beetle outbreak | clear cut and planted | near "Opheliite" hut | 48 ha |
| 2002-2007 | Bark beetle outbreak | clear cut and planted | near "Aleko" hut | 15 ha |
| 2012 | Forest fire | non-intervention | "Bistrishko branishte" reserve | 70 ha |

On May 22, 2001, a storm passed through the "Opheliite" site and the territories of "Torfeno branishte" and "Bistrishko branishte" reserves (Dountchev, 2006; PANAYOTOV & GEORGIEV, 2012). According to our GIS analysis affected were respectively 12 ha, 2 ha and 60 ha of Norway spruce dominated forests. In the "Opheliite" site, the windthrow area was cleared in two years time, while the disturbed areas in both reserves remained without human intervention. In both cases, however, the larger windthrows were followed by outbreaks of *Ips typographus* L., which affected a total of 244 ha of the natural Norway spruce forests in Vitosha Nature Park. Outside the reserves, sanitary measures were implemented on an area of 63 ha, whereby all trees attacked by bark beetles and all trees in their perimeter within a radius of a tree stand height were cut and removed. The logging debris were burned and the cleared areas were planted with Norway spruce, Silver fir (*Abies alba* Mill.) and partly with European beech (*Fagus sylvatica* L.). In "Bistrishko branishte" reserve, the disturbed areas were left to regenerate naturally and a recent study showed that initial regeneration was dominated by Norway spruce (DOUNTCHEV, 2007; Dountchev- pers. comm).

On July 1, 2012, a forest fire in the eastern part of the "Bistrishko branishte" reserve burned an area of about 70 ha, which was previously affected by the windthrow in 2001 and the bark beetle outbreak in 2002-2007.

According to the results of our GIS-analysis (Table 3), in the period from 1940 to 2012 large-scale natural disturbances affected a total of 424 ha (i.e. 28%) of all spruce forests in Vitosha Nature Park. Nearly 139 ha (i.e. 8% of all spruce forests) of the latter were replaced by coniferous plantations, while 285 ha were left to regenerate naturally. Unaffected by natural disturbances are 1103 ha of the natural spruce forests, predominantly in the western part of the park.

**Table 3.** Distribution of spruce forest areas per type of natural disturbance and management approach

| Intact natural spruce forests | | Windthrow areas (incl. fires) | | Areas affected by bark beetle outbreaks (incl. fires) | | Total |
|---|---|---|---|---|---|---|
| 1103 ha (72 %) | | 180 ha (12 %) | | 244 ha (16 %) | | 1527 ha (100 %) |
| Reserves | Managed forests | Natural regeneration | Artificial plantations | Natural regeneration | Artificial plantations | Total |
| 252 ha | 851 ha | 104 ha | 76 ha | 181 ha | 63 ha | 1527 ha |

**Discussion**

Our results show that large-scale natural disturbances have a dominant influence on the development of the natural spruce forests in Vitosha Nature Park in temporal and spatial aspect. During the last 60 years were affected nearly 1/5 of all natural spruce forests in the park. Windthrows occur at about 20 years in average.

From dendro-ecological point of view, the high frequency of windthrows in Norway spruce forests is connected with the limited individual stability of spruce trees (HEURICH, 2001; SCHMIDT-VOGT, 1991). Especially when growing on wet soils, the exceptionally shallow root system of spruce trees tends to be a critical factor for the individual stability of this species. STEFANOV (1939) assumed that this ecological feature of the Norway spruce appears to be an advantage and one of the reasons for the dominance of this species in the high parts of Vitosha Mountain, where soils are shallow and stony. However, these unfavourable edaphic conditions are a critical factor for the growth of spruce trees, not only with respect to its stability, but also with respect to its resistance to pathogens, especially during periods of drought (GANCHEV, 1987; FORSTER *et al*., 2000).

As particularly susceptible to natural disturbances are considered forest stands with low structural heterogeneity (HEURICH, 2001; SCHMIDT-VOGT, 1991), including protected forests which have lost their natural structure and resistibility as result of intensive management activities before their designation as protected areas (WEISSBACHER, 2004; ZATLOUKAL, 1998). This proves to be valid also for the spruce forests in "Bistrishko branishte" reserve. Recent studies (PANAYOTOV & GEORGIEV, 2012; TSVETANOV & PANAYOTOV, 2013) found that natural disturbances in the reserve had affected predominantly homogeneous even-aged stands not older than 140-150 years, with various signs of anthropogenic impacts before the establishment of the protected area.

Our survey results show also that since 2001 the greatest impact on the development of spruce forests in Vitosha Nature Park have the recent outbreaks of *Ips typographus* L. Under favourable conditions such as the presence of large amount of fresh deadwood (e.g. after a windthrow) or mild climate, this species is able to attack and kill sizable areas of spruce stands (FORSTER *et al*., 2004). TSANKOV (2005) assumes that immediate removal of wind felled trees was the decisive

measure for preventing severe bark beetle outbreaks after the windthrows of the mid-1950s. After 2001, however the rate of expansion of the bark beetle outbreak was similar in non-intervention forests as in managed forests (Table 1). GEORGIEV (2006) explained the uncontrollable expansion of the bark beetle populations after 2001 with the extremely dry weather in the period 2001-2005. Alternatively, after 2005, severe weather conditions such as cold and rainy weather during the growing season hampered the development of the bark beetles, increased the number of their natural enemies and finally lead to gradual subsiding of the bark beetle outbreak.

At the same time, numerous authors (FISCHER *et al*., 2002; FORSTER et al., 2000; FREY *et al*., 1995; HALTER, 2011) report that natural disturbances such as windthrows and bark beetle outbreaks affect even larger areas of coniferous forests in Western and Central Europe and Northern America. In essence, climate change is blamed for an increase in the frequency and intensity of devastating storms and extremely unfavourable for coniferous forests climate conditions (FISCHER, 1998; BERG *et al*., 2006; DALE *et al*., 2001; HALTER, 2011; WESTFALL, 2006). On the one hand, periods of extreme drought reduce significantly the resistance of Norway spruce trees against bark beetle attacks (ENGESSER *et a*l., 2008). On the other hand, the higher average temperatures improve the survival rate of bark beetles during the winter and favour the development of more than one and even two generations of bark beetles in the growing season (WERMELINGER & SEIFER, 1999; BERG *et al*., 2006; HALTER, 2011). Limiting the disastrous consequences of bark beetles outbreaks was elusive even when prompt sanitary measures were implemented (FORSTER *et al*., 2004).

The results in Table 2 and Table 3 reveal that except the natural disturbances and the climate change, the distribution and development of natural spruce forests in Vitosha Nature Park is strongly influenced by the type of management of these forests.

On the one hand, sanitary measures were implemented in all managed forests affected by large-scale natural disturbances. As argued by DOUNTCHEV (2006), these sanitary measures were not fully consistent with the ecology of Norway spruce ecosystems. Salvage logging was performed with extreme delay in relation to the fast rates of bark beetle expansion. In the same time, salvage logging reduced the stability of the stands on the edge of the clear cuts, leading to further forest damages. The natural undergrowth was irreversibly damaged, whereas the removal of deadwood compromised the natural regeneration in the long term. Clear cut areas were regenerated artificially with non-native spruce saplings, which were not adapted to the local environmental conditions. As a result, nearly 140 ha of natural spruce habitats were replaced with plantations. These facts indicate worsening of the conservation status of the spruce habitat because its area is decreased and its structures and functions are adversely affected.

On the other hand, despite the die-off of a larger part of the old spruce trees in "Bistrishko branishte" reserve, the non-intervention management applied there allowed preservation of the biodiversity and restoration of the natural character of the spruce habitats (DOUNTCHEV, 2007). This measure, however, lead to uncontrolled expansion of the bark beetle outbreak outside the reserve, affecting also the recreational zone of the park near "Aleko" hut.

When addressing the abovementioned challenges for the conservation of the natural spruce forests in Vitosha Nature Park, good experience could be gained from the German National parks "Bayerische wald" and "Harz" and the forest reserve "Dürenstein" in Austria. The most valuable spruce ecosystems in these protected areas are set aside in core zones where human interventions are limited. In the core zones, spruce ecosystems are left entirely under the influence of natural processes in order to maintain or restore their natural character (FISCHER et al., 2002; JEHL, 2001; KULAKOWSKI & BEBI, 2004). The core zones are surrounded by buffer zones with a

width of 300 to 1 000 m (NATIONALPARK BAYERISCHE WALD, 2005). The buffers are designed in a way which guarantees that the managed forests outside the protected area are not affected by the natural processes (e.g. bark beetle outbreaks) taking place in the core zones. The prevention measures applied in the buffer zone include intensive supervision and prompt treatment of infested trees by peeling and leaving the debarked tree in the forest. Outside the core zones, spruce forests are managed in a way which increases their resistibility by tolerating natural regeneration and forming multi-species and un-even aged forest structures (WERMELINGER, 2004).

Negative experience with regard to the conservation of natural spruce forests is reported in the Czech National park "Šumava" and the Belarusian National Park "Belovezhskaya Pushcha" (PAVLICHKO, 2005; KOZULKO, 2002; SOLAR & GALLAND, 2002). There, similarly to the experience in the "Ophellite" site shared by DOUNTCHEV (2006), the bark beetle outbreaks were counteracted by large-scale sanitary felling, which resulted in serious environmental problems such as excessive clear cuts, erosion and replacement of natural forests with artificial plantations.

**Conclusions**

The research data demonstrates that during the last 60 years large-scale natural disturbances such as windthrows and bark beetle outbreaks played a dominant role in the dynamics of natural spruce forests in Vitosha Nature Park. The occurrence and intensity of natural disturbances was often influenced by the climate conditions, the level of forest heterogeneity and the management types. Implementation of forest protection measures such as salvage logging followed by artificial regeneration lead to worsening of the conservation status of the natural spruce habitats in the protected area. Just the opposite, non-intervention management in the reserve territories guaranteed the preservation of the biodiversity and the natural character of the spruce ecosystems.

On the basis of an analysis of local and international experience in the management of Norway spruce forests in protected areas affected by natural disturbances, we recommend that effective conservation of the natural spruce habitats in Vitosha Nature Park should be achieved by differential zoning of the park so that core zones are surrounded by buffer zones. We suggest implementation of appropriate forest protection measures outside the non-intervention areas, which include prompt treatment of infested trees, leaving sufficient amounts of deadwood in the cutting areas, tolerating natural regeneration and forming multi-species and un-even aged forest structures.

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# *Cicada orni L. in the Food of Wild Boar in the Regional Park Maremma – Toscana, Italy*

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**Abstract.** The study was conducted in the period 1991-2000, in the в Regional Park Maremma – Toscana, Central Italy. The park area is about 9800 ha, covered by Mediterranean vegetation: *Pinus halepensis* Mill., *Pinus pinea domesticus* L., *Quercus ilex* L., *Q. pubescens* Willd., *Arbutus unedo* L., *Phillirea latifolia* L., *Erica multiflora* L., *Pistacia lentiscus* L., *Rosmarinus officinalis* L. *Juniperus phoenicea L., Juniperus oxycedrus macrocarpa L.* During the food analysis (faeces) of wild boar it was established that one of the main parts for it during the winter and spring are the larvae of *Cicada orni* L. In one of the faeces were detected 132 pairs of front legs of those larvae. This was the reason to start a detailed investigation of cicada's life and its role as a food source for wild boar. It was interesting to understand the influence of wild boar on the cicada's life. The initial hypotheses were three: there is a significant influence on its number; there is no influence, or increases its number significantly. In order to achieve the aim the first and the last song of the male cicadas were registered within a period of 10 years. To investigate the number of larvae flying during the summer, in the first spring were randomly chosen 23 pine stems and an area of about 1 m around them. On this area every 15th day after the first registered song (8-10) June the empty skins were collected. This way the number of cicadas was established and their dynamics, which ended about 10-15 August. The last song registered was about 10-15 September. Each year, starting the observation the soil under the trees was divided into two categories - rooted by the wild boar or not. This way was established the influence of wild boar on the cicada's number. T-Student analysis showed that it exists only during some of the years (1995 and 1997). This result gave an answer to the first hypothesis – that the wild boar does not influence significantly the number of cicadas. It was confirmed by the soil samples taken from different places with dimensions 50x50x30 cm.

**Key words:** cicada; Regional Park Maremma; wild boar; food

## Introduction

The interest to *Cicada orni* L. appeared during investigation of wild boar food in the Park (MASSEI & GENOV, 1995; MASSEI *et al*., 1996). This insect is food for the wild boar but not only for it. When it starts flying from the soil it becomes a pray from the ants to the wild boar. But before it goes out from the land for its larvae look the fox and the badger as well (CIAMPALINI & LOVARI, 1985). Despite the many enemies this insect sings around the forests and is part of the food for the wild boar which roots large areas for it under the crowns of *Pinus halepensis* Mill. And *Pinus pinea domesticus* L. It was the reason to start the investigation of the influence of wild boar on cicada's number in the Regional Park Maremma. The initial hypotheses were three: there is a significant influence on its number; there is no influence, or increases its number significantly.

The main aim was to establish the influence of wild boar on the cicada's

number. In order to achieve it the following tasks were set: investigate the density of cicada in the soil depending on the activity of wild boar with soil samples; study the cicada's number during the fly from the land depending on the integrity of the soil under the pine trees chosen.

**Material and methods**

The investigations were realized during the period 1991-2000, in the в Regional Park Maremma – Toscana, Central Italy. The Park is with an area of 9800 ha, covered by Mediterranean vegetation: *Pinus halepensis* Mill., *Pinus pinea domesticus* L., *Quercus ilex* L., *Q. pubescens* Willd., *Arbutus unedo* L., (*Olea europea* L.), *Juniperus oxycedrus macrocarpa* L., *Juniperus phoenicea* L., *Phillirea latifolia* L., *Erica multiflora* L., *Erica arborea* L., *Pistacia lentiscus* L., *Rosmarinus officinalis* L.

In order to study the influence of wild boar, randomly chosen sample plots in the soil under the pine crowns were used, with dimensions 50x50x30 cm – not-rooted 20 and rooted - 30. During the work we had to over-dig the dry soil to reach a depth of 30-40 cm where the pine roots were. Due to the high level of underground water they did not penetrate deeper and the larvae from their cells sucked assimilates from the roots. The larvae found were with three dimensions – small, medium and large, the last one being with well developed head, thorax and abdomen, the front legs adapted for digging, covered by chitin. 23 pine trees were randomly chosen and from an area of about 1 m around their stems the larvae skins were collected during 10 years. The collections took place every 15 days after the first song between 5 and 10 June was recorded or every year 5 controls were done (Fig.). Depending on the integrity of the soil under the crown each year they were divided into two categories – rooted and non-rooted. The collected by tow methods samples (soil and skins) was processed statistically by t-Student test in order to establish if there is a significant difference between them.

**Fig. 1**. *Cicada orni* - empty chitin exoskeleton remain after metamorphosis

**Results and Discussion**

After taking soil samples (Table 1) during 2 years and it was established that there was no significant difference between then. Despite of this we decided that 2 years is not enough long period to have a reliable answer and followed sampling.

**Table 1.** Number of *Cicada orni* larvae found in rooted and non-rooted by wild boar soil.

| Year | 1990 | 1990 | 1991 | 1991 |
|---|---|---|---|---|
| parameters | Rooted | Non-rooted | Rooted | Non-rooted |
| N | 20 | 10 | 20 | 10 |
| Number | 399 | 213 | 368 | 142 |
| Mean | 19.9 | 21.3 | 18.4 | 14.2 |
| DS | 10.97 | 10.17 | 11.35 | 9.06 |
| t-Student | n.s. | | | n.s |

The high drought in the summer sometimes did not allow to wild boat to look for food under the ground. As it was mentioned in the Park it feeds on *Cicada orni*

larvae and in addition with *Melolonta* in 1993, 1995 and 1999 the soil under the trees was not rooted and these years were not included in the analysis.

The influence of the wild boar on the number of cicada was established by both types of soil – rooted and non-rooted. t-Student's t-test analysis showed that it only existed only in some of the years (Table 2). As it could be seen only in two years there is significant difference between both groups of soil. The wild boat feeds on adult insects. *melolonta* L. larvae. This was the reason that The female cicadas lay eggs under the tree bark and from them larvae appear. They fall on the ground and have to penetrate to the tree root to suck assimilates. It is clear that is much easier that if the soil is rooted than the dry and solid substrate. These results give an answer to the three of the hypotheses that the wild boar does not influence significantly the number of cicada. But on the contrary in the dry months it help penetrating the larvae into the soil.

**Table 2.** Difference in the number of *Cicada orni* skins in rooted and non-rooted by wild boar ground.

| Parameters<br>Years | Soil | Number of trees | Number of skins | Mean | SD | P at level 95% |
|---|---|---|---|---|---|---|
| 1991 | rooted | 12 | 767 | 63.9 | 30.7 | - |
| | non-rooted | 11 | 883 | 80.8 | 26.9 | |
| 1992 | rooted | 11 | 420 | 38.2 | 11.5 | - |
| | non-rooted | 12 | 461 | 38.4 | 16.2 | |
| 1995 | rooted | 7 | 247 | 35,3 | 15,5 | P ≤ 0,01 |
| | non-rooted | 16 | 952 | 59,5 | 18,2 | |
| 1996 | rooted | 13 | 1628 | 125,2 | 53,6 | - |
| | non-rooted | 10 | 1739 | 173,9 | 60,0 | |
| 1997 | rooted | 7 | 89 | 12,7 | 8,03 | P ≤ 0,05 |
| | non-rooted | 16 | 413 | 25,8 | 14,11 | |
| 1998 | rooted | 12 | 906 | 75,5 | 23,9 | - |
| | non-rooted | 11 | 828 | 75,3 | 45,0 | |
| 2000 | rooted | 6 | 198 | 33 | 11,7 | - |
| | non-rooted | 17 | 379 | 22,3 | 19,8 | |

**Conclusion**

The above mentioned results give an answer to the hypotheses that the wild boat does not influence significantly the cicada number. Even in the dry months it helps penetrating the larvae into the soil.

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# *Vital Strategy for Cicada orni L. Survival in the Regional Park Maremma (Italy)*

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**Abstract.** The study took place in the period 1991-2000, in the Regional Park Maremma, Central Italy (42˚39′N, 11˚ 05′E). It is with an area of 9800 ha, covered by Mediterranean vegetation: *Pinus halepensis* Mill., *Pinus pinea domesticus* L., *Quercus ilex* L., *Q .pubescens* Willd., *Arbutus unedo* L., *Phillirea latifolia* L., *Erica multiflora* L., *Pistacia lentiscus* L., *Rosmarinus officinalis* L. The larvae of *Cicada orni* L. live in the soil and it with three-year life cycle. When it starts going out of the skin it becomes a pray for some animals, among them ants and wild boar, as it does not move. During one only observation it was established the presence of 222 cicada skins on the sand only some of which reached to fly. On the sand there were also signs from lizards, snakes, birds, hedge hocks, foxes, wild boars, etc. After a deep analysis it was established that their number decreased 30 times from the ground to the trees crowns where the adults live. During 10 years were collected data about the flying dynamics of cicada in order to answer the following hypothesis: what is the life strategy which the insect uses to survive among the numerous enemies. During this analysis there were reached four answers: first – it is a mass; second – it flies in late spring and early summer when the number of other insects is abundant and the enemies feed on them, third – most of the birds are over with the looking after their smalls which need great amount of food, and forth – the mornings in May are colder and more with moisture and this makes the insect slow and it could be a pray fro the birds and mammals as well.

**Key words:** *Cicada orni*, Maremma, survival.

## Introduction

Investigations on cicada density in different habitats in Maremma Regional Park were done by PATTERSON *et al.* (1991), in relation of studying the dimensions, and individual area and food of *Garrulus glandarius* (L., 1758). In 1995 in order to establish the influence of enemies on the number of cicada in the Park a new investigation was realized by PATTERSON *et al*. (1997). In it was established that from the ground to the crown of the trees where the adults live the number decreased by 30 fold. A reason for that was the observation of 222 cicadas went out from the soil and only for few of them the chitin remains were found as an approval that they flew. On the sand there were also signs from lizards, snakes, birds, hedge hocks, foxes, wild boars, etc. During the going out the insect is not in move so it is under the attacks of ants and predator insects. After this investigation the interest to this noisy and numerous insect did not decrease so during the next years were collected data on its flight dynamics in order to answer the following hypothesis:

what life strategy is formed during the evolution in order to survive, because despite the numerous enemies the insect is numerous. To test the life strategy hypothesis related to the species survival despite the strong pressure by enemies. In order to achieve them the following tasks were set: observation of the going out from the skin after going out from the soil; establishment of the cicada density in the soil depending on the holes on the surface; observation of the cicada flight in the summer; establishment of the reproduction behaviour and life span of the adult; analysis of the life cycle and strategy for survival.

**Material and Methods**

The investigations took place in the period 1991-2000, in the Regional Park Maremma – Toscana, Central Italy. The park is with an area of about 9800 ha, covered by Mediterranean vegetation: *Pinus halepensis* Mill., *Pinus pinea domesticus* L., *Quercus ilex* L., *Q. pubescens* Willd., *Arbutus unedo* L., (*Olea europea* L. ), *Juniperus oxycedrus macrocarpa* L., *Juniperus phoenicea* L., *Phillirea latifolia* L., *Erica multiflora* L., *Erica arborea* L., *Pistacia lentiscus* L., *Rosmarinus officinalis* L.

In order to establish the cicada density soil sample were taken (50x50x30 cm). During the sampling it was observed that before they go to the surface the larvae make a small hole which cannot be seen as on it there are needles or grass. It was sufficient to remove the needles or the grass and to count the holes to know the number of ready to fly larvae. In order to compare if the number of holes is the same as the number of larvae 28 soil samples were taken and on 36 samples with the same dimensions the holes were count. The results were compared using t-Student test and they showed no significant difference between them. To study the dynamics of the flight randomly 23 pine trees were chosen. From them during 10 years every 15 days after the first song was registered, were collected all the skins at a distance 1 m around the tree. Many observations were also done – the process of imago going out of the skin, behavior of males during mating, manner of feeding.

**Results and Discussion**

*Observation of imago going out from the skin after going out from the soil*

The cicada is an insect with metamorphosis development and the adult larva looks like the imago. During the soil sampling there were three dimension types of larvae, clearly distinguished so it means that the life cycle is three-year, which is also found in other publications (CIAMPALINI & LOVARI 1985; PATTERSON *et al*., 1991). The larvae live in the soil at a depth of 30-40 cm as the main part of the root system of *Pinus pinea domesticusu* and *Pinus halepensis* L. are at this depth and the larvae feed on the assimilates from the roots. This does not mean that they do not feed on other plant but the samples are taken from this habitat only. In the roots they form a small camera and spend there 3 years. The cuticle is elastic so they can grow without changing the skin.

During the first decade of June (5-10), the song of male cicadas is starting. To the sunset the larva goes out from the soil and starts moving and finds a vertical thing to climb on. The time is limited and if it does not find a thing stays on the ground which is dangerous as to be eaten by enemies. More – the horizontal pose of the skin is not very comfortable because the wings cannot be open normally. After it succeeds to climb it stays fro a moment and the skin is broken. From this part the head is seen, it breaths and the body becomes larger, the front part of the insect appearing. The first pair of legs, which in the larva are now normal, is as in other insects. This way, half appearing, it starts inclining backwards and oscillates from time to time, moving all the legs. This helps to the folded fine and soft wings to become hard and open. After legs, body and wings being hard enough, it stands and fixes with its front legs to the skin and takes the abdomen out. The skin although being inert is so hard fixed that can support the insect weight with doubled volume. In this moment the colour of the insect is pale-rose or pale-green and it was suggested it is related to the sex. But after that is was observed that both sexes have similar colours. The sex ratio of the newly appeared

81 specimens was 34 male and 47 female or 1.0:1.4. After all the parts of the external chitin skeleton being hard enough the insects move to the tree bark. The process lasts 1.5 to 2 h. To the morning the cicada's body becomes grey as the tree bark and one should be very careful to find it. It helps to the survived until this moment insects to rescue to the sunrise being still due to cool temperature, from the numerous birds walking around the tree stems as *Mirops apiaster* L., coming in flocks, followed by *Upupa epops* L. Making noise with the wings and sounds they force the insects to fly and capture them. Over passing all the circumstances they survive and meet the new worm day – in cloudy weather, before and after rain they do not sing (QUARTAU *et al.*, 2000) so in the forest their voices are heard and this lasts about three months.

*Establishment of the cicada density in the soil depending on the holes' number on the soil*

As it was mentioned above, before coming to the surface, the larvae make a vertical hole which cannot be seen as on it there are pine needles or grass. It was sufficient to remove the litter and to calculate the holes to establish the ready for flying larvae. To validate if the number of holes is the same as for the ready to fly insects 28 samples were taken. From 36 samples with the same dimensions the holes were calculated. The results were compared with t-Student test and they showed that no significant difference between both samples. This allowed to evaluate the quantity of ready to fly larvae according to the hoes found (Table 1).

**Table 1.** Difference between holes calculated * and the number of the found in samples (50x50x30 cm) ** ready to fly larvae (1996-1997)

| Number of samples | 36* | 28** |
|---|---|---|
| Number of larvae | 85 | 85 |
| SD | 2,36 | 3,07 |
| t-Student | P = 0.9852; no difference | |

*Investigation of cicada flight dynamics in summer*

During 10 years the first (5-10 June) and the last (8-15 September) song of the male cicada was recorded. Then every 15 days were collected all the skins on the stems at a distance of 1 m around them – 23 pine trees, in order to establish the fly dynamics of cicada. From Table 2 it can be seen that most actively the cicada go out during the second 15-day period and then decreases. During the last period in some years there were no flying adult insects. During some of the years, cicada number dynamics if different despite that the flight cycle is the same, most insects fly during the second period. It could be suggested that it depends on the soil hardness which does not allow some of the larvae to open the surface and to reach the trees roots. There was observed a tendency of increasing the number every third year, with the exception of 1996, in which the highest number of cicada was recorded in the Park. There was established a significant difference between the periods of flying with the exception of the first and third where the activity of flying is the same (Table 3). Possible reasons could be that in June the larvae did not reach their physiological maturity to go out from the soil and the morning temperatures are lower, and the humidity is abundant as the grass is not dry. For the third period the reason is clearer – most of the insects have flown.

**Table 2.** Number of cicada skins collected every 15 days after registration of the first song during the period 1991-2000

| controls | I | II | III | IV | V | total |
|---|---|---|---|---|---|---|
| 1991 | 245 | 693 | 404 | 224 | 60 | 1626 |
| 1992 | 181 | 397 | 230 | 39 | 34 | 881 |
| 1993 | 661 | 915 | 125 | 33 | 0 | 1734 |
| 1994 | 156 | 462 | 102 | 21 | 0 | 741 |
| 1995 | 252 | 597 | 323 | 27 | 0 | 1199 |
| 1996 | 428 | 2299 | 504 | 111 | 102 | 3444 |
| 1997 | 126 | 286 | 84 | 16 | 0 | 512 |
| 1998 | 210 | 1269 | 236 | 17 | 2 | 1734 |
| 1999 | 127 | 415 | 26 | 22 | 0 | 590 |
| 2000 | 94 | 360 | 83 | 28 | 13 | 578 |

**Table 3.** Significant differences in cicada flight dynamics in the period 1991-2000 in cycles of 15 days starting after first song registration

| Controls | I | II | III | IV | V |
|---|---|---|---|---|---|
| I | | 0.02 | n.s. | 0.01 | 0.01 |
| II | | | 0.01 | 0.01 | 0.01 |
| III | | | | 0.01 | 0.01 |
| IV | | | | | 0.01 |
| V | | | | | |

*Establishment of reproductive behaviour and life span of the adult insect*

After being in the tree crown the males sing in order to attract females. Also a competition starts as on the pine there are some males surrounding a female and trying to reach her singing differently and most probably the length and power of it are of vital importance for the female. The power and length of the sound are an indicator for the good condition of the male and the female most probably chooses the ones with more resistance (KREBS & DRAVES, 2002). After the copulation the females lays the eggs in the barks holes, covered by white cotton-like matter. Within some time they enclose and the miniature larvae can penetrate into the hard soil and reach to some roots to suck assimilates. The front legs which serve to dig are about 50% from the body mass. It is not clear yet where the whole energy is found and where from the water drops are coming. Many evening it was observed many cicadas on the bark of *Juniperus phoenicea* L. sucking assimilates.

It was calculated how long do these insects live. The larvae stop going out about 10-15 August as after these dates there were no new skins on the sample trees and the last song was registered around 10-15 September – meaning after a month.

*Analysis of the life cycle and the strategy for species surviving*

During this analysis some answers were reached but not to the main one – what is the species strategy to survive. One of the possible answers could be that it is a massive but this probably could not rescue it in May when all the birds look after their smalls and the insect is large, slow and in addition – noisy. It cannot change (shorten) its life cycle in order to have more generations as three could not be divided into two. There are some comments from KREBS & DRAVES (2002). In some species avoiding increasing of generations is reached by periodical not spatial synchronization which can explain bicycles of some cicadas being 13 or 17 years. These

insects live in the soil as larvae during 13 and 17 years and after that the adults fly. Cicadas with 17-year cycle are 3 species with millions of adults flying at the same time around a large space so to become a pray is not likely. Some scientists had questions why 13 and 17 years but not 15 or 18? A long period between two flights is an advantage to eliminate the predators and parasites – as there are no cicadas during 13 and 17 years, can die or use other prays. These long biological cycles most probably evolved as a form for surviving, as an evolutionary key with which prolonging the cycle cicadas won. Actually this length 13 and 17 years are the first figures which do not allow a predator to synchronize with the cicada using a shorter cycle to coincide with cicada's flight. For example if the cicada is with a cycle of 15 years, the predator with 3 or 5 years could synchronize with cicada and meet it every 3 or 5 generations. More possible is to be extinguished the adults cicadas was observed in the beginning or ending of the flight but not in the middle, when they are very abundant and this is probably their strategy.

The cicada is abundant in mid-summer in pine wood and deciduous wood, in olive wood but at the same time in the open spaces is full of grasshoppers and other insects – so it is not the only one mass victim and the most part of the birds grew their smalls yet, and despite the larger number they only feed but not collect food for the growing and hungry all the time smalls. On the other hand the high temperatures, dry weather and low humidity increase the movement of the insects and they become more difficult pray for some of the low-mobility enemies. More – the fruit mature and there are many plant seeds and some enemies change their diet as for example *Sturnus vulgaris* L. Also the migrations start. All this is in favour of cicada later flying strategy.

**Conclusions**

Flying of the cicada is during the end of spring and summer when there are many other insects and it is not the only pray. High summer temperatures make it very movable and it becomes a difficult pray for the enemies. At the end of summer many fruit mature and some of the enemies move to plant diet.

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# *Forest Fire Impact on the Soil Carbon Content and Stock on the North Slopes of Rila Mountain (Bulgaria)*

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**Abstract.** Forest fires are the major disturbing factor that can affect soil carbon content in a forest ecosystem and may have a particularly persistent effect on the carbon stock in the affected soils. Purpose of the study performed was to investigate the carbon content and stock changes in a soil under coniferous and mixed stands in result of a forest fire occurred on the north slopes of the Rila Mountain (Dolna Bania region). Stands of *Larix decidua* Mill. ranging in age from 25 to 35 years have been affected. Established sampling sites (SS) cover impacts of a crown fire (C1 and C2), surface fire (S1 and S2) and undisturbed stand (control U). Results obtained clearly show the influence of the forest fire on the soil carbon content. For the soil from C1 and C2, where the crown fire prevailed, a decrease in the soil carbon content in comparison with the control values have been observed. Opposite trend was documented for the soil carbon content in sampling sites influenced by surface fire. These values increased with about 1% and reached 3.45% of soil organic carbon (SOC). The SOC stock varied from 1.4 kg/m² to 2.5 kg/m² for the upper 5 cm and from 3.7 kg/m² to 4.6 kg/m² for the lower 15 cm soil layer. Soil from the fire-affected sites accumulated more carbon than the control (unburned) one. This finding is important by confirming the role of forest soil in the global carbon sequestration. The SOC stock values, however, differ depending on the trees species. For the upper 5 cm soil layer, the highest SOC stock was 2.52 kg/m² in the soil of the sampling site, afforested by Scot pine, followed by that below a mixed forest (*Pinus sylvestris* L. and *Quercus cerris* L.).

**Key words**: Forest fire, Soil carbon, Carbon stock, Rila Mountain

## Introduction

Forest fires provoke various changes in ecosystems that affect the composition, structure and patern of the vegetation cover, as well as soil and water resources of ecosystems that are critical for their overall functions (NEARY *et al*., 2005). Forest fires are considered as a driver for global biome distribution and for maintaining the structure and function of fire-prone communities (THONICKE *et al*., 2001; BOND & KEELEY, 2005). From naturally occurring phenomena, forest fires become more often human dependent events during the last two decades. Fire frequency is expected to increase with human-induced climate change, especially where precipitation remains the same or is reduced (STOCKS *et al*., 1998). A general but moderate increase in precipitation, together with increased

productivity, could also favour generation of more flammable fine fuels. MIRANDA (1994) suggests an increase in risk, severity, and frequency of forest fires in Europe. It is well known that the world's forests are the main carbon pool, which is estimated to be 348 Gt C in vegetation and 478 Gt C in soil (to 1 m) (updated since DIXON *et al*., 1994, by BROWN, 1998). Thus, they play an important role for offsetting carbon emissions and preventing (or at least slowing) global warming. Forest fires affect ability of forest ecosystems to store carbon. Typically changes occur in species distribution, net ecosystem production (NEP), net primary productivity (NPP), net biome productivity (NBP), elevated $CO_2$ emissions, as well as in climate variability and weather extremes (SMITHWICK *et al*., 2009). Immediately after a fire, carbon is lost to the atmosphere through combustion. Stand-replacing fires kill living biomass in forests and reduce carbon gains to near zero (KASHIAN *et al*., 2006). Strongest effect of fire on carbon cycling, however, occurs in the changing balance between carbon lost through subsequent decomposition and simultaneous carbon gains through growth of new vegetation. Stand-replacing fires switch ecosystems to being a net source of carbon as decomposition exceeds photosynthesis - a short-term effect (years to decades) that may be important over the next century if fire frequency increases.

Our previous studies showed that forest fires have a significant positive effect on the soil organic carbon (SOC) content in the surface soil layer if it occurs in coniferous forests (VELIZAROVA, 2011; VELIZAROVA *et al*., 2011). Through affecting the more thermo-labile fractions of SOC, the forest fires favour the rate of the mineralisation processes (VELIZAROVA & FILCHEVA, 2011). However, the degree to which forest fire impacts the soil carbon stock has not been explored previously. Our goal was to advance the current knowledge and understanding of fire effects on carbon content and stock in soils on the north slopes of Rila Mountain, under different types of forests.

**Materials and Methods**

Representative sampling sites have been chosen within the coniferous forests on the territory of Dolna Bania Government Forestry Enterprises (GFE). Sampling sites were situated on the north slopes of the Rila Mountain, affected by forest fire in August 2012. Two sampling sites – C1 and C2 with forests, respectively Scots pine (*Pinus sylvestris* L.) and European larch (*Larix desidua*, Mill.), were affected by a crown fire. Both sampling sites were established within the Scots pine (*Pinus sylvestris* L.) forest S1 and mixed forest - Scots pine (*Pinus sylvestris* L.) and Turkey oak (*Quercus cerris* L.) – S2. Control experimental site was established in an unburned Scots pine (*Pinus sylvestris* L.) forest – designate like U. Detailed information for the sampling sites is presented in Table 1.

**Table 1.** Studied areas parameters.

| Sampling sites (SS) | Studied cases | Dominant tree species | Altitude, m | Stand age | Area, ha | Geographical positions |
|---|---|---|---|---|---|---|
| C1 | Crown fire | *Pinus sylvestris* L. | 650 | 25 | 0.2 | 42°19'23.78" N<br>23°46'37.09" E |
| C2 | Crown fire | *Larix desidua* Mill. | 650 | 25 | 9.3 | 42°19'23.77" N<br>23°46'37.10" E |
| S1 | Surface fire | *Pinus sylvestris* L. | 650 | 25 | 4.4 | 42°19'23.75" N<br>23°46'37.11" E |
| S2 | Surface fire | *Pinus sylvestris* L.<br>*Quercus cerris* L. | 650 | 25 | 0.9 | 42°19'23.58" N<br>23°46'36.89" E |
| U | Unburned | *Pinus sylvestris* L. | 650 | 25 | 0.6 | 42°19'25.25" N<br>23°46'36.40" E |

The five 1-m deep soil profiles (till to the rock material) were established using spade – four at the burned forest stands (Sampling sites - C1, C2, S1, S2) and one – at intact forest site (Unburned - U). Soil samples were taken from 0-5 cm and 5-20 cm depth of the mineral part of the soil profile. Additionally four soil samples from the surface mineral soil have been collected randomly at each sampling site. Sampling and sample preparation processing have been performed following the contemporary methodological approach, described in details at ICP Manual - (COOLS & DE VOS, 2010). The soil from observed sites belongs to the group of the Haplic Luvisols (WRBSR, 2006). Identification and classification was performed according to the morphological peculiarities of the established soil profiles and based on the data received from the following laboratory analyses.

Soil organic carbon stock (SOC) for soil and forest litter was calculated according STOLBOVOY *et al*. (2007). For the purpose of our investigation, the reference soil organic carbon stock ($SOC_{ref}$) represents the initial (baseline) amount of the total SOC of the forest plots (unburned in our case). In order to calculate the difference in soil organic carbon stock $\Delta$ $SOC_{stoc}$, provoked by forest fire, the sequence of equations 1 to 4 was used. Soil organic carbon content represents the percentage of carbon (C) by weight (kg C/kg soil) × 100, and thus does not show the absolute carbon mass in the soil, which is sometimes inconvenient for comparing soils differing significantly in their density. Mass of C depends on the soil bulk density (e.g., a soil with a low percentage of C, but with a high bulk density may contain more mass of C compared to a low-density soil with a higher percentage of C).

Soil organic carbon density (SCD) for sampling site can be calculated according to the following equation (1):

$$SCD_{site} = \sum_{layer=1} (SOC_{content} \times BulkDensity \times Depth \times (1 - frag)) \quad (1)$$

Where:

$SOC_{content}$ - SOC content, % of mass $\frac{kg\ C}{kg\ Soil} \times 100$

*Bulk Density* - soil bulk density, $\frac{kg\ Soil}{dm^3}$

*Depth* - thickness of the sampled layer, dm;

*Frag.* - volume of coarse fragments, % of mass or $\frac{m^3 Stone}{m^3\ Soil}$

Parameter $SCD_{site}$ provides an average value for the sampling site, which is derived from a composite sample as indicated in Equation 1.

Next step in the calculation of the difference in soil organic stock is calculation of the mean (arithmetic average) soil carbon density (DCS) for a given plot.

$$\overline{SCD}_p = \frac{1}{n} \sum_{site=1}^{n} SCD_{site} \quad (2)$$

Where:

$SCD_{site}$ is calculated according to Equation 1;

*n* - number of the sampled sites within the plot.

Reference soil organic carbon ($SOC_{ref}$) stock for the plot was calculated according the equation:

$$SOC_{ref} = \overline{SCD}_p \times A_p \quad (3)$$

Where:

$\overline{SCD}_p$ is estimated by Equation 2.

$A_p$ is the area of the plot.

In our case, we assumed that the areas of the plots are equal to **1**ha in order to be able to identify the influence of the forest vegetation.

The changes in organic carbon stock ($\Delta SOC_{stock}$) for the plots were calculated, using the equation:

$$\Delta SOC_{stock} = SOC_{new} - SOC_{refstock} \quad (4)$$

Where:

The $SOC_{refstock}$ is as indicated in Equation 3 and refer to values obtained for unburned reference sampling site.

The $SOC_{new}$ refers to soil carbon stock after the influence of forest fire.

**Results and Discussion**

A summary of the characteristics of soils from the studied sampling sites is given in Table 2.

The measured values for soil pH after forest fire are higher in all experimental variants compared to the control pH values (unburned sites). More pronounced differences was observed for the upper 5 cm of the soil and for all sampling sites, affected by crown fire. The highest difference of about 1 unit of pH was found in the soil after fire for sampling site - C2 - under European larch forest. The burning of the organic materials from tree crowns and above-ground biomass makes nutrient elements such as Mg and Ca more available (MOLINA *et al*., 2007; SCHAFER & MACK, 2010). A similar trend for soil pH changes after a crown fire under 25 years stands of *Pinus sylvestris* L. was found previously (VELIZAROVA *et al*., 2001). Our results show that the soil pH increased also in the layer 5 – 20 cm in all studied variants.

**Table 2.** Basic soil characteristics and results for the soil carbon stock changes

| Sampling sites | Soil depth, cm | pH ($H_2O$) | Soil organic carbon content, (SOC) % | Total soil nitrogen N, % | C/N | Soil bulk density kg soil/dm$^3$ | Soil carbon density (SCD*site*) kg C/m$^2$ | *SCD site*, t/ha (for each studied soil depth) | *SCD site*, t/ha |
|---|---|---|---|---|---|---|---|---|---|
| C1 | 0 - 5 | 6.06 | 1.66 | 0.04 | 39.71 | 1.66 | 1.37 | 13.7 | 56.0 |
| | 5 - 20 | 5.53 | 1.38 | 0.03 | 44.09 | 2.04 | 4.22 | 42.2 | |
| C2 | 0 - 5 | 6.42 | 1.93 | 0.08 | 22.76 | 1.92 | 1.85 | 18.5 | 55.2 |
| | 5 - 20 | 6.12 | 1.24 | 0.07 | 17.74 | 1.97 | 3.67 | 36.7 | |
| S1 | 0 - 5 | 5.81 | 2.35 | 0.10 | 24.59 | 2.15 | 2.52 | 25.2 | 63.7 |
| | 5 - 20 | 5.40 | 1.79 | 0.08 | 21.90 | 1.43 | 3.84 | 38.5 | |
| S2 | 0 - 5 | 6.12 | 3.45 | 0.13 | 26.58 | 1.40 | 2.41 | 24.2 | 70.5 |
| | 5 - 20 | 5.85 | 2.21 | 0.10 | 22.51 | 1.40 | 4.63 | 46.4 | |
| U ($SOC_{ref}$) | 0 - 5 | 5.47 | 2.07 | 0.09 | 22.21 | 1.34 | 1.39 | 13.9 | 43.4 |
| | 5 - 20 | 5.26 | 1.10 | 0.05 | 21.19 | 1.78 | 2.95 | 29.5 | |

The soil pH changes in result of the forest fire depend not only on the fire type – crown or surface, but also on the differences of forest vegetation cover. Besides *Larix Desidua* Mill., the mixed forest of *Pinus sylvestris* L. and *Quercus cerris* L., subjected to fire, provokes a respective increase in the soil pH. The specific chemical composition of the forest litter and tissues of the different forest vegetation are known to cause variability in quantity and quality of burned organic material (KEVIN *et al*., 2003).

The data on soil bulk density and its changes are presented in Table 2 and Fig 1. The fire influenced soil showed increased bulk density values in comparison with that of the control sampling site. This increase is higher for the surface 5 cm layer and a maximal value of 0.81 kg soil/dm$^3$ was established for S1. Rather smaller changes were found for this indicator in the 5 – 20 cm soil layer. Increase in the bulk soil density of up to 0.26 kg soil/dm$^3$ was established for the soil from sampling sites, influenced by crown fire. Surface fire led to decrease in soil bulk density with 0.35 kg soil/dm$^3$ – 0.38 kg soil × dm$^3$ at this depth. BOERNER *et al*. (2009) and CERTINI (2005) found that heat and flames of forest fires transform/destruct the soil aggregates, clay minerals and the biomass, disperse formed ash, filled up soil pores and, thus, decrease soil porosity and permeability. Changes in soil bulk density influence the values of soil carbon density (SCD).

The most significant event occurring during a forest fire is burning of biomass and soil organic matter (CERTINI, 2005).

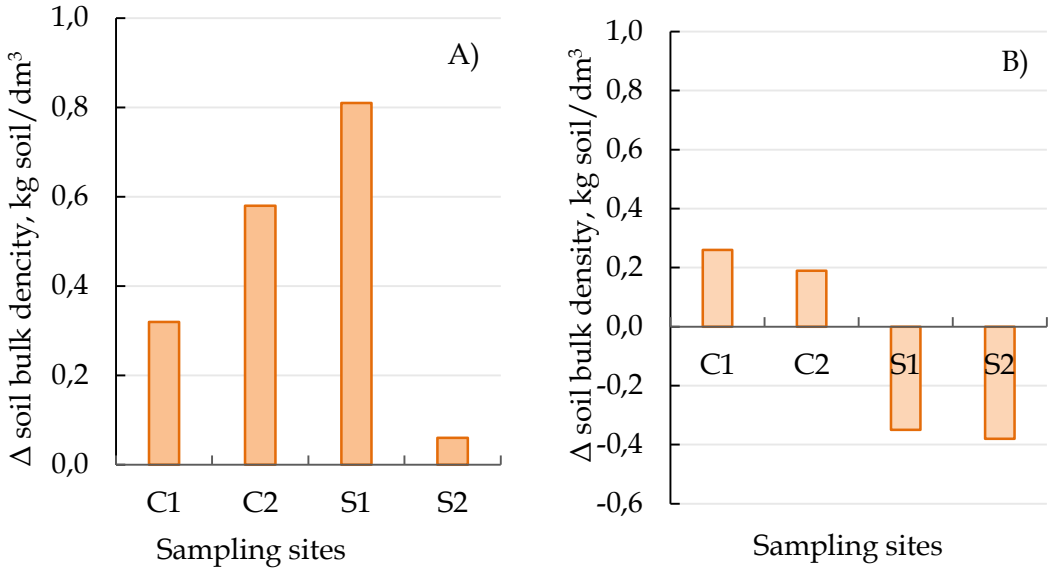


**Fig. 1**. Soil bulk density changes in 0 - 5 cm (A) and 5 -20 cm (B) soil layer.

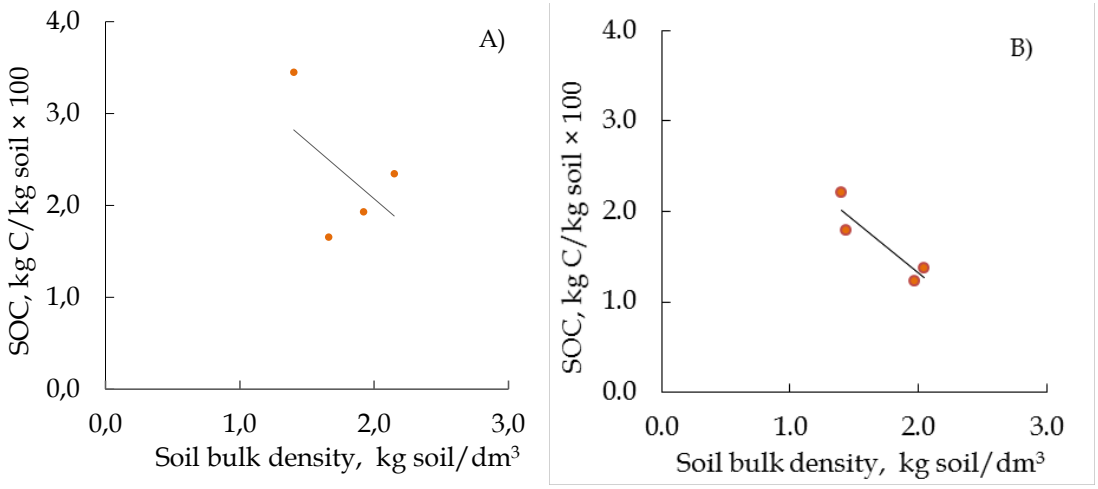


**Fig. 2**. Correlation between the SOC content and soil bulk for 0 – 5 cm soil layer (A) and for 5 -20 cm soil layer (B).

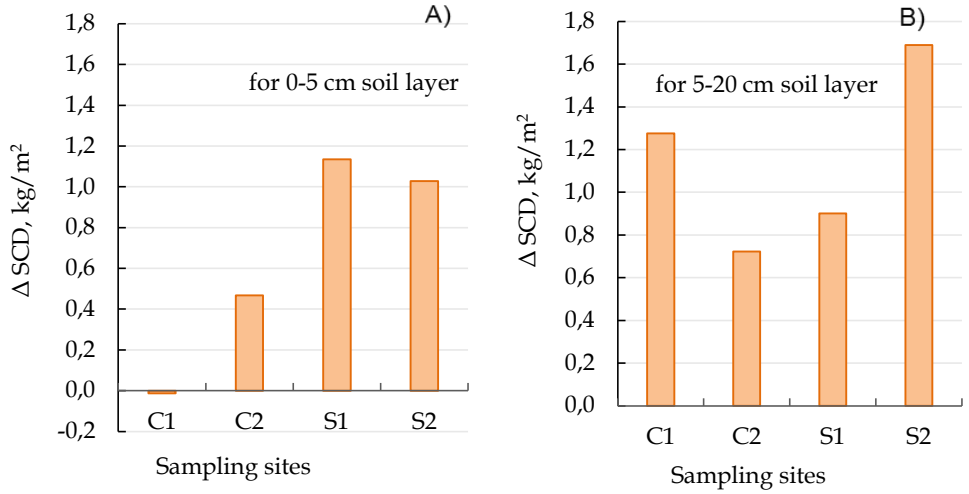


**Fig. 3**. Changes in soil organic carbon stock ($\Delta SOC_{stock}$) for 0 – 5 cm soil layer (A) and for 5 -20 cm soil layer (B).

These processes are of great importance as the forests and soil organic matter are a part of the terrestrial carbon pool, which is in turn – a part of the global carbon cycle (LAL, 2005). In the 0-5 cm layer of the mineral soil, the SOC content varies from 1.65% to 3.45% (Table 2). For the soil from C1 and C2, affected by crown fire, SOC content decreased in comparison to that in control (U). The earlier (HOSKING, 1938) and later studies (DEBANO *et al*., 1998) on heat-induced changes in organic matter have improved our understanding on the specific chemical changes that occur in organic matter during the course of heating. These and other studies demonstrated losses of organic carbon content in result of volatilization and carbonization of organic materials within the temperature interval from 200 °C to 400 °C (SCHNITZER & HOFFMAN, 1964). Contrary, the surface fire, peculiar to S1 and S2 provoked an increase in soil organic carbon amount in the surface soil layer – by up to 3.45% in comparison to the value for the U site. A similar trend for the soil organic concentration in result of a surface fire has been reported for 25 years old Austrian pine plantations (VELIZAROVA, 2000). A slight growth in soil OM content are also reported due to an increased deposition of dry leaves and charred plant materials after fires that affect the tree canopy and forest litter (CHANDLER *et al*., 1983).

The complex interactions among vegetation variety, fire severity, loss or reduction of structure and porosity, water repellency, as well as geomorphic processes influence the organic carbon content distribution along the soil profile. As surface fires suppose greater heat transfer to soil, the organic matter transformation was expected to result in structural changes. The SOC content within the 5-20 cm soil layer showed higher values in all studied variants in comparison with the control site. This increase was evident in the soils from S1 and S2 sampling sites. DEBANO (1981) hypothesizes that because of the steep temperature gradient in the upper 5 cm and deeper soil layers a small amount of organic matter can move downward and condense to form a water-repellent layer that would impede infiltration. Our results showing a negative correlation between the SOC content and soil bulk density for 5 -20 cm soil layer are in agreement with this hypothesis (Fig. 2).

The soil organic matter consists of distracted to a different degree plant materials, wood debris, humified components and other organic fractions and thus it is important to discuss the SOC stock and its changes ($\Delta SOC_{stock}$) for the fire influenced sampling sites (Table 2, Fig. 3). Our results for the SOC stock show that its values vary from 1.4 kg/m$^2$ to 2.5 kg/m$^2$ for the upper 5 cm and from 3.7 kg/m$^2$ to 4.6 kg/m$^2$ for the lower 15 cm. Comparing these values with that for control sampling site, it is evident that the soils from fire affected sites accumulated more carbon than the control one. The SOC stock values, however, differ with the forest types. For example – the soil from Scots pine stand affected by surface fire (S1) showed the highest SOC stock in the surface 5 cm - 2.52 kg/m$^2$, followed by that – for S2 – mixed forest (*Pinus sylvestris* L. *Quercus cerris* L.). The soil of these sampling sites (S1 and S2) showed higher carbon stocks also in the lower 15 cm layer – 3.85 kg/m$^2$ and 4.64 kg/m$^2$, respectively. Standardized SOC stock values for the studied 20 cm layer of the mineral soil exhibit the highest accumulation of 63.7 kg/m$^2$ and 70.5 kg/m$^2$ in the soil from surface fire affected sites. Obviously, the SOC stock in the fire-influenced sampling sites was dependent of fire characteristics (fire type and its severity) and the calculated the changes in soil organic carbon stock ($\Delta SOC_{stock}$) as the difference between the reference soil organic carbon stock ($SOC_{ref}$) and $SOC_{new}$ and use it as a measure for the forest fire influence on the soil organic matter (Fig. 3). The SOC stock differences in the surface 5 cm of the soil were ≈ 1.0 kg/m$^2$ in the S1 and S2 – sampling sites, related to the surface fire influence. Crown fire provoked lower SOC stock changes ≈ 0.4 kg/m$^2$ in the C2. All mentioned changes show positive accumulation trend. A decrease in the SOC

stock - by - 0.01 kg/m² was found for the surface 5 cm of the soil from C1, influenced by crown fire.

Along the soil profile (the 5-20 cm layer), the SOC stock differences vary from 0.6 kg/m² to 1.7 kg/m². Based on the data obtained, we suppose that in deeper soil layers the forest fire influence was less significant and that the carbon stock changes were mainly due to downward movement processes of transformed soil organic compounds, as well as of fine soil particles. RAPALEE *et al*., (1998) found that for boreal soil the SOC stock accumulation is related to the soil permeability, which was confirmed also by other studies (DEBANO, 1981).

**Conclusions**

Our investigation highlights the importance of field-based site measurements in characterizing the soil organic carbon stocks. The SOC stock varied from 1.4 kg/m² to 2.5 kg/m² for the upper 5 cm and from 3.7 kg/m² to 4.6 kg/m² for the lower 15 cm soil layer. The soil from the fire-affected sites accumulated more carbon than the control (unburned) one. This finding is important by confirming the role of forest soil in the global carbon sequestration. The SOC stock values, however, differ depending on the trees species. For the upper 5 cm soil layer, the highest SOC stock was 2.52 kg/m² in the soil of the sampling site, afforested by Scot pine, followed by that below a mixed forest (*Pinus sylvestris* L. and *Quercus cerris* L.). Standardized SOC stock values for the studied 20 cm layer of the mineral soil show that the highest accumulation of 63.7 kg/m² and 70.5 kg/m² is achieved in the soil from the surface fire affected sites. The reasons leading to a higher SOC stock in the fire-influenced sampling sites were related to fire characteristics (type and severity), and to the type of tree species. Changes in soil bulk density induce changes in soil carbon density (SCD).

SOC stock differences stock ($\Delta SOC_{stock}$) vary from 0.6 kg/m² to 1.7 kg/m². Downward movement processes of transformed soil organic compounds and fine soil particles seem to be the main mechanism for SOC stock accumulation. Forest fires influenced to a lesser degree the carbon stock changes in deeper (more than 5 cm) soil layers.

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# *Ecological Assessment of the Rivers Luda Yana and Banska Luda Yana as Based on Selected Biological Parameters*

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**Abstract.** The hydrobiological study of the Luda Yana River, and its right tributary - the Banska Luda Yana River, has been performed seasonally in 2011. Ecological status of the both rivers was analyzed according to the basic biotic indices, characterizing the composition and structure of the macrozoobenthic communities. The taxonomic structure and density showed that the bottom invertebrates community (the macrozoobenthos) was heavily disrupted and the saprobic index fluctuated around α-mesosaprobity probably as a result of municipal organic loads and the industrial wastewaters. The effects of the heavy metals pollution, incidentally occurred in the Banska Luda Yana River and originating most probably from the Assarel-Medet JSC, were discussed.

**Key words:** ecological conditions, Luda Jana River, structure of the macrozoobenthos.

## Introduction

The European Water Frame Directive (2000/60/EU) determines the ecological status of the water bodies as an expression of the quality of the aquatic ecosystem structure and functioning. Its main objective is to obtain a "good status" for all water bodies by 2015 and "good ecological potential" for the artificial and heavily modified water bodies.

In this connection the scope of the present study was to determine the recent ecological status of the Luda Yana River and its right tributary - the Banska Luda Yana River in 2011 year by using a set of metrics, consistent with the included in the WFD, Annex V, biological quality elements (benthic macroinvertebrates) and confirmed with the Ordinance H-4 and Order 412/2012, operating in Bulgaria at the time. Luda Yana River is a left tributary of the Maritsa River. The area along the rivers Luda Yana and Banska Luda Yana is part of the protected area of the National Network NATURA 2000 with number BG0000426, part of the protected by the Habitats Directive zones (92/43/EEC). Along the river the erosion and denudation are very intensive, the water is used for irrigation and part of it is lost in the alluvial

sediments, leading to drying up of the river almost every year. Biodiversity is strongly influenced by the negative anthropogenic impacts - cultivation, mining, settlements, disposal of industrial and municipal waste, flooding, spills, etc. The mines near the town of Panagiurishte and along the Luda Yana River - mine "Radka"; mine "Tsar Asen" and the numerous mines (geological exploration drillings done in the past) affect the ecological condition in the studied area. Some contribution to the overall chemical and ecological status of waters along has the natural metal anomalies in the area, leading to the higher natural background levels of heavy metals in the water.

The studies on the saprobic status of the Maritsa River started in the middle of the 20th century (RUSEV, 1966, 1967; EFREMOV, 1968) and was repeated in ditails in the 80-s by RUSSEV *et al.* (1981); UZUNOV & KOVACHEV (1981); UZUNOV *et al.* (1981); UZUNOV (1981); KOVATCHEV & UZUNOV (1981). In spite of all, its tributaries are not well studied and there is niearly no data about the ecological status in Luda Yana River and no data at all for the Banska Luda Yana River. Scanty data about the ecological status of the rivers Luda Yana and Banska Luda Yana are given by UZUNOV *et al.* (1991). Nowadays the catchment area has been studed in the exectution of the NATURA 2000 project but yet no data was published.

In 2010, after an accidental pollution with wastewaters from the "Asserel Medet – JSC", ressulted in mass fish death, registered in the villages Banya and Buta (Banska Luda Yana River), a new process of stocking has began.

"Asserel Medet – JSC" is the largest open pit mining and primary processing of copper ore in the Balkans. The copper extracting and processing factory has situated several filed stations for operative monitoring of the hydrochemical parameters of the water. Most of them coincided with our sampling points.

## *Materials and Methods*

*Geographical distribution and sampling*

The current study was carried out in 10 sampling point, selected mainly from the area, were death fish was registered, as well as downstream of the Luda Yana River (Fig. 1). During the first sampling season (April 2011) only 4 samples were taken (at st. 2, 4, 5 and 6). During August st. 8 was not sampled and in October.Two new points were added (6′ and 6″) to assess the ecological conditions in the rivers Liuliakovitsa and Mechenska.

Studied sites include sampling points along the rivers Luda Yana, Banska Luda Yana, Liuliakovitsa and Mechenska (Fig. 1). They refer to ecoregion 7 (Eastern Balkan) and the –East-Aegean River Basin. Single time samples were taken in 2011 during the high-water level (April-Jun) and the low water-level period (August - October).

Qualitative samples of benthic macroinvertebrates were taken according to the standard methods ISO 7828:1985/EN 27828:1994 and ISO 9391:1993, included in the methodic for biomonitoring, defined by order RD-412/15.06.2011 of the Minister of Environment and Waters). Field and cameral work was done in accordance with the standard EN/ISO 5667-3:2003/AC:2007. After the laboratory work, a taxonomic determination was done to such level that is enough for determination of the ecological condition, according to the approaches for applying the WFD (Directive 2000/60 EEC) introduced in Bulgaria (CHESHMEDJIEV & VARADINOVA, 2013).

*Physical and chemical parameters*

The physical and chemical parameters of water: water temperature ($^0$C), oxygen concentration (mg.dm$^{-3}$), nitrate nitrogen (N-$NO_3^-$, mg.dm$^{-3}$) and phosphate phosphorus P($PO_4^{3-}$), mg.dm$^{-3}$ were measured *in situ* with portable microprocessor oximeter type WTW 315i/SET and in the laboratory, according to BSS 3758-85 and BSS 7210-838.

*Cenosis structure*

A complex of chosen hydrobiological methods was applied for assessing the biological parameters: methods for investigation of taxonomical composition, abundance and spatial dynamics of the benthic invertebrate fauna: number of taxa

(S) and total abundance (N) (CHESHMEDJIEV & VARADINOVA, 2013); method for evaluation of the correlation between the sensitive and tolerant to pollution organisms: EPT (RESH & GRODHUS, 1983); methods for assessment the general ecological state: Adapted Biotic Index (BI) (CLABBY & BOWMAN, 1979; CHESHMEDJIEV & VARADINOVA, 2013); and EQR (Ecological Quality Ratio).

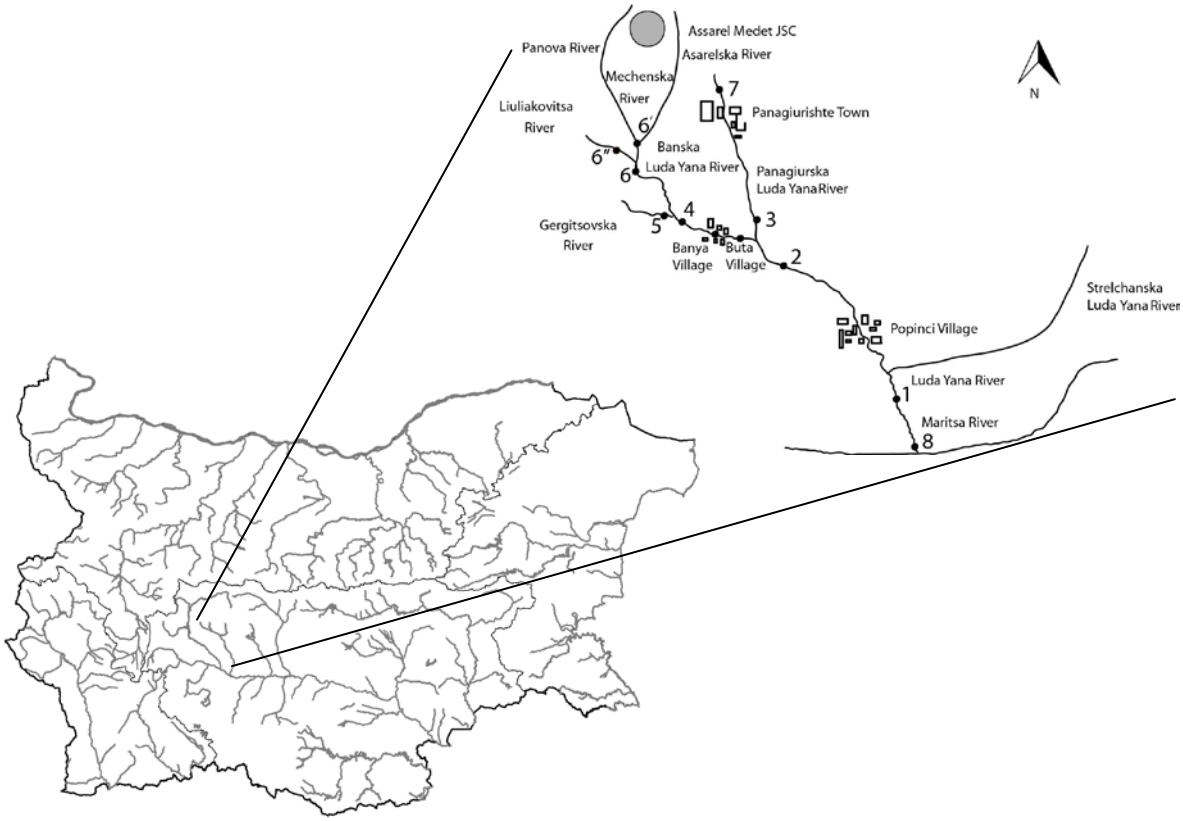


**Fig. 1.** Map of the sampling points.

**Results and Discussion**

*Hydrophysical and hydrochemical parameters*

Hydro-physical and hydrochemical analysis of the water main indicators were conducted before the fish stocking of the Banska Luda Yana River, in order to assess its quality at the beginning of the reconstruction activities of the ichthyofauna. The water samples were taken fourfold in 2011 (Fig. 2). Water sample analyses at st. 8 were done only in two seasons.

The sampling area is within the nitrate vulnerable zones of Bulgaria. Hydro-physical and hydrochemical parameters of the st. 4, 5, 6 and 7 were typical for surface, unpolluted waters, and provide satisfactory conditions for native fish species in the studied areas. The poorest results, especially in August and October, were at the st. 3. At this station this was the collecting point of the water from the rivers Banska Luda Yana and Luda Yana downstream the Panagurishte Town. The low level of dissolved oxygen, high levels of total inorganic nitrogen and phosphate tended to strong anthropogenic impact of different sources. In the direction of st. 3 to st. 8 there was a gradual improvement of the values of the hydrophysical and hydrochemical parameters of the st. 1 and 2, located at the Luda Yana River before and after the Popintsi Village, and the st. 8, before Maritsa River. It was clearly recorded for st. 8, which may be an indicator of good self-purification capacity of the river.

*Biological parameters*

For the whole period of investigation totally 50 taxa with different taxonomic levels (Table 2, appendix) were determined

to such level that is enough for determination of the ecological condition. The total abundance/numbers (N, ind./sample) varied from 5 (st. 4, August) to 1118 (st. 6', October) (Fig. 3a). The low values of the total abundance might have been due to both anthropogenic pollution (including organic and industrial waste) and low water levels at the times of sampling or periods of drying of the river.

The values of the EPT-richness % varied between 0 and 59.54% (Fig. 3b). Only at st. 5 sensitive species occurred in high numbers. In all of the rest stations relatively tolerant macrozoobenthic groups dominated.

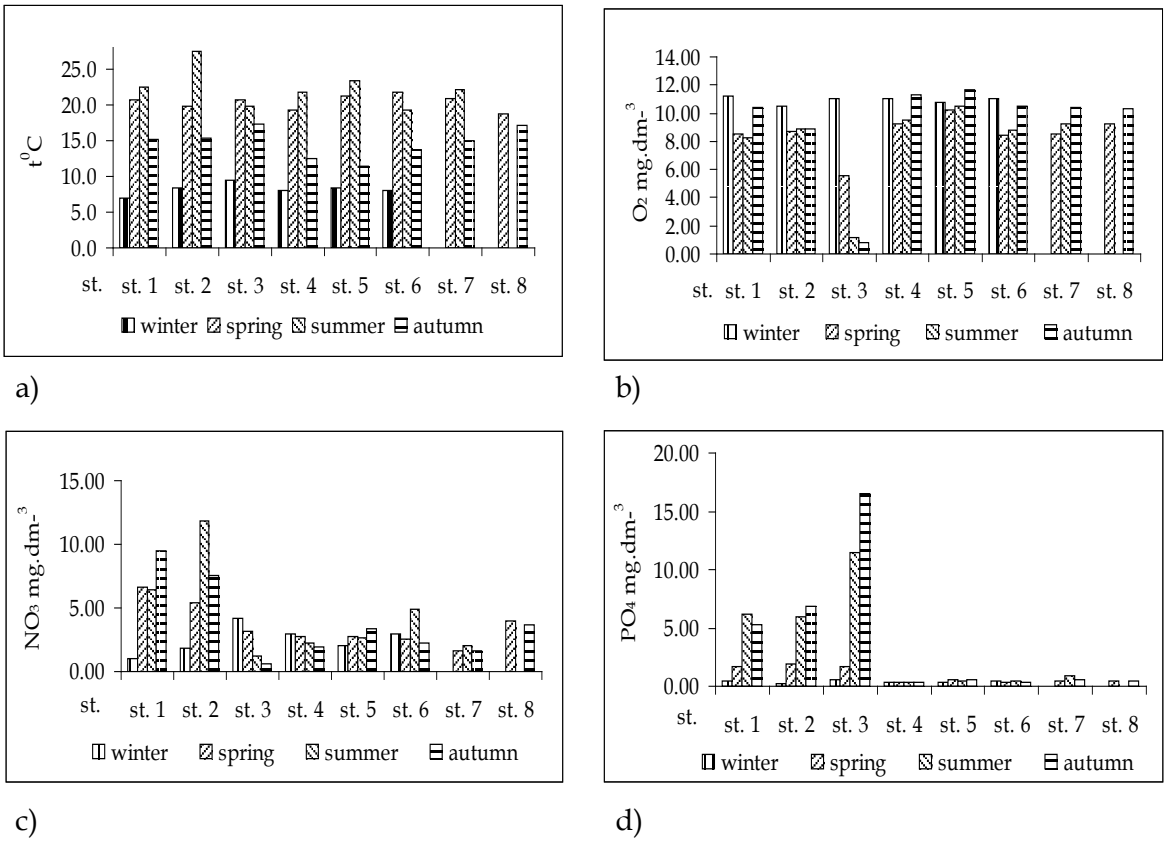


**Fig. 2.** Hydrophysical and hydrochemical parameters: temperature(a); dissolved oxygen (b); nitrate nitrogen (N-$NO_3^-$)(c), and phosphate phosphorus P($PO_4^{3-}$) (d).

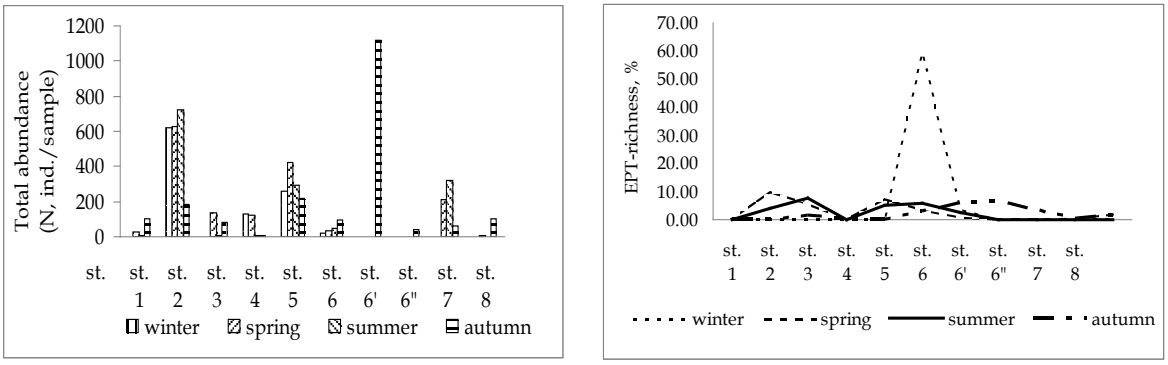


**Fig. 3.** Variation of the total abundance – N ( a) and EPT-richness ( b)

*Biotic index – BI*

The values of the BI (Table 1.) were comparatively constant and varied between 2 and 3, except the values 1 at st. 3 in August and 3-4 at st. 6′ in October.

UZUNOV *et al.* (1991) defined the saprobic status of the rivers Banska Luda Yana (at Banya Village) and Luda Yana (at Rosen Village) as a β-mesosaprobic. According to the East Aegean Basin Directorate –, the BI, in the period 2000-2006, in the section of the Luda Yana River between Popintsi Village (st. 1 and st. 8) and the flows in the Maritsa River, has values of 2-3. Based on benthic invertebrates the ecological status has been assessed as "moderate" to "poor". In this study the saprobic status of the sampled points varied between polysaprobic (at st. 2 and 4 in April, and at st. 1. In August) to β-mesosaprobic (at st. 6′ in October). At the most sites α-mesosaprobic state has dominated.

**Table 1.** Values of BI and color representation of EQR.

| | Station: | st. 1 | st. 2 | st. 3 | st. 4 | st. 5 | st. 6 | st. 6' | st. 6" | st. 7 | st. 8 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **winter** | BI | | 1-2 | | 1-2 | 3 | 2 | | | | |
| **spring** | BI | 2-3 | 2-3 | 2 | 2 | 3 | 2-3 | | | 2-3 | 2 |
| **summer** | BI | 2 | 2-3 | 1 | 2 | 2-3 | 3 | | | 2 | |
| **autumn** | BI | 2 | 2-3 | 2 | 2 | 2-3 | 2-3 | 3-4 | 2-3 | 2 | 2 |

Legend:

red - "very bad status" yellow - "moderate status"

orange - "poor status" green - "good status"

The st. 3 at the Luda Yana River was after the Panagiurishte Town and the variability of the ecological status from "poor" to "moderate" could be as a result of an organic impact of the town and the nearby fields. The higher values of the IBI for st. 2 could be explained with the inflow of purer waters from the Banska Luda Yana River. Some attention had to be paid to the stations 5, 6, 6′ and 6″. St. 5 (Gergitsovska River) was located before the village of Banya and was considered that there was no entry (discharge) of the wastewater plant of the Assarel Medet – JSC. The high level of the BI (3-4) at st. 6′ could be explained with the lake of municipal wastewater.

In 2010, as a result of pollution with wastewaters, discharged by the Mining and Processing Complex "Asarel Medet", a mass fish death had been observed at the Banya and Buta villages. Nevertheless the mining complex discharges wastewater only in Asarelska River, the toxic effect of the rich of heavy metals waters might have caused the fish death. Although it is believed that pollution is accidental and the river is not exposed to a chronic toxicity load, and after one year the analysis of the main hydrochemical and hydrobiological parameters in the river could be done, the ecological status of the river could be assessed so the recovery procedures of the local native ichthyofauna species could begin.

**Conclusions**

Banska Luda Yana River is used for irrigation and in order to reduce the river water velocity, several concrete barrages had been built, some of them with height 3-4 m. They were an insurmountable barrier to the free migration of the fish along the river and led to interruption of the river continuum, drastic changes in the river hydromorphology and turning the river in to a heavily modified water body. . Having in mind this and the fact that the Luda Yana River is assessed as a highly modified river in risk, further monitoring is recommended. It is necessary to monitor the ecological status of the river parallel with the establishment of the barbell stocked in 2011.

The current study revealed the unfavorable ecological situation of/in Luda Yana River, a left tributary of the Maritsa River. It is necessary to identify the contaminants and implement appropriate steps to eliminate their negative impact on riverine environment.

It is recommended biological value and water quality in the Maritsa River to be subjects of further studies.

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# *The Molluscs of the Medieval Settlement at the Village of Zlatna Livada (Bulgaria) with Notes on the Landscape Dominated the Area*

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**Abstract.** In the current study we report a total of 99 specimens of terrestrial snails from which the species *Zebrina detrita* dominated with 76 specimens. All other species were represented by 4-8 specimens each. As a whole the xerophilic species were most abundant – 84 specimens (*Z. detrita* and *Helix figulina*). The rest of the specimens were representatives of mesophilic species. The registered freshwater mussels were from the genera *Unio* and *Anodonta*, and the marine ones were *Ostrea edulis*, *Mytilus galloprovincialis*, and *Lima hians*.

**Keywords:** medieval settlement, shells, Bulgaria.

## Introduction

Molluscs (Mollusca: Gastropoda et Bivalvia) are commonly used as indicator for the reconstruction of environmental conditions in archeology (GULYÁS & SÜMEGI, 2005). Many species are connected to specific habitats and their existence is strongly tied to moisture and vegetation (for terrestrial species), the type of water basin (freshwater) and salinity (marine species). Having hard calcareous exoskeleton they provide abundant materials for paleontological and archaeological research. Many of the larger species of molluscs have been used in the past (and now) by human for food or making tools and jewelry, while other molluscs used to live in the ancient settlements and their surroundings (DOLUKHANOV & SHUKUROV, 2004; LUBELL, 2004; GULYÁS & SÜMEGI, 2005; HAIMOVICI, 2005; DIMITRIJEVIĆ, 2006).

This study was aimed on: 1. determination of the species composition of the molluscs, collected during excavations; 2. analysis of the proportions of different species; 3. collecting data about the village, through the analysis of environmental groups of molluscs; 4. investigation of possible traces of human activity on the shells; 5. obtaining data of the origins of the shells.

## Material and Methods

The investigated shells of molluscs were

collected during the archaeological excavations in the period 1997-2011. The material was stored at the Trakian University, Department "Anatomy" of the Faculty of Veterinary Medicine, Stara Zagora city.

Species were identified using a reference collection of shells. For specimen was considered any gastropod shell or every valve of the shell, except the fragments of *Anodonta* sp. For the specimens of the genus *Unio* is not used the method of minimal number of individuals but were counted only the number of valves or fragments of them.

*Study Area*

Excavations of the medieval settlement in the land of the village of Zlatna livada (municipality of Chirpan town) were organized (with interruptions) from 1997 to 2011 due to the construction of the highway road "Maritsa ". The settlement is located on non-bay terrace, north of the Maritsa River, at the "Byalata Voda" area named on the powerful karst spring, which today supplies with water all the villages surrounding the town of Chirpan. The road in this part of the highway almost repeated the route of the ancient road from Philippopolis, through the Adrianopolis, to the capital of the Empire - Konstantinopolis.

The settlement was not fortified but during the excavations was established the plan of the construction of housing. According the materials, the medieval village origins were around the spring at the end of the X$^{th}$ century. During the excavations were recorded remains of dwellings from the late Neolithic and Chalcolithic stage to and Late Iron age. The settlement has been abandoned by its residents probably before the events related to the Fourth Crusade and the crusader raids at Philippopolis (today Plovdiv) at the beginning of the thirteenth century.

There were several types of housing - with stone foundations, ramshackle semi-digged or ramshackle underground houses. The walls were flimsy in height, and the roofs were made of lightweight materials - straw and probably cane slabs, pressed down under a stone. Furthermore, during the study were recorded the remains of three necropols, multiple outbuildings, common household ovens strongly dug into the ground, and more than 100 pits. Their initial purpose was possibly to preserve the food. Precisely by the pits (after the abandoning) was the majority of osteological material because they were used as a place for garbage.

**Results and Discussion**

During the excavations in the area of medieval settlement near the village of Zlatna livada were registrated a total of 158 specimens of 10 species of molluscs - 5 species of terrestrial snails, 2 species of freshwater mussels and 3 of saltwater musells (Table 1, Fig. 1).

*Terrestrial snails*

We found a total of 99 speciemens of terrestrial snails (Gastropoda), from which strongly dominated *Zebrina detrita*: 76 individuals (76.8% of all gastropods and 48.1% of all molluscs). All other species were represented with approximately similar numbers - 4 to 8 individuals (Table 1). Generally dominate the xerophilous species, inhabitants of open grassy areas (*Z. detrita* and *H. figulina*) - a total of 84 individuals (84.8% of all gastropods). Other gastropods belong to the group of the mesophilic species, inhabitants of the bush or forest communities, which are drought resistant (15.2% of all gastropods). There was lack of shells of highly moisture-loving species.

There was no any human activity on all the shells registered. We assume that the found species were part of the fauna around the settlement. So we can conclude that around the village were prevailed dry and open areas, full of grass or grass-shrub vegetation on limestone rocks (xerophilic dominant species *Z. detrita* and *H. figulina* are also calciphilic). There have been small wetland areas, probably around a river or stream occupied by shrubs and/or trees, habitat for the small number of identified individuals from the mesophilic group (but drought-resistant) of species: *Helix lucorum*, *Cepaea vindobonensis*, and *Monacha* sp.

*Freshwater mussels*

From the freshwater mussels dominated individuals of the genus *Unio* - inhabitants of running freshwaters, usually medium and large rivers with sandy or sandy-muddy bottom. The material represented the shell fragments or whole shells, some of which have been determined as *U. pictorum* (Fig. 2-5,6). Some shells had thick deposits of limestone - a fact showing that they lived in very hard waters. On the external edge of many shells were found damages, caused by human activity - scraping the edge due to some transverse movements opposite of the line of the valves. They may were use as tools, such as tanning animal skins. It should be noted that the rate of wear is significantly less than that found in the Azmashka mound (Neolith) (GEORGIEV *et al*., 2009). During the Neolithic in Lower Volga and Ural rivers species of *Unio* were collected for food (DOLUKHANOV & SHUKUROV, 2004), here we can also assume the use of river mussels in this way.

In Eneolithic settlements in Romania and Moldova (Kukutenska culture) HAIMOVICI (2005) reported that river mussel *Unio* were used for food, and their shells in pottery (which could be also explanation of the specific damages we found on shells). Sporadic use of the three types of river mussels as an alternative food source was reported by DIMITRIJEVIĆ (2006) for the Neolithic culture in Vinča (Belo Brdo, Serbia).

We found two fragments of shells of a swamp mussel *Anodonta* sp. (Fig. 2-7) Widespread today in this area is *A. cygnea* and it can be supposed that the remains we registered could belong to this species.

*Marine mussels*

We have registered remains of four specimens of sea shells belonging to three species: *Ostrea edulis* (edible oyster, Fig. 2-2,3) - 2 specimens, *Mytilus galloprovincialis* - black shell (Fig. 2-4) - 1 specimen, and *Lima hians* - 1 specimen (Fig. 2-1). The large size of the oysters indicated their possible origin from a saltier and warmer sea, and the *Lima hians* is Mediterrenian species which is not found in the Black Sea region. Those facts indicate that they were brought in the village from the nearby Aegean Sea. The Black mussel is found both in the Black and in the Mediterranean seas so the origin of the shell in the settlement was unclear. In the past the Mediterranean mollusc species (and other species) were carried by people in the far north, such as the Romanian part of the Dobrogea (HAIMOVICI, 2007).

**Table 1.** Species, number of specimens and habitats of the molluscs from the excavations.

| Species | Number | Preferred habitat |
|---|---|---|
| **Gastropoda** | **99** | |
| *Zebrina detrita* (Müller, 1774) | 76 | Dry grass and bush open areas |
| *Cepaea vindobonensis* (Férussac, 1821) | 6 | Wet bush and forest areas, often near rivers |
| *Helix pomatia* Linnaeus, 1758 | 4 | Bush and tree areas, resistant to drought |
| *Helix figulina* Rossmässler, 1839 | 8 | Dry grass and bush open areas |
| *Monacha* sp. | 5 | Wet bush and forest areas, resistant to drought |
| **Bivalvia** | **59** | |
| *Unio pictorum* (Linnaeus, 1758) | 42 | Rivers with sandy bottom |
| *Unio* sp. | 12 | Rivers with sandy bottom |
| *Anodonta* sp. | 1 | Standing or slow running waters with muddy bottom |
| *Ostrea edulis* Linnaeus, 1758 | 2 | Salt water (Black Sea, Mediterranean) |
| *Lima hians* (Gmelin, 1791) | 1 | Salt water (Mediterranean) |
| *Mytilus galloprovincialis* (Lamarck, 1819) | 1 | Salt water (Black Sea, Mediterranean) |
| **Total** | **158** | |

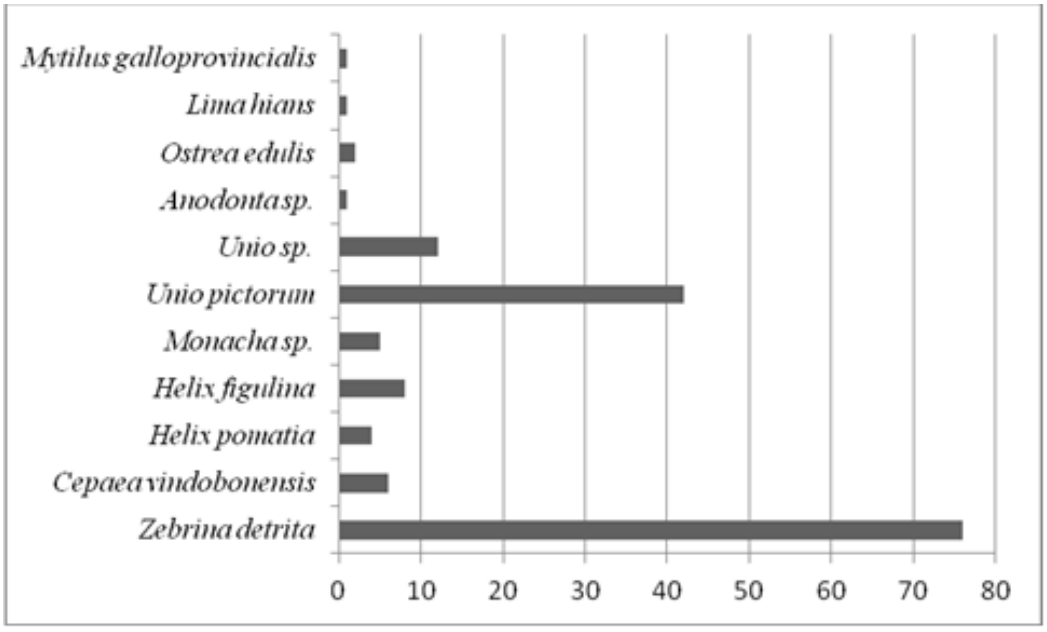


**Fig. 1.** Proportions of the specimens from each mollusc species from the excavations of the medieval settlement near Zlatna Livada.


**Fig. 2.** Mussels from the excavations of the medieval settlement near Zlatna Livada: 1 – *Lima hians,* 2, 3 – *Ostrea edulis,* 4 – *Mytilus galloprovincialis,* 5, 6 – *Unio pictorum,* 7 – *Anodonta sp.*

**Conclusions**

1. In the excavations of the medieval settlement near the village of Zlatna Livada were recorded 10 species of molluscs - 5 species of snails (Gastropoda) and 5 species of mussels (Bivalvia).

2. As a number of specimens found the terrestrial gastropods *Zebrina detrita* and the river mussels *Unio pictorum* dominated.

3. Based on the local ecological groups of mollusc species, it can be assumed that in the areas of the studied medieval settlement

dominated open fields with dry-loving plants - grass or grass-shrub vegetation, with patches of more humid parts from a reduced landscape occupied by mesophilic woody shrubs.

4. The valves of the river mussel (*Unio pictorum*, *Unio* sp.) were used as a tool in the Neolithic and continued to serve in a similar way in the Middle Ages.

5. Sea mussels *Ostrea edulis* and *Lima hians* have a Mediterranean origin and probably were brought to the village from the nearby Aegean Sea.

**Acknowledgements**

We express our thanks to Ulrich Schneppat (Natural History Museum, Chur, Switzerland) for the help on identification of the mussels, and especially the *Lima hians* specimen.

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# *Anatomical Changes in Peach Leaves Infected by Taphrina deformans (Berk.) Tul.*

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**Abstract.** Light microscope study of *Prunus persica* (L.) Batsch. (Fayette cultivar) leaf anatomical structure, naturally infected by *Taphrina deformans* (Berk.) Tul. has been conducted. In the infected leaves histological changes were observed such as increase of the total thickness of the mesophyll and a loss of its differentiation to palisade and spongy parenchyma. An increase in the size of the upper epidermis was established as a result of fungus localization. The results were supported by morphometric and statistical analysis.

**Key words:** leaf curl, *Prunus persica; Taphrina deformans;* leaf anatomy.

## Introduction

Peach leaf curl caused by *Taphrina deformans* (Berk.) Tul. is a disease widely spread and potentially harmful in cool and humid spring seasons. Morphological traits are related to leaf deformation and changes in pigmentation (chlorotic and anthocyanic coloration) of leaves, shoots and occasionally of fruits (Fig. 1). They are due to deep physiological alterations leading to premature aging and leaf abscission. Such are the abnormalities in the leaf gas-exchange, pigment and water content, etc. (RAGGY, 1966; MONTALBINI & BUONAURIO, 1986; RAGGY, 1995; PIPERKOVA & VASILEV, 2000). Changes in the structure of the leaf blade and of the cell shape and structure were described in the papers of MARTE & GARGIULO, 1972; SYROP, 1975; HUANG *et al*., 1993; ADEKUNLE *et al*., 2005. The hormone imbalance leading to hypertrophy and hyperplasia in the infected leaves is in a causal relationship with the changes on a physiological and structural level (SZIRAKI *et al*. 1975; YMADA *et al*. 1990; TSAVKELOVA *et al*. 2006).

Using light and electron microscopic observations, SYROP (1975) and HUANG *et al*. (1993) detected similar changes in almond and peach leaves infected by *Taphrina deformans*. They pointed out that accelerated cell division was the first response of the host. Changes in the palisade tissue, deformation and decreased size of the chloroplasts in the cells surrounded by the pathogen hyphae were found out, followed

by the development of chlorosis. BASSI *et al.* (1984), GIORDANI *et al.* (2012) proved in their studies that the mesophyll layers could not be well-defined in the leaves infected by *Taphrina deformans*. Similar results were also established in the investigation carried out by HANSEN *et al.* (2007) on *Nothofagus pumilio* leaves infected by *Taphrina entomospora*.

Similar studies have not been carried out in Bulgaria. In the present investigation the authors offer their vision on the structural and anatomical changes detected in peach leaves of Fayette cultivar.

**Fig. 1.** Symptoms of naturally infected by *Taphrina deformans* leaves of *Prunus persica* (L.) Batsch.

**Material and methods**

The samples for the analyses were prepared from peach leaves of Fayette cultivar (*Prunus persica* (L.) Batsch), naturally infected by *Taphrina deformans*, as well as healthy leaves (control), collected at the end of April 2011 in the region of Plovdiv.

Both variants were studied following the standard methods of comparative anatomy (NIKOLOV & DASKALOV, 1966; METCALFE & CHALK, 1979). The cross-sections of fresh materials were studied from the middle part of the lamina. Semi-stable microscopic preparations were made. Amplival microscope was used for the light study. Measurements were made with an eyepiece micrometer (10x) and the pictures were taken with a light digital microscope Motic DMBA-210.

Part of leaves of fully development infected and healthy leaves (control) were processed following the protocol, described by DONCHEVA *et al.* (2001). Briefly, the samples were fixed in 5% (v/v) glutaraldehyde in 0.1M Na – cacodilate buffer (pH 7) for 2 h at room temperature and post fixed with 1.3% (w/v) $OsO_4$ in the same buffer. Infiltration and embedding were performed using Durcupan ACM (Fluka, Sigma – Aldrich). Semi-thin section (1 -2 μm), cut from the Durcupan – embedded material with glass knives, were mounted on glass slides, stained with fuchsin and methylene blue and examined under a NU light microscope (Zeiss, Jena, Germany). Metric characteristics of the studied samples included: thickness of the adaxial (ad) and abaxial epidermis (ab) in μm, total thickness of the mesophyll in μm, thickness of the palisade and spongy mesophyll layers in μm. 30 measurements of each characteristic were obtained.

The statistical analysis of empiric data includes point and interval estimation of parameters: mean on each variable (metric statistic), and difference between means for infected and control leaves, at a 95 % confidence level. Null Hypothesis for no effect of infection is proved by a t-test for independent samples at a significance level $\alpha = 0,05$. Programs were used for data processing STATISTICA for Windows (STATSOFT INC., 2007).

**Results and Discussion**

Studies on the anatomical structure of the leaves are of great importance for explaining the adaptive responses of the plants to ecological or pathogenic stress.

By means of light microscope analysis of cross-sections samples of healthy peach leaves (control), it was found out that the epidermal tissue was formed by a single cell layer. The mean height of the adaxial epidermis (ad) was 14.33 µm, and of the abaxial one (ab) – 12.50 µm (Table 1). The mesophyll was of a clearly defined palisade and spongy parenchyma, i.e. dorsoventral type. That structure is typical of most dicotyledonous plants, leaves of which have a flat blade and are horizontally arranged. The palisade parenchyma is structured by oval-cylindrical cells arranged in two rows, with well-developed protoplasma organelles and small intercellular spaces for free gas-exchange, the average thickness being 50.58 µm (Table 1). The cells of the spongy parenchyma are irregular in shape, arranged in several rows, with large intercellular spaces. Its average thickness is 52.41 µm (Fig. 2; 3, Table 1). Vascular bundles are located at the border between palisade and spongy parenchyma.

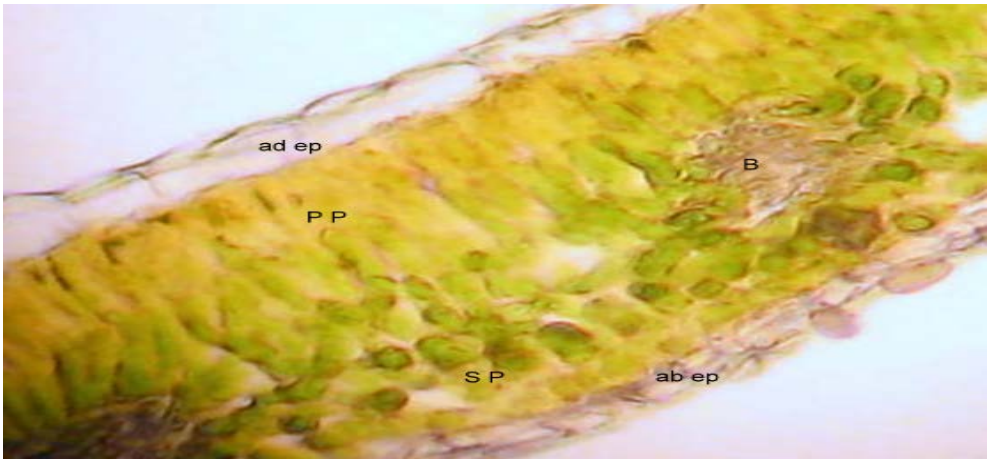


**Fig. 2.** General view of the transverse section of healthy leaves of *Prunus persica* (L.) Batsch. Fayette cultivar, LM at magnification X 100 (ad ep – upper epidermis; ab ep – lower epidermis; PP – palisade parenchyma; SP – spongy parenchyma).

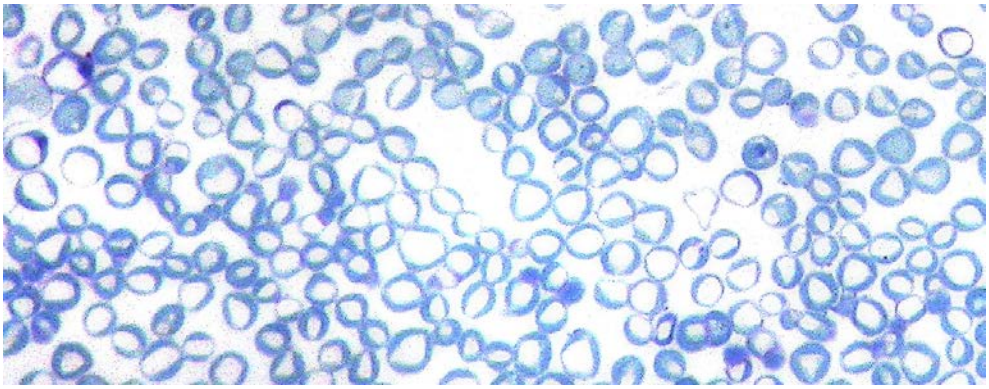

**Fig. 3.** Semi-thin section of healthy leaves of *Prunus persica* (L.) Batsch. Fayette cultivar, LM at magnification X 400.

As it is known, the parasitizing hyphae of *Taphrina deformans* develop in the intercellular spaces of the mesophyll and the epidermis (SYROP, 1975; GIORDANI *et al.* 2012). They cause histological changes like hypertrophy and hyperplasia, provoked by biologically active substances (SZIRAKI *et al.*, 1975; YMADA *et al.*, 1990). Statistically

significant histological changes in the leaf structure were found out in the samples prepared by infected plants. They were detected in the height of the adaxial (ad) and abaxial epidermis (ab), in the thickness of the leaf lamina, in the shape and size of the mesophyll cells. The adaxial epidermis height increased by about 45% compared to the control, i.e. by 6.50 μm in average, while the abaxial decreased from 12.50 to 10.25 μm, i.e. by about 22% (Table 1). The results obtained about the abaxial epidermis height did not agree with those provided by HANSEN *et al*. (2007) in their studies of *Nothofagus pumilio* infected by *Taphrina entomospora*. The reason for that discrepancy in the obtained results is due to the fact that the parasitizing mycelium of *Taphrina deformans* at the end of the pathological process develops subcuticularly, between the adaxial epidermis cells, while the mycelium of *Taphrina entomospora* develops on the abaxial epidermis. Accordingly, the asci are formed on the upper leaf surface in peach and on the lower one in *Nothofagus pumilio*. It could be admitted that thickening of the epidermal layer, spores of the pathogen are bursting through, is a protective mechanism of the host.

Disrupted mesophyll differentiation was established in the areas with parasitizing mycelium, i.e. the leaf lamina changed from dorsoventral to isolateral (equifacial) (Fig. 4.). In result of the processes of hypertrophy and hyperplasia, provoked by the pathogen, the shape of the cells from the palisade layer turned from cylindrical oval to isodiametric. Both, large hypertrophied, strongly vacuolated cells, with organelles located close to the cell wall, with small intercellular spaces were observed, together with cells twice or thrice smaller in size (Fig. 4.).

The morphometric analysis of the cross-section of the control and of the infected leaves confirmed the histological changes (Table 1). The total average thickness of the mesophyll in the first variant was 120.92 μm, while in the infected leaves it reached up to 197.50 μm. The registered difference of 76.58 μm is statistically significant.

The established changes in the structure of the leaf lamina correlated with some changed parameters of the physiological state of the peach leaves infected by *Taphrina deformans*, to which Fayette cultivar is susceptible. The chlorophyll content decreased by 22-29%, the photosynthesis rate was inhibited in the range of 17 to 45% and breathing increased repeatedly (PIPERKOVA & VASILEV, 2000). That confirmed the fact that the histological changes established in the present study by light microscopy, as well as the changes in peach cultivars showing different resistance to the pathogen, found out by GIORDANI *et al*. (2012), are causally related to the disturbed metabolism in the infected leaves.

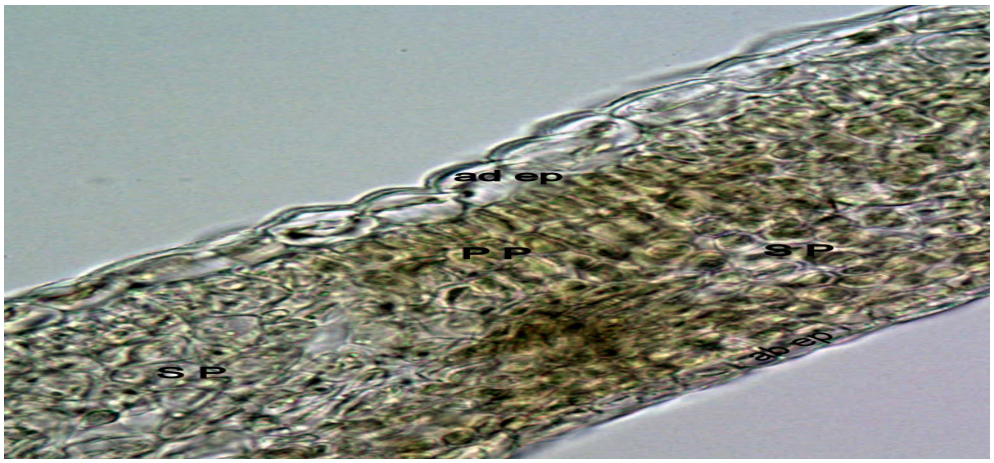


**Fig. 4.** General view of transverse section of infected by *Taphrina deformans* leaves of *Prunus persica* (L.) Batsch. LM at magnification X 100 (ad ep – upper epidermis; ab ep – lower epidermis; spongy parenchyma.

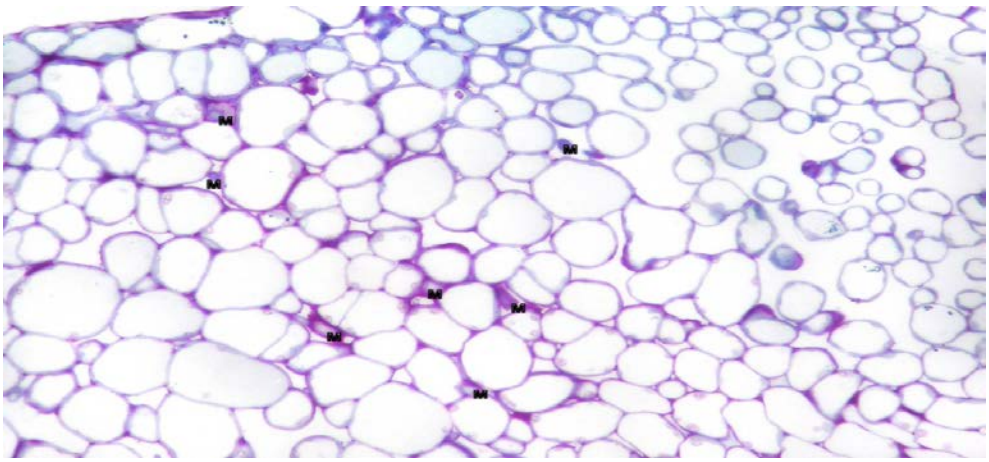


**Fig. 5.** Semi-thin section of infected by *Taphrina deformans* leaves of *Prunus persica* (L.) Batsch. LM at magnification X 400 (M – mycelium).

**Table 1.** Morphometric parameters of healthy (control) and infected by *Taphrina deformans* (Berk.)Tul. leaves of *Prunus persica*(L.) Batsch.

| Parameters | | 95 % Confidence Interval | | |
|---|---|---|---|---|
| | | Mean | Standard Deviation (SD) | Difference between Means |
| Upper epidermis (ad), thickness/µm | control | 14.33 ± 0.65 | 3.9108 | 6.50 ± 0.87* |
| | infected | 20.83 ± 0.62 | 21.3247 | |
| Lower epidermis (ab), thickness/µm | control | 12.50 ± 0.60 | 1.7287 | -2.25 ± 0.84* |
| | infected | 10.25 ± 0.62 | 1.6522 | |
| Mesophyll thickness, µm | control | 120.92 ± 1.46 | 1.6082 | 76.58 ± 8.07* |
| | infected | 197.50 ± 7.96 | 1.6544 | |
| Palisade mesophyll, thickness/µm | control | 50.58 ± 1.09 | 2.9127 | - |
| Spongy mesophyll, thickness/µm | control | 52.42 ± 1.44 | 3.8553 | - |

*statistically significant difference at α = 0.05

**Conclusion**

In result of the study carried out with light microscopy on healthy and naturally infected by *Taphrina deformans* peach leaves (*Prunus persica* (L.) Batsch.) of Fayette cultivar, severe histological changes were established. The leaf structure changed considerably from dorsoventral to isolateral. Under the influence of the processes of hypertrophy and hyperplasia, the plant cells located around the fungal hyphae, were strongly vacuolized, they increased in size and acquired an isodiametric shape. The morphometric analysis confirmed the increase of the adaxial epidermis height by 45% compared to the control and the decrease of the abaxial epidermis height by 22%, which was caused by the *Taphrina deformans* asci formed on the upper surface of the peach leaves. The established changes were statistically significant. Structural differences in the infected leaves were directly related to the changed physiological state of the plant.

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# *New Data about Some Rare and Interesting Agaricales Species in Bulgaria*

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**Abstract.** This paper provides information about the distribution of 27 species of Agaricales. Six of them are of conservation value in Bulgaria: *Agaricus bohusii, Amanita caesarea, A. ovoidea, A. vittadinii, Batarea phalloides* and *Chlorophyllum agaricoides*. Several other species are reported with new localities in the country *(Hymenogaster buliardii*, *H. hessei*, *Hygrophorus piceae*, *Pluteus aurantiorugosus, Coprinus alopecius*, *Stropharia luteonitens* and *Tubaria dispersa)*. New data are reported or confirmations of previous older records are made.

**Key words:** Bulgarian mycota, conservation of fungi, fungal diversity.

## Introduction

The knowledge about the species diversity and distribution of Agaricales in Bulgaria is still scarce. Well known and common threatened fungi are in need of additional information, confirmation of new finds with voucher specimens in new localities. These species are among the most economically important fungi in Bulgaria; therefore they deserve an intensive research on species composition and distribution in the country.

The paper aims to provide information about some recent findings. Special attention is paid to those species, considered to be rare and threatened in Bulgaria, and data about more widespread taxa are included. New localities are reported for 27 species. Among them *Agaricus bohusii, Amanita caesarea, A. ovoidea, A. vittadinii,* and *Batarea phalloides* are of conservation value in Bulgaria (GYOSHEVA *et al.*, 2006). The present article is a contribution to the study of the distribution of the Agaricales in Bulgaria.

## Material and Methods

The investigated specimens were collected from different floristic regions (Forebalkan, Stara Planina Mts, West Frontier Mts, Mt Belasitsa, Rila Mts, Sredna Gora Mts, The Rhodopes Mts, Thracian Lowland, Tundzha Hilly Country and Mt Strandzha) of Bulgaria.

Studied specimens are kept in the Agricultural University (SOA) in Plovdiv. Their accession numbers are given in brackets at the end of each record. Fungal specimens were preserved by drying and documented with color photograph and/or concise description. Air dried specimens were packaged in paper bags. All specimens were collected by the author, unless otherwise stated.

Determination, nomenclature, and taxonomy of the species are in accordance

with MOSER (1967, 1978, 1983), PHILLIPS (1981), WASSER (1980, 2002), CAPPELLI (1984), CLÉMENÇON (1984), HANSEN & KNUDSEN (1992), CONTU (2003) and NEVILLE & POUMARAT (2004). Families and genera are given according to the arrangement of KIRK *et al.* (2008). The author's names of the fungal taxa are abbreviated according to Kirk & Ansell (2004). Wherever indicated, the threat status follows the *Red list of fungi in Bulgaria* (GYOSHEVA *et al.*, 2006). Phytogeographical division of Bulgaria is according to JORDANOV (1966).

*List of collected fungi*

Agaricales
Agaricaceae

*Agaricus bohusii* Bon.

Mt Strandzha: Burgas distr, above the dam of Malko Sharkovo, in communities of *Acer campestre* L., *Quercus frainetto* Ten. and *Q. cerris* L., under *Acer campestre* L., 24 August 2012, leg. & det. M. Lacheva (SOA 60 00344).

*Note. Agaricus bohusii* has been previously reported from Central Stara Planina Mts, The Rhodopes (central), and Thracian Lowland (STOICHEV & ANASTASOV, 1988; LACHEVA, 2006, 2011a, 2012a). Reference is made to Mt Strandzha - near Ropotamo River, leg. Stoyan Beshkov, and Valley of River Strouma (Protected locality Roupite), leg. Dimitar Vasilev.

*Note.* Rare and interesting species which forms fruibodies during the hot summer months (mostly August) and less frequently in autumn. Included in the Red List of Bulgarian fungi under category Critically Endangered (GYOSHEVA *et al.*, 2006).

*Agaricus impudicus* (Rea) Pilát

Thracian Lowland: in the park of the village of Malak Chardak, Plovdiv distr., under *Acer negundo* L. and *Quercus frainetto* Ten., 7 June 2010, leg. & det. M. Lacheva (SOA 60 00346).

Tundzha Hilly Country: in the park of Topolovgrad town, under *Acer negundo,* 20 September 2011, leg. & det. M. Lacheva (SOA 60 00327).

*Note.* The species was reported from Sredna Gora Mts, Thracian Lowland and Tundzha Hilly Country (LACHEVA & STOICHEV, 2004; LACHEVA, 2006, 2011a, 2012a).

*Agaricus cupreobrunneus* (F.H. Møller) Pilát

Thracian Lowland: on soil, among grasses, in the vicinity of the village of Shishmantsi, Plovdiv distr., 22 September 2010, leg. & det. M. Lacheva (SOA 60 00329); on soil, among grasses, in the vicinity of the village of Trud, Plovdiv distr., 22 Sep 2011, leg. & det. M. Lacheva (SOA 60 00332).

*Note.* The species was reported from Sredna Gora Mts, Thracian Lowland and Tundzha Hilly Country (STOICHEV & LACHEVA, 2002; LACHEVA, 2006, 2011a).

*Battarrea phalloides* (Dicks. : Pers.) Pers.

Sredna Gora Mts: on soil with rotted wood, above the village of Bogdan, Plovdiv distr., 13 October 2011, leg. & det. M. Lacheva (SOA 60 00334).

*Note.* The species was reported from Thracian Lowland (DENCHEV & ASSYOV, 2010, LACHEVA, 2012, 2012c). Included in the Red List of Bulgarian fungi under the category Endangered (GYOSHEVA *et al.*, 2006). It is reported for the first time from Sredna Gora Mts.

*Chlorophyllum agaricoides* (Czern.) Vellinga [= *Endoptichum agaricoides* Czern.]

Thracian Lowland: on manure, in the farmyard of the village of Shtarkovo, Pazardzhik distr., 8 September 2011, leg. A. Vuchkov, det. M. Lacheva (SOA 60 00348); on manure, above the village of Trud, Plovdiv distr., 22 September 2011, leg. & det. M. Lacheva (SOA 60 00339); on manure, above the village of Turkmen, Plovdiv distr., 18 September 2011, leg. & det. M. Lacheva (SOA 60 00342).

*Note. Chlorophyllum agaricoides* was reported from Stara Planina Mts (western), Znepole region, Pirin Mts, Sredna Gora Mts, Rhodopi Mts (eastern), Thracian Lowland, Tundzha Hilly Country (DENCHEV & ASSYOV, 2010; LACHEVA, 2011, 2012, 2012c). It is included in the Red List of Bulgarian

fungi under the category Endangered (GYOSHEVA *et al*., 2006).

*Coprinopsis radiata* (Bolton: Fr.) Redhead, Vilgalys & Moncalvo [= *Coprinus radiatus* (Bolton : Fr.) Pers.]

Stara Planina Mts (central): on dung of horse, above the challet of Vejen, 23 June 2011, leg. & det. M. Lacheva (SOA 60 00324).

*Note.* The species was reported from Pirin Mts (VANEV & REID, 1986).

*Coprinus alopecius* Lasch

The Rhodopes (eastern): on fallen leaves, in community of *Fagus sylvatica* L. and *Alnus glutinosa* (L.) Gaertner, above the village of Hrabrino, Plovdiv distr., 11 September 2011, leg. & det. M. Lacheva (SOA 60 00330).

*Note.* The species was reported from Sredna Gora Mts (DIMCHEVA *et al.,* 1992). It is reported for the first time from The Rhodopes (eastern).

Inocybaceae

*Tubaria dispersa* (Berk. & Broome) Singer

Sredna Gora Mts: on soil, in grassy meadow, above the village of Starosel, Plovdiv distr., 13 October 2009, leg. & det. M. Lacheva (SOA 60 00325).

*Note.* Previously this species was reported from Sofia region by DIMCHEVA *et al*. (1992) and GYOSHEVA *et al.* (2012). It is reported for the first time from Sredna Gora Mts.

Amanitaceae

*Amanita caesarea* (Scop.: Fr.) Pers.

Forebalkan: above the village of Gradeshnitsa, near Teteven town, under *Fagus sylvatica* L., 7 June 2012, leg. & det. M. Lacheva (SOA 60 00355).

West Frontier Mts: Osogovo Mt, Kyustendil distr., above the chalet of Trite buki, under *Fagus sylvatica* L., 17 September 2010, leg. & det. M. Lacheva (SOA 60 00328).

Belasitsa Mts: in the vicinity of the village of Parvomai and Zlatarevo, Petrich distr., under *Castanea sativa* Miller, 15 July 2009, leg. & det. M. Lacheva (SOA 60 00326).

Sredna Gora Mts: Lozenska Planina Mt, above the village of Dolni Lozen, near the St. Spas Monastery, under *Quercus* spp., 19 May 2009, leg. & det. M. Lacheva (SOA 60 00336); under the peak Polovrak, in mixed forest of *Fagus sylvatica* L. and *Carpinus betulus* L., under *Fagus sylvatica* L., 26 June 2012, leg. & det. M. Lacheva (SOA 60 00345).

Thracian Lowland: under *Quercus* sp., the village of Draganovo, Plovdiv distr., 6 October 2010, leg. & det. ML (SOA 60 00333); in the oak forest near the village of Dolno Levski, Pazardzhik distr., 18 July 2003, leg. & det. G. Stoichev (SOA 60 00357); in oak forest, near the dam Pyasachnik, Plovdiv distr., 6 October 2010, leg. & det. M. Lacheva (SOA 60 00358).

Tundzha Hilly Country: Mt Sakar, above the village of Lesovo, near Elhovo town, under *Quercus frainetto* Ten., 25 May 2011, leg. & det. M. Lacheva (SOA 60 00337); above the village of Sakartsi, near Topolovgrad town, under *Quercus frainetto* Ten., 25 May 2011, leg. & det. M. Lacheva (SOA 60 00335).

*Note. Amanita caesarea* is included in the Red List of Bulgarian fungi under the category Vulnerable (GYOSHEVA *et al.,* 2006).

The species was reported from Black Sea Coast, Danubian Plain, Forebalkan, Stara Planina Mts (central, eastern), Sofia region, Znepole region, Vitosha region, Rila Mts, Sredna Gora Mts (western), The Rhodopes (western, eastern), Strandzha Mts (DENCHEV & ASSYOV, 2010).

*Amanita ovoidea* (Bull. : Fr.) Quél.

The Rhodopes (eastern): on soil, in oak forest, near the village of Chernichino, Kardjali distr., 13 July 2011, leg. & det. M. Lacheva (SOA 60 00331).

*Note. Amanita ovoidea* is included in the Red List of Bulgarian fungi under the category Critically Endangered (GYOSHEVA *et al.,* 2006).

The species is already known from Eastern Rhodopes (HINKOVA *et al.,* 1979; LACHEVA & GYOSHEVA, 2013) and from Eastern Stara Planina Mts (KUTHAN & KOTLABA, 1989).

*Amanita vittadinii* (Moretti) Vittad.

The Rhodopes (eastern): on soil, among grasses, in glade near the village of Odrintsi,

Kardzhali distr., 28 September 2010, leg. & det. M. Lacheva (SOA 60 00350).

Thracian Lowland: on soil, among grasses, in the vicinity of the village of Markovo, Plovdiv distr., 06 October 2010, leg. & det. M. Lacheva (SOA 60 00347).

*Note. Amanita vittadinii* is included in the Red List of Bulgarian fungi under the category Vulnerable (GYOSHEVA *et al.,* 2006).

The species was reported from Danubian Plain, Sofia region, Znepole region, Vitosha region, The Rhodopes (central, western) and Thracian Lowland (DENCHEV & ASSYOV, 2010; LACHEVA, 2012b; LACHEVA & GYOSHEVA, 2012).

Pluteaceae

*Pluteus aurantiorugosus* (Trog) Sacc.

Sredna Gora Mts: above the village of Krastevich, Strelcha distr., near the chalet of Buntovna, on dead stump of *Quercus* sp., 24 September 2011, leg. & det. M. Lacheva (SOA 60 00338).

*Note.* This second find confirms the occurrence of *Pluteus aurantiorugosus* in Bulgaria. Previously the species was reported only once from Mt Belasitsa (ASSYOV *et al.,* 2006).

*Pluteus petasatus* (Fr.) Gillet

Tundzha Hilly Country: on fallen stump, in community of *Carpinus orientalis* Miller and *Quercus* sp., above the village of Kostur, Svilengrad distr., 11 September 2005, leg. M. Lacheva, det. G. Stoichev (SOA 60 00354).

Thracian Lowland: on rotting bales of straw, above the village of Zhitnitsa, Plovdiv distr., 30 September 2011, leg. & det. M. Lacheva (SOA 60 00359).

*Note.* The species was reported from Black Sea Coast (KUTHAN & KOTLABA, 1989), Eastern Rhodopes (HINKOVA *et al.,* 1979) and Tundzha Hilly Country (STOICHEV & DIMCHEVA, 1987).

Bolbitiaceae

*Conocybe pubescens* (Gillet) Kühner

Rila Mts: on dung of horse, above the village of Krastevich, Strelcha distr., 27 June 2004, leg. & det. G. Stoichev (SOA 60 00340).

*Note.* The species was reported from Pirin Mts (DIMCHEVA & STOICHEV, 1987) and Western Rhodopi Mts (HINKOVA *et al.,* 1979). It is reported for the first time from Rila Mts.

*Conocybe rickenii* (Jul. Schäff.) Kühner

Stara Planina Mts: on cattle excrement, in plantation of *Picea abies* Karst., above the chalet of Mazalat, 12 June 2004, leg. & det. G. Stoichev & M. Lacheva (SOA 60 00349).

*Note.* The species was reported from Znepole region, Vitosha region and Sredna Gora Mts (HINKOVA & ALEXANDROV, 1971, DENCHEV & ASYOV, 2010). It is reported for the first time from Stara Planina Mts.

*Hebeloma anthracophilum* Maire

Stara Planina Mts (central): on coals, in plantation of *Picea abies* Karst., above the chalet of Vejen, 23 June 2011, leg. & det. M. Lacheva (SOA 60 00356).

*Note.* The species was reported from the Rhodopes (STOICHEV & GYOSHEVA, 2005). From Rila Mts it has been previously known only in the work of LACHEVA (2012b). The species is reported for the first time from Stara Planina Mts.

*Hebeloma fusipes* Maire

The Rhodopes (eastern): in oak forest, 16 November 2003, leg. M. Lacheva, det. G. Stoichev (SOA 60 00351).

*Note.* The species was reported from Black Sea Coast (KUTHAN & KOTLABA, 1989), and Eastern Rhodopes (HINKOVA *et al.,* 1979).

*Naucoria alnetorum* (Maire) Kühner & Romagn.

The Rhodopes (eastern): on soil among mosses under *Alnus glutinosa* (L.) Gaertner, above Strazhets village, 23 September 2010, leg. & det. M. Lacheva (SOA 60 00353).

*Note.* The species is known from Vitosha region (STOICHEV & GYOSHEVA, 2005) and from the Rhodopes (LACHEVA, 2012b).

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Cortinariaceae

*Cortinarius bivelus* (Fr. : Fr.) Fr.

Sredna Gora Mts: in beech forest, above the village Karavelovo, Plovdiv distr., 22

September 2011, leg. & det. M. Lacheva (SOA 60 00352).

*Note.* The species was reported from the Rhodopes (HINKOVA *et al.*, 1979; STOICHEV & DRUMEVA, 1987).

*Hydnangiaceae*

*Laccaria bicolor* (Maire) P.D. Orton

Rila Mts: on soil, in the forest of *Alnus glutinosa* (L.) Gaertner and *Pinus sylvestris* L., Chavdar locality, above Belovo town, 6 September 2010, leg. & det. M. Lacheva (SOA 60 00343).

*Note. Laccaria bicolor* has been previously reported from Bulgaria (KUTHAN & KOTLABA, 1981; ASSYOV *et al.*, 2010).

*Laccaria tortilis* (Bolton) Cooke

The Rhodopes (eastern): above the villages of Strajets and Gorny Yurutsy, Krumovgrad distr., under mixed forest of *Fagus sylvatica* L., *Quercus delechampii* Ten. s. str., *Pinus sylvestris* L. and *Alnus glutinosa* (L.) Gaertner, 23 September 2008, leg. & det. M. Lacheva (SOA 60 00341).

*Note.* The species was reported from Znepole region (GYOSHEVA, 1994), Valley of river Strouma (ASSYOV *et al.*, 2012) and Western Rhodopes (STOYCHEV, 1979). The species is reported for the first time from Eastern Rhodopes.

*Strophariaceae*

*Hymenogaster buliardii* Vitt.

Sredna Gora Mts: in culture of *Pinus sylvestris* L. and *Betula pendula* Roth., above the village of Karavelovo, Plovdiv distr., on the ground, under *Betula pendula* Roth., 26 October 2011, leg. & det. M. Lacheva (SOA 60 360).

*Note.* This second find confirms the occurrence of *Hymenogaster buliardii* in Bulgaria. Previously the species was reported only once from Sredna Gora Mts (LACHEVA, 2011b).

*Hymenogaster hessei* Soehner

Stara Planina Mts (eastern): Natural Park „Sinite Kamani", in the Ablanovo locality, in the deciduous forest of *Fagus sylvatica* L. and *Quercus* sp., on the ground, under *Fagus sylvatica* L., 17 September 2010, leg. & det. M. Lacheva (SOA 60 361).

*Note.* This second find confirms the occurrence of *Hymenogaster hessei* in Bulgaria. Previously the species was reported only once from Central Stara Planina Mts (LACHEVA, 2011b).

*Stropharia luteonitens* (Fr. : Fr.) Quél.

Central Rhodopi Mts: On soil among grasses and mosses, above the villages of Mugla, Smolyan distr., 13 Sept 2005, leg. M. Lacheva, det. G. Stoichev (SOA 60 00362).

This second find confirms the occurrence of *Stropharia luteonitens* in Bulgaria. Previously the species was reported only once Central Rhodopi Mts (GYOSHEVA & ANDREEVA, 2000).

Tricholomataceae

*Hygrophorus piceae* Kühner

The Rhodopes (western): the village of Ravnogor, Pazardzhik distr., on the ground, under *Picea abies* Karst., 14 October, 2004, leg. & det. G. Stoichev & M. Lacheva (SOA 60 00365).

*Note.* This second find confirms the occurrence of *Hygrophorus piceae* in Bulgaria. Previously the species was reported only once from Rila Mts (GYOSHEVA, 1997).

*Melanoleuca brevipes* (Bull.: Fr.) Pat.

The Rhodopes (central): In oak forest, above the village of Hrabrino, Plovdiv distr., 18 September 2005, leg. M. Lacheva, det. G. Stoichev (SOA 60 00363); in oak forest, above the village of Brestnik, Plovdiv distr., 23 October 2010, leg. & det. M. Lacheva (SOA 60 00364). The species is reported for the first time from Central Rhodopes.

*Note.* The species was reported from Eastern Rhodopes (HINKOVA *et al.*, 1979) and Thracian Lowland (STOICHEV & NAIDENOV, 1987).

*Tricholoma hordum* (Fr.: Fr.) Quél.

The Rhodopes (western): on soil, in community of *Picea abies* L. and *Pinus sylvestris* L., Chairski ezera locality, above the village of Trigrad, 11 July 2004, leg. M. Lacheva, det. G. Stoichev (SOA 60 00366).

*Note.* The species was reported only once from Western Rhodopes (STOICHEV & GYOSHEVA, 2005).

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# *A Study of Larger Fungi of the Boraka Managed Reserve, Central Rhodopes Mts.*

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**Abstract.** Data on the species composition, distribution and the ecological-trophic structure of larger fungi in Boraka Managed Reserve, Central Rhodopes Mts. are published for the first time. Six species includes in the Red List of fungi in Bulgaria: *Auriscalpium vulgare* Gray, *Hymenogaster luteus* Vittad., *Phallus hadriani* Vent:Pers., *Rozites caperatus* (Pers.:Fr.) P. Karst., *Russula solaris* Ferd. & Winge. and *Sarcosphaera coronaria* (Jacq.) Boud. The aim of the paper is to enrich the information about fungal diversity in the reserves of Rhodopes, as well as in a natural forest of *Pinus nigra* L. in Bulgaria.

**Key words:** Boraka Managed Reserve, conservation value, ecological-trophic structure, fungal diversity, larger fungi, rare taxa, Bulgaria.

## Introduction

Boraka Managed Reserve is situated in the foot of Central-Rhodopes hill Mechkovets, in the land of village of Sarnitsa, Mineralni Bani Municipality, Haskovo distr., close to the Haskovo town. Its altitude ranges up to 850 m a.s.l. The Reserve covers 11.10 ha of the State forest fund. It was founded with the sole purpose of protecting the natural habitat for old, natural, self-reproducing black pine forest and to maintain representative ecosystem for *Pinus nigra* Arn. (Decree 163/22.04.1966 of the Ministry of Agriculture and Forestry). Subsequently, its category was changed to a Managed Reserve (Regulation RD 384/15.10.1999 of the Ministry of Environment and Waters) under the Protected Areas Act (Darzhaven Vestnik 135/1998). The reserve is managed and guarded by RIOSV-Haskovo.

The present work reports on the larger fungi diversity in the Boraka Managed Reserve. This is the first mycological study conducted on the territory of this interesting reserve.

Data on larger fungi in the Boraka Managed Reserve provide information on the species diversity of fungi in the *Pinus nigra* communities in Bulgaria. These communities have a rather limited distribution in the country (MESHINEV *et al.*, 1998), while mycologically they are absolutely unstudied. Any fungus species have been so far published exclusively in relation to cultures, and not in relation to the natural black pine communities in Bulgaria (HINKOVA & DROUMEVA, 1978; HINKOVA *et al.*, 1979; STOICHEV & DIMCHEVA, 1987; GYOSHEVA, 1991, 2000; DIMCHEVA *et al.*, 1992; LACHEVA & GYOSHEVA, 2013, etc.). Information of fungi in the *Pinus nigra* communities, available in the work by GYOSHEVA & ANDREEVA (2000) about fungi of Momchilovski Dol Reserve (Central Rhodopes).

The purpose of the present study was to provide an upgraded inventory of the species composition, distribution and the conservation status of the larger fungi in the reserve. The collected scientific information will be used for analysis and planning activities on the conservation and management of the biodiversity in a secure territory in the future.

**Material and Methods**

The mycological studies were carried out within the 2010-2012 period on the territory of the Boraka Managed Reserve.

The soil is humus-carbonate, sandy-clay, on a base of calcareous rock. The forest litter is composed of needles, cones, bark, dead fragments of grassy plants, etc. The composition of the tree stand is 10 Pn – *Pinus nigra* Arn. Single specimens of *Picea abies* Karst., *Fagus sylvatica* L., and *Quercus* sp. also involved in some places.

Field studies were carried out by the transect method. The specimens were collected from April to November, during each vegetation season. Were collected fungal samples and making observations on the ecology of taxa. The fungal species were determined according to (MOSER, 1963, 1967, 1978, 1983; PHILLIPS, 1981; HANSEN & KNUDSEN, 1992; RYVARDEN & GILBERTSON, 1993, 1994) and the ecological-trophic structure were carried out after direct observations and based on literature data (KALAMEES, 1979; ARNOLDS, 1981; GYOSHEVA & VASILEV, 1994).

Classification of fungal taxa followed KIRK *et al.* (2008).

The author's names of the fungal taxa are given by KIRK & ANSELL (2004) and the plants taxa by BRUMMIT & POWELL (1992).

The ecological-trophic groups are identified by the abbreviated latin names of substrates (ARNOLDS, 1981).

The conservation status is indicated according to the *Red List* of fungi in Bulgaria (GYOSHEVA *et al.,* 2006). The fungus species with conservation value are designed in the list with CV. Designations of ecological-trophic groups: Ad – needle-debris saprotrophs, St – litter saprotrophs, Hu – humus saprotrophs, Br – moss saprotrophes, Mr – mycorrhizal fungi, LeS – wood saprotrophs, LeP – wood parasites.

The characteristics of the reserve were provided by the Haskovo State Forestry.

**Results and Discussion**

In the result of mycological investigations 103 taxa were identified, related to 2 classes (Pezizomycetes and Agaricomycetes), 7 orders, 26 families and 47 genera. The prevailing number of taxa related to the class Agaricomycetes: 6 orders, 25 families and 46 genera and 97 species. Order Agaricales dominated by the number of species (67). Most species abundant were the families: Pezizaceae (6), Agaricaceae and Russulaceae (16 each), Tricholomataceae (21). The greatest number of larger fungi was identified from the genera *Amanita* (3), *Clitocybe* (6), *Cystoderma* (4), *Lactarius* (7), *Russula* (9), *Tricholoma* (4).

All registered fungal species are reported for the first time for the Boraka Managed Reserve. One species are new for the Rhodopes - *Hymenogaster hessei*. Six larger fungi are rare and threatened in Bulgaria and Europe: *Auriscalpium vulgare*, *Hymenogaster luteus*, *Phallus hadriani*, *Rozites caperatus*, *Russula solaris,* and *Sarcosphaera coronaria* (DAHLBERG & CRONEBORG, 2003; GYOSHEVA *et al.,* 2006; DENCHEV & PETROVA, 2011).

Of all species registered in the Boraka Managed Reserve, the following species are characteristic of the pine forests: *Auriscalpium vulgare, Mycena epipterigia, Lactarius deliciosus, L. rufus, L. vellereus, Tricholoma equestre, T. imbricatum, Russula rosea, R. roseipes*.

In the two years of study, the greatest diversity of species and high production of fruiting bodies of fungi were recorded in late summer – in August and September. Dominant in the number of fruiting bodies were identified following: *Boletus edulis*, *Cantharellus cibarius*, *Gomphidius glutinosus, Marasmiellus ramealis* and species of the genera *Lactarius* and *Russula* during August and *Agaricus sylvaticus, Amanita phalloides*, *A. rubescens*, *Lepista nuda*, *Marasmiellus ramealis*, and species of the genera *Lactarius* and *Russula* during September.

The reserve was established fungi from 7 ecological-trophic groups: needle-debris saprotrophs (4), litter saprotrophs (12), humus saprotrophs (53), moss saprotrophs (2), mycorrhizal fungi (18), wood saprotrophs (8) and wood parasites (1). Of fungi belonging to trophic groups and food substrates which are developed are given in Table 1. Despite a short period of study in the pine forests of the reserve was reported right fungal diversity of species of fungi of the most important in forestry terms ecological-trophic groups, namely: mycorrhizal fungi, wood saprotrophs and litter, wood saprotrophs and humus saprotrophs.

Within the reserve were established nine species valuable edible mushrooms - *Agaricus sylvaticus, Boletus edulis*, *Cantharellus cibarius*, *Gomphidius glutinosus, Lactarius piperatus, Lepista nuda, Lycoperdon perlatum, Russula xerampelina* and *R. virescens.* Among the edible mushrooms, most abundant fructification - *Agaricus sylvaticus, Boletus edulis*, *Cantharellus cibarius*, *Lactarius piperatus, Lycoperdon perlatum,* and *Russula xerampelina.*

List of all taxa in a systematic order is presented on Table 1.

**Table 1.** Species composition and ecological-trophic structure of larger fungi in Boraka Managed Reserve

| Taxa | Substrate | Ecological -trophic groups |
|---|---|---|
| **Pezizomycetes** | | |
| **Pezizales** | | |
| **Pezizaceae** | | |
| *Otidea umbrina* (Pers.) Bres. | on soil | Hu |
| *Discina perlata* (Fr.) Fr. | on soil | Hu |
| *Helvella lacunose* Fr. ex Afzelius | on soil | Hu |
| *Peziza repanda* Pers. | on soil | Hu |
| CV *Sarcosphaera coronaria* (Jacq.) Boud. | on soil | Hu |
| *Verpa digitaliformis* Pers. | on soil | Hu |
| **Agaricomycetes** | | |
| **Agaricales** | | |
| **Agaricaceae** | | |
| *Agaricus xanthodermus* Genev. | on soil | Hu |
| *Agaricus sylvaticus* Schaeff. | on soil | Hu |
| *Chlorophyllum rachodes* (Vittad.) Velinga | on soil | Hu |
| *Cystoderma amianthinum* (Scop. : Fr.) Fayod | on soil among mosses | Br |
| *Cystoderma carcharias* (Pers.) Fayod | on the forest litter | St |
| *Cystoderma cinnabarinum* (Alb. & Schwein) Fayod | on soil among mosses | Br |
| *Cystoderma granulosum* (Batsch : Fr.) Fayod | on the forest litter | St |
| *Coprinellus micaceus* (Bull. : Fr.) Vilgalys | on soil | Hu |
| *Lepiota erminea* (Fr. : Fr.) P. Kumm. | on soil | Hu |
| *Lepiota clypeolaria* (Bull. : Fr.) P. Kumm. | on soil | Hu |
| *Lepiota magnispora* Murrill | on soil | Hu |
| *Leucoagaricus leucothites* (Vittad.) Wasser | on soil | Hu |
| *Lycoperdon perlatum* (Pers.) Pers. | on the forest litter | St |
| *Lycoperdon echinatum* (Pers.) Pers. | on the forest litter | St |
| *Macrolepiota excoriata* (Schaeff.:Fr.) Wasser | on soil | Hu |
| *Macrolepiota procera* (Scop. : Fr.) Singer | on soil | Hu |
| **Amanitaceae** | | |

| | | |
|---|---|---|
| *Amanita pantherina* (DC. : Fr.) Krombh | on soil | Hu |
| *Amanita phalloides* (Vaill. : Fr.) Link | on soil | Hu |
| *Amanita rubescens* Pers. : Fr. | on soil | Hu |
| **Cortinariaceae** | | |
| *Cortinarius torvus* (Fr. : Fr.) Fr. | on soil | Hu |
| CV *Rozites caperatus* (Pers. : Fr.) P. Karst. | on soil | Hu |
| **Bolbitiaceae** | | |
| *Hebeloma crustuliniforme* (Bull. : Fr.) Quél. | on soil | Mr |
| *H. sinapizans* (Paulet : Fr.) Gilett. | on soil | Mr |
| ***Hydnangiaceae*** | | |
| *Laccaria amethystine* Cooke | of dead wood | LeS |
| *L. laccata* (Scop. : Fr.) Cooke | on soil | Hu |
| **Inocybaceae** | | |
| *Inocybe asterospora* Quél. | on soil | Hu |
| *Inocybe dulcamara* (Alb. & Schwein. ex Pers.) P. Kumm. | on soil | Hu |
| **Marasmiaceae** | | |
| *Marasmiellus ramealis* (Bull. : Fr.) Singer | on fallen needles | Ad |
| *Marasmius oreades* (Bolton : Fr.) Fr. | on soil | Hu |
| *Marasmius rotula* (Scop. : Fr.) Fr. | on fallen needles | Ad |
| *Mycetinis scorodonius* (Fr. : Fr.) A.W. Wilson | on fallen needles | Ad |
| *Setulipes androsaceus* (L. : Fr.) Antonín | on fallen needles | Ad |
| **Mycenaceae** | | |
| *Mycena epipterygia* (Scop.: Fr.) Gray | on dead wood | LeS |
| *Mycena galericulata* (Scop.: Fr.) Gray | on dead wood | LeS |
| **Physalacriaceae** | | |
| *Xerulla radicata* (Relhan : Fr.) Dörfelt | on soil | Hu |
| **Pleurotaceae** | | |
| *Pleurotus ostreatus* (Jacq. : Fr.) P. Kumm. | on dead wood | LeS |
| **Polyporales** | | |
| *Polyporus squamosus* (Huds. : Fr.) Fr. | on dead wood | LeP |
| **Psathyrellaceae** | | |
| *Psathyrella candolleana* (Fr. : Fr.) Maire | on buried wood | LeS |
| **Strophariaceae** | | |
| *Agrocybe molesta* (Lasch) Singer | on soil | Hu |
| *Agrocybe praecox* (Pers. : Fr.) Fayod | on soil | Hu |
| * *Hymenogaster hessei* Soehner | in soil | Mr |
| CV *Hymenogaster luteus* Vittad. | in soil | Mr |
| *Hypholoma fasciculare* (Huds. : Fr.) P. Kumm. | on dead wood | LeS |
| *Pholiota highlandensis* (Peck) Quadr. & Lunghini | on dead wood | LeS |
| *Stropharia aeroginosa* (Curtis : Fr.) Quél. | on buried wood and in the litter | St |
| *Stropharia coronilla* (Bull. : Fr.) Fr. | on buried wood and in the litter | St |
| **Tricholomaceae** | | |
| *Calocybe gambosa* (Fr. : Fr.) Donk | on soil | Hu |
| *Clitocybe phyllophila* (Pers. : Fr.) P. Kumm. | on soil | Hu |
| *Clitocybe geotropa* (Bull. ex DC.) Quél. | on soil | Hu |
| *Clitocybe gibba* (Pers. : Fr.) P. Kumm. | on soil | Hu |
| *Clitocybe metachroa* (Fr. : Fr.) P. Kumm. | on soil | Hu |
| *Clitocybe nebularis* (Batsch : Fr.) P. Kumm. | on soil | Hu |
| *Clitocybe odora* (Bull. : Fr.) P. Kumm. | on soil | Hu |
| *Gymnopus acervata* (Fr. : Fr.) Murrill | on the forest litter | St |

| | | |
|---|---|---|
| *Gymnopus dryophilus* (Bull. : Fr) Murrill | on the forest litter | St |
| *Hygrocybe conica* (Schaeff. : Fr.) P. Kumm. | on soil | Hu |
| *Hygrophorus* agathosmus (Fr.) Fr. | on soil | Hu |
| *Hygrophorus camarophyllus* (Alb. & Schwein. : Fr.) Dumée, Grandjean & Maire | on soil | Hu |
| *Lepista flaccida* (Sowerby : Fr.) Pat. | on the forest litter | St |
| *Lepista nuda* (Bull. : Fr.) Cooke | on soil | Hu |
| *Melanoleuca melaleuca* (Pers. : Fr.) Murrill | on soil | Hu |
| *Melanoleuca grammopodia* (Bull. : Fr.) Pat. | on soil | Hu |
| *Rhodocollybia butiracea* (Bull. : Fr.) Lennox | on the forest litter | St |
| *Tricholoma equestre* (L. : Fr.) P. Kumm. | on soil | Hu |
| *Tricholoma imbricatum* (Fr. : Fr.) P. Kumm. | on soil | Hu |
| *Tricholoma portentosum* (Fr. : Fr.) Quél. | on soil | Hu |
| *Tricholoma terreum* (Schaeff. : Fr.) P. Kumm. | on soil | Hu |
| **Boletales** | | |
| **Boletaceae** | | |
| *Boletus edulis* Bull. : Fr. | on soil | Hu |
| *Boletus luridus* Schaeff. : Fr. | on soil | Hu |
| **Diplocystidiaceae** | | |
| *Astraeus hygrometricus* (Pers. : Pers.) Morgan | on the forest litter | St |
| **Gomphidiaceae** | | |
| *Gomphidius glutinosus* (Schaeff. : Fr.) Fr. | on soil | Hu |
| **Gyroporaceae** | | |
| *Leccinum scabrum* (Bull. : Fr.) Gray | on soil | Hu |
| **Paxillaceae** | | |
| *Paxillus involutus* (Batsch : Fr.) Fr. | on dead wood | LeS |
| **Suillaceae** | | |
| *Suillus bovinus* (L. : Fr.) Roussel | on soil | Hu |
| *Suillus granulatus* (L. : Fr.) Roussel | on soil | Hu |
| **Cantharellales** | | |
| **Clavulinaceae** | | |
| *Clavulina cinerea* (Bull. : Fr.) J. Schröt. | on soil among mosses | Br |
| *Clavulina coralloides* (Bull. : Fr.) J. Schröt. | on soil among mosses | Br |
| *Cantharellus cibarius* Fr. : Fr. | on soil | Mr |
| **Phallales** | | |
| **Phallaceae** | | |
| CV *Phallus hadriani* Vent. : Pers. | on soil | Hu |
| **Russulales** | | |
| **Auriscalpiaceae** | | |
| CV *Auriscalpium vulgare* Gray | on pine cones in the forest litter | St |
| **Russulaceae** | | |
| *Lactarius controversus* (Pers. : Fr.) Fr. | on soil | Mr |
| *Lactarius deliciosus* (L. : Fr.) Gray | on soil | Mr |
| *Lactarius piperatus* (L. : Fr.) Pers. | on soil | Mr |
| *Lactarius rufus* (Scop. : Fr) Fr. | on soil | Mr |
| *Lactarius semisanguifluus* R. Heim & Leclair | on soil | Mr |
| *Lactarius torminosus* (Schaeff. : Fr.) Pers. | on soil | Mr |
| *Lactarius vellereus* (Fr. : Fr.) Fr. | on soil | Mr |
| *Russula foetens* (Pers. : Fr) Fr. | on soil | Mr |
| *Russula integra* L. : Fr. | on soil | Mr |
| *Russula lilacea* Quél. | on soil | Mr |
| *Russula olivacea* (Schaeff.) Fr. | on soil | Mr |

| | | |
|---|---|---|
| CV *Russula solaris* Ferd. & Winge. | on soil | Mr |
| *Russula rosea* Pers. | | |
| *Russula roseipes* Secr ex Bres. | | |
| *Russula virescens* (Schaeff.) Fr. | on soil | Mr |
| *Russula xerampelina* (Schaeff.) Fr. | on soil | Mr |
| **Thelephorales** | | |
| **Thelephoraceae** | | |
| *Thelephora terrestris* Ehrh. : Fr. | on soil | Hu |

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# *Preliminary Results for the Species Composition and Dynamics of Mosquito Family (Culicidae) in the City of Plovdiv*

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**Abstract.** The mosquitoes from the Culicidae family (Insecta: Diptera) up to this moment comprise more than 3600 species in the world and are found almost in all geographical regions. They are some of the most important insects in terms of epidemiology. They are carriers of more than 50 viral and bacterial infections and parasitoses (malaria, yellow fever, viral encephalitis, dengue fever, tularemia, philariasis etc.). The latest studies of the species composition of the mosquito fauna in the region of Plovdiv date back to 1997. On the basis of research data and own preliminary studies, the entire available information has been compiled and summarized about the species composition, the ecology and the importance of mosquitoes as vectors on the territory of the city of Plovdiv, as well as in the region. The preliminary study was conducted in consecutive years (2011 and 2012) from May to October. In the period from May – December 2011 and 2012, for each of the months, samples were taken, using the method of "hand collection". For the territory of the city of Plovdiv 8 biotopes were identified. The purpose of the study is to investigate the species composition, the morphology and the biology of the mosquitoess from the Culicidae family in the urbanized biocenoses (in the city of Plovdiv). The article provides information about the species composition and the dynamics of the mosquitoes in the city of Plovdiv, about 13 species from 6 genera which have been identified so far, and about the dynamics broken down by months of the period under study.

**Key words:** mosquitoes, Diptera, Culicidae, Plovdiv, Bulgaria.

## Introduction

The mosquitoes (Diptera: Culicidae) are widespread bloodsucking insects. They inhabit almost all regions of the world, except the areas covered with permanent ice and comprise more than 3600 species. In our country there 46 known species from 9 genera (MIKOV, 2011).

The mosquitoes are specific carriers of over 50 transmissible infections and parasitoses, such as malaria, dengue fever, yellow fever, philariasis, tularemia, viral encephalitis, etc. (PETROV, 1984).

In their development the mosquitoes pass through a water environment, in which they develop eggs, larvae and pupae and an air environment – as adult specimen. In its lifespan (on average up to 3 months, rarely 6 months) one female mosquito lays from 80 to 300 eggs. The places which the mosquitoes prefer are permanent or temporary water basins – swamps, river spills, puddles and tree holes etc., places with stagnant water. They are not found only in the fast-flowing waters of big rivers, lakes and the seas.

The male specimen feed on plant juices, while the female are bloodsucking. The blood is necessary for the maturation of the eggs. The female mosquito actively seeks hosts (animals or people) and after filling up with blood finds a peaceful and quiet place where it stays until the maturation of the eggs is complete. They are most active during the evening and early in the morning and the optimal temperature for them is 25–26°C. The distances which the mosquitoes cover while flying in their natural conditions differ. Usually they cover a distance of 3 to 5 km, and some of them, carried by the wind, may fly over a distances up to 80 km.

More than half of the world's human population lives with the risk of infection from mosquitoes, which carry agents of diseases, such as malaria, dengue fever, chikungunya, West Nile fever, Japanese encephalitis or lymph philariasis.

The estimates made by the World Health Organization (WHO) show that 247 million people have become sick in 2006 and around one million people have died (WHO, 1992) from diseases carried by mosquitoes. Although around three quarters of all mosquito species are found in the tropical and subtropical regions, the mosquitoes are not a problem only for those regions. The mosquitoes have a significant impact and can transmit pathogens to people in the temperate geographical latitudes, for example, the West Nile Fever - a viral epidemic in the USA, or high temperature fever, caused by chikungunya in Italy in 2007 (DEPOORTERE *et al*., 2008).

The mosquitoes also cause significant economic damage to agriculture. There are known case in which animals, as a result of large amount of bites, reduce their milk yields with 15–30% or die from intoxications. It has been determined that labor productivity of forest workers during the summer season, when the mosquitoes are most active, may decrease with 30—40% (TURELL *et al*., 2001, MCABEE *et al*., 2008, BECKER, 2003; BECKER *et al.,* 2003).

In the last 100 years the climate in Europe has become warmer on average with 0.8 $^{o}$C as a result of human activity and the greenhouse effect (GITHEKO *et al.,* 2000).

The increase of the average temperature and the warming of the climate would lead to a widening of the ranges, upsurge of the population wave of the carrier to the north. The period which is favorable for the development of mosquitoes would become longer. The epidemic potential of non-endemic or slightly endemic area would increase and in regions where malaria has been eradicated, there would be an increase of the risk of new appearance of the disease (MARTENS *et al.,* 1995; 1997; JETTEN *et al*., 1996; MCCARTHY *et al.,* 2001).

Health problems of importance mosquitoes and measures to prevent and combat it is designed in an article on the problem of PETROV & BILEVA (2010). The interest in mosquitoes, their ecology and the use of appropriate methods for taking samples dates back from the beginning of the nineteenth century with the discovery that mosquitoes can act as carriers of diseases which affect humans and domestic animals.

According to SOUTHWOOD & HENDERSON (2000) perhaps the most important factor in choosing the appropriate methodology for taking samples is careful formulation of the specific ecological problems, which the investigator wishes to take into account. This will determine the time and length of the period for taking samples, the specific program and methodology, whether the study would be wide-reaching, attempting to describe a large scale distribution and abundance of the species composition or whether it would focus on a particular species or population or on the intensive study of a particular population, usually with objective of determining absolute or relative size of the population and of the factors, which regulate its numbers.

The malaria representatives of the mosquito fauna which are found in the region of Plovdiv are: *Anopheles superpictus* Grassi; *Anopheles maculipennis* Mg.; *Anopheles claviger* (= *A. bifurcatits*) Lav.; *Anopheles (myzirrhynchus) pseadopictus* ( = *A. hyrcanus*)

Grassi (MARKOV, 1929). HRISTOVA *et al*. (1969) conducted a study of the species composition of the mosquito fauna in Plovdiv in 1967-1968. The study confirms the studies done so far, summarizing that the most widespread mosquito species in the city of Plovdiv are from the genus *Anopheles* and genus *Culex*.

The last study of the species composition of bloodsucking mosquitoes in the city of Plovdiv was conducted in 1996-1997 (HRISTOVA *et al*., 2000) and the results encompass the following species:

Subfamily Anophelinae

Genus *Anopheles* Meigen

- *Anopheles (Anopheles) maculipennis* Meigen, 1818

Subfamily Culicinae

Genus *Culex* Linnaeus

- *Culex (Culex) pipiens* Linnaeus, 1758
- *Culex pipiens pipiens* Linnaeus, 1758
- *Culex pipiens molestus* Forsk, 1775

Genus *Culiseta* Felt

- *Culiseta (Culiseta) annulata* Schrank, 1776
- *Culiseta (Culiseta) alaskaensis* Ludlow
- *Culiseta (Allotheobaldia) longiareolata* Maqcuart, 1838

This article has been prepared on the basis of the preliminary results from a two-year (2011-2013) study, which aims to investigate the species composition, the morphology, the biology, the ecology of the mosquito population from Culicidae family in urbanized areas (in the city of Plovdiv).

**Materials and Methods**

*Method for collection of imaginal stage specimens.*

In the beginning of the study 4 Malaise traps were made and mounted on the work sites in the city of Plovdiv. To collect and store the trapped insects and specifically the mosquitoes, we used 75% ethyl alcohol ($C_2H_5OH$) for laboratory samples.

The Malaise traps are widely used for collecting flying insects. They operate and provide representative information for the flying insects throughout the season. They are convenient for ecological, faunistic and monitoring studies. Their operation is simple and consists of replacing the container with preserving liquid and providing security for the trap (MALAISE, 1934).

In the beginning of the study specific biotopes were identified to be studied, both in the city of Plovdiv and in the protected area "The River Mouth of the Veleka River" and the Ropotamo river.

In April-May 2011 over 25 biotopes and work sites were surveyed for the upcoming terrain and field practice and from these 4 biotopes and 4 work sites were selected. For the territory of the city of Plovdiv 8 biotopes were identified, of which 4 for positioning of Malaise traps for the entire season from May to October, on one of those we used a mosquito traps BG-Sentinel in two versions - with the addition of $CO_2$ and with placing an insecticide lamp, and 4 biotopes for mosquito larvae, of various types.

The Malaise traps were processed every two weeks for 5 months, two collections of 4 work sites provide a collection of 72 samples with imaginal adult stage specimen, for two years.

On one of the work sites we used a mosquito trap BG-Sentinel. BG-Sentinel ™ in two versions - with the addition of $CO_2$ and with the addition of an insecticide lamp. The catch is in a double bag and is located above a fan, the specimen are not destroyed and are preserved, and can easily be identified and used to identify the disease agents.

Hand collection of adult mosquitoes using the test-tube method for collection of mosquitoes (WHO, 1992).

In the period from May – November 2011 and 2012, for each of the months, samples were taken, using the method of "hand collection". This method was applied at 7 sites, differentiated in three groups:

1. Residential buildings
2. Public buildings
3. Agricultural buildings

*Methods of collection of larvae and pupae.*

Method of submerging of the "white tray" (20 x 15 x 3 cm) for determining of the density at the larval stage of development and method of submerging of hydrobiological net mounted on a circular frame (15 cm diameter) and attached to a wooden or aluminium handle (1.50 m).

Collection of larvae and pupae by means of a trap of automobile tires (SCOTT & CRANS, 2003) and various containers as traps for larvae.

The larval stage specimen were collected from 4 biotopes - biotope 1 is natural and temporary, biotope 2 – artificial and temporary, biotope 3 – artificial and permanent, biotope 3 artificial and temporary.

The characteristic features of each biotope (topography, approximate dimensions, water movement, water purity, water temperature, pH (acidity), hardness, temporary or permanent, natural or artificial) are given in Table 1.

**Table 1.** Characteristic features of the biotopes for collection of larvae.

| Biotopes | Year | Area in sq. m | Water movement | Water purity | Number of checks | pH | Water temperature | Hardness (in German degrees) | Plant life |
|---|---|---|---|---|---|---|---|---|---|
| Biotope No. 1 spills of the Maritsa river near the Food Technologies University | 2011<br>2012 | 1800 | Slow current | Low contamination | 10/3*<br>6/2* | 7,5 | 17 – 30 °C<br>18-32 °C | 14,00 | *Typha* sp. * grassy |
| Biotope No. 2 Aquaduct, Bunardzhika hill | 2011<br>2012 | 70 | Stagnant | Not contaminated | 10/3<br>6/1 | 6,8 | 15 - 34 °C<br>15 - 35 °C | 13,72 | *Typha* sp. |
| Biotope No. 3 fountain in the Tsar Simeon garden | 2011<br>2012 | 650 | Stagnant | Not contaminated | 10/0<br>6/0 | 7,4 | 19 – 31 °C<br>19 – 33 °C | 14,00 | Algae |
| Biotope 4 – automobile tires, residential area "Trakiya" | 2011<br>2012 | 5 | Stagnant | Contaminated | 10/5<br>6/3 | 7,9 | 18 – 35 °C<br>18 – 37 °C | 14,56 | Decaying leaves |

*The first digit shows the total number of the checks, and the second – the number of the checks where mosquito larvae were found.

The species determination is made by morphological characteristics of imago and larvae. The classification of the species is according to SNOW & RAMSDALE (2003).

Works by DRENSKI (1938), BOZHKOV (1991) and HARBAH (2001) are used for the species identification. Species are confirmed by Dr Ognyan Mikov (National Centre of Infectious and Parasitic Diseases - Sofia).

**Results and Discussion**

In the period from May 2011 to November 2012, 20 assemblies of imaginary forms were made from 4 workstations with malaise traps (Fig. 1). 75% of the samples were positive, 15% of the total had no reported samples due to technical or weather reasons.

Using the "hand assembly" method, 228 adult mosquitoes were collected in 14 samples from 7 sites (Fig. 2). Relevant to the research, an increased number of individuals indoors under the constitutional evolutionary factors and adverse abiotic environmental factors of the environment is established in October and November (MARTENS *et al.*, 1995; 1997; JETTEN *et al.*, 1996; MCCARTHY *et al.*, 2001). The preparation for diapause provided a large number of mosquitoes indoors.

Data from the assemblies in one of the workstations, in residential district Trakia with mosquito trap BG-Sentinel ™, is given in table 2. When using two methods, with the addition of $CO_2$ and insecticidal lamp, a greater amount of imaginary forms is established when using light as "bait".

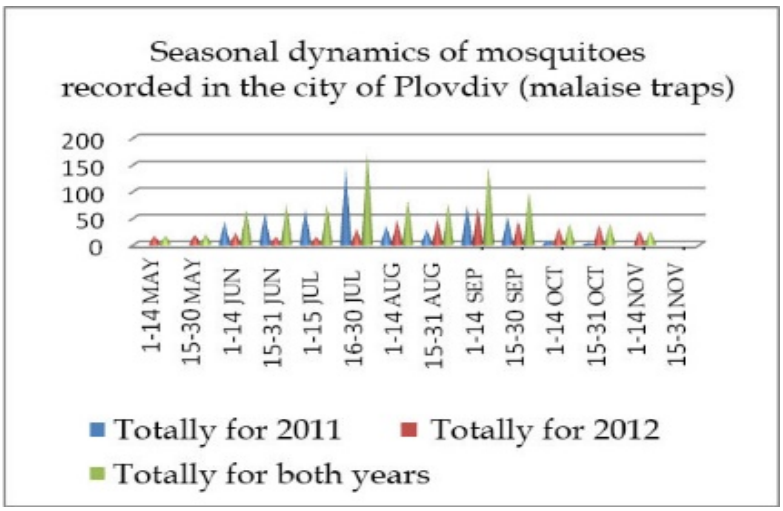


**Fig.1.** Seasonal dynamics of mosquitoes recorded in the city of Plovdiv (malaise traps) - sample distribution by numbers, workstations and weeks.

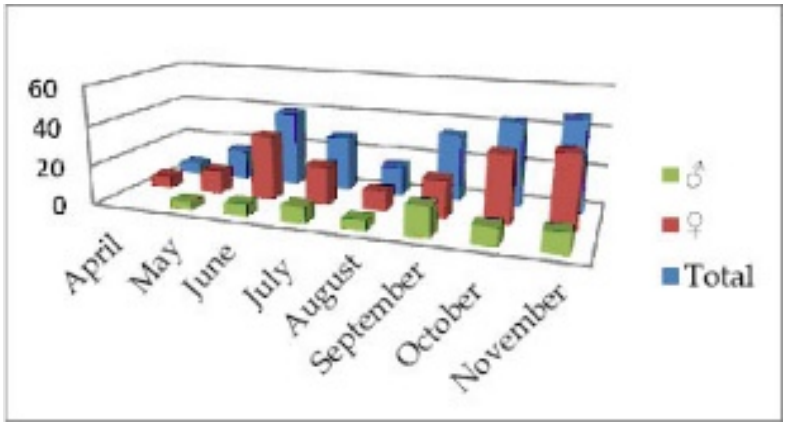


**Fig. 2.** Data on assemblies of imaginary mosquitoes using the "hand assembly" method in the period from 2001 to 2012 by number, sex and date.

**Table 2.** Data on assemblies, total and percentage of collected imaginary forms by BG-Sentinel with $CO_2$ and by BG-Sentinel with insecticidal lamp

| **Assembly by months** | **Total with BG-Sentinel with $CO_2$** | ♀ | ♂ | **%** | **Total with BG-Sentinel with insecticidal lamp** | ♀ | ♂ |
|---|---|---|---|---|---|---|---|
| May | 9 | *7* | *2* | 11,25 % | 12 | *10* | *2* |
| June | 11 | *6* | *5* | 13,75 % | 19 | *14* | *5* |
| July | 11 | *8* | *3* | 13,75 % | 25 | *17* | *8* |
| August | 12 | *9* | *3* | 15 % | 19 | *15* | *4* |
| September | 16 | *12* | *4* | 20 % | 32 | *26* | *6* |
| October | 10 | *8* | *2* | 12,5 % | 18 | *15* | *3* |
| November | 11 | *7* | *4* | 13,75% | 11 | *7* | *4* |
| **Total:** | **80** | **57** | **23** | **100%** | **136** | **104** | **32** |

Seasonal dynamics of mosquitoes recorded in the city of Plovdiv, caught with BG-Sentinel trap, with $CO_2$ and insecticidal lamp indicates that the most favourable months for the development of mosquitoes are June and July, with peaks in numbers in September and the first half of October. (Fig. 3)

1,401 mosquitoes, 853 ♀♀ and 548 ♂♂, were collected during the whole period of study from all biotopes. The collected larvae during this period are 197 and the positive samples are only 27.5%.

Seasonal dynamics of mosquitoes recorded in the city of Plovdiv indicates that the most favourable months for the development of mosquitoes are May, June, July and August, with peaks in numbers in the second half of July, September and early October (Fig. 4).

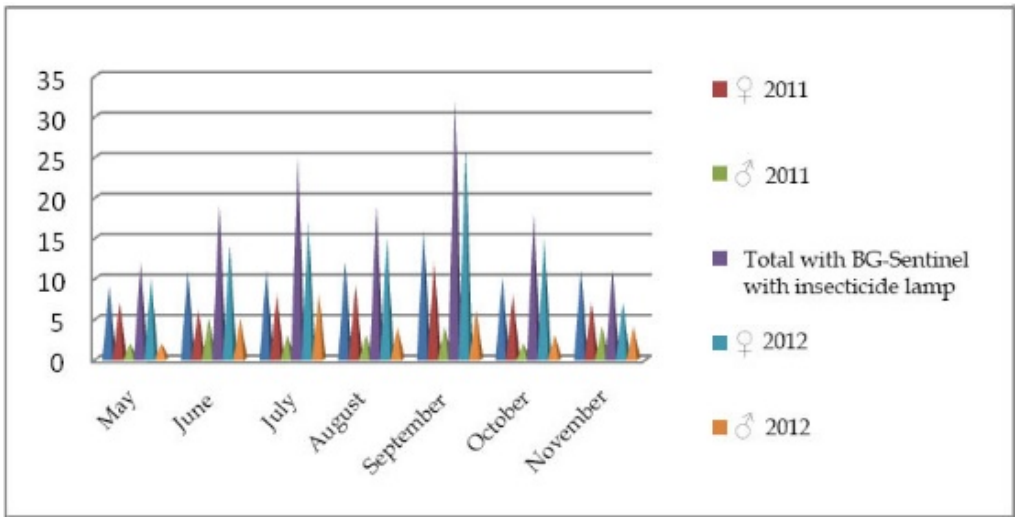


**Fig. 3.** Data on the collected adult mosquitoes with BG-Sentinel by date and sex.

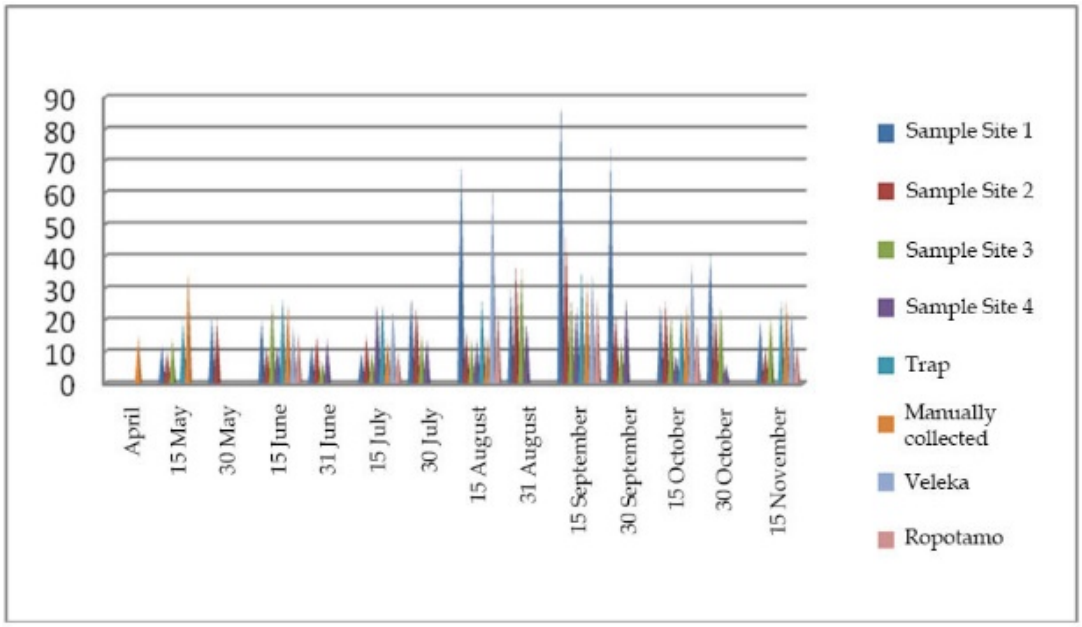


**Fig. 4.** Seasonal dynamics of mosquitoes recorded in the city of Plovdiv and in the control stations.

The amount of mosquitoes is directly dependent on the temperature and atmospheric moisture. The research gives data shift from the previous studies that the greatest occurrence of mosquitoes is recorded in the months that coincide with the second and third generation, namely July and August, while at the end of September and October their number is reduced. These results show that the favorable period for the development of mosquitoes is prolonged, leading to an expansion of the areas and population boom in the north.

Most male individuals prove that the assembly is carried out very close to the place where they proliferate (Fig. 5).

Anthropogenic influences significantly affect populations of mosquitoes in urban areas. Among the bloodsucking mosquitoes in urban areas, the study of the mosquito *Culex pipiens* (VINOGRADOVA, 1997) is of great scientific and practical importance.

During the two years of the study, some part of the collected material was defined. Until now, in the fauna of Plovdiv representatives of 6 genera, including 13 species presented in a systematic list that includes genus, species, author and year of description of the species have been identified (Table 3).

**Conclusions**

As a result of the studies, conducted bet-

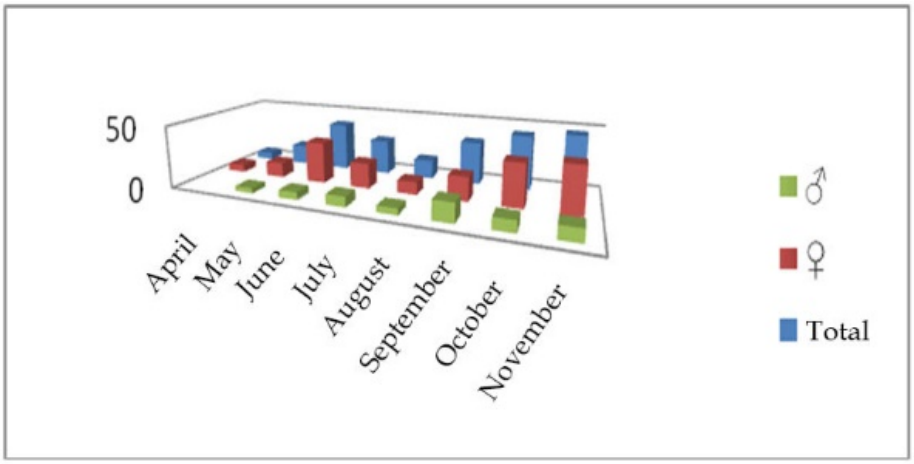


**Fig. 5.** Data on assemblies of imaginary mosquitoes using the "hand assembly" method in the period from 2001 to 2012 by number, sex and date

**Table 3.** Systematic list of the mosquito fauna (Culicidae) in the city of Plovdiv.

| № | Genus | Species | Author | Year |
|---|---|---|---|---|
| 1. | *Anopheles* | *claviger* | Meigen | 1804 |
| 2. | *Anopheles* | *hyrcanus* | Pallas | 1771 |
| 3. | *Anopheles* | *maculipennis* | Meigen | 1818 |
| 4. | *Anopheles* | *messeae* | Falleroni | 1926 |
| 5. | *Anopheles* | *superpictus* | Grassi | 1899 |
| 6. | *Aedes* | *cinereus* | Meigen | 1818 |
| 7. | *Culex* | *pipiens* | Linnaeus | 1758 |
| 8. | *Culex* | *territans* | Walker | 1856 |
| 9. | *Culiseta* | *glaphyroptera* | Schiner | 1864 |
| 10. | *Culiseta* | *longiareolata* | Macquart | 1938 |
| 11. | *Culiseta* | *morsitans* | Theobald | 1901 |
| 12. | *Ochlerotatus* | *cataphylla* | Dyar | 1916 |
| 13. | *Uranotaenia* | *unguiculata* | Edwards | 1913 |

ween 2011 and 2012, on the populations of bloodsucking mosquitoes (Culicidae) in the city of Plovdiv, data on the dynamics, species composition, biological and environmental conditions is obtained.

In the seasonal dynamics of mosquitoes recorded in the city of Plovdiv, there is an extension of the favourable development period (May - October). Increase in population wave is observed in June, July and August, with peaks in numbers in the second half of July, September and early October.

Preliminary results of the study indicate the presence of vertical migration with establishing *Uranotaenia unguiculata*.

According to the preliminary results in the city of Plovdiv, 6 genera (*Anopheles, Aedes, Culex, Culiseta, Ochlerotatus, Uranotaenia*), including 13 species are established. The species composition of the mosquito fauna has been increased by three new genera (*Culiseta, Ochlerotatus* and *Uranotaenia*), in comparison with the recent studies by HRISTOVA *et al*. (2000) and others.

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# *New Information about Polyphenols of Wild (Amygdalus webbii) and Cultivated (Amygdalus communis) Almonds from Southwestern Bulgaria*

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**Abstract.** Plant material (flowers and leaves) of wild *Amygdalus webbii* Spach. and cultivated *A. communis* L. individuals growing in the same area were examined for their polyphenolic composition with aim to receive data about the adaptive potential and relationship between them. Eight flavonoid glycosides and two phenolic acids were detected by thin layer chromatography (TLC). They were identified by co- chromatography with authentic markers as quercetin-3-rutinoside (rutin), isorhamnetin-3-rutinoside, kaempferol-3-rutinoside, quercetin-3-galactoside (hyperoside), quercetin-3-glucoside, kaempferol-3-glucoside, quercetin-3-rhamnoside, kaempferol-3-rhamnoside, chlorogenic and cafeic acids. It is a flavonoid composition of the samples of flowers, the leaf samples showed a simpler qualitative flavonoid composition. Rutin, hyperoside and quercetin-3-glucoside were detected as main components of the leaf samples. The comparative TLC analysis of polyphenolic profiles of all studied samples showed that same polyphenolic compounds were present in each almond individual with small variations in relative levels. Total phenolic and flavonoid content of the studied samples were evaluated by spectrophotometric method. Generally the samples of *A. webbii* displayed higher amount of phenolics and flavonoids than that of *A. communis* but the differences were statistically significant only for phenolics in leaf samples. The received results suggest that adaptive capacity definite by phenolic compounds achieved in the early stage of species formation of *A. webbii* is retained and is effective in the cultivated *A. communis* (Bulgarian origin). To the best of our knowledge this is first report for flavonoid composition of flowers of *Amygdalus*.

**Key words:** flavonoids, phenolic acids, TLC.

## Introduction

Phenolic compounds represent the most abundant and the most widely present class of plant natural products. The development of the ability for synthesis of phenolic compounds is a key evolutionary moment in the successful adaptation of amphibious plants to land. So phenolic compounds have been selected throughout the course of evolution in different plants to help them to adapt to variable biotic and abiotic environment (BOUDET, 2007; CHEYNIER *et al.*, 2013).

Almond is an ancient nut crop of southwest Asia, but its wild ancestor has not been properly identified although *Amygdalus fenzliana* (Fritsch) Lipsky has been indicated as the most plausible almond progenitor (LADIZINSKY, 1999). Almond nuts have been studied extensively for their biological properties and nutritional value including phenolic content (MANDALARI *et*

*al*., 2010; YILDIRIM *et al*., 2010; ESFAHLAN *et al*., 2012; ESFAHLAN & JAMEI, 2012). However, limited information is available concerning phenolics of the leaves of *Amygdalus* sp (MISIRLI *et al*., 2001; BABAEI *et al*., 2008). Furthermore to the best of our knowledge there are no data about flavonoid composition of flowers of *Amygdalus*.

In Bulgaria two wild and two cultivated species of *Amygdalus* L. have been spread respectively *A. nana* L., *A. webbii* Spach and *A. communis* L., *A. triloba* (Lindl.)Ricker (VALEV, 1973). Morphologically *A. webii* is distinguished from *A. communis* only by spinose shoots. The present study aimed to obtain some primary data about the adaptive potential and relationship on the basis of polyphenolic composition between the wild *A. webbii* and cultivated *A. communis* individuals growing in the same areas in relatively similar ecological conditions. For this purpose comparative analysis of extracts of flowers and leaves of five individuals of *A. webbii* and *A. communis* was carried out in relation to their polyphenolic composition.

## Material and Methods

*Plant material.* Plant material (flowers and leaves) of five individuals of *Amygdalus webbii* and *A. communis* were collected in the spring of 2013 year. Detail information about origin of the materials is presented at Table 1.

**Table 1.** Origin of the experimental material.

| Taxon | Country | Region | Soil | Altitude | Exposure |
|---|---|---|---|---|---|
| *Amygdalus webbii* | Bulgaria | Western Border Mountain – Maleshevska Mountain, locality Ljubina skala | dry calcareous | about 400 m s.a.l. | E |
| *Amygdalus communis* | Bulgaria | Struma valley, hills upper the village of General Todorov, locality "Pripechene" | dry calcareous | about 200 m s.a.l. | NE |

*Preparation of the extracts.* Dry, ground plant material (1g) was extracted with 80% methanol by classical maceration for 24 h. After evaporation of the solvent the crude extract was subject to subsequent analysis.

*Thin layer chromatographic analysis of flavonoid glycosides and phenolic acids.* The methanol extracts were examined for flavonoid glycosides and phenolic acids by TLC analysis. Two TLC sorbents and several mobile phases were used. Ethyl acetate:formic acid:acetic acid:MeCOEt: water (50:2:3:30:10) and ethyl acetate:formic acid:acetic acid:water (100:11:11:27) were used as mobile phase for the development of the methanol extracts on silica gel plates Kiselgel 60 $F_{254}$. Acetic acid–water (15:85, v/v) was used for cellulose plates DC-Alufolien Cellulose 5552 (10 x 20 cm, 0.1 mm layer). Chromatograms were viewed under UV light before and after spraying with 1% solution of diphenylboric acid 2-aminoethyl ester complex in methanol. The identification of the compounds was achieved by co-chromatography with authentic markers obtained from Prof. Eckhard Wollenweber.

*Determination of total phenolic content.* Total phenolic content of the methanol extracts was determined by Folin–Ciocalteu reagent and gallic acid as standard (GIORGI *et al*., 2009, NIĆIFOROVIĆ *et al*., 2010). Plant extracts were diluted to the concentration of 1 μg $mL^{-1}$, and aliquots of 0.5 mL were mixed with 2.5 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of $Na_2CO_3$ (6%). After 1 h of staying at room temperature, the absorbances of the samples were measured at 765 nm on spectrophotometer versus

blank sample. Total phenols were determined as gallic acid equivalents (mg GA/g extract).

*Determination of total flavonoid content.* An aluminum chloride colorimetric method was used for flavonoid determination, using rutin as a reference compound (MILIAUSKASA *et al.* 2004). One mL of plant extract in methanol (10 g $L^{-1}$) was mixed with 1 mL aluminium trichloride in ethanol (20 g $L^{-1}$) and diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min at room temperature. Blank samples were prepared from1 mL plant extract and 1 drop acetic acid, and diluted to 25 mL. The absorption of rutin solutions was measured under the same conditions. Standard rutin solutions were prepared from 0.05 g rutin. All determinations were carried out in duplicate. The amount of flavonoids in plant extracts in rutin equivalents (RE).

*Statistical analysis.* Statistical analysis was carried out using excel. Results were presented as a mean value ± standard deviation (SD). Significant levels were defined at $p<0.05$ as analyzed by t-test.

**Results and Discussion**

Plant material (flowers and leaves) of five individuals of *Amygdalus webbii* from wild population and cultivated *A. communis* growing in the same area were examined for their polyphenolic composition. Eight flavonoid glycosides were detected by thin layer chromatography (TLC) and comparison with known compounds. They were identified as kaempferol-3-rutinoside (**1**), kaempferol-3-glucoside (**2**), kaempferol-3-rhamnoside (**3**), quercetin-3-rutinoside – rutin (**4**), quercetin-3-galactoside (hyperoside) (**5**), quercetin-3-glucoside (**6**), quercetin-3-rhamnoside (**7**), isorhamnetin-3-rutinoside (**8**). The leaf samples showed a simpler qualitative flavonoid composition. Rutin, hyperoside and quercetin-3-glucoside were detected as main components as well as quercetin-3-rhamnoside and kaempferol-3-glucoside in trace of the leaf samples (Table 2). Additionally flower samples contain kaempferol-3-rutinoside, kaempferol-3-rhamnoside and isorhamnetin-3-rutinoside. Chlorogenic and caffeic acids were also detected in the extracts of studied samples. The content of chlorogenic acid was found to be significantly higher in the extracts of wild species.

The comparative TLC analysis of polyphenolic profiles of all studied samples showed that same polyphenolic compounds were present in each almond individual with small variations in relative levels. Rutin, hyperoside, quercetin-3-glucoside and quercetin-3-rhamnoside found to be dominant compounds (Table 2).

Total phenolic and flavonoid content of the studied samples were evaluated by spectroscopic method. The extracts of the flowers have a higher content of flavonoids in comparison with the samples of leaves (Table 3). Generally the samples of *A. webbii* displayed higher amount of flavonoids than that of *A. communis* but the differences were not statistically significant. Concerning the phenolic compounds the leaf samples of *A. webbii* displayed twice as much phenolics than that of *A. communis.*

The received data displayed that there are no quality differences in the polyphenolic composition between *A. webbii* and *A. communis*. These results showed that besides morphological proximity both species have similar composition of phenolic compounds. Taking into account that phenolics play a major physiological role, especially in resistance to various stress factors and diseases (TREUTTER, 2007**;** BOUDET, 2007; CHEYNIER *et al.*, 2013) these results suggest that adaptive capacity of the studied species to the changing environment definite by contained in them phenolic compounds is similar. Identical polyphenol composition of the two species supposed that they have common origin. According to DIMITROVSKI & RISTEVSKI (1973), wild almond *Amygdalus webbii* is dwarf rootstock for cultivated almond. The present results are in confirmation of this hypothesis. It is not excluded *A. webbii* to be ancestral species lying in the base line of the long selection of the cultural species.

**Table 2.** Flavonoid glycosides and phenolic acids detected in the flower and leaf samples of examined *Amygdalus* species

| Studied samples | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| *A. webbii* | | | | | | | | | | |
| flowers | X | X | XX | XX | X | X | X | X | XX | XX |
| leaves | | tr | X | X | X | | | tr | | |
| *A. webbii* | | | | | | | | | | |
| flowers | tr | tr | X | X | X | tr | X | tr | XX | X |
| leaves | | tr | X | X | X | | | tr | | |
| *A. communis* | | | | | | | | | | |
| flowers | X | tr | X | X | X | tr | X | X | X | X |
| leaves | | tr | X | X | X | | | tr | | |
| *A. communis* | | | | | | | | | | |
| flowers | tr | X | X | XX | XX | X | X | tr | tr | X |
| leaves | | tr | X | X | X | | | tr | | |
| *A. communis* (out of culture) | | | | | | | | | | |
| flowers | tr | X | X | XX | XX | X | X | tr | tr | X |
| leaves | | tr | X | X | X | | | tr | | |

*Legend:* kaempferol-3-*O*-rutinoside (1), kaempferol-3-*O*-glucoside (2) kaempferol-3-*O*-rhamnoside (3) quercetin-3-*O*-rutinoside (rutin) (4) quercetin-3-*O*-galactoside (hyperoside) (5) quercetin-3-*O*–glucoside (isoquercetin) (6) quercetin-3-*O*–rhamnoside (quercetrin) (7) Isorhamnetin-3-*O*-rutinoside (8); chlorogenic acid (9) caffeic acid (10); tr- trace.

**Table 3.** Total phenol and flavonoid content of the flower and leaf samples of examined *Amygdalus* species

| Studied samples | Total phenolics* mg GAE/g extract | | Total flavonoids* mg RE/g extract | |
|---|---|---|---|---|
| | flower | folia | flower | folia |
| *A. webbii* | 41,72±3,9057 [a] | 64,79±1,0905 [a] | 2,322±0,0177 [a] | 1,95±0,4274 [a] |
| *A. webbii* | 39,61±3,1015 [a] | 58,01±2,2683 [a] | 2,405±0,1233 [a] | 1,79±0,1944 [a] |
| *A. communis* | 29,50±1,9974 [b] | 30,04±0,1484 [b] | 2,066±0,1732 [a] | 1,596±0,0250 [a] |
| *A. communis* | 36,24±3,0033 [a] | 36,89±0,8550 [b] | 2,056±0,1246 [a] | 1,409±0,3950 [a] |
| *A. communis* (out of culture) | 35,01±3,1816 [a] | 35,67±2,0451 [b] | 2,008±0,0951 [a] | 1,542±0,2960 [a] |

*Legend:* * values represent mean ±SD; Values with the same letter are not significantly different, p>0.05; GAE-gallic acid equivalents; RE – rutin equivalents.

## Conclusion

The present study provides data about flavonoid composition of flowers of Amygdalus species for the first time. The received results showed that there is no divergence of *A. webbii* and *A. communis* in respect to their polyphenolic composition. Adaptive and resistant capacity achieved in the early stage of species formation of *A. webbii* is retained and is effective in the *A. communis* (Bulgarian origin).

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# *Effect of Soil Physical Properties on Natural Regeneration of Populus caspica Bornm. and Alnus glutinosa L. in Northern Iran*

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**Abstract.** *Populus caspica,* as endemic and endangered poplar species, and *Alnus glutinosa* as a native species in most of Europe and locally in southwest Asia, are the most important tree species naturally disturbed in plain areas in North of Iran. Although the natural regeneration of *Alnus glutinosa* show good situation, unfortunately natural regeneration, distribution and elite trees of *Populus caspica* were diminished by different reasons. In this study, natural regeneration conditions of these species and their relationships with physical soil properties were carried out in two regions, with poor and good natural regeneration. Sampling have been done randomly in unequal linear transect. In each sampling plot, diameter of breast height for each tree and soil sample was taken. For all of soil samples, soil texture, Bulk density (B.D), Particle density (P.D), and water holding capacity (WHC) were analyzed. Differences of quantitative characteristics of trees and soil properties between two plantations were analyzed based on independent samples t-test, and correlation between variables were determined by Pearson correlation coefficient. Results of this study showed that there was strong relationship between establishments of natural regeneration of *Populus caspica* with soil physical properties. Natural regeneration of *Alnus glutinosa* did not show any relationship with soil physical properties. Natural regeneration of *Populus caspica* was established better in light soil texture with good aeration.

**Keywords**: *Populus caspica*, Natural regeneration, Soil properties, North of Iran.

## Introduction

The majority of the north of Iran is covered by the Caspian forest, a deciduous temperate commercial forest, of about 1.8 million ha located on the northern slopes of the Alborz Mountains overlooking the Caspian Sea (TABARI *et al*., 2011).

*Populus caspica* (Persian poplar), as endemic and endangered poplar species existed naturally in plain areas of Guilan province beside the planted species (ASADI & MIRZAIE-NODOUSHAN, 2011). Hyrcanian Forest is rich in biological diversity, with several endemic and endangered species. It contains the most important natural habitats for in-situ conservation of biological diversity in Iran, including those containing threatened species of outstanding universal value, from the view point of science or conservation (SADATI *et al*., 2010). *Populus caspica* (Salicaceae) is an endangered and endemic tree species (NAJAFI *et al*., 2012)

predominantly distributed in the Hyrcanian Forest. Nevertheless, there is little information about current site conditions for this species. This information is critically important for developing a conservation strategy.

*Populus caspica* species are commonly planted as fast-growing species in the world and some parts of plain areas in the north of Iran. (DICKAMANN & STUART, 1983) declared that *Populus caspica* could grow almost everywhere, but perform up to their full potential only on the best sites. Soil physical properties which play major roles in water holding capacity, aeration and root penetration, have a strong influence on the growth of poplars. As soil can effect poplar growth, these trees also affect different soil properties.

In the last years, the use of natural regeneration increases for following reasons: (1) decrease the costs of regeneration; (2) apply a ''close-to-nature'' kind of forestry, in which the use of smaller clear-cuts and naturally provided seeds are parts; and (3) increase plant density, get denser stands and hence improve the timber quality (HANSSEN, 2003). Light and soil condition are the main factors in natural regeneration and biodiversity of forest stand (AUBINA *et al*., 2008; BARBIER *et al*., 2008). Several researches for conifer stands had well documented that where canopy layer values were high, the cover of vascular plants and ground layer vegetation was reduced through shading (FAHY & GORMALLY, 1998). Overall more studies have reported that plantations of native or exotic timber species can increase biodiversity by promoting woody understory regeneration (PARROTTA, 1995).

JALILI & JAMZAD (1999) stated that in north of Iran the natural regeneration is reduced to different reasons. ASADI & MIRZAIE-NODOUSHAN (2011) stated that the seed breading of *Populus caspica* is found less in natural conditions.

Environmental factors such as temperature, moisture, soil conditions, topography, plant physiological conditions, seeding and seed viability have important roles in different plant species regeneration. ASADI & MIRZAIE-NODOUSHAN (2011) studied a seed collecting from 25 sites in north of Iran, and declared that seed germination and survival of seedling is depend to soil water content and appropriate light. SADATI *et al*. (2010) in their reviews stated that the loam texture soil is the reason of highest germinate.

In this study, natural regeneration conditions of *Populus caspica* and *Alnus glutinosa* and their relationships with physical soil properties were carried out in two regions, with poor and good natural regeneration. It is hypothesized that physical soil properties have effective role on natural regeneration of these species.

**Materials and Methods**

*Study area.* This study was carried out in two nearly regions called Safrabasteh forest park and Parkaposht in Guilan province (37˚19′ N, 49˚57′ E). These regions are on flat and uniform terrain in a plain area with the altitude of 10 m above sea level. Annual mean rainfalls and temperature are 1186 mm and 17.5°C respectively. Soil of the studied regions has neutral to low acidic reaction. Safrabasteh forest park is dominated by the natural forests containing native species such as *Alnus glutinosa, Pterocarya fraxinifolia, Populus caspica, Gleditschia caspica*. Parkaposht region, dominated by *Populus caspica* and *Alnus glutinosa*, is represented by *Populus caspica* regeneration.

Parkaposht is located in the sidelines in Sepidrood River with a significant level of 1.5 to 2 km in length and 300 to 400 m width.

*Sampling method.* Sampling was made randomly using unequal linear transect (AVEVY & BURKHAT, 2002) - 30 plots in each region were determined and total of 60 plots. In each sampling plot, after measuring of diameter of breast height for each tree, the number of regenerations was also recorded. Soil samples were taken in each plot, from 0-20 cm depth, and after mixing, one composed soil sample from each point was prepared (MARANON *et al*., 1999) and transferred to soil laboratory for analyzing.

*Soil properties and statistical analyses method.* All of soil samples were air-dried and passed through a 2 mm mesh. In the

lab, soil texture by hydrometer (BOUYOUCOS, 1962), bulk density (B.D) by clod method, water holding capacity (WHC), and particle density (P.D) by Pycnometer method (GHAZANSHAHI, 1999), were determined. Normality of variables was checked by Kolmogorov-Smirnov test and Levene's test was used to examine the quality of the variances. Differences of quantitative characteristics of trees and soil properties between two plantations were analyzed based on independent samples t-test (Student's t test at $p<0.05$), and correlation between variables were determined by Pearson correlation coefficient ($p<0.05$). For all statistical analysis, SPSS Software (version 17.0) was used.

**Results**

*Number and diameter of trees in study regions.* Mean comparison of the number of trees showed that there are significant differences between *Populus caspica*, *Alnus glutinosa*, *Populus* sp. and *Pterocarya fraxinifolia* in Safrabasteh forest park and region Parkaposht. Results suggest that *Populus caspica* and *Alnus glutinosa* are dominated trees compare to the other tree species in the studied region, although *Populus caspica* in Parkaposht and *Alnus glutinosa* in Safrabasteh forest park were dominated (Table 1). Based on the result, the mean diameter is higher both for *Populus caspica* and *Alnus glutinosa* in Safrabasteh forest park compared to Parkaposht (Table 2). Results of the *Alnus glutinosa* and *Populus caspica* species breeding are shown in Table 3. Based on the data obtained, the regeneration of *Populus caspica* is higher in Parkaposht compared to Safrabasteh forest park. Results did not show significant differences on *Alnus glutinosa* breeding.

**Table 1**. Mean±SD of trees number.

| Species | Region | Mean±SD | t | Significance |
|---|---|---|---|---|
| *Populus caspica* | Parkaposht | 6.97±3.47 | 9.149 | 0.000 |
| | Safrabasteh forest park | 1.03±0.76 | | |
| *Alnus glutinosa* | Parkaposht | 2.7±1.95 | -4.056 | 0.000 |
| | Safrabasteh forest park | 5.4±3.08 | | |
| *Populus* sp. | Parkaposht | 1.27±1.17 | 3.963 | 0.000 |
| | Safrabasteh forest park | 0.00±0.00 | | |
| *Pterocarya fraxinifolia* | Parkaposht | 0.00±0.00 | -2.513 | 0.015 |
| | Safrabasteh forest park | 1.89±0.87 | | |
| *Gleditschia caspica* | Parkaposht | 0.00±0.00 | -1.415 | 0.163 |
| | Safrabasteh forest park | 0.07±0.25 | | |

**Table 2**. Mean±SD of diameter at breast height (DBH)

| Species | Region | Mean±SD | t | Significance |
|---|---|---|---|---|
| *Populus caspica* | Parkaposht | 23.5±8.6 | -21.576 | 0.000 |
| | Safrabasteh forest park | 138.7±51.3 | | |
| *Alnus glutinosa* | Parkaposht | 17.5±5.5 | -4.003 | 0.000 |
| | Safrabasteh forest park | 24.3±11.3 | | |

**Table 3**. Mean±SD of *Populus caspica* and *Alnus glutinosa* breeding.

| Species | Region | Mean±SD | t | Significance |
|---|---|---|---|---|
| *Populus caspica* | Parkaposht | 6.8±1.7 | 3.438 | 0.002 |
| | Safrabasteh forest park | 0.1±0.04 | | |
| *Alnus glutinosa* | Parkaposht | 7.9±5.7 | -1.178 | 0.244 |
| | Safrabasteh forest park | 9.9±7.1 | | |

*Soil physical properties.* Results showed significant differences on some soil physical properties among the regions. Results did not show significant differences on clay and P.D, while the other soil physical properties showed significant differences (Table 4).

*Correlation breeding between Populus caspica and Alnus glutinosa with soil physical properties.* Results of Pearson correlation coefficient between regeneration of *Populus caspica* and *Alnus glutinosa* species with soil physical properties have been shown in Table 5. Among the soil physical properties, B.D and WHC showed positive of negative correlation with *Populus caspica* breeding, respectively.

**Discussion**

Regeneration of *Populus caspica* is significantly higher in Parkaposht region than in Safrabasteh forest park, also should be noted that in both areas the regeneration of *Alnus glutinosa* is happened.

**Table 4**. Mean±SD of soil physical properties.

| Soil properties | Region | Mean±SD | t | Significance |
|---|---|---|---|---|
| Silt, % | Parkaposht | 39.02±3.87 | 4.908 | 0.000 |
| | Safrabasteh forest park | 47.05±7.86 | | |
| Clay, % | Parkaposht | 13.82±3.52 | 1.804 | 0.076 |
| | Safrabasteh forest park | 16.32±6.72 | | |
| Sand, % | Parkaposht | 46.88±6.08 | -5.292 | 0.000 |
| | Safrabasteh forest park | 36.63±8.82 | | |
| B.D, g $cm^{-3}$ | Parkaposht | 1.34±0.13 | -5.901 | 0.000 |
| | Safrabasteh forest park | 1.17±0.09 | | |
| P.D, g $cm^{-3}$ | Parkaposht | 2.28±0.53 | -0.165 | 0.870 |
| | Safrabasteh forest park | 2.26±0.12 | | |
| SP, % | Parkaposht | 37.31±17.00 | 3.183 | 0.002 |
| | Safrabasteh forest park | 47.69±5.46 | | |
| WHC, % | Parkaposht | 45.03±2.57 | 23.781 | 0.000 |
| | Safrabasteh forest park | 84.77±8.87 | | |

*Legend*: B.D-bulk density, P.D-Particle density, WHC-water holding capacity, SP-soil porosity

**Table 5**. Pearson correlation coefficient and significant level between *Alnus glutinosa* and *Populus caspica* breeding with soil physical properties.

| Soil properties | Correlation coefficient | *Populus caspica* breeding | *Alnus glutinosa* breeding |
|---|---|---|---|
| Silt, % | Pearson correlation | -0.178 | 0.249 |
| | Sig. (2-tailed) | (0.173) | (0.055) |
| Clay, % | Pearson correlation | 0.082 | 0.055 |
| | Sig. (2-tailed) | (0.535) | (0.676) |
| Sand, % | Pearson correlation | -0.175 | -0.150 |
| | Sig. (2-tailed) | (0.180) | (0.252) |
| B.D, g $cm^{-3}$ | Pearson correlation | 0.254* | -0.105 |
| | Sig. (2-tailed) | (0.050) | (0.425) |
| P.D, g $cm^{-3}$ | Pearson correlation | 0.011 | -0.066 |
| | Sig. (2-tailed) | (0.935) | (0.617)** |
| SP, % | Pearson correlation | -0.161 | 0.004 |
| | Sig. (2-tailed) | (0.220) | (0.975) |
| WHC, % | Pearson correlation | -0.384* | 0.186 |
| | Sig. (2-tailed) | (0.049) | (0.154) |

*Legend:* **significant level $p<0.001$; *significant level $p<0.05$

Based on the results, it was found that in both regions there are sufficiently trees of *Alnus glutinosa* and *Populus caspica*. Although the *Populus caspica* in Parkaposht is more than 3 times of *Alnus glutinosa*, there are sufficient trees of *Alnus glutinosa* produced each year (270 trees per hectare).

Also in Safrabasteh forest park, the number of *Alnus glutinosa* is about 5 times rather than the number of matures *Populus caspica*. However, there are sufficient mature trees of *Populus caspica* (103 trees per hectare) in this region.

Besides effective environmental factors, appropriate mature trees that are able to produce sufficient seed for the regeneration, are one of the main factors in the regeneration of tree species. This study showed that in both regions, the seed trees are suitable for regeneration, although the mean diameter of *Populus caspica* and *Alnus glutinosa* in Safrabasteh forest park is far higher than in Porkaposht region.

In addition to the origin and characteristics of the seeds, some of the natural factors such as lighting, climate, topography and soil characteristics, are effective in the regeneration. Soil properties such as soil texture, porosity, moisture and soil carbon and nitrogen content, are the most important factors that effect on natural regeneration and seed growth (OLESKOG & SAHLEN, 2000; SALEHI *et al.*, 2011). Differences between physical properties of soils in the two regions could be related to several factors. In this study, it seems that the location of the study regions is one of the main factors influencing the difference soil properties specially soil physical properties.

Parkaposht region has been located near the river and is affected by river sediments. Existed soils have generally evolved on the sediments and have their own characteristics. This type of soil is not very deep; generally have a light texture and are not fertile. Based on the results, soil texture was lighter in Parkaposht region and sand proportion is higher than Safrabasteh forest park. High sand and low clay content results in good aeration, but on the other hand, causes the lack of water and the absorption of nutritional elements. With considering soil conditions and the differences between two areas, it can be stated that the condition of soil properties and specially the existing of nutritious in Safrabasteh forest park are more suitable for the growth of plants than those in Parkaposht area of Safrabasteh forest park. MADSEN & LARSEN (1997) stated that soil moisture has a positive effect on the establishment and growth of European beech seedlings.

According to the study in both Safrabasteh forest park and Parkaposht region, there are enough *Populus caspica* trees. Although in Safrabasteh forest park the regeneration of *Populus caspica* has been less observed, the base of *Alnus glutinosa* is higher and its regeneration is more. In addition to the *Alnus glutinosa* in Safrabasteh forest park region, the tree and the regeneration of *Pterocarya fraxinifolia* and *Gleditschia caspica* can be seen, while it is missing in Parkaposht region.

It can be concluded, that in Safrabasteh forest park the soil has an appropriate condition, especially in terms of content nutrition. Although in Safrabasteh forest park there are enough *Populus caspica* beside other seed trees, the existence of the other species and their regeneration is the better competition against *Populus caspica.* In other words, we can say that in Parkaposht region, where the soil is nearly poor representing fertility, some species such as *Alnus glutinosa* grow less or not at all, but *Populus caspica* has been able to grow better there.

On the base of the results of this study, it can be concluded that the regeneration of *Populus caspica,* as an endemic and useful tree species, does not need a very favorable soil physical properties. It seems that *Populus caspica* can even grow in fertile soils with favorable physical condition, but in comparison to other tree species it have not been able to be established in this situation.

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# *Interesting Lichenized Fungi (Ascomycota) from Struma River Valley and Belasitsa Mts.*

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**Abstract.** New data on 10 interesting or rare lichenized fungi in Bulgaria (*Anaptychia ciliaris, Cetraria islandica, Cladonia ramulosa, Dermatocarpon miniatum, Graphis scripta, Hypogymnia tubulosa, Lobaria pulmonaria, Physconia enteroxantha, Pyrenula nitida* and *Rinodina sophodes*) are revealed. Most of them are documented with digital photographs. *Lobaria pulmonaria* is detected in several localities on old beech and chestnut bark, while *Cetraria islandica* is recorded on soil in Mt Belasitsa at high altitudes. Site description and information on the distribution of each species so far khown in Bulgaria are presented.

**Key words:** biodiversity, lichenized fungi, Struma River valley, Belasitsa, Bulgaria.

## Introduction

The very first data on the lichenized fungi of Belasitsa Mts. can be found in the work of CRETZOIU (1936). Published information, including thorough and more detailed data about the distribution of lichenized fungi from the Struma River valley (including "Prepetscheno" locality) and "Belasitsa", could be obtained from several basic works (POPNIKOLOV, 1937; ZHELEZOVA 1962, 1963; POPNIKOLOV & ZHELEZOVA, 1964) and in the recent published monograph on foliose genera of *Physciaceae* family (ATANASSOVA & MAYRHOFER, 2012). The first monographic study on the morphologically diverse genus *Usnea* Dill. ex Adans. from the mountain regions of the country, comprising Belasitsa Mts., was compiled by MOTYKA & ZHELEZOVA (1962).

During routine field studies in the vicinities of Petrich town (southern part of Strouma Valley and several regions, situated around the lower parts near the villages of Belasitsa Mts., supplied by records from high mountain zone), new data about 10 lichenized fungi of special interest has been revealed. They represent mainly epiphytic, epilitic and epigeous species.

## Material and Methods

Field and laboratory studies were held during 2013 using techniques and methods, accepted and described in DOBSON (2011). The studied specimens were documented macroscopically with the help of digital camera Canon PowerShot A460 in the field or under Boeco B-3500 binocular microscope. Semipermanent microscope squash mounts, made from the cross

sections of the lichen thalli, were prepared in distilled water and observed under the LM Boeco T-SP-180. Data about the localities of the specimens (longitude, latitude and elevation parameters) are received with the help of GPS receiver Garmin Etrex 10. Information on plant substrata and other features of the samples is collected in the field. The identification of the specimens is justified by NIMIS *et al.* (2009), DOBSON (2011) and ELIX (2011).

Data about the distribution of the taxa in the country follows MAYRHOFER *et al.* (2005). The specimens of the lichenized fungi are housed at the Mycological Collection, Institute of Biodiversity and Ecosystem Research, Sofia (SOMF).

**Results and Discussion**

Within the examined lichenized fungi from Belasitsa Mts. and Strouma Valley 10 species are presented here in. Six of them are new records for the studied areas, which is pointed out with an asterisk in the text (*). Below the list of determined species is provided short note on their known distribution in Bulgaria, or important data are discussed briefly. Information on comparison with other closest species, if present, is given too.

***Anaptychia ciliaris*** (L.) Körb.

*Specimen examined:* Bulgaria, Valley of Strouma River (southern): Blagoevgrad distr., vicinity of Petrich Town, above Ruzhdak Village, 41°23’28.4’’N, 023°15’10.8’’E, 346 m a.s.l., 23 May 2013, D. Stoykov, SOMF 28682, on bark of *Quercus pubescens* WILLD., along with *Physconia distorta* (With.) J.R. Laundon; Belasitsa Mts.: above the town of Petrich, far from Paprenitsa border point, along with *Hypogymnia tubulosa* (Schaer.) Hav., *Usnea hirta* (L.) Weber ex F.H. Wigg., *Evernia prunastri* (L.) Ach. and *Physconia distorta* (With.) J.R. Laundon, 41°19’34.3’’N, 023°13’1.9’’E, 1350 m a.s.l., 25 May 2013, D. Stoykov, SOMF 28740, on bark of old *Salix* sp.; ibid., below the mountain road, 41°19’21.6’’N, 023°11’28.5’’ E, 1681 m a.s.l.., 25 May 2013, D. Stoykov, SOMF 28747, on bark of *Fagus sylvatica* L., adjacent to *Sorbus borbasii* Javorka trees. Epiphyte (**Fig. 1**).

*Known distribution:* Black Sea coast, Northeast Bulgaria, Forebalkan, Balkan Range, Sofia and Vitosha regions, Valley of Strouma River, Pirin and Rila Mts., Rhodopes Mts., Toundzha Hilly Country and Strandzha Mts. (see MAYRHOFER et al., 2005).

*Anaptychia ciliaris* resembles morphologically *A. crinalis* (Schleich.) Vězda, but differs mainly in different colour of the upper thalline surface and the wider lobes of the thallus (usually over 1 mm wide).

****Cetraria islandica*** (L.) Ach. - new record for Belasitsa Mts.

*Specimens examined:* Bulgaria, Belasitsa Mts.: above the town of Petrich, below Kongur Peak, near the high mountain road, 41°19’16.3’’N, 023°11’05.9’’E, 1730 m a.s.l., 25 May 2013, D. Stoykov, SOMF 28677, SOMF 28720, on soil, along with *Bruckenthalia spiculifolia* (Salisb.) Reichenb.; on southern slope of Radomir Peak, sine datum, 20 June 2013, coll. S. Stoyanov, SOMF 28741, 28743, 28744; above Samouilovo Village, Lopovo border point region, nearby the high mountain road in both directions – down the southern slope of Radomir Peak, along with *Umbilicaria cylindrica* (L.) Delise ex Duby on surrounding rocks, 41°19’49.9’’N, 023° 02’33.2’’E, 1752 m a.s.l.., 24 May 2013, D. Stoykov, SOMF 28731, on soil among grasses; above the village of Kljuch, along the mountain road, 41°19’53.2’’N, 023°01’18.1’’E, 1752 m alt., ibid., SOMF 28711, on soil, together with *Vaccinium myrtillus* L.; along the mountain road above Kljuch Village in direction Toumbata Peak, 24 May 2013, 41°19’09’’N, 023°06’16’’E, 1725 m alt., D. Stoykov, SOMF 28715, on soil; above the mountain road following Demirkapiya Pass in direction of Kljuch Village, together with *Cetaria aculeata* (Schreb.) Fr., 41°19’34.9’’N, 023°03’43.0’’E, 1766 m a.s.l., 24 May 2013, SOMF 28734, on sandy soil, in communities of *Viola stojanovii* W. Becker. Epigeous species (**Fig. 6**).

*Known distribution:* Balkan Range, Vitosha region, West Frontier Mts.,

Slavyanka Mts., Pirin and Rila Mts., Rhodopes Mts. (MAYRHOFER *et al.*, 2005).

*Cetraria islandica* has a fruticose, small to medium brown thallus, developed loosely or attached in the soil; it is usually tufted, many lobed and irregularly branched. Species is of conservation value in Great Britain, where it is included in the category Least Concerned (LC), WOODS & COPPINS (2012), while in the Red List of the lichenized fungi of Wales is designated as Near Threatened (NT), WOODS (2010). Recently it was confirmed in Bulgaria from Balkan Range (Shipchenska Mts.), from the regions of Malusha Peak (1312 m a.s.l.) in grassy communities, along with *Thamnolia vermicularis* (Sw.) Schaer. var. *vermicularis* and from the mountain zone of Bedek Peak in grassy communities (1488 m a.s.l.) and on soil among mosses in beech forest along the track to Gorski Dom 'Balgarka'.

****Cladonia ramulosa*** (With.) J.R. Laundon - new country record from Belasitsa Mts.

*Specimen examined:* Bulgaria, Belasitsa Mts.: between Kolarovo and Samouillovo Villages, near the old cherry orchard, 7 April 2013, 41°21'57.5''N, 023°05'38.4''E, 376 m a.s.l., D. Stoykov, SOMF 28742 , on bark of old dead trunk from broadleaved tree. Epixylous species (**Fig. 2**).

Probably this species is more frequent in Bulgaria, but seems to be overlooked because of the similarity with another closest species - *Cladonia parasitica* (Hoffm.) Hoffm., which bears squamulose thallus and often inhabits bark of old trees.

***Dermatocarpon miniatum*** (L.) W. Mann var. ***miniatum***

*Specimen examined:* Bulgaria, Belasitsa Mts.: above Skrat Village, along the track to Dubitsata Waterfall, 41°21'25.3''N, 23°00'01.6''E, 616 m a.s.l.., 6 April 2013, D. Stoykov, SOMF 28736, on the surface of big rounded rock, in the vicinity of the settlement. Epilitic lichen.

*Known distribution:* Widespread throughout Bulgaria. Not known in Sofia region, West Frontier Mts., Valley of Strouma River, valley of Mesta River, Sredna Gora Mts. and Tounzdha Hilly Country (after MAYRHOFER *et al.*, 2005).

Confirmed for the first time with dry specimen from Belasitsa Mts. Several recent collections of the closest species *Dermatocarpon luridum* (With.) J.R. Laundon from Rila Mts. (Kostenski Waterfall) and of *D. miniatum* var. *miniatum* in Northeast Bulgaria (Shoumensko Plato Natural Park), made during a period of 2004-2007 years were presented by SHIVAROV & STOYKOV (2010).

****Graphis scripta*** (L.) Ach. - recorded for the first time in Belasitsa Mts.

*Specimens examined:* Bulgaria, Belasitsa Mts.: above Skrat Village, along the track to Dubitsata Waterfall, 41°21'25.3''N, 023°00'01.6''E, 625 m a.s.l., 6 April 2013, D. Stoykov, SOMF 28735, on bark of *Corylus avellana* L.; between Kolarovo and Samouilovo Villages, 41°21'57.5''N, 023°05'38.4''E, 380 m a.s.l., 7 April 2013, D. Stoykov, SOMF 28737, on bark of broadleaved tree. Epiphyte (**Fig. 7**).

*Known distribution:* Black Sea Coast, Northeast Bulgaria, Forebalkan, Balkan Range, Vitosha Region, Rila Mts., Rhodopes Mts.

Characteristic species, often with apothecia in the form of thin curved black lines, more rarely resembling an irregular three line aster on thalline surface, which similarity is used to attach its specific species epithet. Distinguished macroscopically from the closest *Graphis elegans* (Borrer ex Sm.) Ach., which possesses apothecia with raised carbonaceous margin, in the form of small cracks, ± meeting in the centre. Recently *G. scripta* is recorded on old mature beech in the area above 1200 m in Balkan Range (Schipchenska and Trevnenska Mts) in Bulgaria. *Graphis scripta* is known as intermediately pollution tolerant species (BEAVEN, 2008). In Great Britain this lichen, along with *Arthonia radiata* (Pers.) Ach. and *Arthonia cinnabarina* (DC.) Wallr., is common on young smoothbarked trees (ROSE, 1993). RAVERA *et al.* (2010) reported *G. scripta* as an important component of epiphytic climax lichen communities, inhabiting old forests and especially old trees of *Fagus sylvatica* L.

****Hypogymnia tubulosa*** (Schaer.) Hav. – new record in Belasitsa Mts.

*Specimen examined:* Bulgaria, Belasitsa Mts.: above Petrich Town, far from Paprenitsa border point, 41°19′34.3′′N, 023°13′1.9′′E, 1350 m a.s.l. along with *Anaptychia ciliaris* (L.) Körb., *Pseudevernia furfuracea* (L.) Zopf and *Usnea hirta* (L.) Weber ex F.H. Wigg., 25 May 2013, D. Stoykov, SOMF 28739, on bark of old tree of *Salix* sp. Epiphyte (**Fig. 8**).

*Known distribution:* Balkan Range, Vitosha Region, Pirin and Rila Mts., Rhodopes Mts.

*H. tubulosa* is similar in appearance to *Hypogymnia physodes* (L.) Nyl., but bears more erect, tubular and fingerlike thalline lobes and has slightly different colour in the upper part. It is thought to be less resistant species to air pollution than *H. physodes*.

***Lobaria pulmonaria*** (L.) Hoffm.

*Specimens examined:* Bulgaria, Belasitsa Mts.: above Paprenitsa border point, 41°43′45.5′′N, 023°34′20.6″E, 1310 m a.s.l.., 25 May 2013, S. Stoyanov, SOMF 28748, on bark of old beech tree; above Petrich town, far from Paprenitsa border point, abundant on large number (6-7) of very old, mature *Fagus sylvatica* L. trees in the area between 41°19′43.4′′N, 023°12′35.5″E, at about 1410 m alt. untill 41°19′22.4′′N, 023°11′ 47.7′′E, 1640 m a.s.l., 25 May 2013, D. Stoykov, SOMF 28746, on bark of *Fagus sylvatica* L.; above Samouilovo Village, below mountain road after Lopovo border point, in direction to Demirkapiya mount pass, 41°19′12.2′′N, 023°05′16.2′′E, 1706 m alt., 24 May 2013, D. Stoykov, SOMF 28732, along with *Pertusaria pertusa* (WEIGEL) TUCK., on bark of old *Fagus sylvatica* L. - single tree; above the village of Belasitsa, 41°21′33′′N, 023°09′30′′E, 880 m a.s.l., 26 May 2013, D. Stoykov, SOMF 28733, on bark of very old *Castanea sativa* MILL. - single tree; above the village of Kolarovo, 41°35′18.8′′N, 023°13′3.4′′E, 780 m alt., 25 April 2013, coll. S. Stoyanov, SOMF 28743, on bark of old *Fagus sylvatica* L. – single tree. Epiphyte.

*Known distribution:* Black Sea Coast, Northeast Bulgaria, Forebalkan, Balkan Range, Znepole Region, Vitosha Region, Belasitsa Mts., Pirin and Rila Mts., Sredna Gora Mts., Rhodopes Mts., Strandzha Mts. (after MAYRHOFER *et al.*, 2005).

In Bulgaria *L. pulmonaria* can be usually found on bark of broadleaved trees. Occasionally it is recorded from Balkan Range on mossy ground in beech forest (ZHELEZOVA, 1962). Recently was confirmed on bark of single beech tree in Shipchenska Mountain (Ouzana locality), at nearly 1200 m a.s.l. *L. pulmonaria* is an indicator of very old preserved forests. It is widespread, but regionally threatened foliose lichen. Species of conservation value in Great Britain with category Least Concerned (LC), WOODS & COPPINS (2012), while in Estonia nearly 20% of his localities are considered endangered, as their areas are affected by forest management activities (JÜRIADO & LIIRA, 2010). According to the latest known data, in the northern parts of Europe (incl. Great Britain) it is used as an indicator species for natural forest ecosystems and forest areas of long ecological continuity (ROSE, 1993). *L. pulmonaria* have been used also as a model species in ecological, ecophysiological and conservation biological research (SCHEIDEGGER & WERTH, 2009).

***Physconia enteroxantha*** (Nyl.) Poelt

*Specimens examined:* Bulgaria, Belasitsa Mts,: near Petrich Town, after Mladezhki Dom complex, 41°23′24.8′′N, 023°12′49.1′′E, a.s.l. 295 m, 7 April 2013, D. Stoykov, SOMF 28745, on bark of old *Robinia pseudoacacia* L. tree; above Samouilovo village, along the track to Kamenishki Waterfall, 41°21′32.3′′N, 023°04′54.7′′E, 800 m a.s.l., 24 May 2013, D. Stoykov, SOMF 28665, along with *Physconia distorta* (With.) J.R. Laundon and *Parmelia saxatilis* (L.) Ach., on bark of old *Fraxinus* tree. Epiphyte (**Fig. 4**).

*Known distribution:* Balkan Range, Sofia Region, Belasitsa Mts., Rila Mts., Rhodopes Mts.

Recently reported from the vicinity of Belasitsa chalet on bark of *Quercus pubescens* Willd. (ATANASSOVA & MAYRHOFER, 2012).

****Pyrenula nitida*** (WEIGEL) ACH. – new record in Belasitsa Mts.

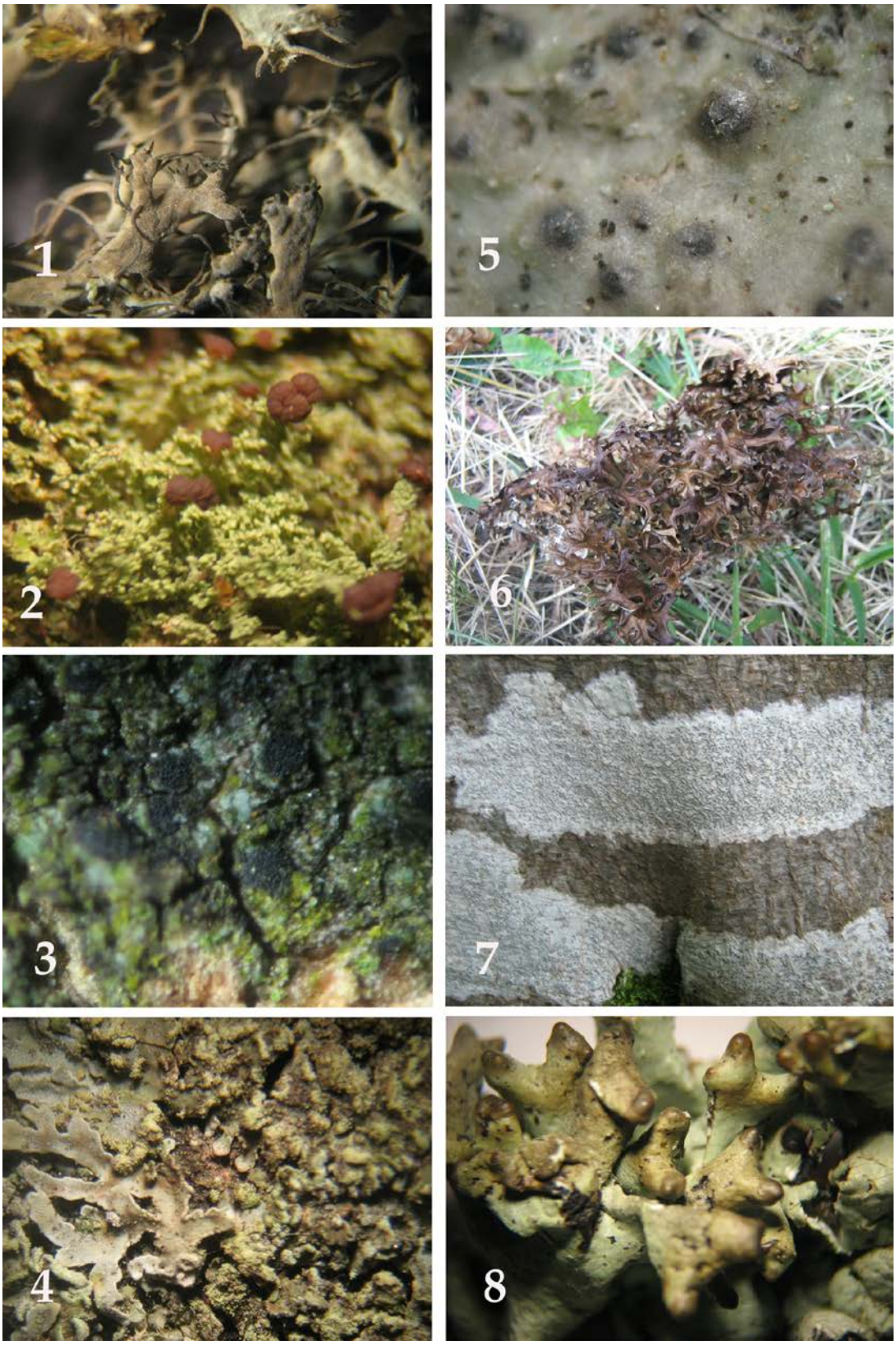


**Fig.1-8**.Thalli of: **1**. *Anaptychia ciliaris* (SOMF 28682), **2**. *Cladonia ramulosa* (SOMF 28742); **3**. *Rinodina sophodes* (SOMF 28725); **4**. *Physconia enteroxantha* (SOMF 28745); **5**. *Pyrenula nitida* (SOMF 28738); **6**. *Cetraria islandica* (SOMF 28731); **7**. *Graphis scripta* (SOMF 28737); **8**. *Hypogymnia tubulosa* (SOMF 28739).

*Specimen examined:* Bulgaria, Belasitsa Mts.: above Petrich town, far from Paprenitsa border point, along the road to Kongur Peak, 41°19'45.4''N, 023°12'44.0''E, 1386 m a.s.l.., 25 May 2013, D. Stoykov, SOMF 28738, on bark of very old *Fagus sylvatica* L. Epiphyte (**Fig. 5**).

Known distribution: Northeast Bulgaria, Forebalkan, Balkan Range, Pirin and Rila Mts., Stranzdha Mts.

* ***Rinodina sophodes*** (Ach.) A. Massal. – new species in Strouma River Valley.

*Specimen examined:* Bulgaria, Struma River Valley (southern): Petrich district, near Ruzhdak Village, above the dung-hill, 41°23'58.9''N, 023°15'20.9''E, 236 m a.s.l., 8 April 2013, D. Stoykov, SOMF 28725, on bark of young *Fraxinus* tree. Epiphyte (**Fig. 3**).

*Known distribution:* Northeast Bulgaria, Sofia region, Vitosha Region, Pirin, Rila and Rhodopes Mts.

**Conclusions**

Five new species for the regions of Belasista Mts. and one new lichenized fungus from Struma River Valley in Bulgaria are recorded for the first time during this study. One of the finds is epigeous (*Cetraria islandica*), while the others are epiphytic (*Anaptychia ciliaris*, *Cladonia ramulosa, Graphis scripta*, *Hypogymnia tubulosa*, *Pyrenula nitida* and *Rinodina sophodes*). *Cladonia ramulosa* is recorded for the first time on dead trunk in Bulgaria. *Rinodina sophodes* is revealed as new species to the Valley of Strouma River. *Lobaria pulmonaria*, an epiphytic species inhabiting very old beech and chestnut trees, is recorded in several localities in Belasitsa Mts. *Dermatocapon miniatum* (an epilitic species) and *Physconia enteroxantha* could serve as fresh specimens for future analysis.

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