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## Landscape Planning for "Kubratovo 1" Sand and Gravel Deposit Located on Alluvial Meadow Soil, Sofia Region, Bulgaria

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**Abstract.** The paper presents results of landscape planning and investigation on Fluvisols. The object is "Kubratovo 1" sand and gravel deposit, Bulgaria. Soil samples were taken from twentyone soil profiles at a depth of 0-20 cm, 20-40 cm, 40-60 cm, 60-80 cm, and 80-100 cm. The changes in physicochemical properties were investigated at different soil depths and the relationships between different soil characteristics were tested. There are statistically significant positive or negative regressions. There are regression relationships between the soil texture fractions and bulk density, total porosity, soil organic matter, total Kjeldahl nitrogen, available phosphorus and available potassium.

**Key words:** Landscape planning, soil, Fluvisols, physical properties, chemical properties, urban green system.

#### Introduction

Rapid urbanization results in the increasing isolation of human populations from the natural world. For the majority of people green public areas are one of the few opportunities for direct contact with the natural environment.

The green spaces in open country are an integral part of the ecosystems of the urban areas and could generate significant as carbon environmental benefits such emission reduction, air pollution reduction, regulation microclimate as well as recreational use. These ecosystems help improve the quality of life and facilitate urban development (JO, 2002; BOLUND & HUNHAMMAR, 1999; JIM & CHEN, 2006; MILLER, 1997; JENSEN et al., 2000; LI & WANG, 2003). Besides having environmental

functions such as air purification, microclimate regulation, noise reduction, biodiversity preservation, etc., urban green areas also have a statistically significant impact on the sales price of real estate properties located in the vicinity of these areas (LIN *et al.*, 2005).

Quarries and ballast quarries, unlike other anthropogenic pollutants, are a source of fine and coarse particles (HINDS, 1999), and post-mining regions are poor in soil organic matter, N, P and K, and no significant changes in soil content are observed even years later. The vegetation consists mainly of grassy annual and biennial species (JIAN-GANG YUAN et al., Quarries that have not 2006). been rehabilitated after mining activities have ceased turn into untapped and

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unsustainable resources, and their reclamation becomes difficult due to the specific environmental conditions, which may vary even within the disturbed area (JAY & HANDLEY, 2001). Gravel sand deposits are located under the soil group of Fluvisols (KOYNOV et al., 1998). Fluvisols are found between Chernozems and Gray forest soils in Northern Bulgaria, along the rivers Danube, Lom, Ogosta, Iskar, Osam, Yantra, Rositsa and Kamchia (TEOHAROV et al., 2015; HRISTOV, 2009). In Southern Bulgaria they are situated between Vertisols and Cinnamonic Forest soils, along the rivers Maritsa, Tundzha, Arda, Mesta, Struma, Iskar, and Erma (ANTIPOV-KARATAEV et al., 1959; Donov, 1993).

Alluvial soils can be represented by a gravel substrate although surface horizons are fine-textured (silty clay loam and sandy clay loam), the subsoil can be finer (clay loam, silty clay or clayey) (CASANOVA et al., 2013), the soil texture depends largely on the speed of the water current where the soils have formed and their location along the river bed (CARATING et al., 2014). The bulk density of these soils varies from 1.2 to 1.35 g.cm<sup>-3</sup> (HUQ & SHOAIB, 2013). Fluvisols are characterized by pH which ranges from moderately acidic to strongly alkaline. The soil organic matter and total nitrogen vary greatly. The moderately supplied soils are with phosphorus (FILIPOVÁ et al., 2010). Soil conditions are one of the factors that determine the species composition of the plant cover (BOGDANOV, 2018a; b).

The purpose of the study was to make a landscape plan for the development of a highly recreational area on an undeveloped sand and gravel deposit site located under the soil group of Fluvisols as well as to characterise their soil properties.

#### Materials and Methods

*The object.* Mollic Fluvisols (FAO, 2014) occurring on the territory of "Kubratovo 1" sand and gravel deposit, located east of the village of Kubratovo, Sofia Region, and west

of the village of Negovan, Sofia Region, Bulgaria was the object of the study. The object is found in the Lower forest vegetation zone (0 - 600 m a.s.l.) of the Moesian forest vegetation area of Bulgaria (ZAHARIEV *et al.*, 1979). The studied area covers about 378.41 ha.

*Methods of study.* Twenty-one profiles at a depth of 0-100 cm were done at representative plots. A systematic sampling design was used (PETERSEN & CALVIN, 1996). The samples were taken at depths of 0-20 cm, 20-40 cm, 40-60 cm, 60-80 cm, and 80-100 cm.

The following soil characteristics were analyzed by using the respective methods:

Bulk density (BD, g.cm<sup>-3</sup>), according to the DIN ISO 11272:1998, 2001; Total porosity (TP, %) by calculation of bulk density and relative density (LORRAINE & FLINT, 2002); Soil texture (Sand - 2 mm - 63 µm, %; Silt - 63  $\mu$ m - 2  $\mu$ m, %; Clay < 2  $\mu$ m, %), using the sedimentation method (ISO 11277), texture groups were determined according to the Soil Survey Staff (1975); Plant available water capacity (PAWC, mm), by a laboratory method, with the calculation of field capacity and permanent wilting point (DONOV et al., 1974); Soil acidity (pH in water extraction) measured potentiometrically by WTW 720 pH meter (ISO 10390:2002); Soil Organic Matter (SOM, %) by ISO 14235:1998; Total Kjeldahl Nitrogen (TKN, %) content, with a modified version of the classic Kjeldahl method (ISO 11261:2002); P<sub>2</sub>O<sub>5</sub> (mg.100g<sup>-1</sup>) extraction with Ammonium Acetate and Calcium Lactate-pH 4.2 (IVANOV, 1984); K<sub>2</sub>O (mg.100g<sup>-1</sup>) – extraction with Ammonium Acetate and Calcium Lactate-pH 4.2 (IVANOV, 1984).

*Data analysis.* Descriptive statistics was applied using Numbers (Apple Co., 2018), Excel (Microsoft Co., 2016) and SPSS (IBM Co., 2016) for Mac. Linear and polynomial regression analysis was applied for testing for a functional relationship between the soil depth and the soil characteristics. Corrected variants of regression coefficients (R<sup>2</sup>) were calculated and their statistical significance (significant difference from zero) was tested at p<0.05 with SPSS (IBM Co., 2016) and Excel (Microsoft Co., 2016).

#### **Results and Discussion**

Soil characteristics. Changes in soil characteristics with depth and soil assessment.

The soil texture at a depth of 0-20 cm falls mainly within the sandy clay loam category and partly within the loam category. At a depth of 20-40 cm it is predominantly sandy clay loam. The soil texture in the other horizons (40-60, 60-80, 80-100 cm) is predominantly sandy loam, where at a depth of 40-60 cm it is in the top half of the textural class (the most clayey), and the samples taken from the other two horizons (60-80 and 80-100 cm) fall within the bottom half (with minimum clay content) of the class. The variation in these classes has also been reported by other authors (ILINKIN et al., 2018). There are significant statistical regressions between the soil texture fractions and bulk density on the one hand and depth on the other (Fig. 1). The sand fraction increases with depth (from 51.48%, which is the mean value in the 0-20 cm layer, to 67.86% in the 80-100 cm layer). The silt fraction ranges between 24.94 and 28.47 (mean values in the 0-20 and 80-100 cm layers). The clay fraction decreases significantly with depth from 23.59% to 3.67% (mean values in the 0-20 and 80-100 cm layers). The data show that the increase in the sand fraction with depth is at the expense of the clay fraction, whereas the silt fraction does not vary greatly. The changes in the sand and clay fractions are properly described by linear regression functions (for both p<0.0001), whereas the silt fraction is properly described by a second order polynomial regression function (p<0.0001). A possible hypothesis for the fact that the changes in the silt fraction are described by a polynomial regression is the nature of soil formation - periodic deposition of parent material. The soil texture variation is consistent with data reported by other authors (CARATING, 2014).



**Fig. 1.** Relationship between depth and soil texture.

The bulk density (Fig. 2) ranges within the averagely compacted class (according to the classification of AG Bodenkunde, 1982) and it gradually increases with depth from 1.26 g.cm<sup>-3</sup> (mean value in the 0-20 cm layer) to 1.45 g.cm<sup>-3</sup> (mean value in the 80-100 cm layer). There is a strong linear regression relationship between depth and bulk density (p<0.0001).

In the soils studied there is a clear trend towards a decrease in total porosity with depth (Fig. 3) p<0.0001, where it changes from averagely porous to slightly porous (according to the classification of AG Bodenkunde, 1982).



**Fig. 2.** Relationship between soil depth and bulk density.

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**Fig. 3.** Relationship between soil depth and porosity.

PAWC in the studied soils varies from medium to high (mean = 170.72) (Fig. 4), which is consistent with other studies on alluvial soils (ILINKIN *et al.*, 2018).

The soil reaction in water extraction for the different horizons is described by a linear regression function (Fig. 5) with high statistical significance (p<0.0001). The variation of pH with depth has also been reported in other studies (DONOV, 1979; ILINKIN *et al.*, 2018).



Fig. 4. PAWC variation.

There are statistically significant (p<0.0001 for both equations) second order polynomial regressions between the content of SOM and TKN on the one hand and depth

on the other. SOM changes from low content to no SOM content (according to the classification of AG Bodenkunde, 1982). TKN in all horizons ranges within the very low availability class (according to the classification by PENKOV, 1996). These data are consistent with the data reported by FILIPOVÁ (2010) on the variation in SOM and TKN, as well as on their decrease with depth.



Fig. 5. Relationship between depth and pH.



**Fig. 6.** Relationship between depth and SOM.

There are statistically significant (p<0.0001 for both equations) polynomial regressions between available phosphorus and potassium on the one hand and depth on the other (Fig. 8 and Fig. 9). The content

of available phosphorus changes with depth from very good availability to low availability (according to the classification by PENKOV, 1996). The content of available potassium changes from very good availability to low availability (according to the classification by PENKOV, 1996).



## **Fig. 7.** Relationship between depth and TKN.





The changes in chemical characteristics (SOM, TKN,  $P_2O_5$ ,  $K_2O$ ) with depth are better described by second order polynomial regression functions than by a linear regression. This could be explained by the decomposition of the organic matter, humus formation and the release of nitrogen, phosphorus and potassium.



**Fig. 9.** Relationship between soil depth and K<sub>2</sub>O content.

Soil characteristics. Regression relationships between some physical and chemical characteristics.

Soil texture is an important factor which affects soil fertility (DONOV, 1979). There are signification statistically regression relationships between some physical and chemical characteristics on the one hand and bulk density and total porosity on the other (Table 1). There is a positive correlation between the soil texture components (sand and silt) and bulk density and an inverse correlation between them and total porosity. In both cases the regression with the silt fraction is statistically significant. The regressions between the clay fraction and bulk density and total porosity are statistically significant (where the clav fraction is inversely correlated to bulk density and positively correlated to total porosity). SOM is strongly negatively correlated with bulk density and strongly positively correlated with total porosity. The correlation between soil texture and porosity has also been reported by other studies (DONOV, 1979; ILINKIN *et al.*, 2018; ILINKIN, 2018).

A well-known fact is the relationships between SOM and other soil characteristics (STOCKMANN *et al.*, 2013). The statistically significant regression relationships between the soil texture components on the one hand and SOM and nutrients (TKN,  $P_2O_5$ ,  $K_2O$ ) on the other are presented in Table 2. There are statistically significant regression relationships between the soil texture fractions (sand and silt) and SOM, TKN,  $P_2O_5$ ,  $K_2O$ , which are inversely correlated. There are statistically significant regression relationships between the clay fraction and SOM, TKN,  $P_2O_5$ ,  $K_2O$ , which are positively correlated.

There are statistically significant polynomial regressions between SOM and

nutrients (TKN,  $P_2O_5$  and  $K_2O$ ) (Table 3). The fact that SOM includes carbon, nitrogen and minerals (DONOV, 1979) as well as the soilforming processes typical of alluvial soils (periodic deposition of sediment) are the most likely reasons for the existence of statistically significant linear regressions between SOM and the other nutrient sources studied. The correlations between SOM and TKN,  $P_2O_5$  and  $K_2O$  have also been reported in other studies (ILINKIN, 2018; ILINKIN *et al.*, 2018).

**Table 1.** Regression relationships between soil texture and SOM and bulk density and total porosity.

		BD (x), g.cm <sup>-3</sup>	TP (x), %
	equation	y = 0.0105x + 0.7297	y= - 0.6989x + 91.044
Sand (y), %	$R^2$	0.7522	0.8544
	p value	< 0.0001	< 0.0001
	equation	y= 0.0081x + 1.1688	y= - 0.6266x + 64.121
Silt (y), %	$R^2$	0.1118	0.1741
	p value	= 0.0004	< 0.0001
	equation	y = -0.0072x + 1.4744	y = 0.4904x + 41.387
Clay (y), %	$R^2$	0.6132	0.7358
	p value	< 0.0001	< 0.0001
	equation	y= - 0.0521x + 1.4477	y= 3.4838x + 43.316
SOM (y), %	$R^{\bar{2}}$	0.7274	0.8392
•	p value	< 0.0001	< 0.0001

Table 2. Regression relationships between soil texture and SOM, TKN, P<sub>2</sub>O<sub>5</sub>, K<sub>2</sub>O.

		SOM (x), %	TKN (x), %	$P_2O_5(x)$ , mg.100g <sup>-1</sup>	K <sub>2</sub> O (x), mg.100g <sup>-1</sup>
Sand (y),	equation	y= - 0.196x + 13.418	y= - 0.0008x + 0.0611	y= - 0.1308x + 8.6411	y= - 1.0499x + 77.576
%	R <sup>2</sup>	0.9712	0.8109	0.8579	0.8674
	p value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Silt (y), %	equation	y= - 0.188x + 6.1707	y = -0.0004x + 0.0221	y = -0.0552x + 2.0877	y= - 0.6065x + 28.956
	R <sup>2</sup>	0.2266	0.0479	0.0387	0.0734
	p value	< 0.0001	= 0.025	= 0.044	= 0.0052
Clay(y),	equation	y= 0.1393x - 0.5311	y = 0.0005x + 0.0048	y= 0.0828x - 0.5127	y= 0.6881x + 3.7234
%	R <sup>2</sup>	0.8582	0.584	0.601	0.6518
	p value	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Γ	ab	le 3	<b>3.</b> ]	R	egressio	on re	lations	hips	between	SOM	[ and	TKN,	$P_2C$	D₅ and	$K_2C$	Э.
												-				

		TKN (x), %	$P_2O_5(x)$ , mg.100g <sup>-1</sup>	K <sub>2</sub> O (x), mg.100g <sup>-1</sup>
SOM (y),	equatio n	$y = 0.0014x^2 - 0.007x + 0.0082$	$y = 0.2652x^2 - 0.2575x + 0.0938$	$y=1.4066x^2+0.501x+7.7685$
%	R <sup>2</sup>	0.8976	0.9697	0.9408
	p value	< 0.0001	< 0.0001	< 0.0001

Mathematical models could be significant even if the R-squared value is low (FARAWAY, 2015). In agrarian sciences authors have presented regression models with R<sup>2</sup><0,5; R<sup>2</sup><0,3 (KOZAR *et al.*, 2002; FANTAPPIÈ *et al.*, 2011; CRESPELL *et al.*, 2006).

A high R-square does not necessarily mean that the model is useful (NETER *et al.*, 1996). The p-value, however, is considered to be a reliable indicator when it is lower than 0.05 (MANGIAFICO, 2016).

The climate has a direct impact on soil temperature and moisture, and an indirect one on the biota. "Kubratovo 1" sand and gravel deposit falls within the European climatic region, continental temperate continental sub-region, the climatic region of the high fields of Mid-Western Bulgaria (TROEVA, 2009). The territories around Sofia are characterized by retention and additional radiative cooling of air masses, and as a result temperatures can drop to -25 °C. In January the average temperatures are around 4 – 5 °C, and in June they are between 20 – 21 °C. The relative air humidity is 70%. The average annual temperature is around 11.5 °C, the average annual rainfall is approximately 837 mm, with a maximum rainfall in May and a minimum in July. The winter is cold and relatively dry with an average temperature in January of 2.6 °C. There is low precipitation (102 mm). The summer is relatively warm with an average temperature in July ~ 23.8 °C. There is considerably more precipitation in the fall than in the spring due to the basic features of this region (TROEVA, 2009).

*Landscape planning*. Public green spaces which include parks, sports grounds,

riparian zones, municipal gardens, etc. (ROY *et al.*, 2012) are heterogeneously distributed within the urban environment. Access to them is often limited due to socio-economic or other reasons, which is becoming a serious problem (ABERCROMBIE *et al.*, 2008; JENNINGS *et al.*, 2012; JOHNSON-GAITHER, 2011; SISTER *et al.*, 2010; BYRNE *et al.*, 2009; MCCONNACHIE & SHACKLETON, 2010). Urban green spaces are multifunctional and have recreational, social, cultural and ecological characteristics (PAULEIT, 2003; PRIEMUS & HALL., 2004).

The conceptual landscape plan for "Kubratovo 1" sand and gravel deposit has been presented in Fig. 10. The proposed conceptual landscape plan aims to meet the need of the city of Sofia for an area with social, cultural, recreational and ecological functions by providing a place for active and passive recreation with increased species diversity (Table 5), and optimal relationship between under-trees spaces and open spaces (consistent with the climatic characteristics of the region and global climate changes). The landscape design also envisages the use of medicinal and aromatic plants (MAPs). Some authors (EFE et al., 2018) recommend the use of MAPs, whereas others highlight their complex influence (cultural connotation, health care and health recovery) (QIAO & ZHANG, 2012).

**Table 5.** Species composition of the conceptual landscape plan for "Kubrotovo 1" sand and gravel quarry. Legend: <sup>1</sup> - species used for tree/shrub groups; <sup>2</sup> - species used for ornamental tree/shrub groups; <sup>3</sup> - species used for hedgerows; <sup>4</sup> - species used for grassing

Abies pinsapo Boiss. <sup>1, 2</sup>	Clematis montana D.C. <sup>2</sup>
Abies pinsapo 'Glauca' <sup>2</sup>	<i>Clematis montana</i> 'Grandiflora' <sup>2</sup>
Abies pinsapo 'Kelleriis' <sup>2</sup>	Clematis montana 'Rubens' <sup>2</sup>
Picea pungens 'Oldenburg' <sup>2</sup>	<i>Clematis patens</i> Morr. <sup>2</sup>
Picea pungens 'Omega' <sup>2</sup>	<i>Clematis patens</i> 'Fortunei' <sup>2</sup>
Picea pungens Engelm. <sup>2</sup>	Clematis patens 'Standishii' <sup>2</sup>
Picea pungens 'Białobok' <sup>2</sup>	Berberis x ottawensis C.K. Schneid <sup>1</sup>
Picea pungens 'Glauca' <sup>2</sup>	Berberis x ottawensis 'Superba' <sup>2,3</sup>
Picea pungens 'Hoopsii' <sup>2</sup>	<i>Berberis julianae</i> Schneid. <sup>2</sup>
Picea pungens 'Koster' <sup>2</sup>	Magnolia kobus DC. <sup>1</sup>
Cedrus libani A.Rich <sup>2</sup>	Philadelphus x lemoinei Lem. <sup>2</sup>
<i>Cedrus libani</i> 'Atlantica Aurea' <sup>2</sup>	Philadelphus x lemoinei 'Erectus' <sup>2</sup>
Cedrus libani 'Fastigiata' <sup>2</sup>	<i>Philadelphus x lemoinei</i> 'Manteau d'Hermine <sup>2</sup>
Pinus nigra Arnold <sup>12</sup>	Platanus orientalis L. <sup>1, 2</sup>

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Pinus nigra 'Fastigiata' 2 Pinus nigra spp. laricio<sup>2</sup> Pinus nigra 'Green Rocket' 2 Metasequoia glyptostroboides Huet Cheng<sup>1</sup> Taxodium distichum Rich<sup>2</sup> Calocedrus decurrens Florin<sup>1</sup> Calocedrus decurens 'Aureovariegata' 2 Thuja orientalis L.<sup>2,3</sup> Thuja orientalis 'Aurea Nana'<sup>2</sup> Thuja orientalis 'Berckmanii'<sup>2</sup> Thuja orientalis 'Fastigiata' 2 Thuja orientalis 'Golden Surprise' 2 Thuja orientalis 'Maturodam'<sup>2</sup> Thuja orientalis 'Nusi' 2 Juniperus sabina 'Blue Danube' 1 Juniperus sabina 'Hicksii' 2 Juniperus sabina 'Rockery Gem<sup>1, 2</sup> *Taxus baccata* L. <sup>1,3</sup> Taxus baccata 'Adpressa Aurea'<sup>2,3</sup> Taxus baccata 'Baccatin' 2,3 Populus alba L.<sup>1</sup> Populus simonii Carr. 1 Salix triandra L.<sup>2</sup> Salix cinereal L.<sup>2</sup> Betula pendula Roth.<sup>1</sup> Betula pendula 'Fastigiata' 1, 2 Betula pendula 'Laciniata' 1 Betula pendula 'Bibor'<sup>2</sup> Alnus glutinosa Gaertner<sup>2</sup> Alnus glutinosa 'Imperialis' 2 Quercus petraea Leibl.<sup>1</sup> Ulmus glabra Huds.<sup>1</sup> Ulmus pumila L.<sup>1</sup>

Spiraea x arguta Zab. <sup>1, 2, 3</sup> Spiraea media F. Schmidt<sup>3</sup> Spiraea x bilardii Hering <sup>3</sup> Spiraea billardii 'Rosea' *Cotoneaster horizontalis* Decne<sup>1, 2</sup> Sorbus aria Crantz<sup>1</sup> Crataegus oxyacantha L.<sup>2</sup> Albizia julibrissin Durazz.<sup>2</sup> Gymnocladus dioicus K. Koch<sup>2</sup> Ailantus altisima Swingle<sup>2</sup> Eunymus europea L.<sup>2</sup> Acer tataricum L.<sup>1</sup> Acer saccharinum L.<sup>1</sup> Acer saccharinum 'Born Gracious' 1 Acer saccharinum 'Wieri' 1 Aesculus hippocastanum L.<sup>1</sup> Aesculus hippocastanum 'Baumannii' 1 Aesculus x carnea Hayne<sup>2</sup> Tilia platyphyllos Scop.<sup>1</sup> Tilia platyphyllos 'Delft' 2 Tilia platyphyllos 'Orebro'<sup>2</sup> Tamarix tetrandra Pall.<sup>1</sup> Syringa vulgaris L<sup>2,3</sup> Syringa vulgaris 'Mrs. Edward Harding' 2,3 Cornus mas L.<sup>2</sup> Agrostis capillaris L.<sup>4</sup> Anthoxanthum odoratum L.<sup>4</sup> Festuca rubra L.<sup>4</sup> Lolium perenne L.<sup>4</sup> Poa compressa L.<sup>4</sup> Poa nemoralis L.<sup>4</sup> Poa pratensis L.<sup>4</sup> Poa trivialis L.<sup>4</sup>



Fig. 10. Conceptual landscape design for "Kubratovo 1" sand and gravel deposit.

#### **Conclusions and recommendations**

Soil characteristics vary with depth. These variations are described by statistically significant linear regressions (for the sand fraction, clay fraction, bulk density, total porosity and pH) and statistically significant polynomial regressions (for the silt fraction, SOM, TKN,  $P_2O_5$ ,  $K_2O$ ). There are regression relationships between the soil texture fractions and bulk density, total porosity, SOM, TKN, available phosphorus and potassium. There are positive linear regressions between SOM and TKN,  $P_2O_5$ ,  $K_2O$ .

The conceptual landscape plan for "Kubratovo 1" sand and gravel deposit shows that if the deposit site is not going to be used, the territory could be turned into a recreational area. The analysis of soil resources and climate data indicate that the species composition can increase significantly.

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### Assessment of Soil Quality in a Copper Mining Region in Bulgaria

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**Abstract.** The paper deals with a mining region of Bulgaria in the Sub-Balkan mountain valleys where there is enrichment of the rock material in copper, gold and other valuable metals and minerals. The study area is localized in the South-west of Chelopech mining - close to the watershed of Topolnitsa River. A monitoring network has been set up. The dominated soil types are Cambisols, Luvisols, Fluvisols, and Phaeozems with shallow surface humus horizon. In the mining region, the anthropogenic activities have different negative impacts over soil cover such as contamination, erosion, acidification, waste disposal, inert and other materials spread over soils surface. The assessment of physico-chemical and agrochemical soil parameters is important for its ecological modelling applications. The study found significant fluctuation of these properties in the area. Acidification affects 45% of the studied points, this accompanied by insufficient nitrogen and phosphorus nutrition.

Key words: soil chemical properties, agrochemistry, soil monitoring.

#### Introduction

The lands around Chelopech mining are parts of the Zlatitsa-Pirdop valley which lies between the Stara planina mountain (The Balkan) and Sredna Gora mountain. The mountains have an influence on the development of soil process, vegetation cover and accumulation of large particles (stony) or fine-grained (clayey) from the surrounding slopes.

The area is characterized by the soils with different stages of development from shallow to deep formed through various geological periods. Contemporary geographic and climatic conditions have a significant effect on their status and agroecological characteristics.

The soils, except for meadow varieties (Phaeozems), can generally be defined as

low to a medium content of humus and nutritional elements. They are also weak developed, acidic, fragmented, heavy, sandy-loamy and loamy by texture, poor physical and physicochemical properties. These soils are difficult for cultivation and agriculture (KOINOV *et al.*, 1974).

The copper enrichment of soils is one of the most significant environmental threats in some parts of Bulgaria. Fluvisols, Cambisols, Phaeozems, Leptosols and Luvisols are the major soil types distributed in the areas around the Cu ore processing plants in Bulgaria such as Elatsite, Chelopech, Assarel-Medet, Aurubis-Pirdop (BENKOVA *et al.*, 2005; ILINKIN *et al.*, 2014; NENOVA *et al.*, 2015; ATANSSOVA *et al.*, 2017).

The lands of the region have been polluted as from the mining industry for

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copper and gold for centuries (MALINOVA, 1998; DONCHEVA-BONEVA et al., 2011; DINEV et al., 2008). Pollution in the region and intensive agriculture in the last decades have drastically reduced soil productive potential and it needs to be constantly remediated and protected to increase soil fertility (BANOV et al., 2010). Because of low pH and high copper-contamination, the soils have been meliorated with calcium and magnesiumrich amendments. These practices have changed the soil reaction, cation exchange capacity and base saturation. Metal contamination in agricultural soils could affect carbon dynamics. This can significantly affect the capacity of the soil microbial communities to degrade organic matter, thereby leading to a loss of soil fertility and modifications in the balance between CO<sub>2</sub> release and long-term SOC storage. The nitrogen cycle is biologically influenced, but as it was mentioned above, the area is highly polluted in the surface horizons with Cu compounds and many populations able to degrade polymerized or aromatic compounds, such as Sphingomonadaceae and Actinobacteria, have been apparently actively involved in degradation in the residue coppercontaminated soils (BERNARD et al., 2009).

The investigation aims to evaluate the soil quality in the copper mining region of Bulgaria by GIS mapping, spatial distribution and physico and agrochemical parameters.

#### **Materials and Methods**

The object of the study is soils is located around the village of Chavdar. They are different soil types- Leptosols, Cambisols, Phaeozems, and Fluvisols. The elevation is about 520 m a.s.l. The vegetation is composed mainly of meadow grasses associations and fields with arable crops such as wheat, sunflower, rape, corn, barley, etc. The study was done by monitoring network with a grid of 0.5 to 0.5 km and sampling to a depth of 0-20 cm (Fig. 1).

The content of soil organic carbon (SOC) was determined using the modified Turin's method (KONONOVA, 1966; FILCHEVA & TSADILAS, 2002; HRISTOVA et al., 2016; FILCHEVA et al., 2018). The content of soil organic matter (SOM) is given as soluble part – humic plus fulvic acids (HA+FA) and an insoluble part called humin. The cation exchange capacity (CEC) and base of saturation (B.S.) were determined by the method of GANEV & ARSOVA (1980). Ammonium and nitrate nitrogen by KEENEY & BREMNER (1966); the mobile forms of phosphorus and potassium by the acetatelactate method of IVANOV (1984). The pH was determined potentiometrically in compliance with ISO 10390: 2010. MS Excel (Microsoft Co. 2010) is used for statistics.

The applied methodology includes a survey by statistical and graphical processing, classification of the studied physical and agrochemical parameters and their mapping in a geographic information system. For mapping, Kriging Interpolation is used-Isoline maps are created by Surfer ver. 8.02 of Golden Software 2002 and Global Mapper v. 16, for a topographic map. The studied area was assessed by classification for soil pH, CEC and bases of saturation (BS), the content of SOM (PENKOV, 1986) and assessment by sufficient amount of nutrients - nitrogen, phosphorus and potassium (ATANASOV *et al.*, 2006).

#### **Results and Discussion**

Descriptive statistic and correlation analysis

The fixed sample points were analyzed for the main physico-chemical properties. The investigated area is best described by using descriptive statistics of main physico-chemical and agrochemical properties (Table 1) and correlation matrix (Table 2). The surface horizons of the survey area show high fluctuations in the soil chemical properties.



Fig. 1. The satellite, topographic map of the area South Chelopech.

Table 1. Descriptive statistics of	of main p	hysico-cl	hemical	and agroc	hemical	parameters.
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	pН	SOC %	HA +FA %	<b>CEC</b> cmol.kg <sup>-1</sup>	<b>ex.H</b> <sup>+</sup> cmol.kg <sup>-1</sup>	<b>ex.Al</b> <sup>3+</sup> cmol.kg <sup>-1</sup>	<b>ex.Ca<sup>2+</sup></b> cmol.kg <sup>-1</sup>	<b>ex.Mg</b> <sup>2+</sup> cmol.kg <sup>-1</sup>	<b>B.S.</b> %	<b>ΣNH</b> <sub>4</sub> <b>+NO</b> <sub>3</sub> mg.kg <sup>-1</sup>	<b>P</b> <sub>2</sub> <b>O</b> <sub>5</sub> mg.100g <sup>-1</sup>	<b>K₂O</b> mg.100g <sup>-1</sup>
Mean	5.94	1.61	0.41	27.25	7.29	0.75	16.90	3.10	72.70	25.64	3.50	22.49
Standard Err.	0.17	0.11	0.03	1.27	0.72	0.16	1.40	0.12	2.89	1.63	0.96	1.59
Median	5.80	1.47	0.38	27.40	7.80	0.40	16.00	3.20	71.40	23.60	1.80	21.70
Mode	5.00	1.54	0.38	24.90	5.50	0.00	16.00	3.20	55.00	23.00	0.20	21.70
Standard Dev.	0.89	0.61	0.15	6.86	3.85	0.84	7.52	0.66	15.58	8.76	5.15	8.59
Sample Var.	0.80	0.37	0.02	47.10	14.84	0.70	56.55	0.44	243	76.78	26.57	73.76
Range	2.40	3.00	0.79	37.40	12.60	2.30	38.70	2.50	49.00	38.10	21.40	27.70
Minimum	5.00	0.73	0.22	18.90	0.00	0.00	9.50	2.00	51.00	8.60	0.20	12.10
Maximum	7.40	3.73	1.01	56.30	12.60	2.30	48.20	4.50	100	46.70	21.60	39.80

**Table 2**. Correlation matrix between main physicochemical and agrochemical parameters.

	тU	SOC	HA+FA	CEC	ex. H <sup>+</sup>	ex. Al <sup>3+</sup>	ex.Ca <sup>2+</sup>	ex.Mg <sup>2+</sup>
	рп	%	%	cmol.kg <sup>-1</sup>				
SOC	0.20	*						
HA+FA	0.05	0.85	*					
CEC	0.18	0.49	0.58	*				
ex. H+	-0.94	-0.13	0.00	0.00	*			
ex. Al <sup>3+</sup>	-0.86	-0.12	0.06	-0.30	0.74	*		
ex. Ca <sup>2+</sup>	0.61	0.52	0.53	0.87	-0.49	-0.60	*	
ex.Mg <sup>2+</sup>	0.55	0.08	0.04	0.47	-0.36	-0.63	0.53	*
B.S.	0.97	0.20	0.04	0.19	-0.96	-0.85	0.63	0.51
$P_2O_5$	0.50	0.02	0.11	-0.10	-0.53	-0.31	0.16	0.23

The statistics show that the parameters studied vary greatly. The content of SOC and its soluble fraction vary only slightly, while the physical and agrochemical parameters vary greatly (Table 1). The strong positive or negative correlations between the parameters are noted in bold in Table 2. Correlation analysis shows that there are stronger trends related to physicochemical than to agrochemicals parameters.

## *Classification of the main pysico-chemical and agrochemical parameters*

The soil results show that pH varies from 5.0 to 7.0 (Table 1) and the mean value is 5.9, which is consistent with other studies in the area which show that the soil reaction is low acidic values pH approximately 5-6 (DINEV *et al.*, 2008). According to the classification for pH: 59 % of the test points are acidic with pH below 6.0; 38 % have a neutral pH with pH between 6. 0 – 7. 3 and 3% are alkalic with a pH above 7.4.

In the acidic points, harmful effects of exchange hydrogen and aluminium ions were found and they have a positive correlation ( $r^2 = 0.74$ ). The negative correlation between pH and hydrogen ion content ( $r^2 = -0.96$ ) is higher than that of aluminium ( $r^2 = -0.80$ ), but both are indicative of a decrease in pH values associated with their raise.

The reasons for having neutral and alkaline pH points are mainly two. First is that in the area the liming is rare practice melioration with calcium and magnesiumrich materials which eliminate high acidity and blockade free heavy metals ions mainly copper (NIKOVA, 2008 - pers. comm.). The second is that there are soils in the region which are rich in carbonates, because of calcium-rich parent rock and the river Topolnitsa which brings base cations from the surrounding area (KOINOV et al., 1974). Sorption capacity is from medium to over high values (Fig. 2), CEC is between 18.9 to 56.0 cmol.kg<sup>-1</sup> (PENKOV, 1986). Over high values of CEC are connected by the content of SOM in a marshy place near river

Topolnitsa. The sorption capacity graph shows that the points with high sorption capacity dominate but they are neutralized no more than 70% by positive calcium and magnesium ions. However, we should note that according to the content of base saturation (BS) all point are "Eutric", they have BS above 50% by WRBS classification (IUSS Working Group, 2006). The content of SOC has significant variation and it's from 0.73% to 3.73% in the studied survey area (Table 1, Fig. 3). Usually, in the cultivation fields, it is about 1.5% which is something typical for arable lands in Bulgaria (BOGDANOV et al., 2015). The high content of SOC is in the area of marshes close to Topolnitza river, which is situated in the south-east part of the survey filed (point 79) in Fig. 1). The type of humus in the region is predominantly humic-fulvic (Ch/Cf = 1)- 0.5; KOINOV et al., 1974; FILCHEVA et al., 2018). Our research confirms that SOM has high sorption capacity and make complexes with calcium (GANEV et al., 1990). That's why correlations between SOC with a soluble fraction (humic and and acids) with CEC fulvic and exchangeable  $Ca^{2+}$  are expected (Table 2). There is no significant correlation between the available forms of nitrogen and the other studied soil parameters. The reason for that could be unequal treatment with different nitrogen fertilizers. The amount of "mineral" nitrogen as the sum of ammonium and nitrate is from low to moderate, ranging between 8.6 to 46.7 mg.kg<sup>-1</sup>, despite the high amount of nitrogen fertilizers that are used in the agricultural lands. The amount of available phosphorus oxide  $(P_2O_5)$  in studied soils is low (Table 2, Fig. 4). The values are ranging from 0.2 to 21.00 mg.100g<sup>-1</sup>. Therefore, the amounts of less than 7 mg.100g<sup>-1</sup> of soil, the plants will suffer a severe shortage of this element (ATANASOV et al., 2006).

The concentration of potassium in the soil solution is an important parameter for assessing the availability of this element



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Fig 2. Exchange cations as % of sorption capacity (CEC  $(T_{8,2})$ ).



Fig. 3. The average content of SOC, humin and HAs by humic classes (%).



Fig. 4. Soil assessment according to NPK thresholds.

for plant nutrition. The potassium's stock  $(K_2O)$ , is in a higher content than the mineral nitrogen and phosphorus (Fig. 4).

#### Mapping with interpolation maps

The distribution of pH, SOC, CEC, available forms of nitrogen, phosphorus, potassium are shown on interpolation maps (Fig. 5, 6, 7, 8, 9 and 10). The acidic points with pH below 6.0 are 59%. It's a big area between points A1, P2, 21, 22, 24, 35, 36, 37, 38, 39, 40, 49, 50, 53, 61, 64, 65, 68 (Fig. 1 and 5). The problem with acid condition is in the same area with pH values below 6.0 and CEC shows insufficient neutralization with calcium, magnesium and harmful amounts of aluminium are present (Fig. 1 and 6).In the studied area there are points of the low humus class and pH below 6.0 but they are only three. Thus a section with degraded soil qualities is drawn by points 21, 37, 68 (Fig. 1 and 8).Insufficient available nitrogen was found at 24.1% of the points, except for p.19 at pH 7.4, all others have a pH below 6.0 (points A1, P2, 22, 37, 40, 61). A major nutrient problem has been identified with phosphorus supply - 75.9% have poor and 10.3% have insufficient phosphorus supply. An exclusion here are the points P2, 19, 25, 52, 79. These points are placed close to one another which confirms the practice that in Bulgaria farmers use very rarely phosphorus fertilizers only in some small spots and only 6.9% of the points have sufficient supply (Fig. 9). The studied soils have a suitable potassium supply. Only 27.6% of sampling points have insufficient available potassium, and at points A1, 35, 40, 50 and 68, it's connected with pH of below 6.0, while points 52 and 79 are not related to acidity. Under the influence of the surrounding mountains in the region and surface waters, potash-containing sediments are weathering and realize good quantities of potassium oxide in low-land fields.

#### Conclusions

An assessment of the quality of soils in a vulnerable area has been carried out. A

monitoring network was used to perform the analysis. The methodology includes an physical establishment of basic and agrochemical indicators. The quantitative evaluation included statistical and correlation analysis. The analysis of complex soil parameters allowed us to point out that the main problem is related to acidity and the lack of nutrient elements. In the studied soils there is a greater need for phosphorus than nitrogen. Mapping indicates zones where there are risks for the environment and human health. In order to overcome the negative effects of the studied degradation processes the meliorative practices such as liming and nutrient fertilization are strongly recommended.

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Fig. 5. Isoline map of Soil pH.



Fig. 6. Isoline map of cation exchange capacity CEC (cmol.kg<sup>-1</sup>).



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Fig. 7. Isoline map of SOC, % .



**Fig. 8.** Isoline map of available nitrogen  $\sum NH_4 + NO_3$ , mg.kg<sup>-1</sup>.

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Fig. 10. Isoline map of mobile potassium oxide - K<sub>2</sub>O, mg.100g<sup>-1</sup>.

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### Forestry Reforestation vs. Spontaneous Revegetation – Soil Changes in Coal Mining Spoil Heaps Across Bulgaria

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**Abstract.** The paper presents results of an investigation of forestry reclamation and spontaneous revegetation on Technogenic soils (Technosols) formed as a result of coal mining activities. The soils are located in "Maksim taban" deposit site, Pernik, Bulgaria. Soil samples were taken at twenty-two profiles at representative plots under spontaneous revegetation - herbaceous plant cover (eleven profiles) and under forestry reclamation - black pine (*Pinus nigra* Arn.) (eleven profiles) at a depth of 0-100 cm. The changes in physicochemical properties were investigated under different vegetation and at different soil depths. The results reveal that most soil characteristics, such as bulk density, total porosity, the content of sand, silt, plant available water coefficient, pH, total organic carbon, total Kjeldahl nitrogen and available potassium are statistically significantly different under different vegetation. There are strong positive or negative correlations among soil characteristics and with soil depth.

Key words: coal mining, soil characteristics, plant cover.

#### Introduction

The mining industry brings drastic changes in the environment which cause a decrease in and/or a complete loss of biodiversity, as well as soil and air pollution (ALEKSEENKO *et al.*, 2017a; b; GIAM *et al.*, 2018; ETTEIEB *et al.*, 2020; AREFIEVA *et al.*, 2019). For dozens of years, environmental changes and subsequent problems related to the extraction of mineral resources have been a major issue worldwide (SKLENICKA *et al.*, 2004; ABAKUMOV *et al.*, 2012; ALEKSEENKO *et al.*, 2017a; b), and no soil formation has begun even after decades (ALEKSEENKO *et al.*, 2017a; b).

Affected areas around mining companies in Bulgaria are a serious

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg environmental problem. The area around the town of Pernik, affected by the mining industry, occupies a territory of about 30 000 ha (KIRILOV & BANOV, 2017). By destroying the flora and fauna, changing the soil characteristics and limiting the soil microbiocenoses, mining activities bring about dramatic changes in the physical and biological characteristics of the mining areas (CORBETT *et al.*, 1996).

Anthropogenically affected areas around mining industry sites in Bulgaria create serious environmental problems, which are particularly conspicuous on the territory of the Pernik coal mines (KIRILOV & BANOV, 2017; KACHOVA & FEREZLIEV, 2018).

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BANOV & PAVLOV (2014) point out that the nature of the technogenic substrates from coal mines requires a comprehensive approach to reducing their negative impact. The open-pit mining technique involves the removal of the layer above the coal seam with geological materials being accumulated in dump areas outside the mine. After mining activities cease, the geological substrates are returned to the extraction pits and reclamation activities are carried out to restore fertility. The techniques used vary depending on the objectives - whether reclamation is done for agricultural, forestry or recreational purposes (BANOV & PAVLOV, 2014; PAVLOV et al., 2015; BANOV & MARINOVA, 2016).

The soils adjacent to mining industry sites are characterized by high bulk density, low pH, low humus content, poor structure, deterioration of soil physical and hydraulic properties and presence of trace element input by anthropogenic activity (SEYBOLD *et al.*, 2004; TSOLOVA *et al.*, 2016; ATANASSOVA *et al.*, 2018).

The spontaneous placement of vegetation in post-mining terrains is extremely slow, heterogeneous communities are created in microhabitats (WHEATER & CULLEN, 1997; PRACH & PYSEK, 2001; TROPEK *et al.*, 2010; PATOVA *et al.*, 2015).

In various studies of disturbed terrains as a result of mineral extraction the state of the soil after recultivation is examined. In terms of vegetation, the most common tree species is mentioned, e. g black pine (DOICHINOVA, 2008). Often the vegetation is divided only into grass and tree - broadleaved and coniferous (HRISTOV et al., 2015), species with slower growth rate and lowered vitality (ALEKSEENKO et al., 2017b). Other studies mention some of the plant species, for example vegetation on old slag-heaps (mining waste dump sites) includes birches and ferns, spare grasses, Portulaca oleracea L., Spergularia rubra (L.) C. Presl, Dysphania botrys (L.) Mosyakin & Clemants and others. The recultivated land has been planted with e.g. red oaks, blue spruces and ornamental

shrubs; self-sowing trees such as black locusts have also spread into these areas (PAJURKOVÁ, 2017).

The purpose of this study is to identify the presence or absence of any differences among the soil characteristics of the technogenic soil formed as a result of coal mining activities under naturally occurring vegetation and under forestry reclamation.

#### Materials and Methods

*The object.* Technosols (FAO, 2014) occurring on the territory of "Maksim taban" deposit site, located to the Northwest next to Klepaloto and Baykusheva mahala districts in Pernik, Bulgaria, were the object of the study. The object of study is found in the Lower forest vegetation zone (0-600 m a.s.l.) of the Moesian forest vegetation area of Bulgaria (ZAHARIEV *et al.*, 1979).

Methods of study. Twenty-two profiles at depth of 0-100 cm were done at а representative plots under spontaneous revegetation (herbaceous plant cover sample plot 1, eleven profiles) and under forestry reclamation (black pine - Pinus nigra Arn. - sample plot 2, eleven profiles). A systematic sampling design was used (PETERSEN & CALVIN, 1996). The samples were taken at depths of 0-5 cm, 5-10 cm, 10-20 cm, 20-40 cm, 40-60 cm, 60-80 cm and 80-100 cm. The following soil characteristics were analyzed by using the respective methods: Bulk density (BD, g.cm<sup>-3</sup>), according to the DIN ISO 11272:1998, 2001; Total porosity (TP, %) by calculation of bulk density and relative density (LORRAINE & FLINT, 2002); Soil texture (Sand (2 mm - 63 μm), %; Silt (63 μm - 2 μm), %; Clay (2 μm), %), using the sedimentation method (ISO 11277), the soil texture classes have been determined according to Soil Survey Staff, 1975; Plant available water capacity (PAWC, mm), by a laboratory method, with the calculation of field capacity and permanent wilting point (DONOV et al., 1974); Soil acidity (pH in water extraction 1:5) measured potentiometrically by WTW 720 pH meter (ISO 10390:2002); Total Organic

Phytocoenotic relevès with area 4 m<sup>2</sup> for grass communities and 100 m<sup>2</sup> for forest communities were made in typical places in the sample plots. Phytocoenotic relevès include: floristic composition; total coverage of the phytocoenotic horizons; species abundance. Species identification was carried out according to the "Guide of Vascular Plants in Bulgaria" (DELIPAVLOV et al., 2011). The quantity of the species at phytocoenosis was assessed through abundance and cover scale (BRAUN-BLANQUET, 1964).

Data analysis. The data were statistically processed using Numbers (Apple Co., 2018), Excel (Microsoft Co., 2016) and SPSS (IBM Co., 2016) in Mac. The ANOVA test was used to demonstrate statistically significant differences (p-value). The ANOVA test was used to determine the significance (p-value) in regression relationships between soil indicators and depth. The minimum and maximum values (min, max), mean, median, standard deviation (SD) were identified.

#### **Results and Discussion**

*Plant cover.* The first plot is occupied by grass phytocoenosis (spontaneous revegetation), consisting of 22 plant species (Table 1). Most of them (77%) are perennial herbaceous plants, and the annuals and biannuals account for 18% and 5% respectively. There are predominantly representatives of the grasses (family Poaceae), as Poa bulbosa L. (3), Poa pratensis L.(2), Festuca valesiaca Scheich. Ex Gaud. (2), Arrhentherum elatius (L.) J. et C. Presl. (2). The Asteraceae family is also represented with more species but with low coverage such as: Cychorium inthybus L. (1), Senecio vulgaris L. (1) and others. The second plot is occupied by forest phytocoenosis (forestry reclamation), consisting of 14 plant species (Table 1). The first phytocoenotic horizon is with coverage 70% and consists mainly of *Pinus nigra* Arn. (5) and single *Fraxinus americana* L. (+). The coverage of the second phytocoenotic horizon is 20% and is represented by the species *Rosa canina* L. (2), *Prunus cerasifera* Ehrh. (2) and *Fraxinus ornus* L. (1). The third phytocoenotic horizon is with 80% coverage. Its composition includes grass species such as: *Geum urbanum* L. (2), *Galium aparine* L. (2), *Ballota nigra* L. (1), *Bryonia alba* L. (+), *Sambucus ebulus* L. (3), *Urtica dioica* L. (3), as well as undergrowth of *Crataegus monogyna* Jacq. (2) and *Quercus frainetto* Ten (1).

The second plot is occupied by forest phytocoenosis (forestry reclamation), consisting of 14 plant species (Table 1). The first phytocoenotic horizon is with coverage 70% and consists mainly of *Pinus nigra* Arn. (5) and single Fraxinus americana L. (+). The coverage of the second phytocoenotic horizon is 20% and is represented by the species Rosa canina L. (2), Prunus cerasifera Ehrh. (2) and Fraxinus ornus L. (1). The third phytocoenotic horizon is with 80% coverage. Its composition includes grass species such as: Geum urbanum L. (2), Galium aparine L. (2), Ballota nigra L. (1), Bryonia alba L. (+), Sambucus ebulus L. (3), Urtica dioica L. (3), as well as undergrowth of Crataegus monogyna Jacq. (2) and *Quercus frainetto* Ten (1).

Soil characteristics. The bulk density (Table 2) of the soil under spontaneous revegetation is higher than the one of the soil under forestry reclamation. There is a statistically significant difference (p=0.002) between the bulk density of the soil under spontaneous revegetation and the one of the soil under forestry reclamation. Both bulk densities under forestry reclamation and spontaneous revegetation increase with depth (Fig. 1). These changes are described using linear regressions with a very high degree of probability (p<0.0001 in both cases). Total porosity (Table 2) decreases with depth (Fig. 2, which is described using linear regressions with a very high degree of probability (p=0.001 under both types of plant cover), which is normal due to the increase in bulk density with depth, but the total porosity under herbaceous plant cover is lower than the total porosity under woody plant cover resulting in a statistically significant difference (p=0.0005).



Fig. 1. Relationship between soil depth and BD.



Fig. 2. Relationship between soil depth and TP.

The data on soil texture are shown in Table 2. The texture of the soils spontaneous revegetation ranges from sandy clay loam, through sandy loam, loamy sand to sand, and the soils under forestry reclamation fall within the following texture categories: sandy clay loam, sandy loam to loamy sand. There are statistically significant differences for the sand and silt fractions (p=0.009 and 0.002 respectively) under the different types of plant cover, but there isn't a statistically significant difference for the clay fractions (p=0.515) under forestry reclamation and spontaneous revegetation. The changes in the fractions of the soil texture with depth are shown in Fig. 3.



Fig. 3. Relationship between soil depth and Soil texture.

The changes in soil texture with depth are described using second-order polynomial regressions (Fig. 3), which are statistically significant (p<0.0001) for all fractions under the two types of phytocoenoses, with the silt fraction exception of the under spontaneous revegetation (p=0.40). Plant available water capacity (Table 2) is higher in the soils under spontaneous revegetation than in those under forestry reclamation, and the difference is statistically significant (p<0.0001).

The soil acidity (Table 3) of the studied forestry soils reclamation and under revegetation spontaneous ranges from moderately acidic to slightly acidic (according Н.-Р. to the von Blume classification), and it increases with depth (Fig. 4), which is described by second-order polynomial regressions with a high degree of probability (p<0.0001 under forestry reclamation and spontaneous revegetation).

These data are consistent with the data reported by other authors on the lower pH values in the root zone in soils formed after coal mining (PATOVA *et al.*, 2015). The soil acidity data show a statistically significant difference (p=0.03) between the two types
Table 1. Plant species on sample plots.

Plant species on sample plot 1	•	Plant species on sample ple					
Plant crosico	Abun-	Phytocoenotic	Abun-	Cover,			
Flant species	dance	horizon/Species	dance	%			
Potentilla argentea L.	2	I phytocoenotic horizon		70%			
Festuca valesiaca Scheich. Ex Gaud.	2	Pinus nigra Arn.	5				
Poa pratensis L.	2	Fraxinus americana L.	+				
Cychorium inthybus L.	1	II phytocoenotic horizon		20%			
Sanguisorba minor Scop.	1	Rosa canina L.	2				
Convolvulus arvensis L.	1	Prunus cerasifera Ehrh.	2				
Plantago lanceolata L.	2	Fraxinus ornus L.	+				
Arrhentherum elatius (L.) J. et C. Presl.	2	III phytocoenotic horizon		80%			
Eryngium campestre Vis. Et Pancic	1	Geum urbanum L.	2				
Senecio vulgaris L.	1	Galium aparine L.	2				
Poa bulbosa L.	3	Clematis vitalba L.	2				
Anthemis arvensis L.	1	Ballota nigra L.	1				
Achillea millefolium L.	1	Bryonia alba L.	+				
Cardaria draba (L.) Desv.	1	Crataegus monogyna Jacq.	2				
Hordeum murinum L.	1	Sambucus ebulus L.	3				
Vicia grandiflora Scop.	1	Urtica dioica L.	3				
Erodium cicutarium (L.) L ,Herit	1	<i>Quercus frainetto</i> Ten.	2				
Trifolium incarnatum L.	+						
Cerastium banaticum (Roch.) Heuffel	+						
Scorzonera laciniata L.	+						
Tlaspi arvense L.	+						
Lepidium campestre (L.) R. Br.	+						

**Table 2.** Physical characteristics of studied soils. Legend: For abbreviations of the names of soil characteristics, see part "Material and Methods". \* For the 0-100 cm layer.

	Depth, cm	n		BD, g.cm <sup>-3</sup>	TP, %	Sand, %	Silt, %	Clay, %	PAWC, mm
			Mean	0.92	61.04	64.14	19.16	16.70	
			Median	0.92	61.06	64.03	22.44	13.24	
	0-5	11	SD	0.02	0.47	2.38	9.94	9.99	
			Min	0.90	60.37	60.40	0.30	4.49	
			Max	0.95	61.84	66.91	30.40	34.35	
			Mean	1.02	58.40	72.94	11.43	15.63	
ы			Median	1.02	58.43	72.37	7.82	20.05	
ove	5-10	11	SD	0.04	1.21	2.07	7.87	8.54	
ţc			Min	0.97	56.84	70.55	1.24	2.58	
an			Max	1.09	60.42	76.33	22.00	27.88	
[d g			Mean	1.10	55.83	82.18	8.73	9.09	
snc			Median	1.10	55.76	81.79	7.58	9.40	
Cec	10-20	11	SD	0.01	0.28	1.48	4.97	5.18	
rba			Min	1.08	55.52	80.37	1.08	0.60	
hei			Max	1.12	56.33	84.79	15.55	18.55	
er	20-40	11	Mean	1.11	54.49	82.38	14.64	2.97	152.71*
pu			Median	1.11	54.49	82.60	16.00	1.40	152.79*
D			SD	0.01	0.25	0.30	0.00	0.30	1.79*

			Min	1.10	54.19	82.03	16.00	1.00	150.01*
			Max	1.11	54.98	83.00	16.00	1.97	154.78*
			Mean	1.13	53.09	82.56	16.00	1.44	
			Median	1.13	53.10	82.51	16.00	1.49	
	40-60	11	SD	0.01	0.62	0.24	0.00	0.24	
			Min	1.12	52.08	82.29	16.00	1.08	
			Max	1.14	53.91	82.92	16.00	1.71	
			Mean	1.20	51.94	82.59	14.00	3.41	
			Median	1.20	52.19	82.92	14.00	3.08	
	60-80	11	SD	0.01	0.87	0.86	0.00	0.86	
			Min	1.19	50.29	81.47	14.00	2.21	
			Max	1.21	52.99	83.79	14.00	4.53	
			Mean	1.28	48.76	86.53	10.90	2.57	
			Median	1.27	48.64	86.47	11.00	2.85	
	80-100	11	SD	0.02	0.56	0.31	0.60	0.61	
			Min	1.25	48.07	86.14	10.04	1.42	
			Max	1.30	49.90	86.97	11.96	3.28	
			Mean	0.81	64.14	68.88	11.25	19.86	
			Median	0.81	64.23	69.46	10.99	19.39	
	0-5	11	SD	0.02	0.43	1.66	9.36	9.61	
	00		Min	0.78	63.32	65.88	0.11	0.24	
			Max	0.84	64.69	71 11	29.73	33.12	
			Mean	0.90	60.34	76.80	12 35	10.85	
			Median	0.90	59.84	75.87	11.00	9 79	
	5-10	11	SD	0.07	1/3	2 30	7 58	7.02	
	5-10	11	Min	0.00	58 74	74.28	0.87	0.23	
			Max	1.00	63.22	79.82	24.93	19.31	
			Mean	1.00	58.85	80.32	11 30	8 38	
			Modian	1.00	50.05	80.41	11.50	4.25	
	10 <b>2</b> 0	11		0.05	1.80	2 52	714	7.55	
er	10-20	11	Min	0.03	56.42	76.08	2 20	0.72	
<b>V</b> 0			Max	1.02	61.90	<u> </u>	2.20	10.12	
ц,			Maar	1.00	E0.02	75.44	20.95	19.13	150.94
lar			Mean	1.02	59.02	75.44	21.05	2.91	159.84
ур	20.40	11	Median	1.00	0.59	74.39	23.78	1.90	160.04
po	20-40	11	<u>5D</u>	0.01	0.59	0.30	0.25	0.46	2.76
ΜO			Min	0.99	58.27	74.00	23.12	1.35	153.78
er			Max	1.02	59.99	74.85	23.93	2.60	162.91
nd			Mean	1.10	56.09	76.49	22.47	1.04	
D	10 (0	44	Median	1.10	56.20	76.48	22.45	0.99	
	40-60	11	SD	0.00	0.64	0.32	0.39	0.50	
			Min	1.10	55.09	76.07	22.02	0.19	
			Max	1.11	56.85	76.92	23.00	1.74	
			Mean	1.21	53.21	80.50	18.38	1.12	
			Median	1.21	52.67	80.46	18.30	1.23	
	60-80	11	SD	0.01	1.10	0.28	0.33	0.53	
			Min	1.19	52.12	80.12	18.02	0.40	
			Max	1.23	54.92	80.98	18.92	1.79	
			Mean	1.26	48.94	78.53	20.49	0.98	
			Median	1.26	49.16	78.60	20.49	1.03	
	80-100	11	SD	0.01	0.66	0.31	0.28	0.39	
			Min	1.25	48.03	78.09	20.13	0.23	
			Max	1.27	49.97	78.87	20.90	1.58	

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of phytocoenoses. Total organic carbon and TKN (Table 3) decrease with depth (Fig. 5) and these relationships are described by polynomial regressions for both indicators with a high degree of probability (p<0.0001).



**Fig. 4.** Relationship between soil depth and pH(H<sub>2</sub>O).



Fig. 5. Relationship between soil depth and TOC and TKN.

However, the TOC and TKN contents are higher under forestry reclamation than under spontaneous revegetation, and the differences are statistically significant (p=0.003 and p<0.0001 respectively).

Available phosphorus (Table 3) decreases with depth under both types of plant covers (Fig. 6), where the changes in depth are described by second-order polynomial regressions with a high degree of probability (p<0.0001 under both types of phytocoenoses). There isn't a statistically significant difference (p=0.425) between the content of available phosphorus under forestry reclamation and under spontaneous revegetation.



Fig. 6. Relationship between soil depth and  $P_2O_5$ .

The availability of potassium (Table 3) in the studied soils under forestry reclamation decreases with depth (Fig. 7, where the decrease with depth is described by secondorder polynomial regressions with a high degree of probability (p<0.0001 under both types of phytocoenoses). The content of available potassium is different (statistically significant difference p<0.001) under forestry reclamation and spontaneous revegetation.



**Fig. 7**. Relationship between soil depth and K<sub>2</sub>O.

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**Table 3.** Chemical characteristics of studied soils. Legend: For abbreviations of the names of soil characteristics, see part "Material and Methods".

	Depth, cm	n		pH(H <sub>2</sub> O)	TOC, %	TKN, %	P <sub>2</sub> O <sub>5</sub> , mg.100g <sup>-1</sup>	K <sub>2</sub> O, mg.100g <sup>-1</sup>
			Mean	5.68	8.63	7.284	8.95	60.24
			Median	5.72	8.46	7.269	8.90	60.73
	0-5	11	SD	0.08	0.48	0.240	0.59	1.52
			Min	5.54	8.00	7.009	8.10	58.22
			Max	5.76	9.43	7.914	9.92	62.45
			Mean	5.36	7.11	4.988	8.11	57.97
			Median	5.33	7.01	5.202	8.11	57.83
	5-10	11	SD	0.12	1.15	0.586	0.14	1.32
			Min	5.21	5.61	4.006	7.89	56.42
			Max	5.54	8.98	5.740	8.38	59.80
			Mean	5.89	3.12	1.477	5.16	52.24
			Median	5.88	3.02	1.620	5.06	52.09
	10-20	11	SD	0.05	0.55	0.317	0.85	1.61
			Min	5.80	2.56	1.036	4.21	50.22
			Max	5.97	3.97	1.904	6.77	55.03
			Mean	6.02	2.45	1.551	3.79	33.62
			Median	6.00	2.23	1.466	3.49	32.16
	20-40	11	SD	0.13	0.32	0.301	0.32	1.70
			Min	5.87	2.07	1.132	3.05	30.14
			Max	6.40	2.92	1.989	3.98	34.94
			Mean	6.31	2.45	1.500	3.42	22.90
			Median	6.30	2.24	1.365	3.45	23.00
	40-60	11	SD	0.05	0.32	0.316	0.31	1.48
uo			Min	6.24	2.11	1.072	3.06	20.01
ati			Max	6.40	2.96	1.997	3.88	24.82
gel			Mean	6.39	1.32	1.059	2.48	12.60
eve			Median	6.38	1.23	1.105	2.43	12.56
S TC	60-80	11	SD	0.14	0.29	0.358	0.28	0.87
no			Min	6.21	1.03	0.551	2.07	11.12
ane			Max	6.59	1.92	1.774	2.97	14.00
nti			Mean	6.57	1.54	0.760	2.35	11.25
spc			Median	6.61	1.49	0.842	2.54	11.27
G	80-100	11	SD	0.11	0.22	0.179	0.45	0.52
pu			Min	6.43	1.20	0.516	1.56	10.17
D			Max	6.77	1.83	0.972	2.73	11.93
			Mean	5.17	10.67	11.886	8.89	31.59
~			Median	5.18	10.60	11.815	8.63	31.61
ior	0-5	11	SD	0.05	0.34	0.582	0.61	1.21
nal			Min	5.10	10.20	11.043	8.20	30.02
lar			Max	5.25	11.20	12.950	9.77	33.57
rec			Mean	5.40	9.18	9.823	7.78	27.49
ry			Median	5.37	9.21	9.642	7.83	27.46
est	5-10	11	SD	0.06	0.25	0.587	0.41	0.86
for			Min	5.32	8.85	9.069	7.09	26.36
ler			Max	5.48	9.50	10.606	8.48	28.95
Jnd	10-20	11	Mean	5.71	4.78	7.075	6.53	23.47
			Median	5.68	4.57	7.078	6.56	23.10
			SD	0.17	0.49	0.488	0.36	0.95

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$ \begin{array}{c c c c c c c c c c c c c c c c c c c $								
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $			Min	5.52	4.29	6.151	6.01	22.05
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$			Max	6.03	5.81	7.761	7.00	24.93
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$			Mean	6.09	3.71	2.79	4.79	21.15
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$			Median	6.11	3.50	2.34	4.65	21.06
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	20-40	11	SD	0.06	0.76	1.27	0.59	1.54
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $			Min	6.02	3.10	2.15	4.04	18.52
$\begin{array}{c c c c c c c c c c c c c c c c c c c $			Max	6.20	5.81	6.56	6.37	24.93
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$			Mean	6.16	3.58	2.241	4.62	17.00
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$			Median	6.16	3.73	2.276	4.69	17.10
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	40-60	11	SD	0.03	0.34	0.176	0.26	1.45
$\begin{array}{c c c c c c c c c c c c c c c c c c c $			Min	6.12	3.12	2.018	4.20	15.07
$\begin{array}{c c c c c c c c c c c c c c c c c c c $			Max	6.20	3.98	2.532	4.96	19.63
$\begin{array}{c c c c c c c c c c c c c c c c c c c $			Mean	6.29	2.49	1.570	2.31	16.52
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$			Median	6.27	2.64	1.493	2.25	16.66
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	60-80	11	SD	0.07	0.30	0.261	0.23	0.73
Max         6.40         2.93         1.977         2.75         17.7           Mean         6.31         2.43         0.771         1.61         12.9           Median         6.34         2.38         0.741         1.65         13.7           Median         6.34         2.38         0.741         1.65         13.7           Min         6.21         2.01         0.536         1.27         10.7           Max         6.39         2.92         0.985         1.96         14.7			Min	6.22	2.03	1.154	2.05	15.63
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$			Max	6.40	2.93	1.977	2.75	17.75
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$			Mean	6.31	2.43	0.771	1.61	12.98
80-100         11         SD         0.07         0.29         0.136         0.22         1.33           Min         6.21         2.01         0.536         1.27         10.33           Max         6.39         2.92         0.985         1.96         14.33			Median	6.34	2.38	0.741	1.65	13.29
Min6.212.010.5361.2710.7Max6.392.920.9851.9614.7	80-100	11	SD	0.07	0.29	0.136	0.22	1.31
Max 6.39 2.92 0.985 1.96 14.7			Min	6.21	2.01	0.536	1.27	10.18
			Max	6.39	2.92	0.985	1.96	14.79

The obtained data from the physicochemical studies show that technogenic soils do not have the most favourable characteristics. This is consistent with data reported by other authors (ZHELEVA et al., 1995) despite the considerably high values of TKN and TOC, which are most likely due to the nature of soil formation from coal mining spoils. This is also consistent with data reported by other authors (KOSTOVA et al., 2013). The soil physicochemical characteristics could be a limiting factor for plant diversity and normal plant development (BOGDANOV, 2018a; b) which is the reason for the long period of time (up to 25 years) necessary for plant cover restoration (ŘEHOUNKOVÁ, 2007). The forestry reforestation of post-mining territories is a significantly more preferable reforestation method than spontaneous revegetation (PRACH et al., 2011; KRÜMMELBEIN *et al.*, 2012). Nevertheless, spontaneous revegetation results in a greater species diversity (HODAČOVÁ & PRACH, 2003), which was also confirmed by the present study.

### Conclusions

The bulk density of the soil under spontaneous revegetation cover is

statistically significantly higher than the bulk density under forestry reclamation. The total porosity under spontaneous revegetation is lower than the total porosity under forestry reclamation, where the difference is statistically significant (p=0.0005). The components of the soil texture (sand and silt) are statistically different under both types of plant cover. The soils under spontaneous revegetation are characterized by higher PAWC compared to the soils under forestry reclamation (p<0.0001).

The phytocenosis dominated by black pine (Pinus nigra Arn.) results in a decrease in the soil reaction –  $pH(H_2O)$  compared to the herbaceous phytocoenosis, which is statistically significant. The high levels of TOC and TKN are due to the technogenic nature of the soils formed as a result of coal mining activities, but higher levels of TOC and TKN are found in soils under forestry reclamation than in soils under spontaneous revegetation (the difference is statistically significant). The available phosphorus and potassium change (decrease) with depth, most likely due to their which is accumulation (mainly in the surface layer) as

a result of the decomposition of organic matter. There aren't any statistically significant differences between the available phosphorus found under the two phytocoenoses. The presence of available potassium is statistically different under both types of plant covers, where it is higher under spontaneous revegetation than under forestry reclamation.

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# Pedo-chemical Perturbations in Soils from Green Ecosystems of the Sofia City (Bulgaria)

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Abstract. In view of the role of cities for ensuring a favorable living environment, it is important to study the urban soils since they are formed and developed under the impact of different by degree and type anthropogenic perturbations. Pedo-chemical studies of urban soils may capture the evolution of different soil components and reveal the different stages of soil matrix transformation. Using pedogenic and chemical analyses, the present article aims to present the trends of perturbations of the mineral and organic matrix of urban soils located along the direction of increasing gradient of urbanization in cursory investigated soil zones belonging to the residential and industrial districts of the Sofia city (Bulgaria). The results obtained show that anthropogenic alterations are predominantly associated with morphological reorganization of some soils rather than soil compaction and structure loss. The increase of exchangeable hydrogen content provoked by fulvic acid production and leaching can be attributed to the current natural perturbations. Anthropogenically induced chemical changes could be linked with increase of the mineral N flux and high ammonium content which will influence the existing acid-base status of Sofian soils.

**Key words**: urban soils; exchange capacity; aqua regia; humic acids specification; nitrogen fluxes.

### Introduction

Globalization processes that started in beginning of 20th century the the transformed cities in a unique assemblage of natural, ethnic, aesthetic, production, commercial, social and tourist symbols but also contributed to the increase of chemical vulnerability soils. The enormous of gathering of population in the cities

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg influences all the environmental components and substantially changes the soil cover. Usually, urban soils significantly differ in properties and organization from natural soils and should be properly managed (LEHMANN & STAHR, 2007). Specific features of urban soils are related to the variation of soil acidity and sorption capacity, enrichment with organic matter mostly in the form of

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non-hydrolysed carbon, low water holding capacity, strong compaction and heavy metals contamination (DOICHINOVA, 2006; DOICHINOVA & ZHYANSKI, 2013).

Metrics such as pH and cation exchange capacity are the most preferable and accessible indicators for initial assessment of chemical status of soils (THOMAS, 1996; DINEV, 2011; NIKOVA, 2009). It is a wellknown fact that the interpretation of data on physico-chemical processes running in the soil adsorbent is a key tool for the sustainable management and protection of soils (HENGLEIN, 1993). Organic matter accumulation and transformation as prime soil-forming processes in the pedosphere are also widely discussed (SARDANS et al., 2012; CHENU et al., 2015; BŁONSKA & LASOTA, 2017; PIERSON, 2017; FROUZ & VINDUŠKOVÁ, 2018; ABDELRAHMAN et al., 2018; FILCHEVA, 2018; STUMPF et al., 2018). The issue of global warming forces the studies, which increase the knowledge of domains, resistance and cycling of chemical elements in and out of ecosystems (FINZI et al., 2011; DELGADO-BAQUERIZO et al., 2013; TSOLOVA et al., 2014; PARTON et al., 2015; YUAN & CHEN, 2015; TAN & WANG, 2016; JIAO et al., 2016).

The scarce data on urban soils in Bulgaria has aroused the interest in studying their pedochemical characteristics as information carriers on modern and relict of formation processes soil and transformation. By studying the cation exchange capacity, base saturation level, content of main exchangeable cations, content and forms of essential nutrients including organic carbon, the present article aimed to present the trends of transformation of mineral and organic matrix of urban soils located along the direction of increasing of gradient urbanization in cursory investigated soil zones belonging to the residential and industrial districts of the Sofia city (Bulgaria).

#### **Materials and Methods**

*Location and morphogenesis of studied soils* Data on 6 soil types, located at the Eastern part of the Sofia city are represented in present publication. All soils form ecosystems with recreational significance and some of them have not been previously studied. They are distinguished for the following morphogenesis:

Anthropogenically overlapped moderately leached Smolnitsa, loamic is characterized by profile 1 located in "Mladost" residential region (Fig. 1). This soil was formed as a result of urbanization and occupies previously unexplored soil zone. In fact, moderately leached Smolnitsa borders this highly urbanized zone according to the previous studies (ACHKOV et al. 1972). The original soil, Smolnitsa (named after organic clays, smolnitsas composing soil) is overlapped by layers of earth calcaric masses, mixed with urban gravel. waste, pebbles and Profile development morphological and organization of new soil includes differentiation of organic matter, which resulted in a bimodal distribution and 3 representative horizons for soil morphogenesis:  $A_{hk}$  (0-15 cm) -  $C_{1k}$  (15-65 cm) - A<sub>b</sub> (65-110 cm).

WRB classification of soil: Urbic Technosol (Eutric, Loamic, Humic, Transportic) over Pellic Vertisol (Chernic, Endocalcaric). Profile 2 characterizes Technogenic soil, moderately deep, loamic, moderately stony (15% coarse surface fragments' content in A<sub>h</sub> horizon, Fig. 2), classified as Urbic Technosol (Amphyskeletic, Calcaric, Mollic, Transportic). This soil is formed by massive pilling of earth calcaric materials onto the moderately leached Smolnitsa, loamic during the "Mladost" district construction. Profile development and morphological organization are results of surface accumulation of organic matter and slow weathering of subsoil that is strongly mixed with urban building artefacts - these processes lead to the formation of a three-layered profile:  $A_{hk}$  (0-21 cm) -  $C_{1k}$  (21-52 cm) - C<sub>2k</sub> (52-85 cm). Parent materials are Quaternary brown alluvial clays and Pliocene sands, usually calcaric (YANEV et

al., 1992; 1995; BOJINOVA-HAAPANEN, 2014). Profile 3 illustrates the morphogenesis in moderately leached Cinnamon forest soil, slightly moderately loamic, to eroded / Chromic Endocalcic Luvisol (Clayic, Differentic, Humic, Profondic)/ located in the periphery of the "Mladost" residential region (Fig. 3). Profile development is a pedogenetic differentiation result of (illuviation) of clay content by depth and leaching of base cations. Morphologically, these processes form the following horizons:  $A_h$  (0-10 cm) -  $B_t$  (10-35 cm) -  $B_{t2}$  (35-72 cm) - $B_{t\kappa}$  (72-86 cm) -  $C_k$  (86-120 cm). The soilforming materials according to YANEV et al., (1992; 1995) and BOJINOVA-HAAPANEN, (2014) are Quaternary diluvial-colluvial materials (non-sorted gravel, boulders and clay-sandy deposits) and Pliocene sediments (yellow-rusty clays with layered structure, usually calcaric, clays with sandy matrix and gravel). Profile 4. Alluvial soil, moderately deep, slightly stony /Hypereutric Fluvisol *Somerimollic*)/ distributed (Loamic, in "Drujba" industrial region (fig 4). This soil is located in an over flooded terrace of the Iskar River, in a virgin district, next to the "Sofia Iztok" Thermal-electric Power Plant. Profile development is limited by coarse fragments abundance in subsoil and therefore the soil formation processes involve only the uppermost 15 cm. They resulted in morphologically simple profile organization:  $A_h$  (0-15 cm) –  $C_1$  (15-40 cm) – C<sub>2</sub> (40-80 cm). Parent materials are mostly large gravels and boulders with a sandy matrix which lie onto a Pliocene stratum composed of sands and grey or green coloured clays (YANEV et al., 1992; 1995; BOJINOVA-HAAPANEN, 2014). Fig. 5 shows profile 5 and Alluvial meadow soil, deep */Hypereutric* Fluvisol (Epiclayic, Endoloamic, Pachic)/ located in "Drujba" residential region. It is formed within the flooded terrace of the Iskar River by fineparticle alluvial sediments of a Quaternary and Pliocene origin (ACHKOV et al., 1972). Profile development and morphological organization is also marked by surface accumulation of organic matter and lithological clay differentiation by depth: A<sub>h</sub> (0-30 cm) - A<sub>2</sub> (30-55 cm) - C<sub>1</sub> (55-105 cm) - $C_2$  (105-155 cm).Profile 6 is in strongly leached Smolnitsa, super deep, moderately clayey / Pellic Vertisol (Pantochernic, *Hupereutric, Relictigleyic)/* distributed in "Mladost" residential region (fig 6). This soil occupies the higher part of the previously unexplored soil zone and neighbours the moderately leached Smolnitsa. This pedon also consists of organic clays (Smolnitsa), which foster the super deep A-horizon development (reaching up to 165 cm depth). Humus horizon directly lies on parent materials - grey-brown Pliocene clays containing calcareous nuts (YANEV et al., 1992; 1995; BOJINOVA-HAAPANEN, 2014).

#### Chemical studies

# The cation exchange capacity

The cation exchange capacity (Equation 1), the base saturation level and the content of the main exchangeable cations in soils were determined under the GANEV & ARSOVA (1980) method. This method determines the contribution of both the permanent, preferential charges (on basal surfaces,  $T_{CA}$ ) and variation charges of soil colloids (basically pH dependent exchange including the lateral surfaces,  $T_A$ ) to the cation exchange capacity by titration of soil extracts (obtained by mixed solution of 1.0 n sodium acetate and 0.2 n potassium maleate having pH 8.25) with 0.04 n sodium hydroxide solution in the presence of determine  $T_A$ phenolphthalein to and subsequent titration of the above eluate with 0.04 n complexon III (after dilution up to 200 cm<sup>3</sup> with deionized water and addition of 10 cm<sup>3</sup> of triethanolamine and 2 cm<sup>3</sup> of 5.0 n potassium hydroxide solution, non-carbonate to achieve pH 12-13) in the presence of chromium-blue to determine  $T_{CA}$  (Equation 1):

$$T_{8.2} = T_{CA} + T_A (cmol/kg)$$
(1)

Exchangeable Al: in 1.0 n calcium chloride filtrate obtained as a soil:extractant ratio 1:25 by titration with 0.04 n sodium

hydroxide in the presence of phenolphthalein. When soil pH <4.0, exchangeable hydrogen ions,  $H_A$ , should be determined first - 1 drop of methyl orange is added to the calcium chloride filtrate and titrated with 0,04 n sodium hydroxide and then this solution is treated to determine the exchange aluminium.

Total acidity (exchange  $H_{8.2}$ ) is calculated by the Equation 2:

$$exch.Al + H_A = exch.H_{8.2} (cmol/kg)$$
 (2)

Exchangeable calcium: 50 cm<sup>3</sup> of the mixed sodium-acetate and potassium-maleate solution is diluted with deionized water to 100 cm<sup>3</sup>. Then 5 cm<sup>3</sup> of triethanolamine (1: 1), 1 cm<sup>3</sup> 5.0 n potassium hydroxide solution and a chromium blue (calcon) are added to achieve intensive purple-red colouring. The solution is titrated slowly with 0.01 n complexon III to a deep blue colour.

Sum of exchangeable calcium and magnesium: A new 50 cm<sup>3</sup> of the filtrate is filled up with deionized water to about 100 cm<sup>3</sup>. Five cm<sup>3</sup> of triethanolamine (1: 1) and a solid mixture of eriochrome black are added to reach pH of 9.5-10.0 and titrated slowly with 0.01 n complexon III to a deep blue colour. Exchange magnesium is determined by the difference between the sum of two alkaline earth cations and exchange calcium.

The base saturation level (V) is calculated in percentages as the difference between the magnitude of total cation exchange capacity ( $T_{8.2}$ ) and total acidity (exchange  $H_{8.2}$ ) relative to the magnitude of total cation exchange capacity.

#### Humic substances content and composition

The content of extractable humus fractions was determined using the Kononova-Belchikova method (FILCHEVA & TSADILAS, 2002) in four extracts at soil: solution ratio 1:20. Total organic carbon was determined by the modified dichromate oxidation method (the oxidation of the soil sample with  $0.4 \text{ N K}_2\text{Cr}_2\text{O}_7$ and concentrated  $\text{H}_2\text{SO}_4$  in a ratio 1:1 at 120  $^{\circ}\text{C}$ for 45 min. in the presence of  $\text{Ag}_2\text{SO}_4$  followed by a titration with 0.2 N Mohr's salt). Humus content is calculated by multiplication of organic carbon content with the coefficient 1.724.

Content of humic (HA) and fulvic (FA) acids – in a mixed solution of 0,1 M  $Na_4P_2O_7$  and 0,1 M NaOH, and separation of FA by 0,5 M  $H_2SO_4$  as an acidifying agent.

Content of free or linked to sesquioxides humic and fulvic acids representing the potentially mobile HA and FA – extracted with 0,1 M NaOH.

Content of the low molecular (aggressive) fraction of fulvic acids – in extracts with 0,05 M H<sub>2</sub>SO<sub>4</sub>.

Optical hallmarks  $(E_4/E_6)$  are determined in HA-fraction as a ratio of the optical densities at 465 and 665 nm.

#### Elemental and speciation assays

Content of ferromagnesian trace elements was determined after sample mineralization with aqua regia (ISO 11466:1995) via AAC (ISO 11047:1998) on a Perkin-Elmer 2100.

Total nitrogen content was quantified by the modified Kjeldahl method (BDS ISO 11261:2002) and the main mineral nitrogen forms – by procedure of BREMNER & KEENEY (1965).

Carbonate content was measured following ISO 10693:1995 protocol which reproduces the Scheibler method.

## Sample pre-treatment and pH determination

Soil samples were pre-treated according to BDS ISO method (11464:2012) and pH was measured in 2.5:1 water soil suspension (1 part soil and 2.5 parts deionized water) according to the protocol given by GANEV & ARSOVA (1980).



Fig. 1. View and location of profile 1 in overlapped moderately leached Smolnitsa.



Fig. 2. View and location of profile 2 in Technogenic soil.



Fig. 3. View and location of profile 3 in moderately leached Cinnamon forest soil.

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Fig. 4. View and location of profile 4 in Alluvial soil.



Fig. 5. Location and view of profile 5 in Alluvial meadow soil.



Fig. 6. Location and view of profile 6 in strongly leached Smolnitsa.

#### **Results and Discussion**

Soils created as a result of urbanization (profiles 1 and 2) are characterized with very slightly alkaline reaction (Table 1) and middle content of carbonates (in the interval 3-5%). They have middle colloidal activity  $(T_{82} \text{ from 30 to 45 cmol/kg})$ , according to the classification given by GANEV (1990) and sorption interactions, transforming the slightly acidic positions of mineral colloids into the hydrogen-acidic complex. Due to the presence of carbonates, this acidic-hydrogen form of T<sub>A</sub>, i.e., exchangeable retention of hydrogen cations upon the slightly acidic positions of mineral colloids can be considered physico-chemical characteristic of soils. which originated from the hydromorphic stage in their genesis and water self-ionization catalytic effect. Signs of hydromorphism are evident in many soil characteristics (clay mineralogy, organic carbon state and transformation) and leave a mark on their current genesis. Concerning clay mineralogy, the predominance of mixed-layered smectite-vermiculite structures was found in Pliocene clays distributed in the "Mladost" residential district (BOJINOVA-HAAPANEN, 2014) which confirm our data and suggestions. This mineral might be indirectly identified through the typically high total magnesium content (or "pseudo" total content determined in the aqua regia extract, Table 1). The sorption capacity which is not very high also suggests a lack of pure smectites and together with transformation of biotite Smolnitsas) (that is present in into vermiculite could explain CEC values. Magnesium, despite of its high content, is not the main exchangeable cation and does not cause magnesium salinization.

Hydromorphism and further transformation of the mineral matrix turn hydrogen in the second abundant exchange ion after calcium. As a result of high hydrolytic acidity (exch.  $H_{8,2}$ ) the base saturation level is under 93%, which is considered a critical minimum for lack of deleterious acidity in soils (PALAVEEV &

TOTEV, 1985; TRENDAFILOV, 1992). The deleterious acidity in soils is not accompanied bv toxic aluminium availability (exch. Al) and possibility for Al desorption in the soil solution - it is limited only to destabilizing role and chemical activity of exchangeable hydrogen.

The content of hydrolytic acidity in (profile 2) sharply Technogenic soils decreases under 21 cm depth (over 2 times) and that positively influences subsoil base saturation status (V). Although the sorption potential in surface horizon of Technosol is generally lower, its hydrolytic acidity content is close to that in topsoil of the overlapped Smolnitsa. Obviously, the hydrolytic acidity in topsoil of these newlyformed soils originated from biogenic processes associated with well-developed meadow vegetation (TSOLOVA & TOMOV, 2018) rather than clay mineralogy, because this is the only profile wherein the vermiculite is not occurred (as we mentioned above the higher content of magnesium than calcium is indicative for vermiculite presence).

The carbonates in the A<sub>h</sub> horizon of moderately leached Cinnamon forest soils (profile 3) take part in neutralization of acid products generated by biodegradable processes. This gradually leads to their depletion and acidification of soil environment to pH 5.9. The strong linear correlations shown on fig 7 reveal the prevalence of exchange hydrogen cations onto slightly acidic positions and pH dependence on the exchange hydrogen content.

The exchangeable acidity (exch. Al) also occurs in topsoil in a concentration that could be toxic for many pasture species (CORANGAMITE REGION "BROWN BOOK"). It usually appears as a result of acid destruction of clay minerals, which can be seen in the ratios:  $T_{8,2}$  in  $A_h/T_{8,2}$  in  $C_k < 1$ and  $T_{8,2}$  in  $B_{t\kappa}/T_{8,2}$  in  $C_k > 1$  (GANEV, 1990). The colloid degradation in  $A_h$  is moderate according to the classification given by GANEV (1990) and shows that this process is still running slowly. The moderately leached Cinnamon forest soil also has moderately high sorption capacity but smaller buffer potential which, as it was mentioned above, decreases as a result of increasing acidity in surface horizons.

Alluvial soils from the Iskar river valley (profiles 4 and 5) are formed of sediments with different coarse fragments contents. The stonier soils (profile 4) are moderately colloidal ( $T_{8,2}$  from 20 to 30 cmol/kg) with high neutralizing potential (V over 80 cmol/ kg). Their acidic systems also saturate the variation charges on colloidal surfaces with hydrogen and evoke slightly acidic reaction (according to ATANASOV et al., 2009 classification) - 6.1-6.9. Studied parameters decrease downwards the profile depth, the distribution resembling in some normally developed, genetically old soils and do not follow the lithological differences between separate horizons.

Alluvial-meadow soils (profile 5) are moderately colloidal ( $T_{8,2}$  from 30 to 45 cmol/kg) and mostly moderately acidic (pH 5,1-6,0). They have the highest hydrolytic acidity among studied soils and respectively the lowest base saturation level within the whole depth (Table 1). The base saturation level in the interval 77-86% defines a middle range of deleterious acidification of soils according to the classification scheme set by Bulgarian legislation (ORDINANCE Nº 4).

The features of strongly leached Smolnitsa (profile 6) reveals the evolution of soils distributed in the peripheral part of this previously unexplored soil zone. They are neutral, highly colloidal soils with high neutralizing potential which is slightly lower in A' and A'' as a result of the listed hypergenic processes. These soils are distinguished with small amount of slightly acidic charges  $(T_A)$  in the humus horizon which is presumably due to the slow in situ transformation of biotite into vermiculite, which suggests a lack of defects in the crystal structures (due to the lack of transportation) and a small formation of lateral surfaces yet. These processes can be

more clearly observed in the last sub-horizon where the content of slightly acidic positions smallest and the content of is the exchangeable magnesium - the highest (Table 1). The content of "pseudo-total" magnesium (from 565 to 607,5 mg/kg) is higher than the content of calcium (420-560 mg/kg) within the whole profile depth which evidences for the strong leaching of carbonates probably in the form of iron carbonates due to the low content of "pseudo-total" iron too (from 1,06 to 1,30%). These data support the opinion of STRANSKI (1936) that a hidden process of podsolization takes place in the black Sofian soils, since it can't be diagnosed by usual morphological features. This phenomenon is also observed by NIKOVA & TSOLOVA (2018) in arable Smolnitsas from the Sofia valley.

# Organic matrix hallmarks

Surface horizon of the Overlapped (Buried) Smolnitsa (profile 1, Table 1) is very rich in organic matter (5.52% humus), despite of soil recent creation (about 45 years ago). The humus is of Mull type, abundant in humic acids ( $C_{HA}/C_{FA} > 2,0$ ) which dominated along the entire depth. Humic acids are strongly condensed  $(E_4/E_6)$ = 3.87), very hydrophobic and slightly mobile polymers. They are strongly bound to the mineral matrix having in mind the dominance of Ca-humates (100%). The low molecular (aggressive) fraction of fulvic acids is also present in descending concentrations (from 0,8 in topsoil to 0,4 g/ kg in buried horizon). The ratios of C:N (14.04-10.40) in this epipedon indicate middle to high enrichment of hydrocarbons with (Fig. 8). These N-dressed Ν compounds are active source of ammonium-N and may provoke the soil toxicity (BRITTO & KRONZUCKER, 2002). Values obtained for main mineral forms of N illustrate this trend (fig 8) considering a principally low content of nitrate-N in soils.

The basic features of organic matter (OM) in surface horizon of Technogenic soils (profile 2) are: morphologically homogeneous humus system of well humificated organic matter (C<sub>HA</sub>: C<sub>total</sub> x 100, in % = 17.3%) formed by soil-biomes interactions and medium humus content (2.59%); Rhizomull type of humus wherein the very strongly condensed and stable humic acids ( $C_4/E_6 = 3,50$ ) are absolutely prevalent ( $C_{HA}/C_{FA}$  = 3,25). The content of organic carbon (OC) sharply drops (up to 1.8 g/kg) in subsoil, where potentially mobile OM is only composed of FA (up to 33% of total C). The degree of OC enrichment with N is very low especially in topsoil (C:N 21.74) and could be primary attached to the features of newly formed organic matter originated from cereal plant species which are dominant in this ecosystem (TSOLOVA & TOMOV, 2018), soil biota activity and low atmospheric inputs of N.

The humus-accumulative horizon  $(A_h)$ of Cinnamon forest soil (profile 3, Table 1) is altered by erosion and this affected carbon stocks - it is moderately rich in organic matter (2.86% humus) likewise Technogenic and Alluvial soils. The prevalence of humic acids is slightly pronounced there  $(C_{HA}/C_{FA})$ = 1.17) and the degree of condensation of their aromatic nuclei is lower ( $E_4/E_6 = 4.08$ ), although this does not change HA hydrophobicity and structure. FA content sharply drops beneath 35 cm and positively influenced the OM humification rate. The increase content of humus acids fractions evokes the naturally occurring leaching process and acidification of pH (5.9). The interaction between pH and potentially mobile fractions of humus acids, respectively fulvic acids is evidenced by the statistically significant correlation between them  $(R^2 =$ 0.73 for both fractions and  $R^2 = 0.85$  for fulvic acids).

C:N ratios (10.44-12.29) in this epipedon indicates high degree of organic matter enrichment with nitrogen and respectively similar rate of release of  $NH_4$ -N.

The status of organic matter in the next three profiles ( $N_{\text{O}}N_{\text{O}}$  4, 5 and 6) differentiates from described above. All of them are characterized with almost equal amount of

HA and FA or lack of HA (like in Alluvial soils - profiles 4 and 5). Fulvic acids are mainly mobile and partially aggressive having in mind the aggressive fractions contents (up to 25% of the total FA fraction). HA are stable, strongly condensed polymers mostly bound to Ca. Organic matter is highly abundant in nitrogen but mineral N content is lower than in profiles 1, 2 and 3 (C:N values fluctuate in the interval 8.6-20.2 with average value 11.54 mg/kg, Fig. 8). The low variation of C:N values by depth reveals the ancient age of humic substances and their stability in diverse soil environments. On the other hand, the older organic colloids have low reactivity (MCBRIDE et al., 1997; BRADL, 2004; COUTRIS et al., 2012) and high resistance to biodegradation and therefore play a minor role in CEC.

The results obtained confirm the fundamental finding that transformation processes of biogenic products in soils are much more intense than those of the mineral components. The presented study shows that biogenic transformation in the modern urban environment is a multifactorial process, dependent on all environmental components.

The elevated NH<sub>4</sub> and NO<sub>3</sub> contents in profiles 1, 2 and 3 can be related to human induced urea saturation of soils (they are also used for strolling pets) which may entail a higher rate of ammonification and amplify the nitrogen cycling. In areas where organic matter is more abundant in nitrogen (profile 4, 5 and 6) the main additional source of N is greenhouse gas emissions (or their precursors - NOx, CO and NMVOCs) which may also affect the cycle of nitrogen transformations. All profiles are located in close proximity to bustling traffic arteries and simultaneously in the direction of prevailing winds (from the north and northeast) which distribute the contamination from the industrial zone known with its strong negative impact on the environment (UZUNOV et al., 1996; FAITONDJIEV et al., 2000; DIMITROVA et al., 2010). The higher temperature of topsoils of profiles 4, 5 and 6 (up to 3-4 °C) supports the assumption for differentiation of mineral nitrogen fluxes during the anthropogenic impact, although the significant correlation between organic nitrogen and carbon (fig 9) shows that humus is the major source of nitrogen. This is also among reasons for alkaline pH values in profiles 1, 2 and 3 regardless of photochemical smog and nitrous oxide (dinitrogen monoxide, N<sub>2</sub>O) acidifying effect.

In urban environments carbon and nitrogen cycles are still coupled (Fig. 9), although the plant diversity in studied ecosystems does not imply substantial nitrogen revenues.

Some more important statistic data regarding the organic carbon abundance can be noted: the established average content of organic carbon in the surface layer of studied urban soils, 19.3 g/kg is almost 2-fold lower than the content of organic carbon in virgin leached Smolnitsas of Bulgaria - 35 g/kg (FILCHEVA, 2007). This content is equivalent to the average content (19.1 g/kg) in the surface horizon of grasslands in Bulgaria (TÓTH *et al.*, 2013) but higher than the content in topsoil of grasslands in the Sofia valley - 12.5 g/kg (LUCAS 2015).

Comparing the results for the mean value of CEC in topsoil of Bulgarian grasslands, extracted for LUCAS (2015) - 36,7 cmol/kg shows a close average value only for moderately leached Cinnamon forest soils (profile 3) and Alluvial-meadow soils (profile 5). The lower content of clay fraction and smectite-vermiculite as well, can explain these lowest CEC values in normally supplied with organic carbon Alluvial soils (profile 4).







Fig. 8. Mineral nitrogen content and C:N ratios in urban soils.



**Fig. 9.** Correlations between total N and C content in root layers of urban soils, and mineral N and pH (average values).

<b>Fable 1.</b> Chemical data on studied urban soils in the city of Sofia.
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Horizons	, pH	T <sub>8,2</sub>	T <sub>CA</sub>	$T_{\rm A}$	exch. H <sub>8,2</sub>	exch Al	exch. Ca	exch. Mg	V (%)	Total C	Humic acids	Fulvic acids	Aggr. FA	Easily mobile HA+FA	C <sub>HA</sub> / C <sub>FA</sub>	$E_4/E_6$	, Ca	Mg
(cm)				C	mol/	kσ					(9	%)		1111111			ARe	xtracts
(- )				-	0	- O Worla	nned r	mode	ratoly l	oacho	d Smol	-) nitea (r	rofilo	(1)				
A bla					0	vena	ppeur	noue	latery i	cache	u Shioi	intsa (F	TOTILE	1)				
0-15	7.10	40.40	34.20	6.20	5.00	0.00	32.50	3.10	88.10	3.20	0.56	0.27	0.08	0.33	2.04	3.87	295.0	700.0
C1k 15-65	7.30	41.20	37.90	4.40	3.00	0.00	34.80	3.20	92.30	1.05	0.21	0.08	0.05	0.07	2.63	3.65	1990.0	800.0
A1b 65-110	7.00	41.10	34.50	6.60	6.00	0.00	32.10	3.40	86.40	1.66	0.40	0.20	0.04	0.16	2.00	3.56	1445.0	675.0
Average	7.13	40.90	35.53	5.73	4.67	0.00	33.13	3.23	88.93	1.97	0.39	0.18	0.06	0.19	2.22	3.69	1243.33	725.00
U						Teo	chnoge	enic so	oil. mo	derate	elv deer	o (profi	le 2)					
Ahk							0		,			- u	)					
0-21	7.25	33.40	28.40	5.00	4.60	0.00	26.00	2.80	86.20	1.50	0.26	0.08	0.04	0.11	3.25	3.50	2700.0	900.0
C1k 21-52	7.45	31.80	-	-	2.10	0.00	26.50	3.20	93.30	0.23	0.00	0.07	0.02	0.04	-	-	2510.0	900.0
C2k 52-85	7.50	32.70	-	-	2.00	0.00	27.50	3.20	93.90	0.18	0.00	0.06	0.02	0.03	-	-	1935.0	880.0
Average	7.40	32.63	28.40	5.00	2.90	0.00	26.67	3.07	91.13	0.64	0.09	0.07	0.03	0.06	1.08	1.17	2381.67	893.33
					Ν	Aodei	rately l	leache	ed Cinr	namoi	n forest	soil (p	rofile	3)				
Ah 0-10	5.85	35.80	28.80	7.00	6.50	0.30	24.50	4.80	81.80	1.66	0.28	0.24	0.04	0.30	1.17	4.08	270.0	575.0
Bt 10-35	6.05	40.00	33.00	7.00	6.30	0.00	29.50	4.60	85.25	1.02	0.22	0.15	0.04	0.14	1.47	4.12	210.0	590.0
Bt2 35-72	7.10	38.00	33.00	5.00	4.00	0.00	29.00	4.60	88.40	0.65	0.18	0.00	0.02	0.08	-	3.64	295.0	600.0
Вtк 72-86	7.30	38.00	35.20	2.80	2.00	0.00	31.80	4.30	95.00	0.48	0.17	0.00	0.02	0.04	-	3.96	835.0	450.0
Ck 86-120	8.00	37.50	0.00	0.00	0.00	0.00	33.10	4.40	100.00	0.37	0.12	0.00	0.02	0.04	-	4.28	5010.0	525.0
Average	6.86	37.86	26.00	4.36	3.76	0.06	29.58	4.54	90.09	0.84	0.19	0.08	0.03	0.12	0.53	4.02	1324.00	548.00

Horizons	рH	T <sub>8,2</sub>	T <sub>CA</sub>	T <sub>A</sub>	exch. H <sub>8,2</sub>	exch. Al	exch. Ca	exch. Mg	V (%)	Total C	Humic acids	Fulvic acids	Aggress FA	Easily mobile HA+	C <sub>HA</sub> / C <sub>FA</sub>	E <sub>4</sub> /E <sub>6</sub>	Ca	Mg
depths (cm)	1			с	mol/]	kg					(	%)		FA			ext	AR racts
						A	lluvial	soil, 1	nodera	tely de	eep (pr	ofile 4)						
Ah 0-15	6.30 2	21.80	16.50	5.30	4.00	0.00	15.00	2.80	81.65	1.57	0.21	0.20	0.04	0.24	1.05	4.06	470.0	552.5
C1 15-40	6.50 2	21.80	17.60	4.20	3.00	0.00	15.80	2.90	85.78	0.43	0.00	0.15	0.01	0.09	-	-	285.0	585.0
C2 40-80	6.70 2	21.40	17.70	3.70	2.00	0.00	16.40	2.80	89.72	0.27	0.00	0.10	0.01	0.08	-	-	475.0	577.5
Average	6.50 2	1.67	17.27	4.40	3.00	0.00	15.73	2.83	85.72	0.76	0.07	0.15	0.02	0.14	0.35	1.35	410.00	571.67
Alluvial meadow soil, deep (profile 5)																		
Ah 0-30	6.103	6.60	29.80	6.80	5.90	0.00	27.50	3.20	83.88	1.52	0.19	0.16	0.03	0.20	1.18	5.80	270.0	570.0
A1 30-55	6.003	37.40	30.80	6.60	5.60	0.00	28.70	3.40	85.80	1.15	0.17	0.12	0.03	0.14	1.42	4.78	260.0	580.0
C1 55-105	6.003	86.70	29.70	7.00	5.60	0.00	28.00	3.10	84.74	0.68	0.09	0.07	0.02	0.11	1.29	3.27	740.0	675.0
C2 105-155	6.003	\$5.00	27.60	7.40	5.80	0.00	26.00	3.00	82.86	0.35	0.00	0.14	0.00	0.10	-	-	550.0	750.0
Average	6.03 3	6.43	29.48	6.95	5.73	0.00	27.55	3.18	84.32	0.93	0.11	0.12	0.02	0.14	0.97	3.46	455.00	643.75
					St	rong	ly leac	hed S	molnits	sa, sup	er deej	p (profi	le 6)					
A' 0-35	6.704	6.20	42.00	4.20	3.40	0.00	38.00	4.80	92.70	2.15	0.32	0.23	0.04	0.21	1.39	3.63	560.0	607.5
A" 35-90	6.704	6.00	41.90	4.10	3.30	0.00	37.60	4.90	92.40	1.52	0.25	0.19	0.03	0.13	1.32	3.34	420.0	595.0
A''' 90-165	6.754	5.60	43.50	2.10	1.70	0.00	34.60	9.30	96.30	0.87	0.15	0.07	0.02	0.10	2.14	3.35	480.0	565.0
Average	6.72 4	5.93	42.47	3.47	2.80	0.00	36.73	6.33	93.80	1.51	0.24	0.16	0.03	0.15	1.62	3.44	486.67	589.17

#### Conclusions

Main alteration of pedo-chemical characteristics of studied urban soils are positive result and negative of transformation of their matrix. Positive changes are mostly related with organic matrix and intensive processes of humusformation and accumulation. Main factors that favour accumulation of organic carbon in studied soils are high content of silt and clay fractions which are typically humus fractions since they contain high amount of humus and humino-mineral complexes; organic clays, smolnitsas, comprising in the parent materials of some soils (because they are rich in organic carbon), as well as the stability of humus acids and their low mobility. Stability of humic acids is related with their dense heterocyclic structure and high nitrogen enrichment. Recently formed

humus is also well humificated and rich in highly condensed humic acids. For this reason, organic colloids are predominantly mature, persistent and slightly active. Simultaneously, in acidic soil horizons even a slight increase of FA content enhances the pH dependence on their content.

Positive changes of the mineral matrix are derived from mineral colloids and slow transformation of biotite into vermiculite – this process may reduce the soil hydrolytic acidity. Mineral colloids predominantly determine the sorption capacity and acidic complexes in studied soils. Negative changes of the mineral matrix are provoked from:

Acid destruction of clay minerals occurring as a consequence of the naturally occurring soil-forming and weathering processes of low intensity and associated processes of slight dispersion and disintegration of the mineral matrix;

Increase of exchangeable hydrogen content above the exchangeable magnesium levels up to the second abundant exchange ion after calcium although the Hdestabilizing role itself is difficult to distinguish.

Anthrogenically induced changes, where they can be identified, can increase mineral N content and fluxes and may influence the existing acid-base status of soils due to the input of neutral, alkaline (urea, ammonia) or acidifying agents (water soluble compounds of  $CO_2$ ,  $NO_2$ ) present in the ground troposphere of the Sofia city.

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# Botanical Composition and Quality Analysis of Grassland of Red Fescue (Festuca rubra L.) Treated with Lumbrical and Lumbrex Biofertilizers

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Abstract. The impact of biofertilizers (Lumbrical and Lumbrex), produced by red earthworm (Lumbricus rubellus), was studied on the botanical composition and forage quality of an artificial grassland of Festuca rubra L. 'Ryder' cultivar, expressed by the fiber composition of dried biomass. The studied bioproducts provide a higher (by 2%) presence of the main crop in the grassland and significantly reduce the weeds. The level of dry forage matter in the fiber structural components is influenced by the biofertilizers. The grasslands treated with Lumbrex 200 ml / da showed the lowest values of NDF (563.02 g kg<sup>-1</sup> DM) and ADL (21.57 g kg<sup>-1</sup> DM). The reduction of the lignin fraction was from 23.1% (Lumbrical 150 ml/m<sup>2</sup>) to 63.6% (Lumbrex 200 ml/da). The foliar treatment with organic fertilizer decreased to the highest degree the lignification process and provided optimal conditions for obtaining ecologically pure agricultural products with improved quality indicators. Treatment of Festuca rubra L. with a granulated substance (Lumbrical 200 ml/m<sup>2</sup>) decreased to the highest degree ADF amount (by 22.3%) in comparison to the control (367.82 g kg<sup>-1</sup> DM). The highest concentration of hemicellulose (284.22 g kg<sup>-1</sup> DM) and the highest in vitro dry matter digestibility (674.10 g kg<sup>-1</sup> DM) was found in the forage mass of that variant. Indicator values exceeded the control by 32.7% and 13.1%, respectively. Bioproducts also reduced the cellulose content by 3.6% (Lumbrex 200 ml/da) to 20.6% (Lumbrical 150 ml/m<sup>2</sup>). The highest energy nutritional value was found in the forage biomass in the variant with Lumbrex 150 ml/da. The compositions of the bioproduct tested increase the amount of gross and exchange energy, respectively, by 0.8 and 1.4% relative to the control.

Key words: bio-fertilization, Festuca rubra L., Lumbrical, Lumbrex.

#### Introduction

Technologies for enhancing the biological and productive potential of crop plants include agro-ecological methods developed on the basis of the optimal use of natural resources. The implementation of measures to preserve and improve the quality of grasslands allows the production of high quality plant and animal products.

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg The distribution of biological products and bio-fertilizers enriched with minerals and easily digestible micro and macroelements favours the biometric performance of forage grass crops (POPOV & YORDANOV, 2012) and the quality of the established grasslands (MASHEVA & MIHOV, 2008; MARINOVA *et al.*, 2019) and is common practice in agriculture in Bulgaria.

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Festuca rubra L. is a major component of grassland associations typical of the Central Balkan Mountain region. According to our research, the application of red earthworm products increases the productivity of hay-making grassland of red fescue in a foothill area and balances the mineral composition in dry feed. The soil application of Lumbrical  $(150 \text{ ml/m}^2)$ in swards of red fescue increased their yield of fresh and dry mass by 34.5 and 37.0% respectively (BOZHANSKA, 2019). The biological activity of humic acids and their ability to form complexes with micro and macroelements, improves soil fertility (ANSARI & JAIKISHUN, 2011; CHAUHAN & SINGH, 2013), supports the development of beneficial soil microflora and facilitates movement the conversion and of organogenic elements in plant-accessible form (ANSARI & ISMAIL, 2012; MAHDI et al., 2010; JAIKISHUN et al., 2014). The granular fraction of Lumbricus rubellus activates the course of biochemical and physical processes in the soil, stimulates plant growth (LALITHA et al., 2000; MAKULEC, 2002; ARANCON et al., 2004; ANSARI & SUKHRAJ, 2010) and improves reducing forage quality, the fiber concentration of grassland by 7.5% and increasing the crude protein content by 20.8% (BOZHANSKA, 2019).

The production of forage with high nutritional and commercial value ensures their full utilization by farm animals. This is the purpose of the present study: to determine the influence of Lumbrical and Lumbrex biotopes on the botanical composition and nutritional value of red fescue sward in the Middle Balkan Mountains of Bulgaria.

# Materials and Methods

The survey was conducted during the period 2014-2016 in the experimental field of the Research Institute of Mountain Stockbreeding and Agriculture – Troyan, Bulgaria.

Biofertilizers Lumbrical and Lumbrex, intended for soil and foliar application, were applied on artificial fly grassland of (Festuca *rubra* L.) 'Ryder' cultivar. The soil substance Lumbrical is an ecologically pure product resulting from the processing of organic waste through red earthworm (Lumbricus rubellis) and applied modern biotechnology. The product is high in nitrogen content 2015) containing (MARKOV, useful microorganisms, macro and micro elements in high concentrations. Organic matter is loose, dark brown and odourless. Biofertilizer is approved for application in organic production with Regulations: №15/03.08.1999 and №22/04.07.2001 of the Ministry of Agriculture and Forests, Bulgaria. Lumbrex liquid fertilizer (foliar application) contains humic and fulvic acids, macro and micro elements in certain concentrations.

We have applied commonly accepted grass meadow cultivation technology, which includes: 1. Soil processing - deep ploughing (23-25 cm) in autumn, immediately after harvesting the preculture and harrowing (in spring) to crush the lumps and level the terrain. 2. Pre-sowing rolling for the creation of a hard bed and better contact of the seeds with the soil. 3. Spring sowing (in the foothills the soil is better moisture preserved) done manually, scattered by the blocking method in 4 replication, with a plot size of 5 m<sup>2</sup> and a sowing rate of 2.5 kg da at 100% purity and seed germination rate. 4. Rolling of sown areas immediately after sowing.

In the year of creation of the swards (2014) the rainfall during the vegetation (March-October) was significantly higher (939.7 mm) compared to the average (514.4 mm) for a 20-year period. In 2015 and 2016, the amount of vegetation rainfall was 683.2 and 536.1 mm respectively. Average monthly air temperatures in the first (15.8°C), second (17.4°C) and third (16.3°C) vegetation were higher at 0.5 to 1.6°C relative to the average multi-year rate. The spring moisture in the years of the experiment created optimal

conditions for the formation of the first crop (Fig. 1).

Experimental variants are: 1. Control /nontreated/; 2. Lumbrical -  $150 \text{ ml/m}^2$  (1 ml = 0.58 g); 3. Lumbrical -  $200 \text{ ml/m}^2$  (1 ml = 0.58 g); 4. Lumbrex - 150 ml/da; 5. Lumbrex - 200 ml/da. The treatment of grasslands by granulated fraction of Lumbrical was performed immediately after the mowing, and foliar application by Lumbrex in tasseling stage.

The soils are light grey pseudopodzolic, low humus content (0.96-1.44%). The stockpile of soil with assimilable phosphorus (1.2-2.4 mg/100 g soil) according to KACHINSKI classification (1958) is very low, and assimilable potassium (5.9-9.9 mg/100 g soil) and mobile forms of nitrogen (8.6-20.2 mg/100 g of soil) very slight. The soil reaction is slightly acidic ( $pH_{H20}$ =5.2-5.5;  $pH_{KCL}$ =4.3-4.4).

We observed the following indicators:

Botanical composition of grassland (%) determined by weight analysis of grass green mass samples taken at each mowing of each variation. Their weighing is carried out in an air-dry state, by weighing the percentage of red fescue and weed species (in total).

Fiber components in plant cell were analyzed at the labaratory: Neutral Detergent Fibers (NDF, g kg<sup>-1</sup> DM); Acid detergent fiber (ADF, g kg<sup>-1</sup> DM) and Acid detergent lignin (ADL, g kg<sup>-1</sup> DM) according to detergent analysis of VAN SOEST and ROBERTSON (1979), and in vitro dry matter digestibility (IVDMD, g kg<sup>-1</sup> DM) by the two-step pepsin-cellulose method of AUFRERE (1982), which is carried out in two steps: I - Pre-treatment with pepsin (200 FIB-U g<sup>-1</sup>), Merck 7190 in 1 N hydrochloric acid for 24 hours (for protein digestion) and II -Treatment in acid medium by cellulase enzyme "Onozuka R-10" that was isolated from Trihoderma viride /Endo-1.4-β-glucanase; 1.4 -(1.3:1.4)- $\beta$ -D-glucan-4-glucan hydrolase/ with enzyme activity of 1.2 U g<sup>-1.</sup>1 g of 1L in 0.05 M acetate buffer with pH 4.6 for 24 hours at 40°C (for cellulose digestibility). The polyosides were empirically calculated: Hemicellulose (g kg<sup>-1</sup> DM) = NDF - ADF and Cellulose ( $g kg^{-1} DM$ ) = ADF - ADL. The lignification degree is expressed through a coefficient as a percentage of ADL and NDF (AKIN & CHESSON, 1990).

The nutritional value of forage was estimated by the Bulgarian system as Feed unit for milk (FUM, in kg DM) and Feed units for growth (FUG, in kg DM) and calculated on the basis of equations according to the experimental values of CP, CFr, CF and NFE, recalculated by TODOROV (2010) digestibility ratios: Gross energy (GE, MJ/kg DM) = + 0.0366\*CF + 0.0209\*CFr 0,0242\*CP 0.017\*NFE - 0,0007\*Zx and Exchangeable energy (EE, MJ/kg DM) =0.0152\*DP (Digestible protein) + 0.0342\*Dft (Digestible fat) + 0.0128\*DF (Digestible fibers) + 0.0159\*DNFENitrogen-free (Digestible extractable substances) - 0.0007\*Zx.

The swards were manually harvested, 20 days after a single foliar application of Lumbrex.

A variance analysis (ANOVA) was used for statistical data processing.

# **Results and Discussion**

Botanical composition of an artificial grassland treated by Lumbrical and Lumbrex biofertilizers.

The biological features of the species, the qualities and adaptability of the variety used, as well as climate conditions in the BARSZCZEWSKI et al., seasons 2007), determine the growth and development of the crop component in the grassland and affect the botanical composition of years. Red fescue and regrowths is characterized by a slow initial growth rate, which allows a higher weed infestation (68-78%) of grasslands in first vegetation (Fig. 2).

In the year of sowing, under conditions of increased soil and air humidity, the share of that grass species exceeded the control by 2.0%, only in the variants of soil application with Lumbrical 200 ml/m<sup>2</sup>. These results can be related to the conclusions of some authors (VLAHOVA *et al.*, 2015), according to which the nutrients imported by biofertilizers favour soil microflora activity, increase soil fertility and stimulate plant growth (TOPRE *et al.*, 2011; VLAHOVA & POPOV, 2018).

In a second vegetation, Festuca rubra L. was characterized by a stronger and more stable turf. The percentage of weeds in the soil treatment variants (Lumbrical 200 ml/ m<sup>2</sup>) was lower reduced (by 3.4% compared to the control) compared to the foliar application (Lumbrex 150 and 200 ml/da) of biofertilizer, where the amount of weeds was reduced by 4.2% and 5.8% respectively, compared to the control. Foliar biofertilizers had a stronger positive effect on the botanical composition of red fescue. The impact of their effect is increased by enhancing the volume of the above-ground matter. The presence of the main crop in the treated grasslands ranged from 82.4% (Lumbrical 150 ml/m<sup>2</sup>) to 93.3% (Lumbrex 200 ml/da).

Red fescue is a typical and persistent meadow grass, with a maximum growth rate in the third, fourth year of the growth cycle. In 2016 (third experimental year), the relative share of *Festuca rubra* L. in the forage mass of treated grasslands was 96.0-97.1%. The highest share of that sown grass was found in the variants of lower dose of Lumbrical and Lumbrex. The excess was 1.3-1.2%, respectively compared to the control.

Data from the analysis above suggests that fertilization (foliar and soil) of red fescue with Lumbrical and Lumbrex provided 2% higher share of main crop in the grassland and significantly reduced weeds. The share of this species is of fundamental importance for the formation and optimum accumulation of fodder biomass. The observed result of the effects of the fertilizers studied can be explained by the higher content of humic acids (2.28 times over fulvoacids - 7%), which stimulate the growth of the root system of the plants and increase the coefficient of nutrient utilization (TANG et al., 2001). The quality and composition of applied organics improves the growth of other organs (ATIYEH et al., probably 2002), which favors the adaptability of the red fescue to the growing conditions. Fiber structural components of cell

walls and in vitro digestibility of dry matter of Festuca rubra L. treated with biotope.

Organic fertilizer introduced in the form of soil and leaf nutrition influences the nutritional and economic qualities of the feed culture and requires detailed consideration of the variation in the content of each structural component individually. The changes in the composition of the structural fiber components of the cell walls and *in vitro* digestibility of dry matter are a major factor for the quality and nutritional value of the feed (NAYDENOVA *et al.*, 2005).

The decrease in the amount of neutral and acid detergent fibers (related to the amount of forage taken), acid detergent lignin and cellulose and the increased content of fully digestible polyoside hemicellulose is an assessment of the effect of the studied biopreparations (Table 1). The positive result of the effect of the root and foliar application with the bioproduct is expressed in reducing the percentage concentration of lignin, neutral and acid detergent fibers, and increasing the amount of hemicellulose compared to the control variant.

On average, for the period of 2014-2016, the dry biomass of the foliar application variant with Lumbrex 200 ml/da had the lowest neutral detergent fiber content (563.02 g kg<sup>-1</sup> DM) and the lowest values of indigestible acid detergent lignin by the animals (21.57 g kg<sup>-1</sup> DM). Decreasing the concentration of both components is essential for the forage quality and the resulting animal production (KRÄMER *et al.*, 2010; MERTENS, 2016).

comparison of The laboratory biochemical data (at a fertilizer dosage of 150 and 200 ml/da) shows that Lumbrex 200 ml/da liquid fertilizer reduced the amount of biochemical characteristics associated with the value of the forage. nutritional The concentration of neutral and acidic detergent fibers and the concentration of the lignin fraction in the forage of Festuca rubra L. were lower than the control by 3.3%, 21.6% and 76.4% respectively.

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Fig. 1. Festuca rubra L. – spring growing, first cut.



**Fig. 2.** Botanical composition of grassland with *Festuca rubra* L. treated by Lumbrical and Lumbrex (%).

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Variante	NDE			Hamicallulosa	Cellulosa	WDMD
v allalits	NDI	ADL	ADL	Heintenutosa	Cellulosa	
Control	581.98	367.82	91.23	214.16	276.59	596.00
Lumbrical 150 ml/m <sup>2</sup>	564.36	289.64	70.12	274.73	219.51	664.83
Lumbrical 200 ml/m <sup>2</sup>	569.88	285.66	33.24	284.22	252.42	674.10
Lumbrex 150 ml/da	582.78	299.62	59.80	283.17	239.81	659.37
Lumbrex 200 ml/da	563.02	288.32	21.57	274.70	266.76	668.03
Average	572.41	306.21	55.19	266.20	251.02	652.47
SD	9.5	34.8	28.1	29.4	22.5	32.0

**Table 1.** Main fiber cell structure components and *in vitro* digestibility of dry matter of *Festuca rubra* L. treated with Lumbrical and Lumbrex biofertilizers on average for the period 2014-2016 (g kg<sup>-1</sup> DM).

In grasslands enriched with granulated fraction of Lumbrical 150 and 200 ml/m<sup>2</sup>, the detergent fiber decreased by 2.1-3.0% (for NDF), 21.3-22.3% (for ADF) and 23.1-63.6% (for ADL) compared to the control variant.

For the experimental period, the cellulose content of forage treated with the tested bioproducts was reduced by 3.6% (Lumbrex 200 ml/da) to 20.6% (Lumbrical 150 ml/m<sup>2</sup>).

The fiber composition of forage plants is a major source of energy for ruminants (HUSSAIN & DURRANI, 2009) and a factor influencing the nutritional value of forage. The biological matter derived from the activity of red earthworm (Lumbricus rubellis L.) and applied to the grasslands of red fescue in a liquid and solid form can be considered as a specialized product that reduces the fiber content of the plant cell and increases the enzyme digestibility of forage biomass. The forage biomass treated with the granular substance of Lumbrical 200 ml/ m<sup>2</sup> had the highest presence (73% - average for the experimental period) of the main crop in the grassland and with the highest *in vitro* dry matter digestibility (674.10 g kg<sup>-1</sup> DM). The excess over the basic option and the average of the indicator is 13.1 and 3.3%. The harvested grassland of that variant is characterized by the lowest content of acidic detergent fiber (285.66 g kg<sup>-1</sup> DM) in dry matter, and the amount of acidic detergent lignin was significantly lower (by 63.6%) than the soil application of Lumbrical 150

ml/m<sup>2</sup>. Our results coincide with that found by AKIN & CHESSON (1990) regarding the content of the lignin fraction as a limiting factor for the digestibility of forage plants.

Hemicellulose is one of the main parameters determining the quality of forage and its weight occupies 20-40% of the composition of fiber components in the plant cell structure (MCKENDRY, 2002). The soil nutrition of red fescue with Lumbrical 200 ml/ m<sup>2</sup> affected the amount of hemicellulose at the highest degree (284.22 kg<sup>-1</sup> DM). Indicator values exceed the control by 32.7%. In the other bio-fertilization variants, the increase in the fully digestible heteropolymer by animals was 28.3% (Lumbrex 200 ml/da) to 32.2% (Lumbrex 150 ml/da). In grasslands with a single foliar application, humic acids increase the permeability of cell membranes (KAYA et al., 2005) and stimulate the biological and physiological processes in plants. Under the influence of the compositions of the studied product (lumbri-culture), lumbrical we observe significant changes in the content of the main fiber components as an energy source for the feeding of ruminants.

Percentage ratio between neutral detergent fibers and acidic detergent lignin determines the lignification degree of the biomass in each of the tested variants. Data analysis shows differences in lignification degree in the forage of grasslands with the application of bio-fertilization (Fig. 3).

On average, for the experiment period, the values in grasslands with reduced

dosage of Lumbrical (150  $ml/m^2$ ) and Lumbrex (150 ml/da) were higher than those treated at higher fertilization rates. The lignification degree is a biological process that influences the economic and nutritional value of the forage enriched. The foliar application of organic matter (Lumbrex 200 ml/da) reduced the lignification process to the highest degree and provided optimum conditions for obtaining environmentally friendly agricultural production with improved quality indicators.

Potential energy nutrition value of forage of Festuca rubra L., treated with Lumbrical and Lumbrex biofertilizers.

The precise identification of the energy value of forage has an important role to play in meeting the animal's food needs and realizing its productive potential. It is a key criterion for the contemporary assessment of the quality of biomass and is determined by the feed units for milk and growth (TODOROV & DARZANOV, 1995). The total energy value of the bio-fertilization variants varied from 17.96 MJ/kg DM (Lumbrical 200 ml/m<sup>2</sup>) to 18.20 MJ/kg DM (Lumbrex 150 ml/da) - Table 2.

In the experimental years, the grassland with a lower dosage of foliar fertilizer had the most pronounced effect on the amount of gross energy. The excess over the control was 0.8%. The forage biomass of this variant had the highest content of physiologically exchange energy that is beneficial for animals (8.21 MJ/kg DM) and the number feed unit for milk (0.76 in kg DM) and growth (0.70 in kg DM) compared to the base and average indicator values. The data correlate with the analysis of the cell wall fiber components and the higher content of neutral (582.78 g kg<sup>-1</sup> DM) and acidic (299.62 g kg<sup>-1</sup> DM) detergent fibers (versus other treated variants) in the dry matter of forage.

Spraying with Lumbrex 200 ml/da and nourishing the grasslands with Lumbrical 150 ml/m<sup>2</sup> result in a higher energy value biomass in terms of the amount of gross energy. The values of the variants exceeded the net control and the mean for the experimental period respectively by 0.5-0.6 MJ/kg DM and 0.2-0.3 MJ/kg DM. The application of the tested preparations did not significantly affect the content of the exchange and net energy (FUM and FUG) in the dry feed mass of the variants with soil and leaf fertilization (150 ml/m<sup>2</sup> and 200 ml/da).



**Fig. 3.** Lignification degree of forage of *Festuca rubra* L. Treated with Lumbrical and Lumbrex biofertilizers (%).

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**Table 2.** Potential energy nutrition value of grass biomass from *Festuca rubra* L. treated with Lumbrical and Lumbrex biofertilizers on average for 2014-2016. *Legend:* GE - gross energy - MJ/kg DM; EE - exchangeable energy (MJ/kg DM); FUM - feed unit for milk (in kg of dry matter); FUG - feed units for growth (in kg of dry matter).

Variants	GE	EE	FUM	FUG
Control	18.06	8.10	0.75	0.69
Lumbrical 150 ml/m <sup>2</sup>	18.17	8.13	0.75	0.69
Lumbrical 200 ml/m <sup>2</sup>	17.96	8.11	0.75	0.69
Lumbrex 150 ml/da	18.20	8.21	0.76	0.70
Lumbrex 200 ml/da	18.15	8.11	0.75	0.69
Averageo	18.11	8.13	0.75	0.69
SD	0.099	0.045	0.004	0.005

### Conclusions

Swards treated with Lumbrex 200 ml/da had the lowest neutral detergent fiber and acid detergent lignin content. The imported foliar organic fertilizer reduced the lignification process to the highest degree by providing optimal conditions for obtaining environmentally friendly agricultural products with improved quality indicators.

Treating red fescue with Lumbrical 200 ml/m<sup>2</sup> lowered the acidic detergent fiber by 22.3%. Forage mass of the variant is characterized by the highest concentration of hemicellulose (284.22 g kg<sup>-1</sup> DM) and in vitro dry digestibility (674.10 g kg<sup>-1</sup> DM). The values of the indicators exceeded the nontreated control by 32.7% and 13.1%, respectively.

The studied biopreparations affected the content of the partially digestible and animal digestible polymer - cellulose in the dry matter of forage. The decrease in the values of the indicator is 3.6% (Lumbrex 200 ml/da) to 20.6% (Lumbrical 150 ml/m<sup>2</sup>).

The highest energy nutritional value is found in the forage biomass of the lower foliar dosage (Lumbrex 150 ml/da). Compositions of the bioproduct tested increased the amount of gross and exchangeable energy, respectively, by 0.8 and 1.4% relative to the control.

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# Histochemical Alterations in Bighead Carp (Hypophthalmichthys nobilis Richardson, 1845) Liver Under Two Pesticides Exposure: A Comparative Study

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**Abstract.** The main aim of the present study was to compare the toxicological effects of a fosetyl-Al and fenamidone based fungicide and a glyphosate based herbicide on the liver lipid accumulation in bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845) in a short-term laboratory conditions (96 hours). A histochemical method with Sudan III staining was applied. We used 30 mg/L, 38 mg/L and 50 mg/L concentrations fungicide, representing 50, 40, 30 times dilution and 20 mg/L, 40 mg/L and 72 mg/L representing 70, 40, 20 times dilution of the fungicide, respectively. These concentrations were considered as real applicable pesticide concentrations in plant protection practices. Overall, we established a different degree of lipid accumulation in the fish liver. In terms to the histochemical alterations, we found that the fungicide had a more sever effect compared to the herbicide.

Key words: histochemistry, liver, fosetyl-Al, fenamidone, glyphosate, fish.

#### Introduction

Pesticide residues in water present a major concern as they pose a serious threat to aquatic ecosystems. According to the authors, pesticides have been directly linked to causing fish mortality worldwide. Moreover, the increase in concentration of pesticides due to its persistent and non-biodegradable nature in the tissues of organisms at each successive level of food chain is known as biomagnification. Due to this phenomenon, organisms at the higher levels of food chain experience greater harm as compared to those at lower levels (GILL & GARG, 2014). In addition, in terms of their

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg persistent, semi-volatile character, as well as lipophilic their high stability and characteristics, which contribute to their bioaccumulation in the adipose tissues of animals and their biomagnification through the food chain (EQANI et al., 2013; PAULINO et 2014; VIEIRA et al., 2019), these al., compounds can promote toxic effects in chronically exposed organisms, such as fish, even at low concentrations (STANLEY & PREETAH, 2016).

In several scientific studies, the authors proposed to better assess the aquatic ecosystems contamination with pesticides, the application of biomarkers, which assess

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the health of organisms (DE LA TORRE et al., 2005; MDEGELA et al., 2006). In addition, the assessment of polluted aquatic ecosystems is related with monitoring their status. The assessment includes identifying the factors that have a negative impact on the ecosystems and measures to limit the pesticide pollution. In this regard, an overall model of the adverse effects of chemicals and their mixtures in contaminated aquatic ecosystems can be developed. On the other hand, stress in the organism caused by the effects of toxicants triggers a series of biological reactions, which can serve as biomarkers of contamination. At the established levels of the reference response, biomarkers assess the rapidly developing stress of the organism. Biological response at a higher hierarchical level is a measure of a late reaction that reflects the state of the entire ecosystem. MINIER et al. (2006) added that biomarkers are important tools since specific present information thev а concerning the biological effects of a particular pollutant. Furthermore, they can be applied in biomonitoring programs, as well as to clarify the negative effects on the organism and the concentration of the toxicant in the health risk assessment.

Changes in fish organism in general allow the determination of water toxicity and the potential hazard associated with anthropogenic substances in aquatic ecosystems. According to MONTENEGRO RAYO (2004) and BRÖNMARK & HANSSON (2005) fish have a direct impact on the function and structure of aquatic ecosystems, including dietary dynamics, zooplankton composition, etc. This impact can be traced mostly in freshwater basins, where they are the largest consumers of the lower trophic levels. As stated by BERNET et al. (1999) criteria for the reliability of histological studies refers to the actual biological significance of the analyzed histological alterations. Moreover, this defines the importance of observed histological changes in different tissues,

expressed to varying degrees in different organs.

The liver is the target organ of chemical intoxication because of the large blood flow. According to STENTIFORD *et al.* (2003) various liver pathological changes may serve as reliable biomarkers for toxic effects of various organic pollutants, including pesticides.

In fish species, lipids and proteins are the main organic constituents, and play many important roles in the fish physiology, which includes growth, reproduction and migration (TOCHER, 2003). According to OLIVARES-RUBIO & VEGA-LÓPEZ (2016) contaminants such as polyaromatic hydrocarbons (PAHs), polychlorinated hydrocarbons (PCBs), pesticides, and pharmaceutical products are the most investigated. Furthermore, these contaminants are hydrophobic, and due to their physicochemical properties are able to accumulate in the lipids of aquatic organism in a dose-dependent manner (KAINZ & FISK, 2009).

According to the Environmental Protection Agency (2010), the tested fungicide is a systemic and a contact pesticide. It contains the active ingredients fosetyl-Al and fenamidone, and belongs to the third category of toxicity. The report of the European Food Safety Authority (2006) does not provide a combined risk assessment of the two ingredients of the tested fungicide. The toxicity data of the fungicide are based on those of the studies carried out with its main ingredient, an ingredient of fosetyl-Al. According to the Environmental Protection Agency (2010), the tested herbicide is a total and systemic herbicide with the substance active glyphosate and belongs to the third category of toxicity. Glyphosate is an aminophosphorus analogue of the natural amino acid glycine. Although, the acute toxicity glyphosate to animals of is considered low (WHO, 2009), the glyphosate-based formulations are generally more toxic for many aquatic species
## (VELASQUES *et al.*, 2016; MELO *et al.*, 2017; ALBAÑIL SÁNCHEZ *et al.*, 2019).

Bighead carp (*H. nobilis*) is a freshwater species, which has the advantages of fast growth, it is strongly resistant to diseases, and has also good meat quality and rich nutrition. However, it has not been that widely studied in terms of the effects of different pollutants compared to other Cyprinids, such as common carp.

Therefore, in the present study we aimed to propose bighead carp as a model for ecotoxicological research. Based on the excessive use of pesticides and their negative impact on the aquatic environment, we tried to determine the effects of real applicable pesticide concentrations in plant protection practices on bighead carp, which is also important freshwater species in aquaculture. Therefore, we applied a histochemical approach to assess the negative effects of the applied pesticides.

#### Material and Methods

#### *Test chemicals*

We used 20 mg/L, 40 mg/L and 72 mg/L herbicide (glyphosate (N-(phosphonomethyl)glycine)) representing 70, 40, 20 times dilution, and 30 mg/L, 38 mg/L and 50 mg/L fungicide (fenamidone (1-anilino-4-methyl-2methylthio-4-phenylimidazolin-5-one) and fosetvl-Al (Aluminium tris-O-ethyl phosphonate), representing 50, 40, 30 times dilution of the stock solution, respectively. The selected dilutions are based on preliminary tests, with dilutions selected where no mortality was found in the individuals. In addition, we aimed to compare the effects of two widely applicable commercial products on the histological structure of bighead carp. We used a stock solution of commercial products actually applicable in agricultural practices. The stock solution was prepared according to the instructions of the company producer. The purpose of the present was to establish the toxicity of the selected products to their applicable concentrations.

#### *Experimental set up*

After transportation, the fish were divided in glass aquaria (100 L) with chlorine free tap water during the acclimatization period for 7 days. The fish were not fed 48 hours prior to the experiment. They were divided into three tested groups (n=15) for each pesticide, including a control group (chlorine free tap water without addition of toxicants) and were treated in static conditions for 96 hours with different concentrations of the tested pesticides. The experimental set up followed MODESTO & MARTINEZ (2010); SANTOS & MARTINEZ (2012) and ALBAÑIL SÁNCHEZ et al. (2019), thus the tested insecticide was added only at the beginning of the experiment and the water was not renewed. The basic physical characteristics of the water such as: pH, temperature, oxygen level and conductivity were followed strictly during the exposure according to a standard procedure (APHA, 2005) with a combined field-meter (WTW, Germany). The experiment, which was performed in triplicates, was conducted in accordance with the national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purposes according to Directive 2010/63/EU (EU, 2010).

#### Histochemical study

Fish dissection was carried out according to the international standard procedures given by ROSSELAND et al. (2003). Histochemical analysis was performed in the laboratory at Medical University of Plovdiv, Bulgaria. Leica Cryostat (Germany) was used to cut the liver samples. Multiple carp sections of 6 µm were prepared according to a standard methodology. They were stained with Sudan III using the method of Daddy (1896) and described by PEARSE (1972). According to the applied method, the lipid droplets in carp liver were presented in vellow staining. Liver histochemical changes of all test specimens, including the control group were appraised individually and semi-quantitatively by using the scale of MISHRA & MOHANTY (2008). Positive Sudan III staining was presented with fat droplets in the hepatocytes cytoplasm. Evaluation of the histochemical lesions was presented as an average value. Each grade represents specific histochemical characteristics and they were categorized as follows: (-) – negative reaction of histochemical staining; (+/-) – very weak positive reaction of Sudan III staining; (+) – weak positive reaction of Sudan III staining; (++) – moderate positive reaction of Sudan III staining; (++) – strong positive reaction of Sudan III staining; III staining; (++) – strong positive reaction of Sudan III staining; (++) – strong positive reaction of Sudan III staining; (++) – strong positive reaction of Sudan III staining; (++) – strong positive reaction of Sudan III staining in the carp liver.

#### **Results and Discussion**

In the control group we found normal histological structure of bighead carp liver in accordance to ROCHA *et al.* (1994). In addition, we found a pale yellow staining in the hepatocytes, which we identified as a very weak positive reaction of the histochemical staining. The obtained results from the conducted histochemical study of bighead carp liver are presented in Table 1 and 2.

**Table 1.** Histochemical changes in bighead carp liver due to the fungicide exposure. Legend: (-) – negative reaction of histochemical staining; (+/-) – very weak positive reaction of Sudan III staining; (+) – weak positive reaction of Sudan III staining; (++) – moderate positive reaction of Sudan III staining; (+++) – strong positive reaction of Sudan III staining in the carp liver.

Fungicide concentration	Control group	30 mg/L	38 mg/L	50 mg/L
Intensity of yellow Sudan III staining	±	±	+	++

**Table 2**. Histochemical changes in bighead carp liver due to the herbicide exposure. Legend: (-) – negative reaction of histochemical staining; (+/-) – very weak positive reaction of Sudan III staining; (+) – weak positive reaction of Sudan III staining; (++) – moderate positive reaction of Sudan III staining; (+++) – strong positive reaction of Sudan III staining in the carp liver.

Herbicide concentration	Control group	20 mg/L	40 mg/L	72 mg/L
Intensity of yellow Sudan III staining	±	±	+	+

At the lowest fungicide concentration (30 mg/L), we found that the intensity of the applied histochemical staining was with the same degree of expression as the control. At the higher fungicide concentrations we found an increase in the intensity of the Sudan III staining. Thereby, in the tested group, exposed to 38 mg/L, we observed a weak positive reaction, expressed in a yellow-orange staining of the cytoplasm of hepatocytes, which indicated accumulation of lipid droplets in the liver cells. At the highest concentration of 50 mg/L fungicide, we found a moderate positive histochemical reaction, which was in expressed in an

intensive yellow-orange staining, indicating the accumulation of a larger amount of lipids in the cytoplasm of hepatocytes (Table 1, Fig. 1).

At the lowest herbicide concentration, we observed a very weak positive reaction expressed in pale yellow. At the higher concentrations of 40 mg/L and 72 mg/L, we found a slight increase in the intensity of the histochemical staining, which showed an increase in the amount of lipid droplets in the cytoplasm of hepatocytes. The degree of the positive Sudan III staining, expressed in a yellow-orange staining in the cytoplasm, was in similar intensity at the higher two concentrations (Table 2, Fig. 2).



**Fig. 1.** Intensity of Sudan III staining in bighead carp liver after the fungicide exposure, x400: A – control group fish; B – Intensity of the histochemical staining at 30 mg/L; C – Intensity of the histochemical staining at 38 mg/L;

D – Intensity of the histochemical staining at 50 mg/L fungicide.



Fig. 2. Intensity of Sudan III staining in bighead carp liver after the herbicide exposure, x400: A – control group fish; B – Intensity of the histochemical staining at 20 mg/L;
C – Intensity of the histochemical staining at 40 mg/L;
D – Intensity of the histochemical staining at 72 mg/L herbicide.

Since, the liver is the first-line of defense against potentially harmful xenobiotics, and it is therefore not surprising that it is also the target organ that is most commonly affected by industrial chemicals (WAHLANG et al., 2013), we established the degree of expression of lipid accumulation in the liver under two widely applicable pesticides in the agricultural practice. As stated by RAMESH & SARAVANAN (2008) the applied histochemical method is an indicator of the accumulation of lipids in cells and allows detection of changes, which occurs at the cellular level under the action of various toxicants, including pesticides. In addition, the liver stores essential carbohydrates in the organism and also participates in the blood glucose homeostasis by maintaining а the processes balance between of glycogenesis and glycolysis.

FABBRINI et al. (2010) added that lipid accumulation in the hepatocytes represents a complex interaction, which includes a balance between triglyceride synthesis (lipogenesis), hydrolysis (lipolysis), and transport. Moreover, XU et al. (2012) found lipid accumulation in goldfish liver due to organophosphate pesticide trichlorfon toxicity. The authors stated that hepatic triglycerides cannot then be transported from the liver and are accumulated in the liver which is associated with changes in apolipoprotein quantity. Fatty degeneration in fish liver was also observed by AL-OTAIBI et al. (2019) under diazinon toxicity. WANG et al. (2019) found lipid metabolism disorders in adult zebrafish due to chlorpyrifos exposure.

Based on the obtained results of the changes in lipid content, we found a tendency towards increasing the lipid inclusions in the cytoplasm, along with an increase in the concentration of the applied toxicants. These results are also confirmed by established fat degeneration by the histological analysis in our previous studies (see STOYANOVA *et al.*, 2014; YANCHEVA *et al.*, 2016). Moreover, the tested fungicide caused more severe degree of fatty degeneration in

the hepatocytes. The large amount of lipid droplets, which were accumulated in the hepatocytes is probably a result of the occurrence of fatty degeneration in the liver cells, which was also found after histological examination of the liver (see YANCHEVA et al., 2016). On one hand, the occurred lipid accumulation in the tested organ is probably due to the increased amount of pyruvate in the liver, and hence by the pyruvate dehydrogenase complex lead to increased amount of Acetyl-CoA, which is used for the synthesis of fatty acids and cholesterol. On the other hand, increased fatty acid synthesis leads to increased triglyceride synthesis and to hyperlipidemia associated with fatty infiltration in hepatocytes. In regard to the fat infiltration in liver cells, these changes can be associated with the absence of the enzyme glucose-6-phosphatase and the inability to release glucose in the blood, which in turn leads to hypoglycemia. Probably, the increased amounts of glucose-6-phosphate lead to increased activity of the pentose phosphate pathway, and hence higher amounts of pyruvate.

on our Based previous study (YANCHEVA et al., 2016), we found increased levels of LDH and ALAT due to the applied fungicide toxicity. Moreover, the changes in the specific enzymatic activity probable lead a stimulation of the process to of gluconeogenesis and accumulation of the glycogen in the liver of the experimental fish species. Along with the obtained results in the present study concerning the lipid accumulation in liver, the increased pyruvate and acetyl-CoA in the liver and lipogenesis pathway lead to accumulation of fatty acids in hepatocytes and thereafter could lead to hyperlipidemia. Thus, the observed changes in the bighead carp liver could serve as protective mechanisms against pesticide toxicity.

In addition, STOYANOVA *et al.* (2014) and YANCHEVA *et al.* (2016) studied changes in the specific enzymatic activity in the liver and changes in the glycogen content under the tested in the present study herbicide. These changes could be linked to the process of lipid accumulation in the hepatocytes, but it may differ from the one, which we supposed occurred under the fungicide exposure. We suggest, similarly to POSTIC *et al.* (2004) that decreased levels in the glycogen storage lead to an increase in the process of glycolysis and thereafter, leads to the process of *de novo* lipogenesis and lipid accumulation in hepatocytes.

#### Conclusion

Overall, we investigated an increase of the intensity of Sudan III staining with accumulation of lipid droplets in the fish liver, which correlates with the increasing concentration of the tested pesticides. Moreover, we could associate this process processes changes the with in of gluconeogenesis and glycolysis. In addition, we found that that the fungicide had a more sever effect compared to the herbicide pronounced expressed in more fatty degeneration. Therefore, this could be considered series of compensatory а mechanisms in the fish liver metabolism in response to the toxic effects of pesticides and the stress they induce. Further investigations in this particular area need to be carried out to better understand the metabolic changes in the liver under the influence of organic contaminants. We consider that these results could be carefully taken into account in monitoring and risk assessment programs, since the tested pesticides are not yet considered as priority substances in surface waters according to EU legislation.

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### Genomic and Chromosome Mutations in Complex with Environmental and Lifestyle Factors as Reasons for Azoospermia and Oligoasthenoteratozoospermia

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Abstract. The aim of the present study was to investigate and characterize the manifestation of genomic and chromosomal mutations in complex with the environmental and lifestyle factors as reasons for azoospermia and oligoasthenoteratozoospermia by applying a classical cytogenetic assay and questionnaire survey. 1540 men were included in the survey. By conventional sperm analysis, 183 of them were diagnosed with azoospermia and oligoasthenoteratozoospermia. Based on the cytogenetic analysis, it was concluded that trisomies, in particular Klinefelter syndrome, and structural chromosome aberrations such as translocations and chromatid fragmentation, are directly related to male infertility. Together with harmful habits such as smoking and alcohol use, they are among the major causes of azoospermia and oligoasthenoteratozoospermia. The results obtained could be successfully used in the implementation of a system of activities for the prophylaxis of male reproductive health.

Key words: mutations, environmental and lifestyle factors, male infertility.

#### Introduction

Human infertility affects between 10% and 15% of couples in reproductive age and male infertility has been associated with approximately half of these problems (YU *et al.,* 2014). Various genetic causes, as well as environmental and lifestyle factors could disturb the course of gametogenesis and the functions of the gametes and to affect the male fertility (SHARPE & IRVINE, 2004; KRAUSZ, 2008; MATZUK & LAMB, 2008; OATES, 2008; GUDELOGLU & PAREKATTIL, 2013). Among them, the role of DNA fragmentations in spermatozoa nuclei (SAKKAS *et al.,* 1999;

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg BENCHAIB *et al.*, 2003; EVENSON & WIXON, 2005; SMITH *et al.*, 2006; YU *et al.*, 2014), as well as the occurrence of genomic mutations, some of which directly associated with male infertility (LANFRANCO *et al.*, 2004; VISOOTSAK & GRAHAM, 2006) have been investigated and analyzed.

In Bulgaria, the relationship between chromosomal diseases and fertility disorders in men is slightly studied. There are incomplete data on the occurrence of Klinefelter syndrome in the Bulgarian population, as well as the relationships between other possible chromosomal aberrations and male

reproductive health, and in particular, the associations of chromosomal mutations with azoospermia and oligoasthenoteratozoospermia as the most severe conditions of male infertility (DZHOGLOV & IVANOVA, 2016; LINEV *et al.*, 2017).

Therefore, the aim of the present study was to investigate and characterize the manifestation of chromosomal mutations in patients with azoospermia and oligoasthenoteratozoospermia by applying a classical cytogenetic assay.

#### Materials and Methods

This investigation was done accordingly ethical principles (Institutional Ethics to Committee Certificate Ν 2/16.01.2019). Informed consent was obtained from each participant entering the study. The study included data from 1540 men (mean ± SD age  $33 \pm 7.4$  years) who visited a reproductive health office in Plovdiv for prophylaxis or for a reproductive problem. All participants have voluntary given answers to a questionnaire providing data on medical history, environmental and lifestyle factors, as well as standardized semen samples.

Among the men with oligoasthenoteratozoospermia and azoospermia (totally 183 -14.1%) 17 patients were subjected to cytogenetic analysis. The following was found for them:

- excessive increase of the boy or man height that is not a family feature;
- abnormalities of external genital organs or anomalies during puberty period;
- reproductive failures (TONCHEVA *et al.,* 2014).

Venous heparinized blood was used for purposes of cytogenetic analysis. Chromosomal preparations were made after cultivating of 72-hour lymphocyte culture. Hypotonic treatment and fixation in Clarke fixation (methanol:glacial acetic acid – 3:1 ratio) was done after the blocking of cell division with cytostatics. In order to denature the chromosomal protein, enzyme (trypsin) or temperature treatment was accomplished before the G-Banding staining. In the course of microscopic investigating Carl Zeiss - Laboval 4 and Olympus BX - 40 microscopes were used. After photograding of quality metaphase plates a karyotype analysis was performed (VALKOVA, 1999).

Descriptive statistics was used to characterize the frequency of the groups compared.

#### **Results and Discussion**

In the whole group of the participants (1540), a diagnosis of oligoasthenoteratozoospermia was established for 96 (7.4%) of them, and azoospermia – for 87 (6.7%).

Data from the survey showed that 15.8% of men who suffer from azoospermia and oligoasthenoteratozoospermia were smokers, 10.9% were alcohol users, 8.2% are receiving medication, 5.5% were working in a harmful professional environment, 4.9% have taken anabolic steroids and 1.1% - drugs.

The conclusions based on the conventional sperm analysis showed that 11 of the participants included in the cytogenetic investigation have diagnosis azoospermia (lack of spermatozoa in the ejaculate), and the other 6 men – a severe form of oligoasthenoteratozoospermia (violation of the three variables - spermatozoa concentration, motility and normal morphology).

Data from the cytogenetic study of the 17 participants indicated that 12 (70.6%) of them have had a normal male karyotype. Among the remaining 5 (29.4%), three cases of Klinefelter syndrome (17.6%), one case of balanced translocation 13/21 (5.9%) and one case of chromatid rupture with dislocations (5.9%) were found - Fig. 1.

Men with Klinefelter syndrome refer to the group of patients with azoospermia and those with structural chromosomal aberrations (translocation and chromatid fragmentation) to the group of patients with oligoasthenoteratozoospermia.



**Fig. 1.** Chromosomal and genomic abnormalities: a) translocation 45, XY, t (13; 21); b) chromatid fragmentation with dislocation; c) Kleinfelter syndrome (47, XXY) – metaphase plate; d) Kleinfelter syndrome (47, XXY) – karyogram.

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In studying problems related to male reproductive health, aneuploidy, leading to an imbalance in the karyotype, has an important clinical significance (TONCHEVA *et al.*, 2014).

The genomic mutation most commonly associated with male infertility is "polysomy X (+ Y)," known as Klinefelter syndrome. Various variants of the syndrome have been described but the most common karyotypes of this group are 47, XXY and 48, XXXY. Its frequency is between 0.1% and 0.2% in the general population and may reach to 3.1% in populations with a higher rate of infertility (GUDELOGLU & PAREKATTIL, 2013).

The results of this study indicate a similar frequency of 0.19% within the general population studied and a frequency among the men with azoospermia – 3.45%, which is significantly lower than in the reported by LINEV *et al.* (2017) results for men with azoospermia, among whom a classic version of Klinefelter syndrome was 10.26%.

Structural aberrations of autosomes are much more common in infertile men than in the general population. There was found, that the Robertsonian translocations are 9 times more common in infertility patients than in the general population and also that reciprocal translocations are 4 to 10 times more common in infertile than in fertile men. The most common Robertsonian translocation associated with male between infertility is found to be chromosomes 13 and 14 of the human karyotype (O'FLYN O'BRIEN et al., 2010).

Data of the present study showed that men with Klinefelter syndrome refer to the group of patients with azoospermia and those with found structural chromosome aberrations - to the group of patients with oligoastenoteratozoospermia. In both cases azoospermia and oligoasthenoteratozoospermia the relationship between the established mutation (genomic or chromosomal) and male infertility is obvious.

In terms of translocations and their relationship to male infertility, the data

from our study supports the findings of other authors (FERLIN *et al.*, 1998; O'FLYN O'BRIEN *et al.*, 2010), with the difference that in our findings translocation is not between 13 and 14, but between chromosomes 13 and 21. The results suggest that structural aberrations affecting the chromosome 13 probably correlate with male infertility, regardless of whether they are translocation exchanges of the chromosome 13 with 14 or 21.

Our earlier studies (DZHOGLOV & IVANOVA, 2016) on the azoospermia showed that for men with Klinefelter syndrome the mean volume of the ejaculate is 0.64 ml less than in the other patients. There was also found a statistically significant correlation between the volume of ejaculate and smoking – the smokers emit an average of 0.61 ml less ejaculate than non-smokers.

It has been also found that in the group of smokers the volume of ejaculate is increased by increasing the number of smoked cigarettes per day (DZHOGLOV & IVANOVA, 2016). The authors associate this with the increased production of seminal vesicles and secretions of prostate gland as an expression of local exudative vascular reaction – a result of the intense smoking. A similar trend has been established for another risk factor – alcohol. No significant difference in the ejaculate volume has been observed between drug users and those who do not use drugs (DZHOGLOV & IVANOVA, 2016).

#### Conclusions

Based on the cytogenetic analysis, it concluded that trisomies, could be in Klinefelter syndrome, particular and structural chromosome aberrations such as translocations and chromatid fragmentation, are directly related to male infertility. Together with harmful habits such as smoking and alcohol use, they are among the major causes of azoospermia and oligoasthenoteratozoospermia. The results obtained could be successfully used in the implementation of a system of activities for the prophylaxis of male reproductive health.

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### Mutagenic and Cytotoxic Effects of Pesticide Lambada 5EK (Lambda-Cyhalothrin) on Sweet and Hot Pepper (Capsicum annuum L.), Beetroot (Beta vulgaris) and Onion (Allium cepa) In Vivo

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**Abstract.** The aim of the present work was to study the potential mutagenic and cytotoxic effects of synthetic pyrethroid insecticide Lambada 5EK in the root meristems of some vegetables. Two concentrations of the pesticide were investigated (0.03% - recommended in agricultural practices, and tenfold lower concentration - 0.003%), and three plant-species, widely used as food, were tested - sweet and hot pepper (*Capsicum annuum* L.), beetroot (*Beta vulgaris* L.) and onion (*Allium cepa* L.). The results obtained regarding the influence of pesticide on mitotic cell division and chromosome status showed that the species have different sensitivity towards the pesticide action. The hot pepper was the most sensitive of all the tested plant species towards the impact of the pesticide in mitosis, and lower mitotic index values were recorded in both tested concentrations compared to the control. The most common observed chromosome fragments, and cells with micronuclei; they were found in all tested plant species, except beetroot treated with 0.003% solution of the pesticide. These results suggested that the beetroot possesses some endurance to the mutagenic action of the insecticide and probably it is an appropriate culture in crop rotation in agricultural practice when lambda-cyhalothrin pesticides have been used.

Key words: genotoxicity, mitotic index, chromosomal aberrations, micronuclei, lambdacyhalothrin, vegetables.

#### Introduction

In a number of cases, classified as harmless pesticides and their degradation products possess increasing toxicity, as they move up through the trophic pyramid; in the high trophic level, their concentration could increase to 1000 times (OWEN & CHIRAS, 1990).

Some of these chemicals are promutagens and they are activated metabolically after getting into the body, and some are only activated in plants. When they get into a human

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg organism with plant food, they may become a real genetic danger to humans. A number of authors have identified cytogenetic, clastogenic and cytotoxic effects of some agro pesticides (ÖZKAN *et al.*, 2009; ASITA & HATANE, 2012; ASITA & MOKHOBO, 2013; MESI & KOPLIKU, 2013; ÖZKARA *et al.*, 2015).

It is really essential to find the proper plant test-systems to evaluate the risk of genotoxicity for examining such mutagenicity compounds. Apart from the recommended pesticide

concentrations, it is also important to examine significantly lower concentrations, because the residual amounts of pesticides do not disappear in the soil; they fall into the groundwater and enter into plants through roots.

Lambada 5EK is a synthetic pyrethroid insecticide with lambda-cyhalothrin (50 g/l) active compound. In agricultural practices, this pesticide is used against vermin as *Eurygaster integriceps* (cereals), *Cydia pomonella* (apples), *Lobesia botrana* (vineyards), *Anarsia lineatella* (apricots), *Myzus persicae* (pepper), *Aphys* spp. (cucumbers), *Leptinotarsa decemlineata* (potatoes), etc.

The purpose of the present study is to estimate the mutagenic and cytotoxic effects of lambda-cyhalothrin on root-tip meristematic cells of four plant test-systems that are widely used as food.

#### Materials and Methods

It was studied two concentrations of Lambada 5EK: 0.03% solution (used in agricultural practices) and 0.003% solution.

In the present study were tested 4 plant's test-systems: sweet and hot pepper (Capsicum annuum L.; cultivars Kurtovska kapia and Biala shipka respectively), beetroot (Beta vulgaris) and onion (Allium cepa; cultivar Density). The seeds were cultivated in Petri dishes with distilled water (control) or pesticide solutions till seed germination. The primary root apex was excised (3-5 mm), washed (in distilled water) and fixed (3:1 ethanol: glacial acetic acid). The fixed roots were washed in ethanol (96% and 70%), hydrolyzed in 3 N HCl (10 min) and treated in 45% CH<sub>3</sub>COOH (30 min) (by STAYKOVA et al., 2005). The roots of beetroot were hydrolyzed for 40 min (in 3 N HCl) and treated for 90 min in 45% CH<sub>3</sub>COOH (authors' modification). The pepper and onion roots were stained with 4% acetic-carmine for 2 h with heating. The roots of beetroot were stained without heating for 3 days (authors' modification). The effects of pesticide were studied on squash preparations and there were 3000 cells of analyzed each variant (magnification 400 ×).

The intensity of cell division was determined by calculation of mitotic index (IM)

(number of dividing cells per total analyzed cells, in percent). The frequencies of mitotic phases were calculated also (phase index - number of cells in concrete mitotic phase per total dividing cells, in percent).

To evaluate the cytotoxicity of the tested concentrations of pesticide, the IM of the treated cells were compared with those of the control.

It was applied an F-test Two-Sample for Variances (ANOVA) to test if the variances between the controls and the treated samples were statistically significant and reliable. If there is a significant difference in a variant, it was applied the t-Test: Two-Sample Assuming Unequal Variances. In other cases, it was used t-Test: Two-Sample Assuming Equal Variances.

The mutagenic effects (genotoxicity) of the tested pesticide concentrations were examined by investigation of chromosome aberrations (chromosome fragments, acentric chromosomes, anaphase- and telophasebridges) and micronuclei in the treated cells and in the controls.

#### **Results and Discussion**

The results of the investigation of the cell division are presented in Table 1. The data showed that IM was higher in all controls than in the treated variants with a 0.03% solution. This indicates that Lambada 5EK slows down the cell division in this concentration in the studied plant species. The difference between IM in control and treated material was the greatest in hot pepper (63.47% and 54.70%, respectively), and the smallest in beetroot (51.60% and 49.77%, respectively). In the tested plant species (except the hot pepper), the concentration of 0.003% of pesticide slightly stimulated cell division and IM was higher than in control. In the sweet pepper, the prophase index (IPph) was higher in treated material than in control and the indexes of metaphase (IMph), anaphase (IAph) and telophase (ITph) were lower. These results indicated retention of dividing cells in prophase after treatment and delayed the transition to the next stages of mitosis. In the hot pepper, there was established a substantial reduction of mitotic

index in the treated material with both doses of pesticide. Higher indexes of telophase were also found in the treated material (1.37% and 1.77% after treatment with 0.003% and 0.03% Lambada, respectively, while 0.16% in control). These results indicate accumulation of cells in prophase probably due to the slower cytokinesis after pesticide treatments. The delay in mitosis was found in beetroot and onion as well (Table 1). Similar mitodepressive actions of lambda-cyhalothrin on the mitotic index in *Allium cepa* as well as in mice have been reported by other authors (MALIK, 2013; ÇAVUŞOĞLU *et al.*, 2014).

Decreasing in the intensity of cell division, after treatment with different pesticides, has been reported in other plants tested (RUFUS *et al.*, 2000; AYDEMIR *et al.*, 2008).

The pesticide concentration is cytotoxic when IM of treated cells is  $\leq \frac{1}{2}$  of the control IM (ASITA & MATEBESI, 2010). Our findings indicated that 0.03% Lambada is not cytotoxic in the investigated plant species. The effects of this pesticide on mitosis in sweet and hot peppers, beetroot and onion showed that the species have a different value of sensitivity. The results showed that hot pepper is the most sensitive to the action of the pesticide among tested species. In the treated cells, the following types of chromosomal aberrations were registered: anaphase (telophase) bridges (intact or broken) (Fig. 1), lagging acentric chromosome and/or chromosome fragments (Fig. 2), and cells with micronuclei (Fig. 3).

The data of chromosomal aberrations, observed in the cells treated with Lambada 5EK, are presented in Table 2. In the controls, there were not any chromosomal disorders identified, which indicates that there is no auto mutagenesis.

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In the treated cells, the following types of chromosomal aberrations were registered: anaphase (telophase) bridges (intact or broken) (Fig. 1), lagging acentric chromosome and/or chromosome fragments (Fig. 2), and cells with micronuclei (Fig. 3).

		Mitotic	Phase Indices					
<b>Plant Species</b>	Samples	Index, IM	IPph	IMph	IAph	ITph		
	Control	64.10	86.84	5.82	4.52	2.86		
Sweet pepper	0.003%	66.03*	93.54*	3.43*	2.27*	0.76*		
1 11	0.03%	59.47*	91.98*	4.32*	3.25*	0.50*		
	Control	63.47	86.82	6.72	6.25	0.16		
Hot pepper	0.003%	56.07*	88.35 <sup>n.s.</sup>	5.35*	4.93*	1.37*		
	0.03%	54.70*	86.35 <sup>n.s.</sup>	5.12*	6.83*	1.77*		
	Control	51.60	92.25	3.17	2.58	1.49		
Beetroot	0.003%	57.17*	92.48 <sup>n.s.</sup>	4.14 <sup>n.s.</sup>	2.80 <sup>n.s.</sup>	0.64*		
	0.03%	49.77*	95.31*	2.61 <sup>n.s.</sup>	1.34*	0.74*		
	Control	60.43	94.93	2.48	1.27	1.32		
Onion	0.003%	63.83 <sup>n.s.</sup>	92.85*	2.77 <sup>n.s.</sup>	2.77*	1.67 <sup>n.s.</sup>		
	0.03%	56.57*	94.87 <sup>n.s.</sup>	2.47 <sup>n.s.</sup>	1.41 <sup>n.s.</sup>	1.24 <sup>n.s.</sup>		

**Table 1.** Mitotic and phase indices (in %) in sweet and hot peppers, beetroot and onion, treated with different concentrations of the pesticide Lambada 5EK. (n = 6; \* indicate significant difference at  $p \le 0.05$  between the treated and control variants; n.s. indicate non-significant difference at p > 0.05).

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**Fig. 1.** Anaphase (telophase) bridges induced in tip root cells in sweet and hot peppers, beetroot and onion treated with Lambada 5EK (magnification 400 ×), scale bar 10 µm.



**Fig. 2**. Chromosomal fragments and acentric chromosomes induced in tip root cells in onion treated with Lambada 5EK (magnification 400 ×), scale bar 10 μm.



**Fig. 3**. Micronuclei induced in tip root cells in sweet and hot peppers, beetroot and onion treated with Lambada 5EK (magnification 400 ×), scale bar 10 μm.

Chromosomal aberrations were observed in the treated variants, except beetroot treated with 0.003% solution of pesticide. Higher values of the total frequency of chromosomal aberrations, as well as the frequency of aberrations in were dividing cells, recorded after with the treatment recommended concentration in agriculture (0.03%). The highest percentages of cells with induced mutagenesis were found in onions (0.50% of

cells analyzed, and 0.88% of dividing cells, Table 2).

After treatment with the higher concentration of the pesticide, there was registered mostly micronuclei in plant cells. Lagging acentric chromosomal fragments and/or chromosomes were observed only in cells of the onion, treated with 0.03% Lambada, however, we consider that they are also formed in the other tested plants, in which micronuclei were observed.

**Table 2.** Frequency of chromosomal aberrations in sweet and hot peppers, beetroot and onion treated with Lambada 5EK. (1 – towards the total number of cells (in %); 2 – towards mitotic cells (in %)).

								Chrom	osomal
Plant		Chrom	osomal	Cells	with	Chrom	osomal	fragmen	nts and
Species	Samples	aberra	ations	micro	nuclei	bric	lges	acen	ıtric
Species								chromo	somes
		1	2	1	2	1	2	1	2
Swoot	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sweet	0.003%	0.08	0.12	0.00	0.00	0.08	0.12	0.00	0.00
pepper	0.03%	0.38	0.64	0.34	0.57	0.04	0.07	0.00	0.00
Hat	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
nonnor	0.003%	0.05	0.09	0.05	0.09	0.00	0.00	0.00	0.00
pepper	0.03%	0.19	0.35	0.16	0.29	0.03	0.06	0.00	0.00
	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Beetroot	0.003%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	0.03%	0.17	0.35	0.14	0.28	0.03	0.07	0.00	0.00
	Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Onion	0.003%	0.09	0.13	0.00	0.00	0.09	0.13	0.00	0.00
	0.03%	0.50	0.88	0.23	0.40	0.11	0.20	0.15	0.27

Similar chromosome aberrations in onion after treatment with lambdacyhalothrin and other pesticides have been observed by other authors (YEKEEN & Anaphase (telophase) ADEBOYE, 2013). chromosome bridges were noted in almost all treated variants. The lowest number of cells with chromosomal aberrations was found in beetroot. After treatment with a diluted solution of the pesticide (0.003%), there were no chromosomal aberrations in cells. These data show that the beetroot possesses somewhat endurance to the mutagenic action of the insecticide.

The highest frequency of cells with micronuclei formed after treatment with a

0.03% Lambada was recorded in sweet pepper (0.34% of cells analyzed and 0.57% of dividing cells), and relatively high - in onion (0.23% of cells analyzed and 0.40% of the dividing cells). The lower pesticide concentration induced micronuclei only in the cells of hot pepper. The highest frequencies of anaphase (telophase) chromosomal bridges were found in cells of onion in both concentrations of pesticide. fragments Chromosome and acentric chromosomes were found only in onions at a higher concentration of the pesticide.

The presented results show that both tested pesticide concentrations have mutagenic effect in the cells of the plants (except beetroot, where lower concentration does not cause mutational changes).

Mutagenic effects of Lambada 5EK in the formation of anaphase occur (telophase) bridges that can be a result from ligated sister chromatids (provoked by a single or double detachment of telomeres) or dicentric and/or polycentric chromosomes. The bridges can break away, with the anaphase progression, and thereby, new acentric fragments, lagging in the equatorial zone of the cell, can be released. Acentric fragments can also be formed as a result of the removal of parts of chromosomes after fragmentations; in daughter cells (next cell acentric fragments generation), or chromosomes form micronuclei observed in the cytoplasm.

It has been reported that Lambdacyhalothrin possess mutagenic effect in animal species as rats, mice, fishes, rabbits, tadpoles and also in human lymphocyte culture (CAMPANA *et al.*, 1999; FAHMY & ABDALLA, 2001; CAMPANA *et al.*, 2003; CELIK *et al.*, 2003, 2005; NARAVANENI & JAMIL, 2005; VELMURUGAN *et al.*, 2006; BASIR *et al.*, 2011; MURANLI & GÜNER, 2011; MALIK, 2013; MURANLI, 2013; XIA *et al.*, 2013; CAVUSOGLU *et al.*, 2014; GADHAVE *et al.*, 2014; FETOUI *et al.*, 2015).

A similar mutagenic effect of various pesticides has been published by other authors (DIMITROV *et al.*, 2006; AYDEMIR *et al.*, 2008; ASITA & MATEBESI, 2010; MARTÍNEZ-VALENZUELA *et al.*, 2017).

#### Conclusions

It was found that the concentration of 0.03% (recommended in agriculture) of the pesticide Lambada 5EK slow down the cell division in root meristematic cells of sweet and hot peppers, beetroot and onion. There was found an accumulation of cells in prophase, and a delay in the transition toward the metaphase and next mitotic phases in the treated materials. Mitosis changes showed that the plants possess a different level of sensitivity. The hot pepper showed the highest sensitivity to the pesticide concentrations. There were no

cytotoxic effects of the concentrations tested in the plant species, however, there were mutagenic effects. Chromosomal aberrations were found in the plants tested, except in beetroot treated with the lower (0.003%),the lowest concentration genotoxicity was found in beetroot, probably to some endurance toward due the mutagenic action of the insecticide.

In the frequent use of pesticides, the residual amounts of them accumulate in the soil. Basing on the results observed in the present study we assume that beetroot is an appropriate culture in crop rotation in agricultural practice when lambdacyhalothrin based insecticides have been used in the previous season.

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### Green System and Air Quality in Sevlievo Town, Bulgaria

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**Abstract.** Green system and urban dendroflora can affect air quality in the following ways: (*i*) converting carbon dioxide to oxygen through photosynthesis; (*ii*) intercepting particulate pollutants ( $PM_{10}$ , dust, ash, pollen and smoke) and absorbing toxic gases such as ozone, sulphur dioxide, and nitrogen dioxide, (*iii*) emitting various volatile organic compounds contributing to ozone formation in cities (*iv*) lowering local air temperatures (*v*) reducing building temperature extremes in both summer and winter and consequently reduce pollution emissions from power-generating facilities. The aim of this study was to investigate the regulating service of urban dendroflora as a depot of carbon and the role of the green system as a reducer of dangerous for people  $PM_{10}$ . In 2017 of the territory of Sevlievo Town were investigated 2555 trees of 45 species taxonomically belonging to 16 families and 30 genera. In 2019 using Huber's simple formula the trees biomass, biomass energy, absorbed  $CO_2$  and accumulated carbon of trees biomass in the streets, quarters and parks were calculated. The total biomass of the dendroflora in Sevlievo Town was found to be equal to 2892.65 t. The total carbon dioxide ( $CO_2$ ) in urban trees was 1446.33 t; the separated oxygen ( $O_2$ ) was 542.37 t and the accumulated carbon (C) was 394.45 t.  $PM_{10}$  in Sevlievo Town have high daily concentrations above the LD50.

Key words: urban dendroflora, CO<sub>2</sub>, climate change, PM<sub>10</sub>.

#### Introduction

According to the urban impacts, the planet becomes more and more an urban system - many cities and their inhabitants are facing heat stress, pollution and growing disconnection with the biosphere. Improving sustainability in urban areas should be thus a major goal on the local and global policy. However, the extent to which urban green system can offer relevant solutions to these challenges is rarely considered in ecosystem service assessments, and therefore unknown to decisionmakers (BARO, 2016). Cities are major hubs for economic and business opportunities and centralize many basic human services such as healthcare and

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg education. Although urban areas still cover a relatively small proportion of the terrestrial land surface of the planet (estimates range from 0.2% to 2.4% circa 2000, according to POTERE & SCHNEIDER, 2007), they have disproportionate environmental impacts well beyond their borders, affecting ecosystems at the local, regional, and global scales (GRIMM *et al.*, 2008; SETO *et al.*, 2012).

Many cities worldwide are vulnerable to the environmental extremes such as droughts, (coastal and inland) flooding or heatwaves because their frequency and magnitude is rising due to climate change (REVI *et al.*, 2014). Pollution and other disturbances (e.g., noise) generated in cities

have also direct and sometimes dramatic health impacts on the urban population (BRUNEKREEF & HOLGATE, 2002; WHO, 2014). Many urban dwellers also suffer the manifold negative effects of sedentary lifestyles, social exclusion and increasing disconnection with the biosphere's ecological dynamics (ANDERSSON *et al.*, 2014).

Human impacts on ecosystems reflect on their functions and processes, as well as – on "their direct and indirect contributions to human well-being" (TEEB, 2010) or providing ecosystem goods and services, which were classified into four main categories: provisioning, regulating, cultural and supporting or habitat services (MEA, 2005; TEEB, 2010).

Provisioning ecosystems services include all the material goods obtained from ecosystems, such as food, fiber, fresh water or medicinal resources. These services of forest ecosystems only contribute directly to the world economy, estimated at 1% of the world's gross product and 3% of world trade. Regulating ones include all the ways in which ecosystems can mediate or moderate ambient the environment, including climate regulation, moderation of extreme events, erosion prevention or biological control. Cultural ones are the nonmaterial outputs of ecosystems that affect physical and mental states of people, for through spiritual example experience, recreation, aesthetic appreciation or sense of place. Finally, supporting or habitat ecosystem services are defined as the ecological processes and functions that are necessary for the production of the previous, including habitat for species and maintenance of genetic diversity.

Urban ecosystems are considered in a good condition if the living conditions for humans and urban biodiversity are good (MAES *et al.*, 2016). This means, among others, a good quality of air and water, a sustainable supply of ecosystem services and a high level of urban species diversity. Important pressures on urban ecosystems are unsustainable land take, air and water

pollution, noise, unwanted and introductions of invasive alien species. (MAES et al., 2018). Air conditions are part of the regulating ecosystem services (HARMENS et al., 2014). The evaluation of climate and air quality related ecosystem services of urban trees is an important task (KISS et al., 2015). On the other hand, urban dendroflora stands modify the city's air quality by sequestration of carbon dioxide and removal of various air pollutants - PM<sub>10</sub>, and by reducing stormwater runoff (KIRNBAUER et al., 2013; NOWAK et al., 2013; JIM & CHEN, 2014). Furthermore, trees in particular are considered to have significant aesthetic and eco-psychological values (O'CAMPO et al., 2009; TYRVÄINEN *et al.*, 2003). Urban dendroflora provides environmental, health, and economic benefits to cities. Urban dendroflora mitigate the effects of urban heat island through evapotranspiration and the shading of streets and buildings. This improves human comfort, reduces the risk of heat stroke and decreases costs to cool buildings (PEARLMUTTER, 2018). Green system improves air quality by absorbing pollutants such as ozone, nitrogen dioxide, ammonia, and particulate matter  $(PM_{10})$  as well as performing carbon sequestration (KONIJNENDIJK, 2018). Urban trees are important to stormwater management. Trees absorb and store rainwater through the canopy, and slow down and filter runoff (United with their roots States Environmental Protection Agency, 2015). Urban green system also encourage more active lifestyles by providing space for exercise and are associated with reduced stress and overall emotional well-being.

Global climate change is already a fact, and the question of how to deal with this change and how to manage the consequences stays in front of humanity. It is clear to all today that cities are one of the most serious heat nuclei and generators of harmful emissions that engage the climate in a vicious circle. One way to catch up or slow down these processes is to increase the quantity and improve the quality of green

areas in urban environments (RANGELOV et al., 2016; RANGELOV, 2019). It is fact that the city does not usually offer trees ideal living conditions. The growth of a tree planted on the street displays important differences as compared to a tree of the same species and age planted in natural conditions, or even planted in a green space in a city. Many the constraints that typical urban environment places on trees limits the average lifespan of a city tree to only 32 years - 13 years if planted in a downtown area - which is far short of the 150-year average life span of trees in rural settings (HERWITZ, 2001). Soil conditions directly affect the growth of street trees. When soil is too compact, due to the weight of asphalt, pavements, vehicles and so on, this results in a reduction in oxygen levels and the ensuing roots asphyxiation of the and the mycorrhizae responsible for nutrition. The same thing happens, when the soil is flooded for a long period of time. Furthermore, as the years go by, the soil in tree pits deteriorates in quality, mainly due to the absence of fallen leaves and dead wood. Therefore, the soil becomes impoverished; it lacks organic matter and the microorganisms that break it down, causing a chemical imbalance in the soil. If this is compounded by a shortage of available water - because most rainwater flows directly into the sewers due to the impermeability of the soil - the result is a tree with a stunted root system and poor growth. The urban environment also contains a series of atmospheric pollutants that may cause damage to trees. For example, there is a great deal of dust in Barcelona (Barcelona City Council, 2011). Trees filter these dust particles, but in excessive amounts. They can form a layer on the leaves and impede the absorption of light and gas exchange. Trees in the urban environment already have a shorter life and smaller dimensions than in the natural environment and these attacks further weaken the tree and reduce life expectancy. Trees, and plants in general, help to attenuate noise pollution in several ways: by

absorption, diversion, reflection and refraction of sound, which reduce the reverberation caused by the noise of cars on the facades (Barcelona City Council, 2011). The recognition of this hierarchical linkage among healthy urban forests and the effectiveness of broader ecosystem protection maintaining goals (e.g., biodiversity and wildlife corridors), highlights the need for scientists and policymakers to gain a better understanding of the socio-spatial dynamics that are associated with tree canopy health at different scales (WU, 2008).

Two of the ecosystem services provided "free of charge" to humanity also from urban green system are its ability to retain / accumulate carbon and PM<sub>10</sub>. Cycling of carbon (C) is essential to processes that provide food, fiber, and fuel for all of the Earth's inhabitants. Carbon dioxide is the second most abundant greenhouse gas after water vapor in the Earth's atmosphere (CHURKINA, 2016). According to the Intergovernmental Panel on Climate Change (IPCC) the anthropogenic impact leads to an increase in greenhouse gases concentration in the atmosphere such as carbon dioxide, nitrous oxide, methane etc., which in turn causes gradual warming of our planet. For this reason, the so-called carbon absorbers, using carbon dioxide from the atmosphere living needs, regulate for their its concentration, which in turn mitigating impact on global climate change.

The aim of presented study was to investigate the regulating service of urban dendroflora as a depot of carbon and the role of the green system as a reducer of dangerous for people PM<sub>10</sub>.

#### Material and Methods

Urban dendroflora analyses

The investigation of urban dendroflora was carried out in 2017. The green system of Sevlievo Town consists of: 60 streets with afforested trees; 4 quarters – "Doctor Atanas Moskov", "Dimitar Blagoev", "Mitko Palauzov/Yug" and "Vazrajdane/Balabanitsa"; 3 parks – "Chernichkite", "Aprilsko vastanie" and "Kazarmite".

The inventory data included: species, genera, family, life form, geo-element, geographical coordinates and altitude, tree height, diameter of breast height (DBH), basal diameter (BD), age of trees, diameter of the crown (max and min), defoliation in % and pest presence. Geographical coordinates and altitude were taken by APS device (Garmin Montana 610). The BD (basal diameter), DBH (diameter of breast height) and tree height have been measured according to DIMITROV (2000).The information about the age of trees was according to the archives of the city. Tree species were determined according to the relevant guides and floras in Bulgaria (DELIPAVLOV *et al.*, 2003, VAKARELOV & ANISSIMOVA, 2010). The floral analyses of dendroflora were published by PETEVA et al. (2018).

Huber's modeled simple formula (DIMITROV *et al.*, 2012) was applied to calculate the stem volume ( $V_{stem}$ ) in m<sup>3</sup>:

$$V_{\text{stem}} = G * H * F, \qquad (1)$$

where G is the circular area,  $G = \pi * (DBH/2)^2$ ; H - the height of tree and F - the species type number. F is based on the height of the predefined scales or the altitude tables (DIMITROV *et al.*, 2012).

Hence, the stem biomass  $(B_{stem})$  in kg is calculated by the formula:

$$B_{\text{stem}}$$
 (Biomass) =  $V_{\text{stem}} * V_{\text{weight}}$  (2)

where V<sub>weight</sub> is volume weight/density in kg.m<sup>-3</sup>, which was found in the corresponding tables (KRASTANOV & RAIKOV, 2012).

Finally, the biomass of one tree is calculated by the formula (LYUBENOVA, 2009; KRASTANOV & RAIKOV, 2012):

$$B_{\text{tree}} = B_{\text{stem}} + B_{\text{branches}} + B_{\text{leaves}}$$
(3)

where  $B_{\text{branches}}$  is the biomass of branches and  $B_{\text{leaves}}$  – the biomass of leaves, that were found in the corresponding tables (KRASTANOV & RAIKOV, 2012).

Following the methodology, the biomass for all trees in the town was calculated using the number of trees per species and arithmetic average  $(X_{av})$  of their traits:

$$\overline{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$
(4)

For the approximate calculation of carbon and energy reserves in biomass, the following ratios (LYUBENOVA, 2009) were taken into account:

$$1 g CO_2 \approx 2 g biomass$$
(5)  

$$1 g CO_2 \approx 10 kcal$$
(6)

In formula (5) we can calculate absorbed  $CO_2$  and in formula (6) - the energy reserves in biomass. The accumulated carbon was calculated using the molecular weight of  $CO_2$ .

Accumulated C = 
$$(CO_2 * 12)/44$$
 (7)

The emitted oxygen (O<sub>2</sub>) was calculated using the following ratio (LYUBENOVA, 2009):

Accumulated 
$$O_2 = CO_2 * 0.375$$
 (8)

The mitigating role of urban dendroflora for  $PM_{10}$ , as described in MANES *et al.* (2016), was obtained using the following equation:

$$Q = F \times L \times T \times 0.5 \times LAI_{i}, \qquad (9)$$

where Q is the amount of air pollutant (in our case  $PM_{10}$ ) removed by trees in a certain time; F - the pollutant flux; L - the total canopy cover in that area; 0.5 - the resuspension rate of particles coming back to the atmosphere (ZINKE, 1967); LAI<sub>i</sub> = 4 is a variable used to refer the removal to 1 m<sup>2</sup> of soil covered by the given functional group. In this case, Q for 2012-2014 was calculated, because there was no recent data and the formula was modeled not only for the conopy cover, but also for the entire green cover of the city including shrubs and grass areas.

Study area. Sevlievo Town (latitude 43° 1' 32" and longitude 25° 6' 48") is situated in the central part of the Fore Balkan, Gabrovo District, Bulgaria. The town (area of 41,244  $\text{km}^2$ ) is an economic center – 1/4 of the town's area is occupied by industrial zones. The city is located mainly on the left bank of the Rositsa River in the center of the Sevlievo Valley at the altitude of 196 - 210 m a.s.l. (Development Plan of the Municipality of Sevlievo, 2014-2020).

#### Results

The street dendroflora of Sevlievo Town is constituted of totally 2555 trees referring to 45 species. The inventory containing the characteristics of the trees and the most useful data for maintenance work is basic to good tree heritage and sustainable management. The trees upon the streets (52%) are followed by these in the quarters -30%. The interesting fact is that only 18% of trees are located in the parks (PETEVA *et al.*, 2018).

The collected data (Table 1) of height (H) and diameter of breast height (DBH) was

statistically processed to calculate  $X_{av}$  for each trait and species.

The calculated trees biomass in streets, quarters and parks is about 3 Mt with energy equivalent stock of 60.5 GJ (Table 2). The absorbed  $CO_2$  by photosyntesise is about 1.4 Mt, and carbon reserves - about 0.4 Mt. The emitted  $O_2$  stock was about 0.5 Mt, destributed mainly in quarters (Table 2).

According to the analyzed data the tree species that provide the greatest accumulation of carbon (including carbon dioxide) and oxygen release in the atmosphere are: Acer campestre L., Acer negundo L., Acer pseudoplatanus L., Aesculus hippocastanum L., Betula pendula Roth, Juglans regia L., Picea abies L., Robinia pseudoacacia L., Tilia cordata Mill. and Tilia tomentosa Moench. (Table 1).

According to the Sevlievo Municipality (2016), there are no permanent posts for air quality control on the territory of Sevlievo Town in 2014. The measurement of the main atmospheric pollutants (PM<sub>10</sub>) levels was carried out by the mobile station for emission air control at EEA (RL) - Rousse on schedule and on-site - monitoring station (PM) with a location approved by the MOEW (2019). For the period up to 2014, the town of Sevlievo is implemented by the PM - "OSC" located in the central part of the town in the parking lot next to the building of the Municipality of Sevlievo.

Table 1. Tree traits for the biomass calculation in Sevlievo Town.

Ν	Species in Sevlievo Town	Number of trees (n)	Average height, m	Average DBH, cm	Total C, t	Total O <sub>2</sub> , t
1.	Abies alba Mill.	15	13.0	29.6	1.14	1.57
2.	Acer campestre L.	74	11.4	36.7	10.47	14.40
3.	Acer negundo L.	109	10.3	37.7	13.39	18.41
4.	Acer platanoides L.	12	14.7	43	3.81	5.24
5.	Acer pseudoplatanus L.	148	9.8	31.9	11.83	16.27
6.	Acer saccharinum L.	2	9.0	52.0	0.36	0.49
7.	Aesculus hippocastanum L.	136	11.8	31.0	11.53	15.85
8.	Ailanthus altissima Mill.	7	7.4	34.6	0.30	0.42
9.	Albizia julibrissin Durazz	42	8.5	28.9	1.65	2.27
10.	Betula pendula Roth.	333	14.1	34.0	35.74	49.14
11.	<i>Caragana arborescenc</i> Lam.	26	5.5	11.0	0.15	0.21

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12.	Castanea sativa Mill.	81	11.1	33.0	6.29	8.64
13.	Catalpa bignonioides Walt.	30	10.0	30.0	1.41	1.95
14.	Cupressus sempervirens L.	78	7.2	24.4	1.10	1.51
15.	Elaeagnus angustifolia L.	12	3.0	12.0	0.01	0.01
16.	Ginkgo biloba L.	12	14.1	47.2	3.63	4.99
17.	Juglans regia L.	67	11.6	39.7	9.83	13.52
18.	Koelreuteria paniculata Laxm.	20	4.4	40.0	0.32	0.44
19.	Liquidambar styraciflua L.	20	7.0	32.5	0.47	0.65
20.	Malus baccata L.	14	11.6	35.7	1.11	1.52
21.	Malus domestica Borkh.	19	6.5	29.8	0.35	0.47
22.	<i>Morus alba</i> L.	18	12.5	55.1	5.39	7.41
23.	Morus nigra L.	21	10.0	25.3	0.86	1.18
24.	Paulownia elongata L.	20	7.7	31.0	0.68	0.94
25.	Paulownia tomentosa L.	15	12.6	39.3	2.11	2.90
26.	Picea abies L.	147	12.1	34.6	14.52	19.96
27.	Pinus nigra L.	26	10.1	23.3	0.95	1.31
28.	Pinus sylvestris L.	15	12.8	25.7	0.91	1.25
29.	Plananus orientalis L.	18	14.4	31.1	2.79	3.84
30.	Populus alba L.	13	8.5	40.0	0.73	1.00
31.	Populus nigra L.	17	10.0	39.5	1.27	1.74
32.	Prunus armeniaca L.	14	5.4	31.6	0.15	0.21
33.	Prunus avium L	26	11.5	24.5	0.71	0.98
34.	Prunus cerasifera Ehrh.	11	4.8	22.0	0.05	0.06
35.	Prunus domestica L	20	5.1	32.3	0.20	0.28
36.	Prunus persica (L.) Batsch	16	5.6	18.6	0.06	0.08
37.	Pyrus elaeagrifolia Pall.	20	5.9	26.0	0.17	0.24
38.	Quercus robur L.	67	11.2	33.8	7.66	10.54
39.	Robinia pseudoacacia L.	358	10.7	35.2	23.57	32.42
40.	Salix babyloniva L.	33	12.1	50.6	4.98	6.85
41.	Sophora japonica L.	12	3.0	9.5	0.004	0.005
42.	Thuja acidentalis L.	12	1	4.8	0.0001	0.0002
43.	<i>Thuja orientalis</i> L.	14	14.1	48.8	2.11	2.91
44.	Tilia cordata Mill.	234	13.5	43.5	37.78	51.95
45.	Tilia tomentosa Moench.	151	13.8	40.0	21.51	29.58

**Table 2.** Calculated indicators for the dendroflora of the town of Sevlievo, Bulgaria.

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Objects in Sevilieurs Tourn	Total bior	C +	0.1	CO 1			
Objects in Sevilevo Town	t	kJ	GJ	- C, l	$O_{2,l}$	$CO_{2,l}$	
Streets (60)	1224.85	25 633 090	25.63	167.03	229.66	612.43	
Quarters (4)	1254.90	26 261 919	26.26	171.12	235.29	627.45	
Parks (3)	412.90	8 640 981	8.64	56.30	77.42	206.45	
Total (67)	2892.65	60 535 995	60.54	394.45	540.50	1446.33	

According to research of VLAKNENSKI *et al.* (2016), also confirmed by the results of the municipality itself, the greatest contribution to the air pollution is from household sector during the heating season of the year using coal and firewood - 37% for the town of Sevlievo. The contribution is very high of local background pollution from resuspended particulate matter. According to Sevlievo Municipality (2016) the location of the polluted area and population in the area exposed to atmospheric air pollution with PM<sub>10</sub>, practically covers the city's territory in its central part and the surrounding residential areas that are most affected.

All this means that there is a big problem with the  $PM_{10}$  for the population of the city which is 20 464 people according to data of the National Statistical Institute of the Republic of Bulgaria for 2019 year (NSI, 2019). Based on these results can be said that the dendroflora, respectively green system, can't compensate the higher PM<sub>10</sub> (Table 3, Fig.1), which is dangerous for health and life expectancy (United States Environmental Protection Agency, 2019). The obtained results highlight a relevant contribution of urban vegetation to the ES of  $PM_{10}$  removal. Unfortunately, the green system proves to be insufficient to compensate for the large amount of  $PM_{10}$  in the air and therefore these particles have somehow affected the health of the residents of Sevlievo Town.

#### Discussion

In recent years, the interest in the analysis and assessment of ecosystem services has been

extremely The European strong. Biodiversity Strategy by 2020 entrusts Member States to assess the economic value of the benefits of ecosystem services on their territory and to organize its integration into reporting and reporting systems at European and national level. The scope of ecosystem services is extremely broad and diverse: protecting biodiversity, providing water supplies, reducing the effects of natural disasters, increasing climate capacity to tackle change, safeguarding genetic resources, and so on. This complicates the determination of their economic value and is underestimated for a long time. Economic assessment is mostly based on financial value and misses the social and environmental benefits for which there is no official market and no pricing (KAZAKOVA-MATEVA & PENEVA, 2015). The cities must be part of the solution if an urbanizing world is to grapple successfully with ecological challenges such as climate change. In concentrated urban areas, it is possible for environmental economies of scale to reduce the impact of human beings on the Earth. This has already started to happen in Europe (SHIELDS & LAGNER, 2009). According to the UN Population Division, 72% of the continent's population is urban but the European Environment Agency (EEA) says that its cities and towns account for just 69% of energy use.

The comparative assessment of carbon dioxide emissions in different European cities is presented on Table 4.



**Fig. 1.** Amount (Q) of the air pollutant (in our case  $PM_{10}$ ) removed in a certain time.

**Table 3.** Measured amount of  $PM_{10}$  for the town of Sevlievo (after Sevlievo Municipality, 2016). Legend: (\*) - LD50 is 50 µg/m<sup>3</sup>.

Vor	Measured above norms	Affected	Population exposed to pollution
Teal	Average Daily Concentration (*)	area	with PM <sub>10</sub>
2012	51 - 77.2 μg/m <sup>3</sup>		20 464 inhabitants of the town of
2013	$50.5 - 85.6 \mu g/m^3$	2.5 km <sup>2</sup>	Sevlievo (about 63% of the
2014	55 - 80.1 μg/m <sup>3</sup>		population of the Municipality)

City	Population	CO <sub>2</sub> emissions per head	Reference
Sevlievo Town, Bulgaria	20 464	0.07 tonnes	
Sofia City, Bulgaria	1 269 384	4.42 tonnes	SHIELDS et al., 2009
Amsterdam, Netherlands	743 000	6.66 tonnes	SHIELDS et al., 2009
Athens, Greece	3 400 000	5.92 tonnes	SHIELDS et al., 2009
Belgrade, Serbia	1 700 000	3.85 tonnes	SHIELDS et al., 2009
Istanbul, Turkey	12 600 000	3.25 tonnes	SHIELDS et al., 2009

Table 4. Assessing the human impact of Europe's major cities.

According to Table 4, Sevlievo Town had the smallest share of all European cities compared, including the capital Sofia. The data showed that Sevlievo Town had no problem with this greenhouse gas as opposed to PM<sub>10</sub>. The registered data from the CAA measurements in Sevlievo Town made within the NASEM for the period 2007 - 2010 do not show any exceedances of the average annual rates for the main atmospheric pollutants. With regard to the PM<sub>10</sub> measurements, periodic accidental exceedances of the daily average PM<sub>10</sub> values were observed. Air pollution is а consequence of the typical urban anthropogenic activity in the area, with heavy road traffic on the road network and the presence of localized production areas in the town of Sevlievo.

On the first place the major source of PM10 are the households (43%), then the industry (37%) and lastly – transport (20%). All this mean that the major factor are the town's citizens, that use unsustainable heating methods in the winter season like woods or coals.

The dendroflora cover is about 33% of all green cover of the town and cannot compensate this higher levels of  $PM_{10}$  in the

atmospfere. In this 33 % are included absolutely all trees in the town's territory. In 2008, Barcelona City Council (2011) introduced a street tree management programme to meet the new needs of street tree management in the city. In Sevlievo Town, such program will be a good point for the sustainable ecosystem services ecosystem management. The services provided by trees are on going and could become more valuable in the future as external factors changes. For example, there is an increasingly urgent need to reduce levels of PM<sub>10</sub>. Poor air quality associated with a congested road network and the port was an increasing problem, resulting in the recent designation of a 'Clean Air Zone' in the city (DEFRA, 2015). Planning tree stocks to maintain a high level of ecosystem service of paramount delivery is, therefore, importance (DAVIES et al., 2017a; b). Dendroflora of the city can be a decisive factor. Now for the town of Sevlievo it is 33% of the green cover and system which can't compensate the high PM<sub>10</sub> dose. This means that enough trees must be planted in the town's territory and the percentage of dendroflora must be at least 60% of the green system and cover. Thus will reduce the amount of  $PM_{10}$  in the air and the pollution will be captained.

#### Conclusions

Trees are probably the type of greenery that makes its presence nicest in the life of the people. Many researchers talk about street dendroflora and the percentage distribution of trees between streets, quarters and parks. The obtained results are a good start for other investigations on the same topic. These results will give an idea to the authorities in order to take the necessary precautions.

Air pollution in Sevlievo Town is a consequence the typical urban of anthropogenic activity in the area, with heavy road traffic on the road network and the presence of localized production areas in the city. Sources of atmospheric pollution with PM<sub>10</sub> in Sevlievo Town are the household sector, industry and transport. The quantitative results of the inventory of the main  $PM_{10}$  emission sources in the ambient air of the town of Sevlievo show that the household sector has the largest contribution to the atmospheric pollution of the area, with the tendency to decrease the local PM<sub>10</sub> emissions. There is an urgent need to reduce levels of PM<sub>10</sub> in Sevlievo Town. Therefore, planning a methodology for this as an ecosystem service is paramount. Modeling dendroflora in the city can be a deciding factor. At present, for Sevlievo Town, the dendroflora with its 2555 trees or 33% of the green system cannot compensate higher levels of PM<sub>10</sub>. This means that enough trees need to be planted throughout the town. According to our results if the dendroflora is at least 60% of the total green coverage, it will lead to a natural reduction of PM<sub>10</sub> and improved ambient air quality for the town's residents.

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### Features of Landscape Fires Occurrence (Based on the Example of Lviv Region of Ukraine)

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**Abstract.** The article is devoted to the investigation of peculiarities of landscape fires occurrence, namely: forest fires, peat fires, steppe fires and landfill fires. Publications related to the dynamics of these fires development, the conditions of their occurrence and the impact of these fires on the environment are analyzed in this paper. The analysis of fires, based on the example of Lviv region (Ukraine), revealed the following: the largest number of fires occurs at the beginning of the fire-dangerous period that coincides with the ascent of snow cover, as well as during harvesting. The frequency of fires in landfills closely depends on weather parameters (temperature and relative humidity); the frequency of steppe and forest fires occurrence is less dependent on these indicators. Constructing of a Voronoi Diagram for coordination of fires occurrence allows us to study the largest accumulation of fires and their impact on the environment during specific period, as well as to determine the locations of firefighting equipment for quick response to these fires.

Key words: Landscape fires, forest fires, peat fires, steppe fires, landfill fires, Voronoi Diagram.

#### Introduction

Currently there are tendencies in the world that the number and scale of fires in natural ecosystems are increasing; in particular, it concerns forest fires (DENNISON *et al.*, 2014; JAFARZADEH *et al.*, 2017). The research (ALBERS, 2012) shows disappointing statistics that every year in Europe 600 thousand hectares of forest are damaged and destroyed because of fires.

The problem of fires occurrence in ecosystems is global and is being researched by scientists around the world. In India fires happen in the area of 5 thousand hectares of forest every year. In particular, fires occur annually in 445 out of 647 districts of the country (DOGRA *et al.*, 2018). In the study (ULLAH *et al.*, 2013), the dynamics of forest fires was described in the Sanming forestry,

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg Fujian Province, China, and within 2000 and 2009 on these territories there were 818 fires on an area of more than 8,500 hectares. The increasing frequency of fires in natural ecosystems in central Europe is also specified in research (MAJLINGOVÁ, 2015).

Statistics on forest fires and dry grass fires in Slovakia indicate their roughly equal numbers (an average of 2,000 per year). On the territory of the Czech Republic in the period from 1992 to 2004, there were about 15,985 fires on a total area of about 8 hectares (KULA & JANKOVSKÁ, 2013). The most shocking statistics on fires is in the United States. According to statistical data for 1992-2011 (REID *et al.*, 2016a; b), on average 80,000 fires have occurred in the United States and about 2 million hectares of forest were destroyed; and in 2006, there were about

140,000 fires on an area of more than 4 million hectares. In addition, the study of dynamics over the past 5 years has been conducted in Portugal (MATEUS & FERNANDES, 2014), Canada (LE *et al.*, 2014), USA (MAUDLIN *et al.*, 2015; REID *et al.*, 2016a; b) and other countries of the world, where also the dynamics of growth is established.

Taking into consideration the above statistics, there is a need for a detailed analysis of the conditions and causes of landscape fires for a particular region, based on which it would be possible to develop and introduce preventive measures for their occurrence, spreading and negative impact on biota.

Many works were devoted to the dynamics of forest fires. In particular, the distribution of forest grassland and steppe fires was investigated in (BURASOV, 2016). It is established that the rate of fire distribution increases linearly with the wind speed increase, and decreases with an increase in the humidity of the combustible material. Due to stronger winds steppe fires spread faster because of the large inflow of oxygen into the combustion zone and a more powerful convective heat transfer by wind. It is established experimentally and theoretically (GRYSHYN, 2008) that the front of any natural (forest, peat, steppe) fire consists of a heating zone, drying and pyrolysis of natural (vegetative) combustible materials, as well as a flame burning zone of gaseous pyrolysis products. Using the information about the structure of the front, the authors managed to obtain analytical formulas for determining the distribution rate of forest and steppe fires.

Scientists (ADAB *et al.*, 2013; FILKOV, 2005) developed a geoinformation expert system for forecasting forest fires, which allows to determine the possibility, time and place of the fire, taking into consideration following factors: soil structure and the type of plant combustible material, precipitation, wind direction and radiation of the Sun, the dynamics of the drying of plant combustible material, anthropogenic loading, and

lightnings. Moreover, the software for forecasting forest fires distribution was developed for the Mediterranean pine forests of Greece based on weather data and the state of plant combustible material (MITSOPOULOS *et al.*, 2016). The modelling of velocity of the highland coniferous forests spread was carried out in (CRUZ & ALEXANDER, 2017) using the Monte Carlo method.

What concerns the transition from downhill to highland fires, the most dangerous and complicated in terms of firefighting are the fires in highlands. Despite the fact that the percentage of upland forest fires is insignificant, the area they passed in comparison with the downhill fires is larger. Main reasons of this transition (HABIBULLIN, 2017) is an increase of superficial combustible materials mass on uncleansed areas of felling or the allocation of quarrying residues in heaps, low-down crowns of coniferous trees, as well as the development of coniferous growth and undergrowth. The transit process is described in (LAUTENSHLEGER, 2016). It is established that the downhill fire in this process consists in the heating, drying, pyrolysis and inflammation of pyrolysis products in the crowns of trees, as a result of the strong wind rapid spread of the fire front on the crowns of trees occurs.

In the paper (JAFARZADEH et al., 2017) there were presented theoretical aspects of determining the risk of forest fires in the western Iran using the Apriori algorithm and the Fuzzy Clustering Algorithm (FCM). The risk of forest fires was estimated with the following factors: distance from settlements, population density, distance from the road, slope, dryness, temperature, soil cover and distance from agricultural land. The authors prove that the main cause of fires in natural ecosystems in 95% is human negligence (SYPHARD & KEELEY, 2015). The largest number of fires occurs in forests near the populated areas, in places where there is no entry ban, and also near agricultural lands of common ownership (CHAS-AMIL et al., 2015). According to the study (KULA & JANKOVSKÁ, 2013), the increase in the number of fires is
the result of an increase in the attendance of forest, and the main reasons become abandonment of the bonfire without supervision, smoking and disadvantages of forestry management. Another problem that directly affects the growth of fires in forests is the change in land use in recent decades (FERNANDES *et al.*, 2014).

It is necessary to indicate that forest behavior leads to the loss of life in this area, significant carbon emissions, deterioration of air quality and significant extinguishing costs (ABATZOGLOU & WILLIAMS, 2016). Even lowlevel low-intensity fires can destroy nests, eggs and kill young animals that cannot avoid the effect of fire (DOGRA *et al.*, 2018).

The hazard indicator is the concentration of fine particulate matter PM2.5 in air contaminated with fume of forest fires biomass. This particular matter includes carbon (soot and coal), organic carbon, sulfates, nitrates, potassium carbonate and silicon (LARSON & KOENIG, 1994). Authors of study (LE et al., 2014) indicate that an increase in PM2.5 concentration due to forest fires can affect the health of elderly people thousands of kilometers away from these fires. In addition, combustion of plants, peat and especially solid household wastes accompanied with the formation of toxic combustion products remain in soil and water. Toxic combustion products are hazardous to human health, can cause respiratory diseases (LIU *et al.*, 2017), respiratory complications and children athma children (HEHUA et al., in 2017), cardiovascular diseases, etc. (REID et al., 2016a; b). The most dangerous are fires in forests located in the Exclusion Zone near Chornobyl (EVANGELIOU et al., 2014), as well as in landfills, as they can cause cancer (WIWANITKIT, 2014). In their studies (O'DONNELL & BEHIE, 2015), scientists have found the impact of fires in ecosystems on the weight of male newborn children.

The effect of fires quantity and their intensity on the regeneration of tropical deciduous forests is not sufficiently studied (THEKAEKARA *et al.*, 2017). In the study of fires

in the Nilgiri Biosphere Reserve, Western Ghats (India) (MONDAL & SUKUMAR, 2015), the survival rates of young tree species do not differ between the areas that were affected by fires and other areas. Fires in tropical deciduous forests have impact that is more negative. At the same time due to the fire, there is a smaller number of wood species and a lower density of plantings (KITTUR et al., 2014). Instead, subtropical pine forests are more adapted to fires (DOGRA et al., 2018). In the work (TROCHTA et al., 2012) there was investigated the effect of fire on vegetation, in particular on defoliation of pine trees on the territory of the Bohemian Switzerland National Park, which was exposed to fire. A year after the fire that has happened in this area, pine plantings, which were in close proximity to the fire, were gradually defoliated. Forest fires, according to studies (KONSAM et al., 2017), do not have effect on significant changes in vegetation regeneration compared to areas not exposed to fire. However, in (SEIFERT et al. 2017), a significant reduction in the growth of the radiant pine (Pinus radiate) was revealed after the lowland fire in the year when the fire occurred, as well as in the following year.

Fires in ecosystems lead to changes in the qualitative composition of soils. Thus, studies (HOLDEN & TRESEDER, 2013) show that the high temperature effects of the fire lead to a decrease in the large number of microbes, while fires with higher intensity deplete the soil, depriving it of nutrient organic substances, and exhaust the soil, depriving it of nutrient organic matter (CHANDRA & BHARDWAJ, 2015). Moreover, the drainage properties of forest soils are reduced, which lower their infiltration and increase erosion (VERMA & JAYAKUMA, 2012).

Also, forest fires make influence on the quality of underground and terrestrial water sources, lead to difficulties in supplying drinking water (BLADON *et al.*, 2014; BUSTAMANTE *et al.*, 2016), reduce soil fertility and pollute the air.

Dangerous impact on the environment is made by peat fires. In the study (DOGRA *et al.*,

2018) authors present the results of the mathematical modeling of peat fires, in particular, they describe the process of transition of grass forest fires into peat fires due to radiation and thermal heat transfer from the flame front of a lowland fire through the underlay of the forest. Theoretical and experimental studies (HUANG & REIN, 2015; HUANG et al., 2016) confirm the theory of distribution of peat combustion considering its area and volume. It leads to long-lasted fires and continuous release of combustion products into the atmosphere. Therefore, the quality of air in the area is deteriorating, which leads to a human health deterioration, the sustainability of ecosystems and changes in the global carbon cycle (PAGE & HOOIJER, 2016). Smoldering of peat can continue in wet weather (TURETSKY et al., 2015). Climate change leads to a decrease in water levels in peat bogs and, consequently, to an increase in the number of peat fires (TURETSKY et al., 2015). The authors (PUTRA et al., 2016) indicate that in order to reduce the risk of peat fires it is necessary to maintain groundwater level.

It should be noted that the fire hazard of landfills and the impact on the environment and human health have been investigated less. In particular, the concentration of landfill gas, microclimate and gas well temperature (MILOSEVIC et al., 2018) have effect on the fire hazard of landfills. Research on the morphological composition of garbage in landfills **BUSTAMANTE** et al., 2016; WIWANITKIT, 2014) and the qualitative composition of combustion products released into the atmosphere have shown that smoke number carcinogenic contains а of compounds that increase the risk of human cancer (EVANGELIOU et al., 2014; ROVIRA et al., 2018).

## Materials and Methods

Lviv Oblast is located in the west of Ukraine, located within the limits of 22°43'-25°24 'E and 48°45'- 50°46' N latitude. The total area of the region is 2.18 million hectares. The region is located in three zones: forest, steppe, foothills and mountainous areas of the Carpathians. Forests cover almost a one fourth of the total region area.

The climate is temperate continental, humid: winters are soft with thaws, wet springs, warm summers and warm autumns. The average January temperature is -5°C, July temperature is + 18°C in the central part of the region and + 12°C in the mountains. Annual precipitation varies from 600 mm on the plain to 1000 mm in the mountains.

Data on landscape fires from 2016 to 2018 has been provided by the State Emergency Service of Ukraine in Lviv Oblast. This information includes the place and time of the fire occurrence, their area, the causes of fires, consequences. The weather data, which includes the temperature and relative humidity of air, was obtained from the government web-resource of the Ukrainian Hydrometeorological Center at the station (49°48' N 23°56' E).

Statistics data are processed using mathematical statistical methods such as method of least squares to build trend lines, definition dependence between two quantities calculated using Pearson's correlation coefficient.

In order to study the largest accumulation of landscape fires on the territory of Lviv region, reasons of their occurrence, as well as the use of preventive measures, methods of mathematical statistics and Voronoi diagrams were used in the research. The Voronoi diagram is a special type of partition of the metric space, defined by the distances to a given discrete set of isolated points of this space, to the *n*-th number of convex polygons. In the simplest case, we have a plurality of points in the plane *S*, which are called vertices of the Voronoi diagram. Each vertex *s* belongs to the Voronoi cell, also known as the Dirichlet cell, V (s), formed from all points closer to s than to any other vertex. The boundaries in the Voronoi diagram are all points in the plane, which are equidistant from the two nearest vertices. The Voronoi vertices (nodes) are the points equidistant to three (or more) sites. In Computational geometry, Voronoi the

diagram is needed first of all to solve the problem of proximity of points (OKABE *et al.*, 2009).

Voronoi diagrams are used in various fields, in particular, in geolocation systems for different search and location analysis, in cartography to delineate the boundaries of the regions and further analyze on its basis. For instance, Voronoi diagram was used (KUZYK, 2014) to determine the area of proximity of trees in the forest, and behind it - the local forestry indicators of the trial area where the fire hazard was investigated.

## **Results and Discussion**

Taking into consideration that the causes of the vast majority of landscape fires are carelessness and human negligence, the conditions that contribute to their occurrence and distribution are considered. The main conditions for the emergence and spread of landscaped fires on large areas are weather conditions such as temperature, relative humidity, rainfall, wind speed (ULLAH et al., 2013). Therefore, it is necessary to analyze the influence of seasonality and weather parameters on the frequency of the occurrence of fires in ecosystems in Lviv region.

The distribution of forest, peat, steppe and fires in landfills monthly has been analyzed during 2016-2018. Fig. 1 shows graphical dependencies in changes in the number of fires in ecosystems for three years.

Exploring the occurrence of steppe fires throughout the year, it should be noted that most often they take place in February-April and August-September (Fig. 1a). In particular, in 2018 the peak of steppe fires was in April (more than 450 fires), for 2016-2017 years –in March (about 300). The second peak of fires was reached in August: in 2016 - 252 fires, in 2017 - 74, in 2018 - 26.

The dynamics of peat fires is somewhat different from the steppe ones (Fig. 1 b).

During 2016-2017, there was a gradual increase in the number of fires by exponential dependence from February to August-September. Within 2018 the peak of peat fires occurred in May. According to Fig. 1 c) and d) the conclusion can be made that there are no clear patterns of their occurrence during the year. These fires start to happen in March-April and ends in October.

The analysis of fires during the last 3 years has shown that there is no close correlation between the number of fires and the months of their occurrence. The highest number of fires in Lviv region is observed at the beginning of the fire-dangerous period when the snow cover melts, as well as during the harvest period.

The fire danger period is a part of the year when forest fires occur (from the moment the snow cover goes down to the onset of durable humid autumn weather or the formation of snow cover).

In China, the research of influence of weather conditions on increase of the forest fires has been conducted by the scientists (ULLAH *et al.*, 2013). They have analyzed the dependence of frequency of forest fire occurrence upon weather parameters (Fire weather index). The results showed that in 2000 - 2009 the number of forest fires and their area were larger when the average FWI value was greater than 50. With an average FWI of less than 27, very few forest fires occurred.

In order to determine the patterns of landscape fires occurrence in Lviv region, the weather parameters of this region and the frequency of these fires were analyzed. Statistical data on landscape fires in Lviv region during 2016-2018 (for each type of fires), as well as the average monthly weather parameters (relative humidity and air temperature) during this period were taken.

To study the influence of weather conditions on the occurrence and spread of fires, the dependence of the fire numbers on the average monthly temperature is considered.

Graphical dependencies in Fig. 2 show that as the average monthly temperature increases, the number of fires grows. This dependence is clearly visible in Fig. 1 b and d (for peat and landfill fires). The Pearson correlation coefficient between the amount of peat fires and the average monthly temperature is R = 0.533, and between the number of fires in landfills and the temperature is R = 0.525. It means that in this particular case a high correlation between the peat fires and the average monthly temperature exists, as well as landfill fires and temperature.

It is also established the mutual influence of the number of fires in natural ecosystems and the average monthly relative humidity of air.

According to Fig. 3, the greatest correlation is found between the average monthly relative humidity and fire numbers in landfills (R = 0.552). As the average monthly relative humidity increases, the amount of landfill fires decreases. Having compared the

study results of the dependence between fire numbers and weather parameters, it was established that the frequency of fires in landfills closely depends on weather parameters (temperature and humidity). The frequency of occurrence of steppe and forest fires less depends on these parameters. Sufficiently significant influence of weather conditions exists on the occurrence of peat fires.

Fig. 4 shows the Voronoi diagrams in places where fires occur in natural ecosystems. The points show the places where fires have occurred most often during 2016-2018. For generating a Voronoi diagram from a set of points in a plane was used by Fortune's algorithm, an  $O(n\log(n))$  algorithm.



**Fig. 1.** Distribution of the number of fires in Lviv region: a) steppe fires; b) peat fires; c) forest fires; d) landfill fires.



**Fig. 2.** Dependence between the amount of fires and the average monthly temperature in Lviv region: a) steppe fires; b) peat fires; c) forest fires; d) landfill fires.



**Fig. 3.** Dependence between the amount of fires and the average monthly humidity in Lviv region: a) steppe fires; b) peat fires; c) forest fires; d) landfill fires.

## Features of Landscape Fires Occurrence (based on the example of Lviv region of Ukraine)

According to Fig. 3, the greatest correlation is found between the average monthly relative humidity and fire numbers in landfills (R = 0.552). As the average monthly relative humidity increases, the amount of landfill fires decreases. Having compared the study results of the dependence between fire numbers and weather parameters, it was established that the frequency of fires in landfills closely depends on weather parameters (temperature and humidity). The frequency of occurrence of steppe and forest fires less depends on these parameters. Sufficiently significant influence of weather conditions exists on the occurrence of peat fires.

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Because of the Voronoi diagrams, a plane is split and each region forms a set of points. It gives us an opportunity to study the biggest accumulation of fires and their environmental impact over a period. The vertices of cell rebars are points that are equidistant from 3 or more (depending on how many ribs intersect at this point) centers of fire origin.







Fig. 4. Voronoi diagrams: a) steppe fires; b) peat fires; c) forest fires; d) landfill fires.

It means that in areas of greatest concentration of fires it is necessary to place firefighting equipment in order to respond in a timely manner to a fire.

#### Conclusions

Having examined the connection between the occurrence of landscape fires and climatic parameters, we have a possibility to predict the peak periods of such fires and with developed preventive measures we can prevent its occurrence, as well as prepare state and voluntary fire brigades. In order to respond in a timely manner to a fire, it is necessary to place firefighting equipment at fire safety points and forest fire stations in such a way that one checkpoint could service several populated areas where fires in ecosystems often occur. The optimal location of these points (taking into account the existing fire departments and community fire units) will be an intersection of the Voronoi chart cells rebars, provided that the distance from the most remote settlement to the point of reference will not exceed 3 km, as required by the State Construction Norms of Ukraine. Features of Landscape Fires Occurrence (based on the example of Lviv region of Ukraine)

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# Soil Biogeochemical Features of Nadym-Purovskiy Province (Western Siberia), Russia

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Abstract. An elemental composition of soils has been studied on the example of northernmost merge of taiga zone in Westerns Siberia, namely in Naym-Purovskiy district. The trace elements (As, Cd, Cu, Mn, Ni, Pb, Zn) content has been determined with the use of atomic absorption spectrometry method. Chemical soil composition was determined with the use of X-ray fluorescence analyzer. It was shown that the silica content in studied soils were about 69-74%, which indicated the long cryogenic transformation and related weathering alteration. The indexes of soil fertility were low due to percentages of nitrogen, which is in maximum is about 0.02 %. The pyrogenic podzols are considered as most fertile, while the C/N ratios here are minimal and the pH indexes are highest. The content of all studied trace elements were low in sampled soils. An average clark values were fixes as following Zn - 0.73; Cu, Mn - 0.32; Ni -0.31; Pb 0.24; Cd and for As was lower than 0.1. The studied province are characterized by extremely low content of elements even if one compare with low background concentration, which is typical for West-Siberian geochemical province. The Cu and Mn concentrations are comparable with the lowest level, described by V.V. Kovalskiy or lower. The biological accumulation of the elements is expressed in low rates in soils of both taxonomy trenches: organogenic and postlithogenic ones (namely in the superficial horizons). Cd is fixed been accumulated in the topsoils of peat eutrophic soils (Dystric Hystosols). Pb was dominated in Histic Gleysols. Other elements did not showany trends of biological accumulation. There is evident eluvial-illuvial profile distribution of iron in Podzols. The differences in elemental composition of organogenic and mineral horizons are low due to penetration of the fine earth into superficial soil horizons.

Key words: soils, trace elements, Western Siberia.

## Introduction

The biological role of trace element is well-known, the low concentration or elevated content of these biochemically active compounds can cause adverse reactions of organisms, even presented by developing of endemic diseases. In the system of biogeochemical regionalization of KOVALSKY & ANDRIANOVA (1970) the

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg territory of the North of Western Siberia is classified as a taiga- forest biogeochemical zone which main properties are the lack of calcium, phosphorus, potassium, cobalt, copper, iodine, molybdenum, a pine forest, zinc and elevated concentration of strontium. At the same time on sites in the affected the wide range of elements belonging to pollutants are accumulated in

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topsoils and this result formation of geochemical anomalies in landscapes. The evident accumulations of Ba, Sr, Pb, Cu on gas fields is noted (OPEKUNOV et al., 2012; Моѕкоvснепко, 2013; Сискоо, 2016). Тhe pollution by nickel and mercury on the southern Yamal (II et al., 2019) is also confirmed. Distant transfer and translocation of pollutants from areas with high concentration of the population and industrial production has a certain impact even on merged areas of the Russian Arctic. In the north of Siberia atmospheric losses of Cu, Ni, Pb which sources are industrial regions of the Urals and Norilsk are comparable with transport of these elements by the Ob River (VINOGRADOVA et al., 2009).

The formation of biogeochemical structure of tundra and taiga landscapes of Western Siberia is substantially defined by active biogenic and water migration of the key of trace elements. The soils of the region are characterized by an acid reaction which promotes *in situ* soil weathering and further mineral part alteration of the parent material components. This result in translocation of trace elements (TM) to soil solutions with the following replacement of the cations in to waters of the rivers and lakes, into plants, and, on the component of the food chains including the animals and human. The intensive immobilization of soluble substances from friable rock mass and soils is typical for cold and over moisted environment and is caused by genesis of taiga soils (TARGULYAN, 1971). It is possible serious accumulation of trace elements in amounts which are higher than threshold concentration due to intensive weathering of mineral of initial parent materials in could and humid pedoenvironments. In tundra plants the are known as intensively uptaken and accumulated (MOSKOVCHENKO, 1995; DOBROVOLSKY, 2003). Due to intensive biogenic migration of the trace elements even at low concentration in soils excess of threshold concentration in live organisms is possible. The low concentrations of the low level of content of Cd and Hg in soils of

Yamal region is fixed simultaneously with increased concentrations of this element in region (AGBALYAN the plants of & LISTISHENKO, 2017). The increased content of mercury is revealed in blood at 41.7% of the investigated the Yamalo-Nenets particular Autonomous Area, in at indigenous representatives of people (Agbalyan & Shinkaruk, 2018; Agbalyan *et al.*, 2018).

The necessity of the assessment of geochemical state the benchmark of ecosystems in the North of Western Siberia is especially relevant in the conditions of strengthening of technogenic influence. In the territory of Yamal region more than 20% of world reserves of natural gas are concentrated. It is proved that the pollution environment caused by of the gas production is hazardous to health of people and can lead to developing of congenital anomalies. Understanding of biogeochemical features of the region is necessary at recultivation of the disturbed sites, calculation of indicators of geoenvironmental risk and development of management methods of this risk management (BASHKIN, 2014; 2017).

In this context, the aim of this research is to estimate the biogeochemical state of the territory of Nadym-Purovsky geochemical region, located in the southern part of Yamal autonomous region. This region is key and very important both for industrial development of the all Russia, and for preservation of traditional environmental management of indigenous ethnic groups of the Far North. Here the considerable part of the local indigenous population of Yamal autonomous region is concentrated, winter reindeer's pastures are located and intensive natural gas exploration is implemented. The following objectiveshave been formulated for achievement of the stated aim of the research: (1) to analyze the concentration of the trace elements in the soil fine earth samples, and (2) to evaluate possible threshold concentration of the trace elements in geochemical region mentioned.

#### **Materials and Methods**

Soil profiles in surrounding of benchmark ecosystems of the Nadym city and Pangody settlement (Fig. 1) were describe and sampled. Sites are located on Arctic peats and taiga landscape. The soils of this area are acid and show the stagnant and gley properties of water migration and nitric type of biogeochemical circulation prevail (NECHAYEVA, 1990). In the surroundings of Nadym a pleistocene-holocene sediments of alluvial genesis prevail. Close to the Pangody settlement the alluvial and marine deposits of the fourth sea terrace dominate (Atlas, 2004). The sampling was conducted in the profiles illuvial podzols (including their pyrogenic suborders), peat soils and gley soils. Sampling was made from the soil horizons within an active layer which thickness varied from 35 cm in peat soils to > 1 m in podzols. On the texture class the soils were mainly classified as sandy class.

Determination of content of trace element concentrations (Cd, Pb, Cu, Zn, Mn, As, Ni) and iron content was carried out by method of an atomic absorbing spectroscopy. microelements The content was of determined by method of the X-ray fluorescent analysis on the SPEKTROSKAN MAKS-GV device in the center of collective use of Institute of physical and chemical and biological problems of soil science of RAS (Pushchino). In samples from four soil pits values of the pH a salt extract (CaCl<sub>2</sub>), hydrolytic titrated acidity and content of carbon and nitrogen were determined according to routine methods procedures.

Statistical treatment of results – arithmetic-mean value of concentration of elements (M), a median (Me) and a mean square deviation (SD), are calculated with use of the SigmaPlot software package. For assessment of biogeochemical features of the surveyed territory clarks of concentration of KK - the relation of maintenance of elements in the soil horizons to clark of the top part of continental crust on (RUDNICK & GAO, 2003) and Shaw's coefficient – average value of clark of concentration of separate elements are calculated. The influence of acidity levels and content of organic matter on elemental composition was estimated with use of the correlation analysis (coefficients of correlation of Spirmen).

The following coefficients has been calculated for evaluation of the soil minerals transformation degree:

-coefficient of an elluviation of Ke (LIU, 2009)

 $Ke = SiO_2/ (MnO + CaO + K_2O + MgO + Na2O)$ (1)

-the index of chemical change of CIA (chemical index of alteration) reflecting a ratio of primary and secondary minerals and characterizing leaching process (NESBITT & YOUNG, 1982):

CIA = 100xAl2O3/(Al2O3 CaO Na2O K2O), (2)

- coefficient of oxidation of Kok = (Fe<sub>2</sub>O3 MnO)/Al<sub>2</sub>O<sub>3</sub> (3)

-index of potential soil fertility of FI (Taylor et al., 2008)

 $FI = (CaO MgO 10P_2O5)/SiO_2 \qquad (4)$ 

#### **Results and Discussion**

The soils investigated belong to trench the Podzols, Histosols and Gleysols according to current Russian soil taxonomy (2004) and WRB (2014). In some soils at few horizons demonstrate the signs of the pyrogenic influence are traced. Results of definition of physical and chemical indicators, amounts of the total carbon and nitrogen in the surveyed soils are given in Table 1.

The soils investigated have acid and strong acid reaction of the fine earth. The pH in salt extracts values changes within 3.8-5.7. The most acid samples are in peat soils (average value pH = 4.1). An acid reaction of soils of the tundra and forest-tundra was repeatedly noted by various authors for the Yamal Peninsula (VASILYEVSKAYA et al., 1986), Nadym-Pur-Tazovsky region (CUCKOO, 2016). In the conditions of low temperatures and over moistening transformation of the plant remnants transformation results in formation of mobile organic acids which are deeply capable to penetrate into the mineral

layers (TARGULYAN, 1967). The pyrogenic podzol are less acid, than their undisturbed mature analog, in particular in the superficial horizons that producing organic acids after the fire is limited. In Podzols the gradient of increasing of pH values with a depth in proportion to reduction of content of organic matter is not revealed. The much less acid reaction was noted the horizon of E as it was described in Poodzols of Siberian Ridge (SMOLENTSEV, 2002), and in BF horizon. The hydrolytic acidity values have a similar trends of profile distribution. Thus, the acidity depends not only on intake of organic acids, but also on processes of fine earth leaching and sesquoxides transformation.

Total organic carbon in peat soils makes more than a half of a bulk composition while in the suprapermafrost horizons of Gleysols and Podzols its contents does not exceed the parts of percents. The decreased mineralization rates of carbon of over moisted soils resulted in accumulations of organic carbon in high values, especially in case of acid soil reaction. The mineral horizons of soils contain extremely small amount of nitrogen which is not exceeding 0.02%. The maximum content of nitrogen (0.28%) is characteristic of the superficial layers and peat horizon Histic Glevsol, while in the suprapermafrost layers the nitrification is practically absent. The amount of nitrogen in the surveyed soils varying within 0.0001 -0.28% is less than values given for soils of the tundra of Fennoscandia (MASLOV, 2015) that demonstrates weaker activity of microorganisms. Extremely low content of nitrogen in soils of the tundra of Western Siberia, in particular in the Arenosols, was published earlier in (TOMASHUNAS & ABAKUMOV, 2014). C/N ratio in the surveyed soils significantly varies. The low C/N values is typical to fertile soils with an intensive mineralization of organic matter are noted in the near-surface mineral horizons of the pyrogenic podzols. In undisturbed podzol of C/N value in the

eluvial horizon is significantly higher that demonstrates to weak biogeochemical circulation that together with the low maintenance of mineral elements does them extremely infertile.

Aeration substantially defines geochemical properties of polar soils and the parent materials. Owing to intensive implementation of soil and weathering prevailing processes, the mineral constituents of soils are friable deposits is the quartz making usually about 70%, and the content of the silicates and alumosilicates presented by potassiumsodium feld-spars, no more than 15-20% (DOBROVOLSKY, 1994). The research of bulk soil composition showed that as a part of the mineral horizons prevail silica dioxide which content varies in small limits, from 69 to 74% (on average 71.2%). Then, in decreasing order, Al<sub>2</sub>O<sub>3</sub>, Fe<sub>2</sub>O<sub>3</sub>, K<sub>2</sub>O (table 2) follow.

The dominance of silica dioxide can be considered as the evidence of long cryogenic transformation and aeration of rocks (DOBROVOLSKY, 1994). In cold and dry environments here is increasing of the relative contents of silica (VITAL & STATTEGGER, 2000; LIU *et al.*, 2015). The total maintenance of SiO<sub>2</sub> prevailing as a part of and Al<sub>2</sub>O<sub>3</sub>, makes 82-85% that soils corresponds to the sizes peculiar to the Arctic soils in general (80-90% of fine earth (DOBROVOLSKY, substance) 1994). In organogenic soils the content of silica dioxide averages 9.3%. Accumulation of SiO<sub>2</sub> in peat soils happens, first of all, to the form of the dust mineral particles brought by wind or melt water. The enrichens of peat in a tundra zone by silty and dusty sized particles was noted by many authors **IGNATENKO** & DRUZIN, 1972; VASILYEVSKAYA, 1980). It was noted (DOBROVOLSKY, 2003) that the maximum content of mineral admixture in peat is characteristic of flat sites on which there is a drain of melt water. Ashes of a peat layer in the top part of a soil profile in the subarctic tundra can reach about 28% (DEDKOV, 1995)

that testifies to dust Aeolian accumulation. Penetration onto the deep into mineral particles, judging by silica dioxide distribution, variously - from 5 cm in the oligotrophous peat soils of peatlands to 20-25 cm in the dry Histic moor horizons of well drained landforms spaces. Sharp decreasing of silica values at depths of 20-25 cm is observed. So, in the dry Histic horizons soil the values of SiO<sub>2</sub> were 15.7% at a depth of 18-22 cm but decreased to 3.7% for 28-32 cm. The values of the index of chemical change of CIA vary in mineral soils in small limits, from 67.6 to 77.7 (average value of 73.9 units). Within the depth extent of chemical change decreases (Fig. 2). It is known that not weathered parent materials are characterized by values of CIA about 50, but in well altered materials values of CIA reaches 100 units (SYSO, 2007). Thus, the surveyed material is characterized by average extent of aeration. In sandy Podzols the values of CIA is higher, than in loamy Cryosols. The coefficient of an eluviation of Ke is also much higher in Podzols that is explained by carrying out of oxides Ca, K, Mg, Na as a result of mobilization by organic acids.

In a reduction pedoenvironment the lower horizons of Cryosols showed the of coefficient of oxidation value significantly decreased, while in Podzols these values were significantly higher and are distributed more homogenous (Fig. 2). It is obvious that the topsoils are aerated highly, then lower, but gradient reduction of sizes oxidation coefficient with a depth it is not revealed. In the superficial layers of Cryosols and Podzols of value coefficient of oxidation increase in comparison with a middle part of a profile a little. Possibly, it is connected not with strengthening of intake of oxygen in the lower part of a profile, and c accumulation of Fe<sub>2</sub>O<sub>3</sub> on a permafrost geochemical barrier during an illuvial redistribution of soil components. In general, according to gross structure eluvial

and illuvial differentiation in soils of a postlitogenic type of profile organization is expressed rather poorly in comparison with Podzols of Siberian Ridge where it, it agrees 2002), where SMOLENTSEV, it was substantiated was very sharply changed in profile. The greatest distinctions characters for CaO which percentages in the horizon E Podzols make 0.91%, and in BC horizon -1.33. The content of  $Fe_2O_3$  changes in a profile of podzol from 4.3 to 5.0%. Possibly, weak differentiation is connected with weaker development of preferential flow in soils because of a smaller amount of precipitation, and youth of soils which development began after more southern analogs.

In the dry Histic soil of value of Ke and CIA in the organogenic horizons up to the depth of 20 cm are similar to values of these coefficients in mineral soils that testifies to considerable impurity of fine earth. The index of potential soil fertility determined mainly by amount of phosphorus in organogenic soils repeatedly above, then in soils of a postlitogenic trunk among which the least fertile are sandy podzols. Content of bulk phosphorus as much as possible in the dry Histic soil where in the top 30 cm exceeds 1%. Low temperatures also lead to decreasing microbiological activity and a mineralization of phosphorus, especially during the winter period that leads to its accumulation (BOWMAN et al., 2003). In the mineral horizons the average content of  $P_2O_5$  was about 0.12%. Distinctly expressed gradient of reduction of content of bulk phosphorus with a depth is observed. Thus, a considerable part of phosphorus is involved in biological circulation, collects in the live phytomass, peat, the superficial horizons of soils. Down a profile phosphorus migrates with organic acids (MASLOV, 2015). According to YANG et al. (2016) content of organic phosphorus is 73%-83% of its general contents in soils of the tundra.

Soils	Hori zon	Depth, cm	pH CaCL <sub>2</sub> .	Hydrolitic acidity, cMP+/100 p	TOC, %	N,%	C/N
	0	0-10	4.1	96.5	38.27	0.07	547
	Е	10-18(20)	3.8	5.73	0.8	0.03	27
Podzol	BF	18-60(65)	4.6	6.25	1.1	0.03	37
	BC	60(65)-90	4.6	6.25	0.6	0.02	30
	С	>90	4.4	5.98	-	-	-
Histososl, Eutric	$TE_1$	0-30	3.9	116	59.86	0.21	285
	$TE_{2h}$	30-75	4.2	116	58.01	0.13	446
	OT	0-10	4.7	106	58.45	0.28	209
	$TE_1$	10-15	4.5	106	57.09	0.21	272
Histic Gleysol,	$TE_{2h}$	15-20(28)	4.8	98.7	56.44	0.19	297
Antric	$G_1$	20(28)-40	4.9	13.3	0.39	0.009	43
	G <sub>2ox</sub>	40-55	4.9	11.7	0.2	0.004	50
	$G_3$	55-80	5.2	10.3	0.17	0.001	170
	Opyr	0-3	5.4	78.8	24.57	0.11	223
	Е	3-10(20)	5.1	4.92	0.19	0.02	10
Podzol, Pyrogenic	$BF_1$	10(20)-25	5.7	7.28	0.28	0.02	14
	$BF_2$	25-40	5.6	7.28	0.40	0.001	400
	$BF_3$	40-50	5.4	6.81	0.31	0.019	16
	BC	50-65	4.9	6.38	0.32	0.001	320
	С	65-100	5.0	6.38	0.16	0.02	8

Table 1. Soil basical chemical indexes

 Table 2. The bulk soil chemical composition.

Oxide	Mineral Layers			Organic layers			
s	М	Me	SD	М	Me	SD	
Na <sub>2</sub> O	0.85	0.83	0.12	0.53	0.54	0.07	
MgO	1.17	1.20	0.13	0.09	0.02	0.13	
$Al_2O_3$	11.8	11.9	0.44	2.51	0.99	2.90	
$SiO_2$	71.2	70.2	2.12	9.3	2.8	14.48	
$P_2O_5$	0.12	0.10	0.07	0.70	0.39	0.57	
S	0.017	0.017	0.007	0.38	0.35	0.12	
K <sub>2</sub> O	1.99	1.91	0.17	0.14	0.02	0.46	
CaO	1.15	1.23	0.18	2.9	2.8	2.1	
TiO <sub>2</sub>	1.01	1.02	0.02	0.19	0.11	0.23	
MnO	0.045	0.040	0.012	0.024	0.025	0.026	
$Fe_2O_3$	4.15	4.31	0.66	3.9	3.7	0.9	

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Fig. 1. The study sites locations



Fig. 2. Geochemical indexes values for various soil horizons.

The bulk content of the trace elements for soils studied is given in Table 3. The percentages of all studied elements in soils below the average world values. Extremely low concentration are noted for Cd, Pb, Fe, As. In Clark's size of concentration elements are ranged as follows: Zn 0.73 > Cu 0.32 > Mn 0.32 > Ni 0.31 > Pb 0.24 > Fe 0.16 > CD 0.08 > As 0.02. The similar results testifying to the low content of heavy metals in soils were received for the adjacent regions of the Yamal Peninsula (TOMASHUNAS & ABAKUMOV, 2014). Lower concentrations of elements were revealed during the research of structure of soils of the Beliy Island (the Arctic tundra of West-Siberian sector) where the average content of elements in the Gleysols of watersheds was: Mn-212; Cu-3.5; Fe-8595; Zn of-20.9 mg/kg (MOSKOVCHENKO et al., 2017). The low portions of minerals which have Clark of concentration < 1 is the general property of weathering layer of parent materials and soils of the Arctic environments (DOBROVOLSKY, 1994) The average content of copper is at the level of the lower threshold concentrations of this element in the soil (KOVALSKY & ANDRIANOVA, 1970), 6-15 mg/kg, and the content of manganese is lower than this border (400 mg/kg). Content of zinc is close to an optimum value.

The average value of coefficient of Shaw for soils Nadym - Purovsky region was 0.3. Earlier when studying element structure of soils of YaNAO it was revealed that sizes of coefficient of Shows average 0.64 (SOROKINA et al., 2007). Thus, even in comparison with poor soils of the North of Western Siberia the surveyed area is characterized by extremely low concentrations of elements in soils. The data obtained confirm a conclusion that soils of Nadym-Pur-Taz region are characterized by the content of heavy metals of 3-9 times below the Clark of values and considerable dispersion of values (CUCKOO et al., 2018). The reasons of low contents are the dominance of sandy textured parent materials with absolute prevalence of silica dioxide and the chemical, mechanical and biogeochemical barriers which are grown poor by minerals, intensive

leaching in the conditions of acid reaction and over moistening and simultaneous low expression of chemical barriers.

The lowest concentration are peculiar to Cd and As that is connected with a lithology the parent materials and intensive leaching. It was noted that in the parent materials of the north of Western Siberia contents the Ca and As of elements is decreased (MOSKOVCHENKO, 2013). Intensive leaching of elements under the influence of organic acids is confirmed by the correlation analysis which showed existence of positive relation between the values of Cd, Fe, Cu and the pH values. Most strongly the percentages of Cu (r=0.85) are affected by acidbase conditions of the fine earth. Only the portions of As (Table 4) are connected to amount of carbon and nitrogen - key elements of organic matter. The content of Cu, on the contrary, decreases at increase within soil organic matter increment.

Comparison of elements content in the organogenic and mineral horizons of the surveyed soils (Table 3) demonstrates biogenous accumulation not only of As, but also Cd while concentration of Fe, Cu, Pb, Ni is higher in the mineral horizons. However distinctions are small and statistically not evident, except for Fe which concentration in the mineral horizons 1.3 times higher (value of criterion Student of t =-2.4;  $\alpha$ =0.05).

The weak relationship between the concentration of minerals on organic matter and small distinctions of element structure of the organogenic and mineral horizons are not typical for soils in general and tundra soils especially. Normally trace elements content are connected with organic matter of soils (KABATA-PENDIAS, 2011). In tundra gley soils distribution of the biogenic elements Mn, Zn, P, has an appearance of the decreasing curve with a maximum of concentration in the top part of the peat horizon (MOSKOVCHENKO, 1995). It was noted that peaks of content of the majority of heavy metals in soils of Yamal coincide with maximal content of soil organic matter (TOMASHUNAS & ABAKUMOV, 2014). It should be noted that the superficial and accumulative distribution of trace element proportional to amount of organic substances content is characteristic not of all soils and in the tundra it is shown mainly fo Ca and As and manganese. Thus, in alluvial sod soils of the Yamal Peninsula in the superficial horizon phosphorus, zinc is typical, while in Histic-Gley soils Zn and Mn accumulate intensively (MOSKOVCHENKO, 1995, 2013).

Data of profile distribution of trace elements are given on the fig. 1 The lack of significant correlation between the content of metals and organic matter (carbon and nitrogen) and also statistically reliable differences between element structure of the organogenic and mineral horizons of soils is connected with a variety of reasons. The presence of fine earth at the superficial peat horizons of soils described result in leveling the impact of biogenous accumulation. Also, rather weak accumulation of trace elements by green mosses - the main precursor of Histic materials in oligotrophous peatland. It green mosses noted that differ was characterizes by the smaller biogeochemical activity in comparison with those typical dominants subarctic tundra as bushes of S. Betula nana, Salix glauca, lanata (MOSKOVCHENKO, 2011).

For more clear understanding of features of biogenic accumulation of elements and their illuviation, the graphs of profile distribution has been created (Fig. 3)

distribution Profile of chemical elements in Podzols shows distinct eluvial and illuvial differentiation of distribution of Fe which minimum content is noted in E horizon, maximum in BF horizon. Cd also accumulated in the illuvial horizon at with minimal concentration in Histic horizon. Pb Cu show moderate decrease in and concentration in E horizon. Content of Zn decreases with a depth slightly. Distinct eluvial and illuvial differentiation of sesquioxides, distribution silicates, of alkaline and alkaline-earth metals in ironilluvial podzols was previously described (SMOLENTSEV, 2002). However, distribution of elements in a profile of Podzols can be essentially changed by the processes of a cryogenic mass exchange leading to changes of stratification of the horizons and further change of distribution of elements. Cryogenic mass exchange does not change the typical profile distribution of elements, however, the general regularities of distribution of elements - falling of concentration of Fe in the horizon of E, the maximum concentration of CD in the top part of the illuvial horizon (BF1) (Fig. 3)

In a Histic Gleysol the sharp increase in percentages of Cu in the gley horizon is noted that indicates weak biogenic concentration of this element. At the same time lead content as much as possible in peat topsoil layers, this is probably, connected with accumulation of this element mosses and lichens in the of increase in atmospheric conditions emissions sites of anthropogenic on influence.

In soils of an organogenic taxonomy trunk the small concentrations of Cd is typical, while in opposite, the Fe, Pb and Zn are increased in mineral layers. Decrease in concentration of Fe in the horizon of BCg of the peat oligotrofic soils with features of cryogenic perturbations soil is caused by leaching of iron from the gley horizons, typical for soils with а reduction environment. It is important to note that processes of a cryogenic mass exchange can change the nature of profile distribution of trace elements, depending on a ratio of an organic and mineral component. Thus, biological accumulation of elements is shown for Cd in peat oligotrofic soils, while the Pb increased concentration was typical for a Histic Gleysols.

It is obvious that the botanical composition of peat defines its chemical composition. There is a number of estimates of intensity of biological accumulation of elements plants of the West Siberian tundra. It was noted high biogeochemical activity of dominant of the subarctic tundra – bushes of *Betula nana, Salix glauca* while green mosses play low role in biochemical transformation processes (MOSKOVCHENKO, 1995). TENTYUKOV (2010) revealed vigorous accumulation by tundra plants of Yamal of such elements as Zn, P, Mn, and the polar birch acts as the most active concentrator of zinc. In the tissues of plants of the Urengoy tundra an intensive accumulation of Cu, Pb, Zn, and weak – the siderofilic elements (Fe, Co) has been recorded (MOSKOVCHENKO *et al.*, 2017).

We have conducted a comparison of elemental composition of oligotropous and autotrophous peats at Histic topsoil horizons of the Podzols (Fig. 4)



Fig. 3. Vertical soil profile distribution of key trace elements (Cd, Fe, Pb, Cu, Zn)



**Fig. 4.** Elemental composition of topsoil organic layers: 1 – oligotrofic peat, 2 – eutrofic peat, 3- organic topsoils of Podzols.

Parameter	Cd	Pb	Cu	Zn	Mn	Fe	As	Ni	
Mineral layers									
M	7.1	5.0	14.1	50.8	264	8574	0.10	16.3	
Me	6.1	4.7	9.6	45.6	262	8221	0.08	16.2	
SD	4.9	1.8	12.2	15.7	119	4653	0.05	5.8	
n	41	27	27	27	27	27	27	27	
Organic layers									
M	12.5	4.1	10.1	50.0	288	6563	0.12	15.4	
Me	8.4	3.9	8.2	46.2	275	5999	0.12	14.2	
SD	17.9	1.8	6.8	13.6	153	2601	0.06	6.6	
n	44	40	40	40	40	40	40	40	
The soil Clark (KABATA–PENDIAS, 2011)	410	27	38.9	70	488	-	6.83	29	
The Lithosphere Clark (RUDNICK & GAO, 2003)	90	17	27	67	774	39180	4.8	47	

**Table. 3.** Statistic parameters of data obtained (mg/kg, Cd- $\mu$ kg/kg).

**Table 4.** Values of Spearmen correlation coefficients.

Parameter	Cd	Cu	Fe	As
TOC		-0.53		0.55
Ν				0.51
_pH	0.66	0.85	0.52	

According data obtained, to oligotrofous peat demonstrate higher concentrations of Cd, Cu, Fe. Eutrophic peats demonstrate higher portions of Pb. Zn poorly is fixed on a biogeochemical barrier. In the topsoil Histic layer of Podzols the concentrations of elements is less, than in peat materials of organogenic soils. This can be connected with peculiarities of mineral substrate and the weak accumulation of elements by the Cladonia lichens dominating in structure of a vegetation on sites with domination of Podzols. It was noted that superficial soil lichens of the Cladonia genera accumulate significantly less elements, than epiphytic lichen Hypogymnia an of (VALEEVA & MOSKOVCHENKO, 2002). Thus, accumulation weak of elements on geochemical barriers, including biogeochemical barriers is characteristic of the surveyed territory.

#### Conclusions

The investigations of main types of soils of Nadym-Purovskiy region, located in the

southern part of Yamal autonomous region showed the dominance of the representatives of following soil types: Podzols, Cryosols and Oligotrophic and Eutrophic peat soils. Data obtained for the first time give important information about the biogeochemical peculiarities of the territory. Soils of the Nady-Purovskiy region are formed under the effect of long-term cryogenic alteration and it-situ soil weathering. These processes resulted in accumulation of the silica oxides in main mineral horizons. The content of SiO<sub>2</sub> varies from 69 to 74% while the  $Al_2O_3$  varies from 11,1 to 12,1% in soils investigated. Soils investigated characterizes by acid or strong acid reaction. This is a reason of intensive leaching of trace elements and their low accumulation degree on the geochemical barriers. The low content of main element (which is close to deficit) in comparison with Clarks and average content in soils has been fixed. An average value of the concentration coefficient was 0.73, 0.32, 0.31, 0.24, 0.16, Cd and lower than 0.1 for Zn, Cu, Mn, Ni, Pb.

Fe. Cd As correspondingly. The profile distribution of elements in soil profiles showed that Cd was accumulated in the superficial soil layers of oligotrophic peats. The lead concentrations were increased in peat-glevic soils. There was not an essential accumulation of the trace elements in organic topsoils of the pits investigated. The differences between organic and mineral soil horizons are not essential because the fine earth penetration of the mineral particles into superficial layers of the peat soils. Evident eluvial-illuvial differentiation is characteristics only for iron in soil profiles investigated. The podzols, affected by pyrogenic impact have much lees acids pH values. The region surveyed is characterized by very low concentrations of the trace elements, even one compare with other soils of West-Siberian geochemical province. At the level of threshold border and below it there were concentrations of Cu and Mn. Thus, the Nadym-Purovskiy region can be classified as a specific geochemical province of the North-Western Siberia, which is characterizes by very low concentration of all trace element investigated.

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# Methods of Calculating the Emissions of Greenhouse Gases from Cattle and Buffalo Housing in Bulgaria

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**Abstract.** A methodology for measuring greenhouse gases from mixed excrements from cattle and buffaloes was developed. A universal formula was used to calculate them. The authors gave an example of a calculation. They reflected the typical excrement distribution based on the most used animal breeding technologies and the net energy in feed and excrements in Bulgaria.

Key words: cattle farming, greenhouse gases.

## Introduction

Over the years, the European legislation requires more decisive measures to limit pollution in the continent caused by waste products from both air and liquid emissions. In this respect, a number of emission limit values have been established and implemented for all Member States -Environmental policy review, 1996/62; Directive 2001/80/EC (EC, 2001); Directive 2009/29/EC (EC, 2009).

All levels of the practical training of future environmental professionals should include the implementation of strategies for objectively reporting any problems. In this way, the learners will acquire basic skills that will form the basis for good practices of limiting emissions and maintaining the ecological balance in nature (VAKLEVA, 2014; 2017).

Cattle farming and buffalo farming are considered some of the major greenhouse

gas pollutants (FAO, 2017). At the same time, these livestock sub-sectors are the most developed in Bulgaria. This is why measuring the gas emissions in them accurately is the basis for finding more accurate technological solutions for limiting them. Another important point is that accurate emission calculations are the basis for a fairer allocation of greenhouse gas emission allowances.

The development and implementation of up-to-date methodologies for calculating of livestock emissions has been the subject of previous publications of ours. These works observed other livestock sub-sectors (PENKOV *et al.*, 2012; 2014). Similar principles of calculation are shown in our previous publications for sheep and goats (PENKOV *et al.*, 2019), but the technique of calculation is different due to the different technologies and terms of cattle housing.

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg Union of Scientists in Bulgaria – Plovdiv University of Plovdiv Publishing House The aim of the publication is to offer an objective methodology for calculating the fertilizer emissions from cattle/buffalo breeding in Bulgaria, based on "volatile solid excretion".

#### Material and Methods

For the general separation of greenhouse gases from all excrements we use the adopted by us formula of the MEW which converts all emitted gases (base – methane) into a conditional "pollutant" (IPCC, 2001):

VS = GE\*(1 kg-dm/18.45 MJ)\* (1-DE/100)\*(1 - ASH/100)(original formula for 24 hours per 1 kg DM)

VS=(DMI\*18.45)\*(1-DEI/100)\*(1-%ASH/100) (adopted formula on base total DM intake and total DE intake per 1 animal/24 hours)

where: VS- volatile solid excretion per 24 hours on a dry matter weight basis – kg DM (dry matter) per 24 h; DMI- dry matter intake – kg/animal/24h; 18.45 – mean gross energy content in 1 kg dry matter of the fodders – MJ; DEI – digestible energy intake from 1 animal/24h (as coefficient, or percentage) from the gross energy intake; %ASH – percentage of ash in the DM of the excrements

For greater clarity we are going to demonstrate our reasoning with the following example: define the volatile solid excretion for 1 year (365 days) in a farm with 100 dairy cows, average annual fertility of the cow herd - 90% (90 calves born alive per 100 cows). All heifers stay in the farm until they become 18 months old (2 months into their first pregnancy). All bull calves are sold for fattening when they are 120 days old. The example is chosen as typical for technological conditions of cattle in Bulgaria, but the numbers can be changed to any specific technologies.

The average data for emitted mixed excrements (faeces and urine) as well as for the dry matter and ash content in them is taken from PETROV *et al.* (1983). The data for the content of percentage/coefficient of the energy exchange as well as for the dry matter intake in each animal category is calculated on the basis of the new energy system for assessment of fodders in Bulgaria (TODOROV, 1997). Because of the similarity in the utilization of nutrients in cattle and buffalos, we are assumed that the principle of the calculation will be the same in both species.

#### Results

Defining the quantity of the manure waste from the animals in the farm and the chemical composition of the faeces and the urine:

According to the aforementioned source for a 24-hour period 25 kg of faeces and 15 kg of urine are emitted from a cow (live weight of 500-550 kg). 12.5 kg of faeces and 7.5 kg of urine are emitted from a calf (live weight of 250 kg).

Although the data for the dry matter content in the faeces may vary greatly depending on the fodder intake, PETROV et al. (1983) provide us with general values which are 16.66% for cows (variations from 13.77% to 18.75%) and 18.86% for calves with a live weight of 250 kg on average with variations from 16.46% to 22.16%. Bearing in mind that suckling calves are freely given concentrate as well as hay from the first week, in the calculations we assume that at the end of the first month the dry matter in their faeces will not be any different from the aforementioned data. The ash content in the cow faeces are 1.1% total in fresh faeces (PETROV et al., 1983) or 6.6% when recalculated on the basis of dry matter (DM). For calves the data is 1.3% and 6.97% respectively.

For the urine the data cited by the same source is respectively: For cows: DM – 4.18% (variations from 2.91% to 6.98%); calves (250 kg) – 4.68% (variations from 3.63% to 5.61%). The average ash content in the cow urine is 0.103% (2.46% on the basis of dry matter), and for calves – 1.74% (37,2% on the basis of

dry matter). We believe that the percentage values do not vary substantially depending on the age and the physiological condition of the different cattle categories.

Defining the quantity of the dry matter intake of the animals, according to the formulas given by TODOROV (1997).

For dairy cows with a live weight of 500 kg and an average annual milk yield of 7300 kg (average milk yield throughout the year of 20 kg), the daily consumption of dry matter is the following: DMI =  $(0.093*500^{0.75})$  + 0.3 \* 20 = 15.83 kg, where 500<sup>0.75</sup> – metabolizable mass of the animal.

For heifers with a live weight of 250 kg and an average daily growth of 0,75 kg, the daily consumption of dry matter (in kg per 24 hours) is: DMI =  $6/ \{(25/250) + (0.34*0.75) + 0.57\} = 6.49$  kg. Since this is the average live weight between the birth of the calf and its transformation into a pregnant heifer – second month, we assume that the average consumption per day from the birth to the 548 day after the birth will be 6.49 kg.

For bull calves with a live weight of 250 kg and an average daily growth of 1.0 kg, the daily consumption of dry matter (in kg per 24 hours): DMI =  $6.1/\{(25/250) + (0.34*1.0) + 0.57\} = 6.04$  kg, but the bull calves are sold at the end of the third month on the basis of a live weight of 125 kg, thus the average consumption of dry matter before the sale will be 3.02 kg. From the birth the bull calves begin their consumption from 0 kg, so the average consumption of dry matter per every 24 hours at the farm will be 1.51 kg.

Defining the correlation expressed in percentage between the gross energy intake and the nett energy intake. We offer replacement of metabolizable energy with nett energy, because it is - an objective indicator of the distribution of energy in the body (TODOROV, 1997).

For dairy cows with a live weight of 500 kg and a daily milk yield of 20 kg – the daily nett energy intake is 87 MJ, and the gross energy intake is – 15.83\*18.45 = 292.06 MJ, therefore DEI = 87/292.06 = 0.298.

For heifers for breeding purposes and young cows: the daily intake of nett energy

is 34.2 MJ, and of gross energy – 6.49\*18.45 = 119.74 MJ, therefore DEI = 87/292.06 = 0.298.

For bull calves for fattening with a final weight of 125  $\kappa$ g (bred for additional fattening in other farms): the daily intake of nett energy is 19.2 MJ, and of gross energy – 1.51\*18.45 = 29 MJ, therefore DEI = 18.45/29 = 0.636.

Calculating the greenhouse emissions from the different cattle categories in the farm.

Recalculation of the average percentage of ashes in mixed excrements of dairy cows:

- Faeces: 25 kg \* 16.66% = 4.165 kg DM \* 6.6% = 0.275;

- Urine: 15 kg\*4.18% = 0.627 kg \* 2.46% = 0.016 or the total percentage of ashes in the excrements is 0.291% in the DM.

Released emissions from 100 dairy cows, per 365 days: VS =  $\{(15.83*18.45)*(1-0.298/100)*(1-0.291/100)*100*365\} = 11294299 kg dry matter (11,294.30 tons).$ 

Recalculation of the average percentage of ashes in mixed excrements of calves (250 kg LW):

Faeces: 12.5 \* 18.86% = 2.3575 kg DM \* 6.97% = 0.164.

Urine: 7.5\* 4.68% = 0.351 kg DM \* 37.2% = 0.131 or the total percentage of ashes in the excrements is 0.297% in the DM.

Released emissions from heifers for breeding purposes and young cows until the age of 18 months and recalculation for 1 year.

For 1 year with 90 calves born, 45 will be female and 45 will be male, therefore: VS =  $\{(6.49*18.45)*(1-0.298/100)*(1-0.297/100)*45*365\} = 1995053 \text{ kg dry matter (1995.05 tons).}$ 

Released emissions from bull calves during their time at the farm (approximately 120 days) – this will also be for the whole year: VS =  $\{(1.51*18.45)*(1-0.636/100)*(1-0.297/100)*45*120\}$  = 149041 kg dry matter (149.04 tons).

The total quantity of volatile solid excretion per the whole year on a dry matter weight basis from the farm activity under the stipulated conditions will be 13438.39 tons.

#### Discussion

The proposed calculations are rather broad, but they represent to the greatest level the correlation between the fodder intake (forage: concentrate) as well as their average energy transformation in the body of the ruminant animals (correlation gross energy: nett energy). All formulas included in the calculations are revised and adapted by us for the needs of the current examination. Apart from the data used from the aforementioned sources, we conformed the basic examinations of the energy transformation from gross energy content of the fodders to its specific transformation to metabolizable energy separately in the organism of full-grown dairy cows and young grooving calves (INRA, 1987). The daily consumption of dry matter of the different cattle categories in defined on the official basis in Bulgaria (TODOROV, 1997).

The methods which are proposed are compatible with the modern ideas of defining the emissions from manure waste from cattle and can be used for defining the quotas for each European Union member state (on the basis of the statistical data about the number of cattle/buffalos in the different categories) and also have at least two advantages:

1. The methods can be used for recording the emissions in investment programs for setting up and modernizing cattle breeding farms – mainly in the dairy cattle breeding, but they can be used as a basis for creating methods for the field beef (for meat production).

2. The methodology as a whole (or parts of it) can solve practical problems in ecology education in the specialised agricultural secondary schools and universities and also in the educational establishments where ecology is taught.

We believe that the last 2 activities in particular will fill a void and bring the Bulgarian and European specialised education near the level of the modern ecological requirements. Last, but not least, there is the fact that the aforementioned calculations can be digitalised (in Excel) and developed into an automated software programme for calculating emissions.

It is proper to mention that apart from the manure waste the ruminant animals also release a significant amount of fermentation gases (mainly methane) which should be added. There gases are described in the literature according to their quantity and age and they are not the subject of this article.

Recalculation based on 365 days instead of 24 hours is more acceptable because emission allowances are determined for 1 year.

## Conclusions

The proposed methodology is compatible with the used principles of greenhouse emission's recording. It could be for developing practical cases for the people who study domestic animal's ecology/breeding and for all professionally concerned parties.

The basic calculations described in this article can easily be digitalised and developed into a software package for automatic calculation of greenhouse emissions.

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# Comparative Leaf Epidermis Analyses of Micromeria frivaldszkyana (Degen) Velen. and Clinopodium vulgare L. (Lamiaceae) from Bulgarka Nature Park, Bulgaria

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**Abstract.** The present work presents a comparative anatomical study of the leaf epidermis of the Bulgarian endemic *Micromeria frivaldszkyana* (Degen) Velen. and *Clinopodium vulgare* L., belonging to the family Lamiaceae. In both species they occur diacytic and anomocytic stomatal type, while the indumentum is presented by multicellular, linear covering trichomes and multicellular, stacked glandular trichomes. The results obtained from the statistical data processing contribute to the distinction between the two species with respect to the peculiarities of the leaf epidermis. Taxonomically significant anatomical features for their determination are: number, width and length of stomata on the abaxial epidermis; width of on the adaxial epidermis; number, width and length of basic epidermal cells along both epidermis and thickness of upper and lower cuticle.

Key words: Micromeria frivaldszkyana, Clinopodium vulgare, leaf epidermis, Bulgarka Natural Park.

## Introduction

Globally, the Lamiaceae family includes about 258 genera and 3,500 species (DUARTE & LOPES, 2007). According to HARLEY *et al.* (2004) and SINGH (2010) it comprises about 236 genera with 7173 species. Many of them have medicinal properties due to the essential oils contained in their various parts and are widely used as a raw material in the cosmetic industry.

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg Others are used in the official medicine of Anatolia, Europe, China and others (BAYTOP 1999; CUI *et al.*, 2003).

Genus *Micromeria* Benth. belongs to the Nepetoideae subfamily, the Menthae tribe. According to MORALES (1993) and BRÄUCHLER *et al.* (2005), it includes about 70 species distributed from the Himalayas to the Macaronesian Archipelago, and from the

Union of Scientists in Bulgaria – Plovdiv University of Plovdiv Publishing House Mediterranean to South Africa and Madagascar. ARABACI *et al.* (2010) describe the genus as a complicated complex comprising about 54 species, 32 subspecies and 13 varieties. In Europe, the genus is represented by 21 species (TUTIN *et al.*, 1972), of which *M. frivaldszkyana* and *M. juliana* are also found in our country.

There are 4 species of Bulgarian flora, (STOJANOV *et al.*, 1967; KOZHUHAROV, 1992; DELIPAVLOV & CHESHMEDZHIEV, 2003), of which 1 Balkan endemic – *Micromeria dalmatica* Bentham ssp. *bulgarica* (*Velen*) Guinea and 1 Bulgarian endemic – *Micromeria frivaldszkyana* (*Degen*) Velen.). The species *M. frivaldszkyana* is included in Biological Diversity Act (2002), Red list of Bulgarian vascular plants (PETROVA & VLADIMIROV, 2009) with a category "endangered", Red Data Book of the Republic of Bulgaria. Vol. 1. Plants and Fungi (PEEV *et al.*, 2011).

The genus Clinopodium L. is cosmopolitan (HARLEY et al., 2004). Recently, based on biosystematic studies, a number of taxa have been added to the genus Clinopodium, thus making the species composition in the genus approximately 100 (FIRAT et al., 2015). *Clinopodium vulgare* L. is the only one represented in Bulgaria (DELIPAVLOV & CHESHMEDZHIEV, 2003). The species is ubiquitous in the Bulgarian flora, known for its medicinal properties and included in the Bulgarian Medicinal Plants Act (MPA, 2000).

A number of molecular and morphological studies have shown a close relationship between the genera *Micromeria* (section Pseudomelissa) and genus *Clinopodium* (BRÄUCHLER *et al.*, 2005; 2008).

highly Although influenced by environmental factors, anatomical features, including epidermal structures, are an important marker for determining the boundaries of taxa from different plant families (METCALFE & CHALK, 1950; 1979; STENGLEIN et al., 2003; SATIL & KAYA, 2007; SALMAKI et al., 2011; CELEP et al., 2014).

The highest taxonomic value for the representatives of the Lamiaceae family are the structure and distribution of the trichomes covering their vegetative organs, as well as the type of stomatal apparatus (METCALFE & CHALK, 1972; KAHRAMAN *et al.*, 2010; VENKATESHAPPA & SREENATH, 2013).

According to METCALFE & CHALK (1985) at labiate plants have covering and glandular trichomes. According to the authors, the glandular trichomes can be in the form of unbranched papillae, stellate and others.

A number of studies show that there are mainly two types of stomatal apparatus in the Lamiaceae family – anomocytic and anisocytic (HARUNA & ASHIR, 2017).

The data on anatomical studies of both genera are relatively small. One of the recent studies is that of KAYA (2016), in which the stem and root anatomy of 6 species of the genus is affected *Clinopodium*. A year earlier AL-ZUBAIDY *et al.* (2015) gives a full description of the stem and leaf anatomy of the genus, also describing the type of trichomes covering the vegetative organs.

There are a number of studies for representatives of the genus *Micromeria* providing information on their morphological characteristics (ARABACI *et al.*, 2010; KREMER *et al.*, 2012; MARIN *et al.*, 2013). SLAVKOVSKA *et al.* (2017), analyzing the leaf and stem anatomy of 9 species of the genus *Micromeria*, they find that the anatomical features reflect deeper genetic differences and have a taxonomic value. In addition, some anatomical features of the genus are also affected in the studies of KOCA (1996; 2002) M MOON *et al.* (2009).

The main objective of the present work is a comparative anatomical study of the leaf epidermis of the taxonomically controversial species *M. frivaldszkyana* and *C. vulgare*, with the aim of separating the features serving to distinguish the two taxa.

## Material and Methods

The plant material (leaves) of the two studied species was collected during the 2019 growing season from the territory of Bulgarka Nature park – Stara planina floristic region (Central). The species were identified at the Department of Botany and Methods of Teaching Biology, Faculty of Biology, University of Plovdiv "Paisii Hilendarski" according to DELIPAVLOV & CHESHMEDZHIEV (2003). The voucher materials were deposited in the Herbarium of Agricultural University - Plovdiv (SOA).

The leaf material is fixed in 70% ethanol, then histological preparations of leaf epidermis are made. The following qualitative and quantitative features were followed: hair type; type, number (1 mm<sup>2</sup>), width ( $\mu$ m) and length ( $\mu$ m) the stomata on the adaxial and abaxial epidermis; number (1 mm<sup>2</sup>), width ( $\mu$ m) and length of base cells on both epidermis; cuticle thickness ( $\mu$ m) on both leaf surfaces.

The results for quantitative traits are based on a review of 50 fields of view. Lightmicroscopic images of each of the scars examined were taken using a microscope *Magnum T*, equipped with photo documentation system *Si5000* at magnification x100 до x400 in Department of of Botany and Methods of Teaching Biology, Faculty of Biology, University of Plovdiv" Paisii Hilendarski". The processing of the quantitative data received for each sign is done using a statistical processing program IBM SPSS, ver. 20 (IBM, 2018). The critical level of significance used is p=0.05.

## **Results and Discussion**

In the analysis of the adaxial and abaxial leaf epidermis of the two species studied, a single-layered structure was found, with main cells having different sizes and degrees of folding of anticlinal walls (Fig. 1). According to the classification of ANELI (1975) they refer to zig-zag folded cell walls, but according to the classification of SVESHNIKOVA (1970) are

defined as curved to highly curved. In addition to these cells, the epidermis of *M. frivaldszkyana* and *C. vulgare* also include stomata cells, subsidiary cells and trichomes cells.

The leaf surface is covered with a welldefined cuticle of varying thickness, with different anatomically distinct trichomes.

Stomata

In the epidermis of the two analyzed taxa predominates diacytic stomatal type, but in places they are also found anomocytic stomatal type. In the first type, there are two around the stomatal cells perpendicular to the ostiolum, while for anomocytic stomata are characteristic subsidiary cells, which do not differ in shape from the underlying epidermal cells (Fig. 2). The location of the stomata on both epidermis defines the leaves as amphistomata. These results are in line with the statements of METCALFE & CHALK (1950) and HARUNA & ASHIR (2017) for leaf type and stomatal apparatus with members of family Lamiaceae.

Trichomes

Two types of trichomes were found on the surface of the adaxial and abaxial epidermis of the two species studied - covering and glandular (Fig. 3). The glandular trichomes are multicellular, stacked. These results contradict the data obtained from YA'NI *et al.* (2018), for other species of family Lamiaceae, in which the authors establish multicellular glandular trichomes with unicellular head. Established by AL-ZUBAIDY *et al.* (2015) two cell structure of the glandular trichomes of genus *Clinopodium*, was not found in this study.



Fig. 1. Basal epidermal cells of Micromeria frivaldszkyana (a) and Clinopodium vulgare (b).

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**Fig. 2.** Anomocytic and diacytic stomatal type of *Micromeria frivaldszkyana* (a, b) and *Clinopodium vulgare* (c, d).



**Fig. 3.** Covering and glandular trichomes of *Micromeria frivaldszkyana* (a, b) and *Clinopodium vulgare* (c, d).
### Abaxial epidermis

The results of the statistical processing of quantitative data by using Students` T-Test, for the abaxial epidermis in both species are presented on Table 1.

The table shows that a statistically significant difference (p≤0.001) between the two types exists with respect to all seven quantitative features. The number of stomata at M. frivaldszkyana value (mean of 201.95 number/mm<sup>2</sup>) is significantly larger than that of *C. vulgare* (mean value of 100.49 number/mm<sup>2</sup>). In proportion to this trend is the reduction in the size of the stomata at C. vulgare. M. frivaldszkyana shows higher mean values for width and length of the stomatal cells. The statistical differences with the highest significant difference ( $p \le 0.001$ ) regarding these three quantitative dimensions of the stomata apparatus between the two species necessitate further ecological studies of the two

taxa. The need for these studies is based on the claims made by ZARINKAMAR (2007), who reports that regulation of the size, density and placement of the stomata is a major way of adapting plants to environmental conditions. A smaller number is found in the major epidermal cells of C. vulgare (696.10 number/mm<sup>2</sup>) at the expense of larger sizes (width and length) than at M. frivaldszkyana (P≤0.001). One of the major contributing factors of small cells according to NINOVA & DUSHKOVA (1981) are the lower temperatures in the habitat. Mean cuticle thickness at M. frivaldszkyana (1.81 µm) are twice as large as those of C. vulgare (0.91  $\mu$ m). The statistically significant difference between the two species for this sign is again of the highest significance (p≤0.001).

#### Adaxial epidermis

Table 2 shows the results obtained from the treatment of adaxial epidermis data for both taxa.

**Table 1.** Comparison of the mean values of anatomical characteristics in *Micromeria frivaldszkyana* and *Clinopodium vulgare* for abaxial epidermis. Legend:  $X_1$  – Mean value of *M. frivaldszkyana*;  $X_2$  – Mean value of *C. vulgare*; SE – Standart Error of Mean value; T – Students' T-test; *p* – Accuracy indicator; *p*≤0.001 - \*\*\*.

Feature	<b>M. frivaldszkyana</b> X1±SE	C. vulgare X2±SE	Т	p
Stomata (Number)	201.95±3.34	100.49±3.53	20.87	0.000***
Stomata (Width)	4.02±0.06	0.95±0.03	44.15	$0.000^{***}$
Stomata (Length)	5.87±0.07	$5.05 \pm 0.05$	9.92	0.000***
Epidermal cells (Number)	865.37±8.46	696.10±6.04	16.29	0.000***
Epidermal cells (Width)	5.41±0.07	5.86±0.06	-4.73	0.000***
Epidermal cells (Length)	14.81±0.27	18.07±0.26	-8.71	0.000***
Cuticle (Thickness)	$1.81 \pm 0.03$	0.91±0.03	22.43	0.000***

**Table 2.** Comparison of the mean values of anatomical characteristics in *Micromeria frivaldszkyana* and *Clinopodium vulgare* for adaxial epidermis. Legend:  $X_1$  – Mean value of *M. frivaldszkyana*;  $X_2$  – Mean value of *C. vulgare*; SE – Standart Error of Mean value; T – Students' T-test; *p* – Accuracy indicator; *p*≤0.05 - \*; *p*≤0.001 - \*\*\*.

Feature	M. <i>frivaldszkyana</i> X1±SE	C. vulgare X2±SE	Т	p
Stomata (Number)	42.93±2.57	37.56±2.33	-1.55	0.125
Stomata (Width)	3.03±0.04	2.87±0.05	-2.51	$0.014^{*}$
Stomata (Length)	6.10±0.04	4.83±0.05	-21.08	0.000***
Epidermal cells (Number)	714.15±6.43	465.37±7.97	-24.30	0.000***
Epidermal cells (Width)	6.34±0.07	7.96±0.03	20.04	$0.000^{***}$
Epidermal cells (Length)	17.09±0.18	13.01±0.07	-21.23	0.000***
Cuticle (Thickness)	1.91±0.03	1.12±0.03	-19.72	0.000****

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Analyzing the upper leaf surface, it was found that there is no statistically significant difference between the number of stomata in the two species, which defines the sign as unstable. The least significant difference  $(p \le 0.05)$  is the difference between the width of the stomata, where M. frivaldszkyana shows higher mean values (3.03 number/ mm<sup>2</sup>). The trend in the mean values for the other features is similar to that of the abaxial epidermis. The only difference is in the length of the epidermal cells, which in the epidermis adaxial is larger in М. frivaldszkyana. The statistically significant difference (p≤0.001) for the sign length, number, width, and length of the major epidermal cells and thickness of the upper cuticle defines them as stable and suitable for the correct determination of the two taxa.

## Conclusions

A comparative anatomical study of the epidermis in the species leaf М. frivaldszkyana and C. vulgare was made for the first time in Bulgaria. The two species are indistinguishable in terms of the quality characteristics tracked - the type of stomata apparatus (diacytic and anomocytic) and type of trichomes (multicellular, linear covering trichomes and multicellular, glandular stacked trichomes). The differences between the values of the quantitative marks (number, width and length of the stomata on the lower epidermis; length of the stomata on the upper epidermis; number, width and length of the main epidermal cells on the two epidermis; thickness of the upper and lower cuticles) are significant, which defines them as suitable for distinguishing the two taxa.

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# Floristic Diversity of Certain Wetlands in Southern Bulgaria

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**Abstract.** The two-year study on the species composition of higher plants was conducted in three moisture zones in the Maritsa river valley, Bulgaria: "Rice-field Plovdiv", protected zone (PZ) "Rice-field Tsalapitsa" and protected area (PA) "Martvitsata Zlato Pole". The analysis was done, using the floristic methods. There were 154 species of vascular plants identified, which belong to 125 genera and 43 families. The highest floristic diversity was found for PA "Martvitsata Zlato Pole" – 74% of the total number of species found, followed by "Rice-field Plovdiv" (47%) and PZ "Rice-field Tsalapitza" (36%). The families Asteraceae, Poaceae, Fabaceae and Lamiaceae have the largest number of representatives. The total floristic composition of the three tested areas showed the predominance of dicotyledonous taxa. The comparative analysis of the biological types showed the prevalence of the perennial herbaceous plants, followed by the annual plants.

Key words: species composition, vascular plants, moisture zones, floristic diversity.

### Introduction

At the beginning of the 20<sup>th</sup> century, the wetlands in Bulgaria covered 2% of the country's territory and now they have decreased 20 times. Many of them were drained, ploughed or construction took place on those sites, without evaluating their importance (National Plan for Conservation of the Most Significant Wetlands in Bulgaria 2013-2022). As a result, a number of plants spread only in riparian areas, have been included in the Red data book of the Republic of Bulgaria, Vol. I (PEEV *et al.*, 2015) and the Biological Diversity Act (2002) with an endangered status endangered [EN], such as *Nymphaea alba* L., *Utricularia minor* L.,

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg *Aldrovanda vesiculosa* or regionally extinct [RE], *Caldesia parnassifolia* (L.) Parl.

The great species diversity of flora and fauna in the riparian wetlands is the reason for their putting under some form of legal protection – protected areas within the meaning of the Protected Areas Act (1998) and/or Natura 2000 protected areas within the meaning of the Biological Diversity Act (2002). When determining the conservation status of most of the wetlands along the Maritsa River, particular attention is paid to the specific species composition of the fauna. The floristic composition of higher plants in those areas has been comparatively poorly studied until now.

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VIHODTSEVSKI (1963) mentioned individual plant species along the Maritsa River valley. The author pointed out some new species for the flora of Bulgaria and some new habitats of rare species. A detailed floristic description has not been made.

Describing the flora of the Maritsa River banks, BONDEV (1991) mentioned the existence of black alder, willow and poplar forests, combined in some places with artificially planted poplar trees and hygrophytic grass communities.

Partial data about the species composition of higher plants along the Maritsa River were found in the plans and reports developed for the conservation of that territory (MESHINEV & APOSTOLOVA, 2006; MARINOV et al., 2007; Management Plan for the protected area for birds BG 0002086, "Rice Fields Tsalapitsa", 2013). The following species were determined as hygrophytes: common Potamogeton berchtoldii Fieber, Potamogeton nodosus Poir. The presence of helophytes as Sparganium erectum L., Lythrum salicaria L., Polygonum hydropiper L., Typha latifolia L., Epilobium *hirsutum* L. along the riverside is significant. The river banks are overgrown with ruderal grassy hygrophytic species and single trees of white willow (Salix alba L.).

The lack of detailed floristic studies of the riparian wetlands is the main reason for carrying out the present research, which aims at making an inventory of the species composition and assessing the floristic diversity of three selected representative areas along the Maritsa River valley, subject to different anthropogenic impacts.

## Material and Methods

The present study is part of a project of the Faculty of Biology at the University of Plovdiv "Paisii Hilendarski", related to the complex eco-biological assessment of the status of wetlands in southern Bulgaria. The project identified three territories along the Maritsa River and they can be related to two types of standing water ponds – rice paddies and old beds of large lowland rivers – "martvitsi" (oxbow lakes). Two areas – "Rice Fields Plovdiv" and the protected zone (PZ) BG0002086 "Rice Fields Tsalapitsa" – were floristically explored in detail for the first time. The two territories constitute a complex of wetland areas used at present for rice production, surrounded by low dikes, canals and uncultivated deserted lands. They are located in close proximity to densely populated areas and are subjected to strong anthropogenic pressure.

The second type included the protected area (PA) "Martvitsata" near the village of Zlato pole, Municipality of Dimitrovgrad. It was declared as a protected area by Order RD-476 of MOEW (State Gazette 73/2001). It is the largest wetland of a natural origin along the Maritsa River, its conservation status implying a weaker anthropogenic pressure (Fig. 1).



**Fig. 1.** Indicative map of the locations of the three wetlands in southern Bulgaria.

The study of the species composition of higher plants in the three selected areas was carried out by field surveys and systematic collection of materials in the period 2017-2018. Each of the three territories was labelled as a 5 km long transection, at the center of which a GPS coordinate point was put: "Rice Fields Plovdiv" – N42010'14", E24040'31"; "Rice Fields Tsalapitsa" – N42012'02", E24035'59"; "Martvitsata – Zlato pole" – N42002'12", E25042'55". Locations and sizes of the transects are consistent with the requirement for the highest possible representativeness and comparability in the analyses of the selected zones.

The taxonomic approach was chosen as basic for the analysis of the floristic composition in the three zones. The ecological spectrum of the flora of these three zones was stated in a previous study - RADOUKOVA *et al.* (2018).

The identification of plant species composition was carried out on-site and under laboratory conditions. The following sources were used for identifying the plant species and their biological type: Flora of NR Bulgaria (YORDANOV, 1963-1979), Guidebook to the higher plants in Bulgaria (KOZHUHAROV, 1992), Key to the Plants in Bulgaria (DELIPAVLOV & CHESHMEDZHIEV, 2003).

The list of the taxa found was alphabetically arranged by the names of families, genera and species.

The quantitative assessment of the floristic diversity was carried out according to the methods of SHMIDT (1980) and the works of KAMELIN (1973) and TOLMACHEV (1986).

The number of species, genera and families of the Bulgarian flora follows the DELIPAVLOV & CHESHMEDJIEV (2003) and ASSYOV & PETROVA (2012).

The species-genus ratio in the families found was calculated according to ASSENOV (2014). The names of the species were updated following The Plant List (2019).

The unified methodology used in collecting and analyzing data from the three zones provides an opportunity for good comparability of the findings.

## **Results and Discussion**

As a result of the two-year inventory carried out in the three studied areas, a total of 154 species of higher plants were found, belonging to 125 genera and 43 families (Appendix 1). The largest number of species was found in the PA "Martvitsata – Zlato Pole" – 115, belonging to 105 genera and 39 families. "Rice Fields Plovdiv" ranked second for the number of species – 72 species of 67 genera and 31 families. The PZ "Rice Fields Tsalapitsa" had the smallest number of species – 58 species, 54 families and 25 genera (Appendix 1). In a 41-kilometer stretch in the middle course of the Maritsa River, between Plovdiv and Parvomay, MARINOV *et al.* (2007) found about 200 species of higher plants belonging to 139 genera and 57 families. GEORGIEV (2012) found 222 species of higher plants of 168 genera and 58 families in the protected area "Nahodishte na blatno kokiche" in Vinitsa village, municipality of Parvomay (18.6 ha).

three studied In the areas, the Magnoliophyta species were represented, as follows: 128 Magnoliopsida species (83.1% of the total number of taxa and 4.4% of the species of Bulgaria) and the 26 Liliopsida species (16.9 % of the total number of taxa and 4% of the species of Bulgaria). Upon the Maritsa River (PA "Nahodishte na blatno kokiche" in Vinitsa village), TASHEV et al. (2014) found 46 species belonging to Class Liliopsida, distributed in 31 genera and 11 families, and 175 species belonging to Class Magnoliopsida, distributed in 136 genera and 46 families (Table 1).

The highest number of species was found in the PA "Martvitsata - Zlato Pole" and respectively the number of the representatives of both classes in that area was the highest (98 Magnoliopsida and 17 Liliopsida). Although "Rice Fields Plovdiv" ranked second for the number of species found, the monocotyledonous plants in that area were less in number than those in the PZ "Rice Fields Tsalapitsa" – 11 and 13, respectively (Table 1). The reason is the higher percentage of species of the Poaceae family in the PZ "Rice Fields Tsalapitsa" (Appendix 1).

The ratio of the dicotyledonous to the monocotyledonous plants in the three areas is 4.9:1. It is close to the ratio, known for our country, between the number of the species in the two classes 4.4:1, found by GUSEV *et al.* (2004). The largest share of the dicotyledonous plants was found in the PA "Martvitsata – Zlato Pole" (5.7:1), followed by "Rice Fields Plovdiv" (5.5:1) and the PZ "Rice Fields Tsalapitsa" (3.4:1).

**Table 1.** Taxonomic structure of the vascular flora in "Rice-field Plovdiv", PZ "Rice-field Tsalapitsa", PA "Martvitsata Zlato Pole" and in total for the three observed areas.

Taxon	Quantitative characteristics of the studied flora	Magnoliophyta	Magnoliopsida	Liliopsida
	Number in general of the three observed areas	43	36	7
	Number for "Rice-field Plovdiv"	31	27	4
lly	% of the total number found for the three zones	72.1	75	57.1
E	Number for PZ "Rice-field Tsalapitsa"	25	20	5
Fa	% of the total number found for the three zones	58.1	55.6	71.4
	Number for PA "Martvitsata Zlato Pole"	38	31	7
	% of the total number found for the three zones	88.4	86.1	100
	Number in general of the three observed areas	125	104	21
	Number for "Rice-field Plovdiv"	67	58	9
ns	% of the total number found for the three zones	53.6	55.8	42.8
eni	Number for PZ "Rice-field Tsalapitsa"	54	42	12
G	% of the total number found for the three zones	43.2	40.4	57.1
	Number for PA "Martvitsata Zlato Pole"	104	88	16
	% of the total number found for the three zones	83.2	84.6	76.9
	Number in general of the three observed areas	154	128	26
	Number for "Rice-field Plovdiv"	72	61	11
es	% of the total number found for the three zones	46.8	47.7	42.3
eci	Number for PZ "Rice-field Tsalapitsa"	58	45	13
$\mathbf{S}\mathbf{p}$	% of the total number found for the three zones	37.7	35.2	50
	Number for PA "Martvitsata Zlato Pole"	115	98	17
	% of the total number found for the three zones	74.7	76.6	65.4

**Table 2.** Families with the highest participation and relative share of species and genera in "Rice-field Plovdiv", PZ "Rice-field Tsalapitsa", PA "Martvitsata Zlato Pole" and in total for the three observed areas.

Family	Asteraceae	Fabaceae	Poaceae	Lamiaceae						
Species										
"Rice-field Plovdiv"	Number	18	5	8	3					
	% for three observed areas	62.1	38.5	47.1	30					
$DZ #D; (: -1.1 T_{-1} - 1; ) "$	Number	15	3	9	1					
FZ Rice-neiu Isaiapitsa	% for three observed areas	51.7	23.1	52.9	10					
PA "Martuitanta Zlata Pala"	Number	22	9	11	9					
rA Martvitsata Ziato role	% for three observed areas	75.9	69.2	64.7	90					
In general of the three observed areas	Number	29	13	17	10					
In general of the three observed areas	% for three observed areas	6	4.5	5.2	6.5					
% of the total number of species for	or Bulgaria	13.8	7.9	7.6	4					

	Genus				
"Diag field Dlanding"	Number	17	5	6	3
Rice-neid Plovalv	% for three observed areas	70.8	55.6	42.8	37.5
PZ "Rice-field Tsalapitsa"	Number	15	2	8	1
TZ Rice-neiu Isaiapitsa	% for three observed areas	62.5	22.2	57.1	12.5
PA "Martuiteata Zlata Pala"	Number	21	7	10	8
I A Waltvitsata Ziato I Ole	% for three observed areas	87.5	77.8	71.4	100
In some and of the other schedured areas	Number	24	9	14	8
in general of the three observed areas	Index Species/Genus	1.1	1	1.3	1

Describing the specific characteristics of flora, it is necessary to identify the families' richest in species and genera (TASHEV & ANGELOVA, 2005). The families represented by the largest number of genera and species in the three studied areas were Asteraceae (24 genera and 29 species), Poaceae (14 genera and 17 species), Fabaceae (9 genera and 13 species) and Lamiaceae (8 genera and 10 species). The number of species and genera in those four families reached 44.8% of the species and 44% of the genera for the three studied territories (Table 2).

As should be expected, the highest percentage of species and genera for those four families was reported in the PA "Martvitsata – Zlato pole" (Table 2). The lowest percentage of those indicators was reported for the PZ "Rice Fields Tsalapitsa", with an exception of Poaceae family, in which the share of the species is 52.9% and the genera – 57.1%, i.e. significantly higher than the values for "Rice Fields Plovdiv" – 47.1% and 42.8%, respectively (Table 2).

The comparison of the most richly represented families in the three areas and the flora of Bulgaria shows a certain difference (VASSILEV & ANDREEV, 1992; GUSEV *et al.*, 1997). Poaceae family, which ranked third in the flora of Bulgaria after Asteraceae and Fabaceae, in the three studied areas was the second for the number of genera and species. The Lamiaceae family, ranking eighth in a relative share of species in the whole flora of Bulgaria, occupied the fourth place in the studied areas, surpassing the families Rosaceae (7 genera, 7 species), Caryophyllaceae (3 genera, 4 species),

Brassicaceae (7 genera, 8 species), Scrophulariaceae (2 genera, 3 species).

A relatively large number of species and genera were reported for the families Apiaceae (6 genera, 6 species), Brassicaceae (5 genera, 5 species), Rosaceae (7 genera, 7 species), but only in the PA "Martvitsata – Zlato Pole" (Appendix 1).

In total for the three areas, 58.1% of the families are represented by 1 genus, 18.6% by 2 genera, 6.9% by 3 genera. 7 of the families are represented by more than 5 genera, i.e. 16.3%. The largest number of families, represented by only 1 genus, was found in the PA "Martvitsata – Zlato pole" – 25, followed by "Rice Fields Plovdiv"– 20 families (Fig. 2). In "Martvitsata – Zlato Pole" the largest number of families with more than 5 genera were identified – 7, or 16.3% of the total number of species found in the protected area.

With regard to the number of species in a family, 44.2% of all the families found in the three areas, are represented by 1 species. The families with more than 5 species are 6 or 13.9%. The largest percentage of families with 1 species was reported in PA "Martvitsata - Zlato Pole" - 67.4% of the total number of families found in the three areas or 74.4% for the protected area. The largest number of families with more than 5 species was reported for the same area - 7 or 16.3%. In the other two studied areas "Rice Fields Plovdiv" and "Rice Fields Tsalapitsa", the number of the families represented by 1 species was the same – 17 or 39.5% of all the species found and 54.5% and 68%,

respectively, of the species composition in each of the two areas (Fig. 3).

The found species/genus ratio was about 1.5 because most of the genera were represented by 1 or 2 species only (Table 2).

A total of 15 species of trees and shrubs and 139 species of herbaceous plants were found in the flora of the three studied areas. The trees and shrubs belong to 11 genera and 6 families and the herbaceous plants to 115 genera and 37 families. In the middle course of the Maritsa River between Plovdiv and Parvomay, MARINOV et al. (2007) found 23 species of trees, 11 species of shrubs, 5 species of lianas and 160 species of herbaceous plants. In PA "Nahodishte na blatno kokiche" on the Maritsa river, TASHEV et al. (2014) found that the perennial herbaceous species were most represented -117 or 52.7%, followed by the annual - 43 species (19.4%), trees - 14 species (6.3%), shrubs – 12 species (5.4%).

In PA "Martvitsata - Zlato Pole" and "Rice Fields Plovdiv" the tree and shrub species found belong to 5 families. In the first area, the genera found are 11 and in the second one - 6. Five species of trees and shrubs, belonging to 2 families, were identified in the PZ "Rice Fields Tsalapitsa". Deforestation of riparian wetlands, used for rice growing, was included in a number of management plans (Ministry of Environment and Water. Regional Inspectorate of Environment and Water - Plovdiv). They state that the riparian vegetation on the banks of the rice paddies is dominated by low and high herbaceous vegetation - 85%. Shrub communities are 5% and trees with a height of less than 10 m – 10%.

The highest number of tree and shrub species was found in the PA "Martvitsata – Zlato pole" – 12. In "Rice Fields Plovdiv" and PZ "Rice Fields Tsalapitsa" the same number of those species was found, i.e. 6 in each area.

Tree plants found in the three areas are divided into four groups by their biological type, the largest being the number of trees. The biological type of semi-shrub – shrub was found only in the PA "Martvitsata – Zlato pole" (Table 3).

The herbaceous species identified in the three studied areas refer to 6 biological groups (Table 4). Perennial species are prevailing. They represent 51.4% of the total number of herbaceous plants and 46.1% of the total number of species found in the three areas. The largest share of perennial herbaceous plants was found in PA "Martvitsata - Zlato pole" (50% of the group of herbaceous plants, 33.1% of the total number of species in the three areas and 44.3% of the total number of species in that concrete area). The second group of herbaceous plants is that of the annual species. They occupy 29.7% of the herbaceous plants found in the three areas and 26.6% of the total number of species. Comparing the three studied areas, the largest share of annual herbaceous plants was found in the PZ "Rice Fields Tsalapitsa" (40.4% of the herbaceous species, 36.2% of the total number of species in that area). The fact that the annual herbaceous species are of big number is indicative for the strong anthropogenic impact, due to deforestation, erosion and agricultural activity.

## Conclusions

Anthropogenic pressure on the flora was reported in the studied areas PA "Martvitsata – Zlato pole", PZ "Rice Fields Tsalapitsa" and "Rice Fields Plovdiv", expressed in a reduced share of tree species and a high percentage of annual herbaceous plants.

The most significant anthropogenic impact was found in PZ "Rice Fields Tsalapitsa", where the lowest total number of species and the largest share of annual herbaceous plants were reported compared to the flora of the other two studied areas.

The largest number of higher plants and the highest percentage of tree species found in PA "Martvitsata – Zlato pole" compared to the other two areas, define the protected area as the least affected by the anthropogenic impact.



**Fig. 2.** Relative participation of families with different number of genera in the flora of "Rice-field Plovdiv", PZ "Rice-field Tsalapitsa", PA "Martvitsata Zlato Pole" in total for the three observed areas.



**Fig. 3.** Distribution of families with different number of species in the flora of "Rice-field Plovdiv", PZ "Rice-field Tsalapitsa", PA "Martvitsata Zlato Pole" and in total for the three observed areas.

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**Table 3.** Distribution of tree plants in the flora of "Rice-field Plovdiv", PZ "Rice-field Tsalapitsa", PA "Martvitsata Zlato Pole" and in total for the three observed areas. Abbreviation: t- Tree, s - Shrub, h-s -Half-shrub.

Zone	Biological type	t	S	t-s	h-s
In gaparal of the three	Number		4	3	1
observed areas	% of the total number in the group	43.8	25	18.8	6.6
	% of the total number of species	4.5	2.6	1.9	0.6
	Number	3	1	2	
"Rice-field Plovdiv"	% of the total number in the group	50	16.6	33.3	
	% of the total number of species	1.9	0.6	1.3	
	% of the total number of species in that area	4.2	1.4	2.8	
	Number	3	2	1	
D7 "Disc field Teclerites"	% of the total number in the group	50	33.3	16.6	
rz kice-neiu Isaiapitsa,	% of the total number of species	1.9	1.3	0.6	
	% of the total number of species in that area	5.2	3.4	1.7	
	Number	5	4	2	1
PA "Martvitsata Zlato	% of the total number in the group	38.4	30.8	15.4	15.4
Pole"	% of the total number of species		2.6	1.3	1.3
	% of the total number of species in that area	4.3	3.5	1.7	1.7

**Table 4**. Distribution of herbaceous plants in the flora of "Rice-field Plovdiv", PZ "Rice-field Tsalapitsa", PA "Martvitsata Zlato Pole" and in total for the three observed areas. Abbreviation: ph - perennial herbaceous; a - annual; b – biennial.

Zone	Biological type	а	b	a-b	a-ph	b-ph	ph
In general of the	Number	42	5	13	2	6	71
three observed	% of the total number in the group	29.7	3.6	9.4	1.4	4.3	51.4
areas	% of the total number of species	26.6	3.24	8.4	1.3	3.8	46.1
	Number	23	5	5	1	2	30
"Rico-field	% of the total number in the group	34.8	7.6	7.6	1.5	3	45.5
Ploydiy"	% of the total number of species	14.9	3.2	3.2	0.6	1.3	19.5
riovalv	% of the total number of species in that area	31.9	6.9	6.9	1.4	2.8	41.7
	Number	21	1	3	1	1	25
D7 "Dice field	% of the total number in the group	40.4	1.9	5.8	1.9	1.9	48.1
Tealapitea"	% of the total number of species	13.6	1.5	1.9	1.5	1.5	16.2
1 Salapitsa	% of the total number of species in that area	36.2	1.7	5.2	1.7	1.7	43.1
	Number	33	6	9	1	3	51
DA "Martuiteata	% of the total number in the group	31.4	5.9	8.8	0.9	2.9	50
71ato Polo"	% of the total number of species	20.8	3.9	5.8	1.5	1.9	33.1
Liato Pole"	% of the total number of species in that area	27.8	5.2	7.8	0.9	2.6	44.3

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Familia	Genus	Species	Biological types	Rice-field Plovdiv	Rice-field Tsalapitsa	Martvitsata Zlato Pole
	Anthriscus	A. sylvestris (L.) Hoffm.	ph			+
	Chaerophyllum	Ch. temulentum L.	a - b			+
Aniaceae	Conium	<i>C. maculatum</i> L.	а	+	+	+
ripiaceae	Daucus	D. carota L.	a-b			+
	Heracleum	H. sibiricum L.	b - ph			+
	Torilis	<i>T. arvensis</i> (Huds.) Link	a			+
Aristolochiaceae	Aristolochia	<i>A. clematitis</i> L. <i>A. crithmifolia</i> Friv. ex Hampe	ph ph	+	+	+
	Achillea	A. setacea Walds. et Kit.	ph			+
	Anthemis	A. austriaca Jaca.	a – b			+
	Arctium	A. lavva L.	b	+		+
		A. absinthium L.	ph	+		
	Artemisia	A. vulgaris L.	ph	+	+	+
	Bidens	B trinartita L	г а			+
	Carduus	C acanthoides L	a	+	+	+
	Carthamus	C lanatus L	a		+	+
	Centaurea	C. solstitialis L.	a	+	+	+
	Chamomilla	<i>Ch. recutita</i> (L.) Rauschert	a	+	+	+
	Chondrilla	Ch. juncea L.	a			+
	Cichorium	C. intubus L.	ph	+	+	+
	<i>c</i> : <i>i</i>	C. arvense (L.) Scop.	ph	+		+
	Cirsium	C. vulgare (Savi) Ten.	b		+	+
Conyza		C. canadensis (L.) Cronquist	а	+	+	+
	Crenis	C. biennis L.	a – b	+		
Asteraceae	Стеріз	<i>C. tectorum</i> L.	ph			+
	Erigeron	<i>E. acer</i> L.	a – ph	+		
	Filago	F. arvensis L.	a	+		
	0	F. vulgaris Lam.	а			+
	Helminthotheca	H. echioides (L.) Holub	а		+	+
	Inula	I. ensifolia L.	ph		+	
	Lactuca	L. serriola L.	a – b	+	+	+
	Ononordum	$\Omega$ acanthium I		+	+	
	Picris	P hieracioides L	a - ph		+	+
	Taraxacum	T. officinale F.H. Wigg	ph	+		+
	Тинихисит Тragonogon	T dubius Scop	P <sup>11</sup>	+	+	+
	Xanthium	X strumarium I	a	+		+
	Anchusa	A officinalis I	nh			+
Boraginaceae	Cunoolossum	C officinale L	Pil			+
Doruginaceae	Echium	E italicum L	b	+		+
	Cansella	<i>C. bursa-pastoris</i> (L.) Medik.	a – b	+	+	+
	Cardaria	C draha (L.) Desv	nh	+	+	
	Descurainia	D conhig (I) Webb ex Prantl	a = b			+
	Frusimum	<i>E. sophia</i> (E.) Webb ex I failin	a = b b = bb			+
Brassicaceae	Rorinna	R prolifera Simonk	a = b			+
	Sisumbrium	S officinale (L.) Scop	a - b	+		
	sisymorium	S. orientale I.	a - h	'		+
	Thlaspi	T. arvense L.	h	+		
Butomaceae	Butomus	B. umbellatus I.	ph			+
	Cannabis	C. sativa L.	а	+		+
Cannabaceae	Humulus	H. lupulus L.	ph	+		

**Appendix 1.** List of taxa found in "Rice-field Plovdiv", PZ "Rice-field Tsalapitsa", PA "Martvitsata Zlato Pole" and in general of the three observed areas.

Caprifoliaceae	Sambucus	S. ebulus L. S. nigra L.	ph s – t         +				
	Dianthus	D. armeria L.	a – b		+		
Carvophyllaceae		D. campestris M. Bieb.	ph			+	
	Herniaria	H. hirsuta L.	a – b			+	
~ .	Silene	S. vulgaris (Moench) Garcke	ph	+			
Chenopodiaceae	Chenopodium	Ch. album L.	a	+		+	
Convolvulaceae	Calystegia	<i>C. silvatica</i> (Kit.) Griseb.	ph	+			
_	Convolvulus	C. arvensis L.	ph	+	+	+	
Cuscutaceae	Cuscuta	C. europaea L.	a			+	
Cyperaceae	Cyperus	C. longus L.	ph		+		
51	Scirpus	S. lacustris L.	ph			+	
Dipsacaceae	Dipsucus	D. luciniutus L.	D h mh	+		+	
-	Scuolosu Europortia	S. columburu L.	b – pn	т		т	
Euphorbiacea	Бирногош	E. cypurissus E. E. salicifolia Host	ph	+		т	
	Amornha	A fruticosa I	pii s	1		+	
	Dorucnium	D herhaceum Vill	nh			+	
	Galega	G. officinalis L.	ph	+			
	Lotus	L. corniculatus L.	ph	+	+	+	
	Medicago	<i>M. lupulina</i> L.	b	+		+	
	Melilotus	<i>M. albus</i> Medik.	а	+			
		T. affine C. Presl	а			+	
	Trifolium	T. arvense L.	а			+	
Fabaceae	1,19011111	T repens L	ph			+	
	Trioonella	T caerulea (L.) Ser	P <sup>11</sup>	+		+	
	Vicia	V cracca L	nh			+	
	VICIU	V. dalmatica A. Kern.	ph		+	-	
		V lathuroides I	2		+		
	Eradium	E cicutarium (L) L'Hor	a	т	_	т	
Commission	LIUUUU	C. dissectum I	a	+			
Geraniaceae	Geranium	C. nalustra I	a nh	+			
Halamanaaaa	Mani cula Ilena	G. putustre L.	ph	т			
Hudrocharitacoao	Niyriophyllum	M. spicatum L.	pn			+	
Hypericaceae	Hypericum	H nerforatum I	ph	+	+	+	
Trypericaceae	Ballota	B vigra I	ph	+	+	+	
	Clinenedium	D. migru E.	pii	1			
	Cimopouium	C. outgare L.	pn			+	
	Glechoma	G. hederacea L.	ph	+		+	
	Lamium	L. garganicum L.	pn			+	
Lamiaceae	Luconus	L. purpureum L.	a = b	т		+	
	Lycopus	L. europueus L. M. neregrinum I	ph			+	
	Marrubium	M miloare I	ph			+	
	Mentha	M aquatica L	ph			+	
	Scutellaria	S. altissima L.	ph			+	
Lemnaceae	Lemna	L. minor L.	ph	+	+	+	
Lythraceae	Lythrum	L. salicaria L.	ph	+		+	
Malvaceae	Malva	M. sylvestris L.	b – ph	+	+	+	
Oleaceae	Fraxinus	F. americana L.	t	+			
Onagraceae	Epilobium	E. hirsutum L.	ph	+	+		
Papaveraceae	Papaver	P. rhoeas L.	a	+	+	+	
	Aegilops	Ae. cylindrica Host	а			+	
	Agropyron	A. repens (L.) P. Beauv.	ph	+			
	Avena	A. fatua L.	а		+	+	
	Bromus	B. mollis L.	а	+		+	
		<i>B. tectorum</i> L.	а	+		+	
		B. sterilis L.	а	+	+		

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Urticaceae	Urtica	U. dioica L.	ph	+	+	+
			1			
1 y priaceae		T. latifolia L.	ph		+	
Typhaceae	Typha	T. angustifolia L.	ph	+	+	+
Sparganiaceae	Sparganium	S. erectum L.	ph			+
		S. nigrum L.	hs			+
Solanaceae	Solanum	S. dulcamara L.	hs			+
	Datura	D. stramonium L.	а			+
Simarubiaceae	Ailanthus	A. altissima (Mill.) Swingle	t			+
Sapindaceae	Koelreuteria	K. paniculata Laxm.	t	+		
		S. purpurea L.	s			+
Sancaceae	Salix	S. fragilis L.	t	+	+	+
Calianana	r Calin	S. alba L.	t		+	+
	Populus	P. nigra L.	ť		+	+
r	Verbascum	V. speciosum Schrad.	ph		+	
Scrophullariaceae		V. blattaria L.	b			+
	Linaria	L. vulgaris Mill.	ph			+
		G. verum L.	ph			+
Rosaceae Rubiaceae	Galium	G. palustre L.	ph			+
	- <i>u</i>	G. aparine L.	а	+		+
	Rubus	R. caesius L.	S	+	+	+
	Rosa	R. canina L.	s		+	+
	Pyrus	P. pyraster (L.) Burgsd.	t			+
Kosaceae	Prunus	P. cerasifera Ehrh.	s - t	+	+	+
D	Potentilla	<i>P. reptans</i> L.	ph			+
Rosaceae	Crataegus	C. monogyna Jaca.	s - t			+
	Agrimonia	A. eupatoria L.	ph			+
Potamogetonaceae	Potamogeton	P. nodosus Poir	ph		+	
Determent	Deterrite	P. crispus L.	ph	+	+	+
Portulacaceae	Portulaca	<i>P. oleraceae</i> L.	а		+	
	пител	R. pulcher L.	ph			+
rorygonaceae	Rumer	R. crispus L.	ph	+	+	
Polygonaccas	Polygonum	P. aviculare L.	а	+	+	+
	Persicaria	P. hydropiper (L.) Spach	а	+	+	+
- minuginaceae		P. major L.	ph	+	+	+
Plantaginaceae	Plantaoo	P. lanceolata L.	ph			+
	Sorgnum	5. nalepense Pers.	ph		+	
	Setaria	<i>S. viridis</i> (L.) P.Beauv.	a		+	
	с :	S. glauca (L.) P.Beauv.	a		+	
Poaceae	Poa	P. pratensis L.	ph			+
-	Phragmites	P. australis (Cav.) Steud.	ph	+	+	+
	Lolium	L. perenne L.	ph	+	+	+
	Hordeum	H. murinum L.	a	+	+	+
	Festuca	<i>F. pratensis</i> Huds.	ph			+
	Dasunurum	D. villosum (M.Bieb.)Maire	a			+
	Cunodon	C. dactulon (L.)Pers.	ph	+	+	+
	Bothriochloa	B. ischaemum (L.) Keng	ph		+	

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# Floristical Investigation of "Yazovir Konush" NATURA 2000 Site (BG0002015), Southern Bulgaria

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Abstract. "Yazovir Konush" NATURA 2000 site (BG0002015) is situated near Konush village, Plovdiv district and occupies an area of 0.376 ha. It was established for protection of the habitats of bird species, included in Biological Diversity Act and Bird Directive as a part of NATURA 2000 network of Bulgaria. Vascular plant diversity was investigated during 2012-2014 field working seasons using transect and semi-stationary sampling methods. All species were categorized by biological groups, life forms and floristic elements. The plants were classified by ecological groups according to their preference to water, light and heat conditions. A total of 172 vascular plants were identified. They belong to 50 families and 133 genera. One hundred and three species are medicinal plants, which comprise more than 59.8% of the total flora of the study area. The richest families are Fabaceae, Asteraceae, Lamiaceae, Poaceae, Brassicaceae and Rosaceae. Among the richest genera are Vicia, Carex, Veronica, Rumex, Lathyrus, Trifolium and Potentilla, which comprise more than 14.5% of the total flora of the study area. Only 2 species in the floristical composition with conservation status were identified (Cephalanthera damasonium and C. rubra). The ecological analysis showed that the flora of the investigated area is dominated by hemicriptophytes (41.3%), followed by terophytes (26.7%). Mesophyte and heliophyte species are predominant, whereas Euro-Asiatic and Euro-Mediterranean floristic elements are the most widely distributed floristic elements in the study area.

Key words: Protected zone "Yazovir Konush", NATURA 2000, vascular plants, wetland flora.

#### Introduction

"Yazovir Konush" NATURA 2000 Site (BG0002015) was declared with Order № RD-367/16.06.2008 from the Ministry of Environment and Water (MOEW) aiming to protect, support and restore habitats of bird species, included in the Biological Diversity Act. It is situated in the Thracian plane, near Konush village, Asenovgrad municipality (Fig. 1) and covers an area of 0.376 ha.

According to the morphografic division of Bulgaria the study area falls into Southern part

(Thracian lowland) of the Transitional zone of mountains and basins (STEFANOV, 2002). The area belongs to the Transitional-Continental climate zone, which is characterized by mild climate (VELEV, 2002). The precipitation maximum occurs in July and November and its minimum is in August and February (VELEV 2002). The average annual rainfall is 551 l/km<sup>2</sup>, which is relatively low, compared to other areas. The average annual temperature is around 12.1° C, whereas the average air humidity is 72%.

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# Floristical Investigation of "Yazovir Konush" NATURA 2000 Site (BG0002015), Southern Bulgaria

According to the floristic division of Bulgaria (JORDANOV, 1963-1979) the territory of "Yazovir Konush" NATURA 2000 site falls into the Thracian Lowland floristic region. On the territory of this wetland area the natural vegetation is presented by macrophyte communities of *Phragmites australis, Typha* 

*angustifolia* and *T. latifolia*. This NATURA 2000 site is surrounded by farmlands, former landfill, orchards, pastures and meadows.

The aim of this study is to reveal the floristical diversity on the territory of "Yazovir Konush" NATURA 2000 Site (BG0002015), which has not been investigated before.



Fig. 1. Indicative map of the studied area.

## **Material and Methods**

Vascular plant diversity was investigated during the 2012-2014 field working seasons (February-September) using transect and semi-stationary sampling methods. Transects and representative sites were selected, in order to cover all habitat types. About 150 herbarium specimens from different parts of the protected area were collected.

Species determination was carried out according to DELIPAVLOV & CHESHMEDZHIEV (2011), KOZHUHAROV (1995), JORDANOV (1963-1979), VELCHEV (1982-1989). The processing of herbarium materials and characteristics of flora were carried out by standard methods (GUSEV et al., 2004; APOSTOLOVA-STOYANOVA & STOYANOV, 2009). The biological spectrum of the flora was determined by life forms according to RAUNKIAER (1934). Determination of floristic elements followed ASSYOV & PETROVA (2012). Analysis of the flowering period for identified species all was conducted according to DELIPAVLOV & CHESHMEDZHIEV (2011), Kozhuharov (1995), Jordanov (1963-1979), VELCHEV (1982-1989). The conservation significance of the species was determined according to: Red List of Bulgarian vascular plants (PETROVA & VLADIMIROV, 2009), List of Balkan endemics in Bulgaria (PETROVA & VLADIMIROV, 2010), List of Bulgarian endemics (PETROVA, 2006), Biological Diversity Act (2002), Red Data Book of the Republic of Bulgaria (PEEV, 2015), CITES (2009), Directive 92/43/EEC (EC, 1992) and Bern Converniton (1979). Alien species were determined according to PETROVA et al. (2012) and PETROVA & VLADIMIROV (2018), whereas medicinal plants were determined according to Medicinal Plants Act (2000).

General information about the number of families, genera and species from Bulgarian flora (e.g. PETROVA & VLADIMIROV, 2018) was used for comparison of the local floristic diversity vs. national floristic diversity.

The floritstic list was arranged alphabetically by family names, within the framework of the respective classes and

divisions (Table 1). Information about the life form type, flowering period, floristic element, medicinal plant affiliation and ecological characteristics was provided for every species using ecological indicator values for 6 parameters (light, temperature, continentality, moisture, pH and value). They were determined according to ELLENBERG et al. (1991), PIGHATTI et al. (2005), CHYTRÝ et al. (2018). For the species which were not included in those literature sources ecological indicators were determined using information from Delipavlov & Cheshmedzhiev (2011) and personal knowledge. When there is insufficient data about a species it is marked with a ND abbreviation in Table 1.

#### **Results and Discussion**

As a result of the current study 172 vascular plants (without bryophytes), belonging to 49 families and 130 genera were determined (Table 1). They account for 4.23% of all of the species in Bulgaria, 14.1% of the genera, and 30.8% of the families. Most of the determined species belonged to Magnoliopsida, which is represented by 39 families (79.6 % of the total number of families in the investigated studied), 102 genera (78.5% of the total number of genera) and 137 species (79.7% of the total number of species). Liliopsida is represented by 10 families (20.4 %), 28 genera (21.5 %) and 35 species (20.3 %).

The families with the highest number of genera (Table 1) are: *Asteraceae* (16), *Poaceae* (14), *Lamiaceae* (12), *Fabaceae* (8), *Brassicaceae* (7), *Rosaceae* (6) and *Boraginaceae* (4). The species richest families are (Table 1): *Fabaceae* (18), *Asteraceae* (17), *Poaceae* (15), *Lamiaceae* (15), *Brassicaceae* (8), *Rosaceae* (8), *Cyperaceae* (7) and *Boraginaceae* (6).

The species-richest genera (number of species  $\geq$  3) are: *Vicia* (5), *Carex* (4), *Veronica* (4), *Rumex* (3), *Lathyrus* (3), *Trifolium* (3) and *Potentilla* (3).

In the life-form spectrum of the flora of "Yazovir Konush" hemicriptophytes are widest distributed group presented by 75 species (43.6% of all species found in the investigated area), followed by therophytes (44 species/25.6%), therophytes-hemicryptophytes (30/17.5%) and phanerophytes (12/7%).

Geophytes and chamaephytes are represented respectively by 10 and 1 species or 5.8 and 0.6%.

**Table 1.** A list of the vascular plants found in "Yazovir Konush" NATURA 2000 site (BG0002015). The following abbreviations were used: Floristic elements – FE; Life form – LF; Flowering period – FP; Medicinal plants - MP; Light – L.; Temperature – T; Continentality – C; Moisture – M; Nitrogen – N; G – geophytes; H – hemicryptophytes; Ch – chamephytes; Ph – phanerophytes; TR – terophytes.

Tayon	ЕС	IE	ED	MD	т	т	C	М	ъЦ	N
	ГЕ	LF	ГГ	IVII	L	1	C	IVI	рп	1
<u>Lillopsiuu</u> Alliaceae										
Allium rotundum L	Euro-OT	G	V-VI	М	7	7	6	4	8	4
Araceae	Luio Ci	C	, ,,	1,1			Ũ	-	U	-
Arum elongatum Steven	Pont-OT	G	IV-VI		3	6	2	7	7	8
Butomaceae		-			-			-	-	
Butomus umbellatus L.	Eur-As	G	VII-IX	М	6	6	5	1	ND	7
Cyperaceae		_			-	-	-			
<i>Carex acutiformis</i> Ehrh.	Kos	Н	IV-VI		7	5	3	9	7	5
<i>C. caryophyllea</i> Latourr.	Boreal	Н	II-V		8	5	3	4	ND	2
<i>C. hirta</i> L.	Boreal	Н	IV-VI		7	6	3	6	ND	5
C. vulpina L.	Eur	Н	V-VII		9	6	5	8	ND	5
<i>Cyperus glomeratus</i> L.	Eur-As	Н	VI-IX		9	8	5	1	1	5
Pycreus flavescens (L.) Rchb.	Kos	Н	VII-IX		9	6	4	7	5	4
Iridaceae										
Crocus flavus West.	Euro-Pont	G	II-IV		9	5	5	1	ND	ND
Iris pseudacorus L.	Eur	G	V-VI	Μ	7	6	3	9	ND	7
Juncaceae										
Juncus atratus Krock.	subMed	Н	VII-IX		8	7	8	9	7	4
J. effusus L.	subBoreal	Н	VI-VIII		8	5	3	7	3	4
Liliaceae										
Gagea villosa (Bieb.) Duby	Eur-As	G	II-IV		6	7	5	4	6	5
Ornithogalum umbellatum L.	Pont- subMed	G	IV-V		6	6	4	5	7	7
Muscari botryoides (L.) Mill.	Med	G	III-V		7	5	4	5	ND	ND
Orchidaceae										
<i>Cephalanthera damasonium</i> (Mill.) Druce	subMed	G	V-VII		3	6	2	4	7	4
C. rubra (L.) Richard	Eur-As	G	V-VII		4	5	4	3	8	4
Poaceae										
Agrostis capillaris L.	Boreal	Н	VI-IX		7	5	3	6	4	4
Alopecurus pratensis L.	Eur-As	Н	V-VII		6	5	5	6	6	7
Avena fatua L.	Boreal	TR	V-VII		6	6	6	5	7	ND
Bromus arvensis L.	Eur-As	TR-H	V-VII		6	6	4	4	8	4

B. sterilis L.	Boreal	TR	V-VII		7	6	4	4	ND	5
Chrysopogon gryllus (L.) Trin.	. Pont-Med	Н	V-VIII		9	7	8	3	7	3
Cynodon dactylon (L.) Pers.	Kos	Н	VI-IX	Μ	8	7	3	4	ND	5
Dactylis glomerata L.	Eur-As	Η	V-VIII		7	6	3	5	5	6
<i>Festuca valesiaca</i> Schleich. ex Gaudin	Pont	Н	VI-VIII		8	7	7	2	7	2
Hordeum murinum L.	Boreal	TR	V-VI		8	7	4	4	7	5
Lolium perenne L.	Eur-As	Н	VI-IX		8	6	3	5	7	7
Paspalum paspaloides (Michx) Scribn.	Adv (Paleo)	Н	VI-IX		8	8	ND	1	0	8
<i>Phragmites australis</i> (Cav.) Trin. ex Steud.	Kos	Η	VII-IX		7	5	ND	1	7	7
Poa trivialis L.	Boreal	Η	V-VIII		6	ND	3	7	ND	7
Triticum aestivum L.	Adv	Tr	V-VI		ND	ND	ND	ND	ND	ND
Typhaceae										
Typha angustifolia L.	Kos	Η	VI-VII	Μ	8	7	5	1	7	7
T. latifolia L.	Kos	Η	VI-VIII	Μ	8	6	5	1	7	8
<u>Magnoliopsida</u>										
Aceraceae										
Acer tataricum L.	subMed	Р	V-VI	Μ	5	5	ND	4	ND	ND
Apiaceae										
Conium maculatum L.	Eur-As	TR-H	VI-VIII	Μ	8	6	5	6	5	8
Eryngium campestre L.	Pont-Med	Η	VI-VIII	Μ	9	7	5	3	8	3
<i>Orlaya grandiflora</i> (L.) Hoffm	. Ap-Bal	TR	V-VII		7	7	2	3	9	4
Aristolochiaceae										
Aristolochia clematitis L.	Euro-Med	Η	V-VII	Μ	6	7	3	4	8	8
Asteraceae										
Achillea millefolium L.	Euro-Sib	Η	V-IX	Μ	8	ND	ND	4	ND	5
Anthemis arvensis L.	Euro-Med	TR	VI-IX		7	6	5	4	6	6
<i>Arctium minus</i> (Bernh.) Hill.	Eur-As	TR-H	VI-VIII	М	9	5	3	5	ND	8
Artemisia vulgaris L.	subBoreal	Η	VI-VIII	Μ	7	6	8	6	ND	8
Carduus acanthoides L.	Eur	TR-H	VI-VIII	Μ	9	5	6	4	8	7
Carlina vulgaris L.	Euro-Med	TR-H	VI-IX	Μ	7	5	3	4	7	3
Centaurea calcitrapa L.	Med	TR-H	VI-IX	Μ	8	7	3	5	ND	6
<i>C. solstitialis</i> L.	Euro-Med	Η	VII-IX	Μ	8	6	6	4	7	6
<i>Chamomilla recutita</i> (L.) Rausch.	Eur-As	TR	V-VIII	М	7	5	5	6	5	5
Cichorium intybus L.	Euro-Sib	Η	VI-IX	Μ	9	6	5	4	8	5
<i>Cirsium arvense</i> (L.) Scop.	Eur-As	Η	V-X		8	5	ND	4	ND	7
Inula germanica L.	subMed	Η	VI-IX	Μ	8	7	6	3	8	2
Lactuca serriola L.	Eur-As	TR-H	VI-IX	Μ	9	7	7	4	6	4
Matricaria trichophylla (Boiss.) Boiss	Med	TR-H	V-VII	М	8	6	ND	5	ND	ND

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Taraxacum officinale L.	Euro-Med	Η	IV-IX	Μ	7	ND	ND	5	ND	7
Tragopogon pratensis L.	Euro-Med	H-TR	VI-VIII	М	7	6	3	4	7	6
Tussilago farfara L.	Eur-As	Н	VII-X	М	8	7	3	6	8	7
Boraginaceae										
Anchusa officinalis L.	Pont-Med	H-TR	VI-VII	М	9	7	5	3	7	5
A. thessala Boiss. et Spruner	rPont-Med	TR	V-VI		9	8	ND	1	ND	ND
Cynoglossum creticum Mill.	Med-CAs	H-TR	V-VI		9	9	5	3	ND	7
Echium italicum L.	subMed	H-TR	V-VI	М	9	8	5	3	3	4
<i>Myosotis cyanea</i> (Boiss. & Heldr.) Peev et N. Andreev	Med-SAs	H-TR	V-VII		6	5	3	5	ND	7
M. ramosissima Rochel	subMed	TR	V-VI		9	6	5	2	7	1
Brassicaceae										
<i>Arabidopsis thaliana</i> (L.) Heynh.	subBoreal	TR-H	IV-V		6	6	3	4	4	4
Brassica nigra (L.) Koch	Kos	TR	V-X	М	8	7	5	8	8	7
<i>Capsella bursa-pastoris</i> (L.) Medicus	Kos	TR-H	I-XI	М	7	ND	5	5	5	6
Cardaria draba (L.) Desv.	Euro-Med	Н	IV-X	М	8	7	7	3	8	4
Erophila verna (L.) Chevall.	Euro-Med- CAs	TR	III-V		8	6	3	2	4	2
Rorippa amphibia (L.) Besser	Eur-As	Н	V-VII		7	6	7	1	ND	4
<i>R. sylvestris</i> (L.) Besser	Eur-As	Н	V-X	Μ	6	6	5	8	8	6
Thlaspi arvense L.	Eur-As	H-TR	IV-VI	М	6	5	5	5	7	6
Campanulaceae										
Campanula sparsa Friv.	Bal	TR	V-VII		5	5	ND	5	ND	ND
Cannabaceae										
Cannabis sativa L.	Adv	TR	VI-VII	Μ	8	7	5	5	5	5
Humulus lupulus L.	Euro-Sib	Н	V-VIII	Μ	7	6	3	8	6	8
Caprifoliaceae										
Sambucus ebulus L.	Euro-Med	Н	V-VIII	Μ	8	6	3	5	8	7
S. nigra L.	Euro-Med	Р	IV-VI	Μ	7	5	3	5	ND	9
Caryophyllaceae										
Holosteum umbellatum L.	Eur-As	TR	II-V		8	6	5	3	2	2
Silene italica (L.) Pers.	Euro-Med	H-TR	V-VII		5	7	5	4	6	5
Stellaria media (L.) Vill.	Kos	TR-H	I-XII	Μ	6	ND	ND	4	7	8
Chenopodiaceae										
Chenopodium album L.	Kos	TR	VI-IX	Μ	7	7	5	4	5	7
Convolvulaceae										
<i>Calystegia sylvatica</i> (Kit.) Griseb.	Med	Н	V-VIII		7	8	6	7	5	7
Convolvulus arvensis L.	Kos	Н	V-X	Μ	7	6	5	4	7	5
Cornaceae										
Cornus sanguinea L.	subMed	Р	V-VI	Μ	7	5	4	5	7	ND

Dipsacaceae										
Scabiosa argentea L.	Bal-Anat	H-TR	VI-IX		9	8	6	2	7	2
Euphorbiaceae										
Euphorbia helioscopia L.	Eur-As	TR	IV-X		6	9	3	5	7	7
Fabaceae										
Astragalus onobrychis L.	Eur-As	Н	V-VIII		8	7	6	2	9	1
Lathyrus aphaca L.	subBoreal	TR	V-VII		7	7	3	3	8	3
L. cicera L.	subMed	TR	V-VI		8	8	5	3	5	2
L. hirsutus L.	Euro-Med	TR	IV-VII		7	6	4	4	7	ND
Lotus corniculatus L.	Euro-Med	Н	V-IX	Μ	7	ND	3	4	7	3
Medicago minima (L.) Bart.	Eur-As	TR	V-VI		9	7	3	3	8	2
M. sativa L.	Adv (CAs)	Н	VI-VIII		8	6	6	4	7	3
Melilotus alba Medicus	subBoreal	TR	VI-IX	М	9	6	6	3	7	4
M. officinalis (L.) Pall.	Eur-As	TR	VI-VIII	М	8	6	6	3	8	3
Onobrychis gracilis Besser	Pont-Med	Н	V-VII		9	7	ND	5	ND	ND
Trifolium hybridum L.	Euro-Med	Н	V-IX		7	6	5	6	7	5
<i>T. pratense</i> L.	subBoreal	Н	V-VII	М	7	ND	3	5	ND	ND
T. repens L.	Euro-Sib	Н	V-X	М	8	ND	ND	5	6	6
Vicia cracca L.	Eur-As	Н	VI-VIII	М	7	5	ND	6	ND	ND
V. grandiflora Scop.	subMed	TR-H	IV-VI	М	7	7	6	4	5	4
V. hirsuta (L.) Gray	Euro-Med	TR	V-VII		7		5	4	ND	4
V. peregrina L.	Eur-As	TR	IV-VI		7 7		4	4	5	4
V. striata Bieb.	Euro-Med	TR	V-VII		7		6	4	6	5
Fagaceae										
Quercus robur L.	subMed	Р	V	М	7	6	6	6	5	6
Papaveraceae										
Fumaria officinalis L.	Euro-Sib	TR	IV-VI	М	6	6	3	5	6	7
Papaver rhoeas L.	Euro-Sib	TR	IV-IX	М	6	6	3	5	7	6
Geraniaceae										
Erodium cicutarium (L.) L'Her	subBoreal	TR	IV-IX	Μ	8	6	4	4	5	4
Geranium dissectum L.	Eur-As	TR	IV-VII		6	6	3	5	8	5
G. molle L.	Euro-Med	TR-H	IV-VII		7	6	3	4	5	4
Hypericaceae										
Hypericum perforatum L.	Kos	Н	V-VIII	М	7	6	5	4	6	4
Lamiaceae										
Acinos suaveolens (Sibth. et	1 7 6 1				0	0		2	-	•
Sm.) G. Don	subMed	Н	V-V11	Μ	8	8	6	3	7	2
Ajuga chia Schreber	Pont-Med	TR-H	IV-VII	Μ	7	8	2	4	9	2
<i>A. reptans</i> L.	Euro-Med	Н	IV-VI	М	6	ND	2	6	6	6
Ballota nigra L.	Euro-Med	Н	VI-IX	М	8	6	5	5	ND	8
Betonica officinalis L.	subMed	Н	VI-VIII	Μ	7	6	5	6	4	3
Clinopodium vulgare L.	subBoreal	Н	V-VII	Μ	7	5	3	4	7	3
Glechoma hederacea L.	Eur-As	Н	IV-VI	М	6	6	3	6	5	7

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Lamium purpureum L.	Euro-Med	TR	III-VIII	Μ	7	5	3	5	7	7
<i>Mentha aquatica</i> L.	Boreal	Н	VI-X	Μ	7	5	3	9	7	5
M. pulegium L.	Eur-As	Н	VI-IX	Μ	8	7	5	7	ND	2
Salvia aethiopis L.	Eur-As	H-TR	V-VIII	Μ	7	6	6	3	3	2
S. nemorosa L.	Euro-OT	Н	V-IX	Μ	7	7	6	4	9	4
Stachys palustris L.	Boreal	Η	VI-VIII		7	5	ND	7	7	6
Teucrium chamaedrys L.	subMed	Η	IV-V	Μ	7	6	4	2	8	1
T. polium L.	Pont-Med	Η	IV-VIII	Μ	9	8	4	2	Х	1
Thymus pannonicus All.	Eur	Η	V-IX	Μ	7	6	6	4	5	3
Lythraceae										
Lythrum salicaria L.	subBoreal	Η	VI-VIII	Μ	7	5	5	8	6	ND
L. virgatum L.	Eur-As	Н	VI-VIII	Μ	7	8	6	7	3	4
Malvaceae										
Malva sylvestris L.	Kos	H-TR	V-IX	Μ	8	6	3	4	7	8
Plantaginaceae										
Plantago lanceolata L.	Kos	Н	V-VIII	Μ	6	7	3	ND	ND	ND
P. major L.	Boreal	H-TR	VI-IX	Μ	8	ND	ND	5	ND	6
Polygonaceae										
Persicaria hydropiper (L.) Opi	zEur-As	TR	VII-X	Μ	7	6	ND	8	5	8
Polygonum aviculare L.	Kos	TR	V-X	Μ	7	6	5	4	6	6
Rumex acetosella L.	Euro- subMed	Н	V-VII		8	5	5	5	1	2
R. crispus L.	Boreal	Н	VII-VIII	Μ	7	5	3	7	ND	6
R. palustris Sm.	Eur-As	TR	VI-IX		8	7	3	9	9	8
Primulaceae										
Lysimachia nummularia L.	Eur	TR-H	VI-VII	Μ	4	6	4	6	ND	ND
Ranunculaceae										
Clematis vitalba L.	Eur	Р	VI-VIII	Μ	7	6	3	5	7	7
<i>Consolida hispanica</i> (Costa) Greuter et Burdet	Med	TR	VI-VIII	М	9	7	ND	2	ND	ND
C. regalis S. F. Gray	Euro-Med	TR	V-IX	Μ	6	7	6	4	8	5
Ranunculus repens L.	subMed	Η	V-VIII	Μ	8	6	ND	1	8	2
R. sceleratus L.	Euro-Med	TR-H	IV-VIII	Μ	9	6	ND	9	7	9
Resedaceae										
Reseda inodora Rchb.	Euro-Med	H-TR	VI-VII	Μ	8	7	ND	2	ND	ND
Rosaceae										
Agrimonia eupatoria L.	Euro-Med	Н	VI-VIII	Μ	7	6	4	4	8	4
Crataegus monogyna Jacq.	subBoreal	Р	V-VI	Μ	7	5	3	4	8	4
Geum urbanum L.	subBoreal	Н	V-VII	Μ	4	5	5	5	6	7
Potentilla argentea L.	SPont	Η	V-IX	Μ	9	6	3	2	3	1
P. pedata Willd.	Med	Η	V-VII		9	7	3	3	7	3
<i>P. reptans</i> L.	Kos	Η	VI-VIII	Μ	6	6	3	6	7	5
Rosa canina L.	subMed	Р	V-VIII	Μ	8	5	3	4	ND	ND

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Rubus caesius L.	Eur-As	Ch	V-VIII	М	6	5	4	7	8	7
Rubiaceae										
Galium aparine L.	Eur-As	TR	V-VI	М	7	6	3	4	6	8
G. verum L.	Eur-As	Н	VI-VIII	М	7	6	6	4	7	3
Salicaceae										
Populus deltoides H. Marsh	Adv	Р	IV-VI		ND	ND	ND	ND	ND	ND
Salix alba L.	Eur-As	Р	III-V	М	5	6	6	8	8	7
S. fragilis L.	Eur-As	Р	III-V	М	5	5	3	8	6	6
Scrophulariaceae										
Verbascum blattaria L.	Euro-Sib	TR-H	VI-VII		8	7	7	3	7	6
Veronica anagalis-aquatica L	.Boreal	Н	V-VIII	М	7	6	3	9	7	6
V. hederifolia L.	Euro-Med	TR	II-V		6	6	3	5	7	7
V. sublobata M. A. Fisch.	Eur	TR	IV-V		5	4	5	5	5	5
V. triloba (Opiz) A. Kern.	subMed	TR	III-IV		7		5	3	7	2
Solanaceae										
Datura stramonium L.	Am (Adv)	TR	VI-X	М	8	6	5	4	7	8
Hyoscyamus niger L.	Eur-As	TR-H	V-VIII	М	8	6	5	4	7	9
Solanum dulcamara L.	Eur-As	Η	VI-VIII	М	7	5	ND	8	ND	8
S. nigrum L.	Kos	TR	VI-IX	М	7	6	3	5	7	8
Ulmaceae										
Ulmus minor Mill.	Euro-Med	Р	III-IV	М	5	7	5	ND	8	ND
Urticaceae										
Urtica dioica L.	Boreal	Н	VI-IX	Μ	ND	ND	ND	6	7	9
Valerianaceae										
Valerianella microcarpa	Furo Mod	TP	IV VII		Q	Q	4	2	5	1
Loisel.	Luio-Meu	IK	1 v - v 11		)	)	т	2	0	1
Verbenaceae										
Verbena officinalis Voss.	Kos	Η	V-VIII	М	9	6	3	5	7	7
Violaceae										
Viola arvensis Murr.	Eur	TR	IV-X		6	5	5	2	ND	ND
Vitaceae										
Vitis vinifera L.	subMed	Р	V-VI	Μ	9	8	3	4	7	6
Zygophyllaceae										
Tribulus terrestris L.	Eur-As	TR	V-VIII	М	8	8	6	2	5	3

A total of 27 floristic element groups are found in the flora of Yazovir Konush NATURA 2000 Site (Table 2). Euro-Asiatic and Euro-Mediterranean groups dominate in the flora with 39 species (22.7%) and 26 (15.1%). Groups of Boreal and subBoreal species are also well-presented with 13 species (7.6%) and 11 (6.4%) although low altitude because they includes specie which have wide range of distribution in the country such as *Carex hirta*, *Juncus effusus*, *Artemisia vulgaris*, *Erodium cicutarium*, *Plantago major*, etc.

In the floristic composition only 2 species with conservation concern were identified (*Cephalantera damasonium* and *C. rubra*), which are included in Appendix 2 of CITES (2009). Also totally 103 or 59.8% of all identified species are determined as medicinal plants (Table 1).

**Table 2.** Floristic elements in the flora of "Yazovir Konush" NATURA 2000 Site (BG0002015).

Eloristic alamont	Number	% of all		
rioristic element	of species	species		
Adv	3	1.7		
Adv (CAs)	1	0.6		
Adv (Paleo)	1	0.6		
Am (Adv)	1	0.6		
Ap-Bal	1	0.6		
Bal	1	0.6		
Bal-Anat	1	0.6		
Boreal	13	7.6		
Eur	8	4.7		
Eur-As	39	22.7		
Euro-Med	26	15.1		
Euro-Med-CAs	1	0.6		
Euro-OT	2	1.2		
Euro-Pont	1	0.6		
Euro-Sib	7	4.1		
Euro-subMed	1	0.6		
Kos	18	10.5		
Med	6	3.5		
Med-CAs	1	0.6		
Med-SAs	1	0.6		
Pont	1	0.6		
Pont-Med	7	4.1		
Pont-OT	1	0.6		
Pont-subMed	1	0.6		
SPont	1	0.6		
subBoreal	11	6.4		
subMed	17	9.9		

The distribution of the vascular plants according to their period of flowering shows that the most active period is from May to July (Table 1). During that period 135 (78.5%) are blossom. Among them, most species blossom in June-August – 17 species, May-August & June-September – 13, May-June & May-September – 7, etc.

Researched flora shows quite diverse floristic composition. The flora of "Yazovir Konush" NATURA 2000 site includes some wetland plants such as *Typha latifolia*, *T. angustifolia*, *Phragmites australis*, *Butomus*  *umbellatus, Iris pseudacorus, Juncus effusus,* etc. On the other side some ruderal species were also found (e.g. *Capsella bursa-pastori, Cirsium arvense, Centaurea calcitrapa, Papaver rhoes, Centaurea cyanus*) and mesophyric and xerophytic species widespread in neighbouring habitats.

The ecological structure of the flora of "Yazovir Konush" NATURA 2000 site shows the following characteristics:

- Dominance of half-light (sciophytes) and (heliophytes) species full-light (presenting 157 species or 81.9%), which are developed on fresh to average moisture soils (119 species or 69.2%). The group of full-light species has widest distribution. The group of half-light species includes predominantly species, which are found in low-herb layer in macrophyte and grassland communities or ruderal species, which are periodically shade from field crops neighbouring with "Yazovir Konush" NATURA 2000. On the other hand mesophytic and xeromesophytic species are dominants in the species composition.
  - According to nutrient availability of species most common are moderately nutrient rich and nutrient rich species (95 or 55.2%).
  - In the species composition a great variety exists in terms of soil reaction preference (pH). The group of slightly acidic to slightly basic species (pH = 7) is presented with 51 species or 29.7%. Groups of acidophilous and calciphilous species includes similar number of species 36 (20.9%) and 30 (17.4%) respectively. Data is missing for 42 species (24.4%).
  - Group of moderately thermophilous to thermophilous flora is presented by 141 species (81.9%). Those species are restricted to warm habitats in Bulgaria and southern Europe.

## Conclusions

"Yazovir Konush" NATURA 2000 site (BG0002015) covers a relatively small area of

0.376 ha, but is characterized by a great variety of vascular plants diversity presented by 172 species, belonging to 50 families and 133 genera. Of these, 59.8% are medicinal plants and only 2 species (*Cephalantera damasonium* and *C. rubra*) are with conservation concern.

A total of 27 floristic element groups are found in the flora of "Yazovir Konush" NATURA 2000 Site. Euro-Asiatic and Euro-Mediterranean groups dominate in the flora, which shows strong Mediterranean influence in the study area.

The ecological analysis showed that the flora of the investigated area is dominated by hemicriptophytes (41.3%), following by terophytes (26.7%). From ecological point of view predominate mesophytes and heliophytes species.

The study of the flora and vegetation of the wetlands is important for restoring and maintaining natural habitats of this type, which are subjected to strong anthropogenic influence. This is closely related to the protection of the populations of the animals found in them.

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# Agro-Ecological Assessment of Ovcharitsa Dam (Bulgaria) Water Used For Thermal Power Plant Cooling

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Abstract. Ecological (as a natural source) and agricultural (as a resource for fish farming and irrigation of crops) assessment of Ovcharitsa Dam water, used for thermal power plant (TPP) cooling was carried out in one monitoring point by measurement of 12 physicochemical parameters (temperature, transparency, pH, EC, DO, COD, BOD<sub>5</sub>, unionized NH<sub>3</sub>, NO<sub>2</sub>, NO<sub>3</sub>, total N and P-PO<sub>4</sub>), one biological parameter (chlorophyll-a), 9 pesticides and volatile organic compounds /VOC/ (atrazine, simazine, diuron, 1,2,3-, 1,2,4- and 1,3,5-trichlorobenzene, tetrachloroethane, trichloromethane, hexachlorobutadiene) and 6 microbiological parameters (aerobic mesophilic microorganisms, coliforms, total coli titer, Escherichia coli counts, E. coli titer, Salmonella spp. counts), stipulated in Bulgarian legislation in 2016-2017 (REGULATIONS: No. 4, 2000; No. 18, 2009; On EQS for priority substances and certain other pollutants, 2010 and No. H-4, 2012). The water samples were taken periodically during a two-year period: for physicochemical and biological analysis - in February, April, June, August and November; for microbiological observation - in June, August and November; for pesticides and VOC - in April. Water sampling, sample preparation and analysis were performed according international ISO and BSS standards. It was found that: a) the dam water ecological status was determined as "poor" by chlorophyll-a content and "very poor" by orthophosphates content based on the lowest estimates for the monitored parameters; b) with regard to the content of pesticides and VOC, the dam water was defined as water "in good chemical status"; c) the values of all monitored parameters were within the ranges (recommended and mandatory) for carp fish water with exception of temperature and unionized NH<sub>3</sub>, which exceeded the norms during some months of the year; d) according to measured water transparency, the trophic state of dam water was determined as hypereutrophic; e) the microbial status of the analyzed water demonstrated that it was not suitable for irrigation because it exceeded norms for total coli-titer and E. coli-titer, and due to the presence of intestinal pathogens (Salmonella spp.), which are not allowed in the water for irrigation.

**Key words:** dam water, physicochemical and microbiological parameters, priority pollutants, water quality, natural source, fish farming, irrigation.

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## Introduction

On the territory of Bulgaria around 2200 dams are built. These surface water bodies have different ecological characteristics due to the different environmental conditions and level of anthropogenic pressure. According to Water Framework Directive (WFD) 2000/60/EC (EC, 2000) all water bodies had to achieve a "good status" and "good ecological potential" up to 2015. To date, for a number of reasons legislative, political, absent or deficient enforcement mechanisms and effective control, insufficient funding, etc., this goal has not been achieved yet in Bulgaria. Despite the existing tendency towards improving surface water quality during the period 1996-2016, there are still water bodies at risk (NRSPEB, 2018). Such an object is the Ovcharitsa Dam, one of the 25 largest dams in the country. It is rather different from the other dams as its water is used mainly for thermal power plant (TPP) cooling and also for fish farming, fishing sport and irrigation of agricultural crops. Despite the strong anthropogenic pressure on the dam, its location (on the flyway of migratory birds), suitable climate conditions and warm water make it an appropriate habitat for many birds (native and migratory) and fish species.

Ovcharitsa Dam as a part of the protected area (43.062 ha) of the National Ecological Network NATURA 2000 (No. BG0002023) is an important habitat for 76 species (of which 22 are included in the Red of Bulgaria) of migratory Book and waterfowl (about 45,700 birds annually) (SG, 2008). It is one of the suitable places in the world where the great cormorant (Phalacrocorax carbo L., 1758), the Dalmatian Pelican (Pelecanus crispus Bruch, 1832) and the big White-fronted Goose (Anser albifrons Scopoli, 1769) stay for the winter. Moreover, the lake is inhabited by more than 18 fish species, such as common carp (Cyprinus carpio silver L., 1758), carp (*Hypophthalmichthys* molitrix Val., 1844), bighead carp (Hypophthalmichthys nobilis Rich., 1845), grass carp (Ctenopharyngodon idella Val., 1844), black carp (Mylopharyngodon Rich., piceus 1846),

European catfish (Silurus glanis L., 1758), channel catfish (Ictalurus punctatus Raf., 1818), pike-perch (Sander lucioperca L., 1758), perch (Perca fluviatilis L., 1758), pike (Esox lucius L., 1758), Prussian carp (Carassius gibelio Bloch, 1782), bream (Abramis brama L., 1758), bleak (Alburnus alburnus L., 1758), pumpkinseed (Lepomis gibbosus L., 1758), vimba bream (Vimba vimba L., 1758), chub (Squalius cephalus L., 1758), roach (Rutilus 1758), rudd rutilus L., (Scardinius erythrophthalmus L., 1758), etc. The basin also harbors different species of mollusks, of which the commonest is the invasive "Zebra" mussel (Dreissena polymorpha Pall., 1771).

During the last 10 years, a number of studies have been carried out on many surface water bodies in the country, including Ovcharitsa Dam. They were focused on different aspects of water monitoring and water quality assessment but did not provide an integral picture for their state. In the summer 2009 CHESHMEDJIEV et al. (2010) investigated 80 lakes/reservoirs on the basis of four main metrics (phytoplankton biovolume, Algae Groups Index, transparency, chlorophyll-a) and found that more than half of the them were in compliance with the WFD (EC, 2000) good requirements for ecological state/potential, and Ovcharitsa Dam was determined being "moderate" as in ecological status.

In the period 2012-2013, large-scale studies were conducted on the abiotic and biotic parameters of the Ovcharitsa Dam water and the area around it, with the aim to develop and implement in 2013 a Plan for the management of the dam as a protected area (DULEV et al., 2013). Data on the physicochemical indicators of water, the qualitative and quantitative composition of phyto- and zooplankton and benthos, the species composition and the status of fish stocks and populations of amphibians and birds have been collected and processed. Based on the obtained results the water quality status was determined as "moderate",

despite the measured above-threshold values for phosphates and transparency (TRAYKOV, 2013; UZUNOV, 2013).

ATANASOV et al. (2012) established high concentrations of heavy metals (Fe, Mn, Cu, Cr, Ni, Zn, Pb and Cd) in water and liver of carps (Cyprinus carpio L.) from dams with high anthropogenic pressure in Stara Zagora District, incl. Ovcharica dam. VALKOVA et al. (2015) found the highest Cd content in the sediment followed by aquatic plants, water and muscles of carps from different surface water bodies in Stara Zagora region (Bedechka River, Sazliyska River, Tundzha River, Jrebchevo Dam). In two other papers VALKOVA (2014; 2015) reported significantly higher levels of Zn, Pb, Cd and Ni in the muscle of "Zebra" (Dreissena polymorpha) from Ovcharitsa Dam than in the water, allowing the author suggesting the use of this mussel as an indicator of heavy metals pollution of surface water bodies. The studies of DOCHIN (2014), DOCHIN et al. (2015) and DOCHIN & IVANOVA (2017) in dams with different levels of anthropogenic load (Kardzhali, Dospat and Konush dams) revealed that the most significant abiotic factors affecting the quantitative, seasonal and spatial distribution of phytoplankton in water were pН, DO, transparency and electrical conductivity.

All these studies, for the most part, concern the water quality and assessment of the surface water bodies as a natural resource. There is no up-to-date integral scientific information on water bodies, heavily modified by strong anthropogenic pressure, used for different human activities. Ovcharitsa Dam falls into this group and the above mentioned argument motivated the present study, whose purpose was to assess the dam water quality by means of physicochemical, biological and microbiological parameters in three aspects - as a natural source, as a resource for fish farming and as a resource for irrigation of agricultural crops.

## **Materials and Methods**

*Study area.* The study was carried out during 2016-2017 in Ovcharitsa Dam (altitude 135 m, area 6550 acres, water volume 62.4 million m<sup>3</sup>, fed by Ovcharitsa River) located in ecoregion 7 (Eastern Balkan), the East Aegean River Basin, Southeast Bulgaria, Stara Zagora district, 50 km southeast from town of Stara Zagora. According to Regulation H-4 (2012) for characterization of surface water in the country, the Ovcharitsa Dam type is a L15 – large planar medium deep dam. The dam water is used for cooling of the TPP "Maritza East 2" EAD (1630 MW) in the largest energy complex of Bulgaria.

Sampling and sample preparation. Water samples were collected from surface water in one Monitoring Point (MP) - N42.263781° E26.146924° in the same months for the two years of the study as followed: 5 times for physicochemical and biological parameters August (February, April, June, and November, n=10), 3 times for microbiological parameters (June, August and November, n=6) and once for priority substances (pesticides and VOC) - April, n=2. For water sampling and sample preparation for analyses, international references (ISO 5667-1, 2, 3; ISO 27828) were used. The samples for physicochemical analysis were collected in dark containers with chemically pure glass beakers (3 L) and for microbiological analysis were taken in sterile bags.

Parameters and methods for analysis. The following parameters, characterizing surface determined: water quality were physicochemical parameters - temperature (T, °C) by Bulgarian State Standard (BSS) 17.1.4.01:1977, transparency by Secchi (TS) by Routine laboratory method (RLM)-2010, pH by ISO 10523, electroconductivity (EC) by BSS EN 27888 and dissolved oxygen (DO) by BSS ISO 25814 - in situ, with field Multi-340i/SET meter; COD by ISO 15705, BOD<sub>5</sub> by BBS EN 1899-2, unionized NH<sub>3</sub> by BSS 17.1.4.10, nitrite (NO<sub>2</sub><sup>-</sup>) by BSS EN 26777, nitrate (NO<sub>3</sub><sup>-</sup>) by BSS 17.1.4.12, total nitrogen  $(NH_3, NO_2^-, NO_3^- and N_{org.})$  by BSS EN 12260 and orthophosphates /P-PO4/ by BSS EN ISO 6878; biological parameter - chlorophylla by BSS ISO 10260; pesticides and volatile

organic compounds (VOC) - pesticides containing nitrogen and phosphorus by RLM 1026/2014, alkylurea pesticides by ALM 1026/2014, VOC by BSS EN ISO 15680; microbiological parameters aerobic mesophilic microorganisms (AMO) (MORITA, 2003), sanitary indicator microorganisms (Escherichia coli, total coliforms) and pathogens (Salmonella spp.) were determined by plating of 1 mL of the sample solutions or appropriate dilutions on selective, chromogenic culture sheets medium (Rida<sup>®</sup>Count Total; Rida<sup>®</sup>Count E. Rida<sup>®</sup>Count coli/Coliforms; Salmonella/ Enterobacteriaceae, **R-Biopharm** AG, Germany). The sheets were inoculated in duplicate, incubated at 35 °C for 24-48 h and the colonies were counted. Specific microorganisms form colonies of different colour on the specific test cards. To confirm the results for Salmonella spp., suspected colonies were subcultured onto Bismuth sulfite (BS) agar (HiMedia, Mumbai, India) at 35 °C for 24 hours. If typical colonies were present (brown, gray, or black sometimes with a metallic sheen), 2 or more of them were transferred onto triple sugar iron (TSI) agar (HiMedia, Mumbai, India) and lysine iron agar (LIA) (HiMedia, Mumbai, India). If, atypical reactions were observed after 24 h of incubation the result was considered as negative for Salmonella spp. The results are expressed in colony forming units (CFU/ml).

*Water quality assessment.* It was carried out in three aspects: the dam water as a natural source, as a resource for fish farming and as a resource for irrigation by requirements of Bulgarian standards (Regulation No. 4, 2000; Regulation No. 18, 2009; Regulation on EQS for priority substances and certain other pollutants, 2010 and Regulation No. H-4, 2012).

*Statistical analysis.* All data were analyzed by STATISTICA 6.0 for Windows (StatSoft Inc., 2001).

## **Results and Discussion**

*Ovcharitsa Dam water quality as a natural source* 

*Physicochemical parameters*. This group includes 12 parameters (Table 1). The monitored parameters demonstrated different dynamics of variation during the different months of the year as followed:

*Temperature.* The water temperature of Ovcharitsa Dam depended on the season and largely on the mode of TPP operation. As expected, the lowest temperatures were measured in February (10.4-12.5 °C), and the highest - in summer – June and August (28.3-31.0 °C). These higher temperatures and the continuous flow of warm water prevent the dam from freezing and make it unique for wintering fauna (DULEV *et al.*, 2013).

Transparency. Transparency values varied within a very narrow range for both years – from 1.0 to 1.2 m, with higher values in 3 out of 5 months for 2016 and in 4 out of 5 months in 2017. As the indicator is directly related to the phytoplankton quantity, it can concluded that amount be the of phytoplankton increased during the summer (June and August 2016 and August 2017) compared to other seasons. According to the data for this indicator, the status of the "moderate". waters was defined as CHESHMEDIJEV et al. (2010) assessed the Ovcharitsa Dam water quality on the same metric as "good" in July-September 2009 at water transparency 2.3 m.

*pH*. The distribution of surface water pH by months was relatively even – from pH 7.20 to 8.83, 2016 and from pH 7.51 to 9.02, 2017. The pH values in February, June, August and November for 2016, and in August and November 2017 fell within the range corresponding to "good" ecological status of the water (pH 6.5-8.7) are.

*EC.* The results obtained determined the water quality for this parameter as "moderate" for all monitored period. Highest levels of EC were measured in April followed by slight decrease in following months. TRAYKOV (2013) explained the dynamics in EC values during the different seasons with the dying of "flowering" in the spring and subsequent decomposition of the

biomass, that releases a large amount of soluble compounds into the water.

DO. This parameter indicated the level of eutrophication and depends on various factors: abiotic (temperature, vertical and horizontal circulation of water) and biotic (development of photosynthetic organisms, putrefactive processes, etc.). The maximum DO values were measured in February (10.5-11.2 mg/l) at lowest water temperature (10.4-12.5 °C) and the minimum DO values in August - 7.6 mg/l (2016) and June - 7.5 mg/l (2017) at highest water temperatures (30.0-31.0 °C). similar А relationship between dissolved oxygen and water

temperature was reported by TRAYKOV (2013) and UZUNOV (2013) again for Ovcharitsa Dam in 2012-2013. Our results characterized water quality by DO content as "excellent" in June, August and November 2016 and in April, June, August and November 2017.

*COD*. This is not a standard indicator of surface waters quality, but it gives an idea of their pollution, especially with substances that are not biodegradable. The parameter's values varied significantly (12-22 mg/l, 2016 and 14-25 mg/l, 2017) with a fluctuating trend towards decrease from February-April to November during the surveyed period.

**Table 1.** Physicochemical and biological parameters of Ovcharitsa Dam water, 2016 – 2017. *Legend:* \* R - Recommended norms; M - Mandatory norms (for carp water); \*\* MPC - Maximum permissible concentrations; *Ecological status: E* – excellent, *M* –moderate, *G* – good, *P* –poor, *VP* – very poor.

Parameters	Years	Parameters values and Ecological water status by months			Regulation H-4/2012	Regula No.4/20	tion 100	Regulation No.18/2009 MPC**		
		II	IV	VI	VIII	XI		R*	<b>M</b> *	
Physicochemical parameters										
т⁰С	2016	10.4	20.5	29.0	30.0	15.1	_	28.0	10.0	28.0
1, C	2017	12.5	24.7	31.0	28.3	17.9	-	20.0	10.0	20.0
Transpa-	2016	1.2	1.2	1.0	1.0	1.2	1.0-2.0			
rency, m	2017	1.2	1.2	1.2	1.0	1.2	М	-	-	-
рН	2016	8.65	8.22	8.83	8.62	7.20	6.5-8.7	6	0	6.9
pm	2017	8.71	9.02	9.01	8.34	7.51	G	0-	-9	0-9
EC,	2016	750	770	760	756	759	> 750			2000
μS/cm	2017	790	821	763	800	765	М		-	2000
DO,	2016	11.2	10.1	9.1	7.6	7.9	9.00-7.00	> 8	>7	> 2
mg/l	2017	10.5	9.1	7.5	8.1	7.8	Е	- 0	- 1	~ 2
COD,	2016	22	18	20	17	12				100
mg/l	2017	19	25	16	14	16	-		-	100
BOD <sub>5</sub> ,	2016	1.5	1.3	2.2	2.7	1.8	2-4	16		25
mg/l	2017	1.7	2.8	2.5	2.9	3.5	G	<b>&lt;</b> 0	-	25
Uniopized NH mg/l	2016	0.076	0.077	0.025	0.072	0.020	< 0.1	<0.05	<0.025	5
Onionized W13, mg/1	2017	0.092	0.150	0.031	0.064	0.030	Е	<0.05	<0.0 <u>2</u> 5	5
NO <sub>2</sub> -,	2016	0.005	0.005	0.006	0.003	0.015	< 0.03	<0.03		
mg/l	2017	0.007	0.002	0.006	0.006	0.030	Е	<b>NO.05</b>	-	-
NO <sub>3</sub> -,	2016	0.54	0.49	0.32	0.17	0.30	< 0.8			
mg/l	2017	0.73	0.03	0.05	0.18	0.21	Е		-	20
Total N,	2016	1.28	1.21	0.92	1.15	0.92	0.7-2.5			
mg/l	2017	1.34	0.89	0.76	1.06	1.27	G		-	
P-PO <sub>4</sub> ,	2016	0.07	0.08	0.10	0.10	0.12	> 0.06, P	0	4	2
mg/l	2017	0.08	0.08	0.10	0.19	0.16	> 0.10, VP	0.	.4	3
			В	Siologic	al parai	neter	_			
Chlorophyll 2 ug/l	2016	10	7	30.6	17	10	10.1 <b>–</b> 20.0, M			
α, μg/1	2017	6	12	32	20	9	20.1-50.0, P	-	-	-

 $BOD_5$ . The measured  $BOD_5$  values also varied significantly (1.3-2.7 mg/l, 2016 and 1.7-3.5 mg/l, 2017), but at relatively low levels throughout the monitored years. The water organic load was higher in 2017 compared to 2016, from 1.07 times in August to 2.15 times in April, revealing that this parameter was time-dependent. With regard to this characteristic, water quality was defined as "good" throughout the study period.

Biogenic elements. Nitrogen compounds (Unionized NH<sub>3</sub>, NO<sub>2</sub>, NO<sub>3</sub> and total N). Unionized NH<sub>3</sub> values varied significantly (0.020-0.150 mg/l) with higher levels in February and April compared to the other monitoring months (June, August and November). The  $NO_2^-$  concentrations also demonstrated large fluctuations throughout the year (0.002-0.030 mg/l), but with higher levels in November than in other months. The established  $NO_2^-$  levels in the water, as an intermediate product between the oxidized and reduced nitrogen states can be defined as low. NO<sub>3</sub><sup>-</sup> concentrations showed a similar monthly dynamics as unionized  $NH_3$  and  $NO_2^-$  concentrations. The measured  $NO_3^-$  values were much higher in February than in other months, especially in 2017. Significant fluctuations of the three indicators confirmed the influence of many environmental factors, among which most important are probably the processes of nitrification and denitrification. The content of unionized NH<sub>3</sub>,  $NO_2^$ and  $NO_3^$ determined the quality of dam water as "excellent".

Total N values varied between 0.76 and 1.34 mg/l during the different months of the two years of the study (Table 1). The highest levels were measured in February and the lowest - in June and November (2016) and in June (2017). According to the results for this parameter, the water quality was assessed as "good".

*Orthophosphates (P-PO*<sub>4</sub>). The parameter values fluctuated from 0.07 to 0.16 mg/l and showed an upward trend from February to November, more pronounced in 2017 compared to 2016. The water quality

assessed on the basis of orthophosphates was determined as "poor" in February-August 2016 and in February-June 2017, and as "very poor" in November (2016) and August and November (2017).

The location of the dam (on lands contaminated or at risk for pollution by biogenic elements, Order No. RD-146/2015) and the recirculation of the water used to cool the TPP contributes not only to the accumulation but also to the increase in the concentrations of the pollutants in the water body. In the same time, the concentrations of nitrogen compounds were maintained low, which determined the "good" and "excellent" water quality of the dam, while the content of orthophosphates was high and determined a "poor" and "very poor" water quality. According to TRAYKOV (2013) and UZUNOV (2013), the main reason for this contradiction is the higher temperature of the water as a result of TPP cooling. Higher water temperature, especially during the summer months, accelerates the metabolic degradation of the accumulated organics, especially of nitrogen compounds and enhances the processes of denitrification. This leads to a decrease in the concentrations of the various forms of nitrogen without substantially altering the amount of phosphorus. The results show that the nitrogen compounds were more environmentally sensitive, as they are converted more quickly than orthophosphates. Therefore, practices to limit contamination of the dam with biogens should be implemented.

*Biological parameter*. The values of the only determined biological parameter - chlorophyll-a, showed similar and significant dynamics of variation over the different months of the studded period (Table 1). The lowest levels of chlorophyll-a were determined in February and November (6-10  $\mu$ g/l), the highest - in June and August (17-32  $\mu$ g/l) and intermediate – in April (7-12  $\mu$ g/l) (Table 1). The established seasonal differences in the amount of chlorophyll-a are logical, being determined by the seasonal
dynamics in the amount of phytoplankton. During the warmer months of the year, especially in the summer, the biogenic elements accumulated in the water body from the catchment area of the dam and by the organic wastes from the two fish farms and other human activities, combined with the higher water temperatures, create conditions for intensive development of phytoplankton, respectively the amount of chlorophyll-a.

On the basis of chlorophyll-a content, the ecological status of Ovcharitsa dam water was determined as "moderate" in February and November 2016/2017 and in April 2016, and as "poor" in June and August 2016/2017, and in April 2017. In July-September 2009 CHESHMEDJIEV *et al.* (2010) assessed the dam water quality on this metric as "good" (chlorophyll-a =  $7.32 \mu g/l$ ).

Based on the estimates for all physicochemical and biological metrics monitored, the majority of which were in the ranges between "moderate" and "excellent" status, the final estimate for Ovcharitsa dam water for the period 2016-2017 was "poor" by chlorophyll-a content and "very poor" by orthophosphates, i.e. on the lowest estimates according to the Regulation H-4 (2012).

When comparing the results obtained in the present study and the estimates for the monitored indicators with those established by DULEV (2013), TRAYKOV (2013) and UZUNOV (2013) on the same parameters for Ovcharitsa Dam water in 2012-2013, no significant differences were found. The estimates authors' for the different parameters varied between "moderate" and "very good", with exception of the phosphates and transparency, characterizing the water quality as "poor". Therefore, it can be concluded that phosphorus compounds and, to a lesser extent, the other biogens continue to be a problem for dam water quality.

*Pesticides and volatile organic pollutants.* The dam water contained negligibly low concentrations of some of the pesticides and volatile organic pollutants (Table 2). Their levels were much lower than the permissible of the average annual values limits according to Regulation on EQS (2010) as followed: for atrazine by 12 times; for simazine by 100 times; for 1,2,3-, 1,2,4- and 1,3,5-trichlorobenzene by 4 times; for trichloromethane by 6.25 times and for hexachlorobutadiene by 15 times (calculated by MPC). Ovcharitsa Dam water quality, in terms of all these priority pollutants was defined as water "in good chemical status". This assessment is an indirect indicator for the good operation of the local waste water treatment plant at the TPP "Maritza East 2" EAD, which is a source of priority and priority hazardous substances. The National System for Environmental Monitoring of Water has neither found an excess of the individual emission norms for pollutants and priority substances in the treated waste water from the TPP in 2017 (RSSW-EAB, 2017).

*Ovcharitsa Dam water quality as a resource for fish farming* 

Dam water quality assessment as a resource for fish farming according to Regulation No. 4 (2000) was made based on the analysis of data obtained for physicochemical parameters, pesticides and volatile organic compounds (Tables 1, 2). Here, we included additionally coliform counts, which is a required microbiological indicator (Table 3) and water transparency, which is not regulated by the standard, but is important indicator for fish water quality. In this case, we used the legislation for carp fish farming because the water temperature of the Ovcharitsa Dam is more suitable for cyprinids rather than for salmonids or acipenserids. The dam water is inhabited by various carp species such as common carp, silver carp, bighead carp, grass carp, bleack carp and Prussian carp, as well as some other bottom and warm-loving fish species - European catfish, channel catfish bream, bleak, pumpkinseed, vimba bream, chub, roach, rudd, etc.

**Table 2.** Content of pesticides and volatile organic compounds (VOC) in Ovcharitsa Dam water, 2017. *Legend:* \* MPC - Maximum permissible concentrations (norms for carp water); \*\*AAV-EQS: Average annual value - Environmental quality standards; \*\*\*MPC- EQS: MPC - Environmental quality standards.

	Concentration	Regulation	Regulation	on EQS/2010
Parameter		No. 4/2000	AAV-	MPC-
	μg/I	MPC*	EQS**	EQS***
C. Nit	rogen and phospho	rus containing p	pesticides, n =	2
Atrazine	< 0.05	-	0.6	2.0
Simazine	< 0.01	-	1.0	4.0
	D. Alkylurea	pesticides, n = 2		
Diuron	< 0.03	-	0.2	1.8
	E. Volatile Organi	c Compounds, 1	n = 2	
1,2,3-trichlorobenzene	< 0.1	0.4	0.4	Not applied
1,2,4-trichlorobenzene	< 0.1	0.4	0.4	Not applied
1,3,5-trichlorobenzene	< 0.1	0.4	0.4	Not applied
Tetrachlorethane	< 0.4	-	-	-
Trichloromethane				
(chloroform)	< 0.4	12.0	2.5	Not applied
Hexachlorobutadiene	< 0.04	0.1	-	0.6

**Table 3.** Microbiological parameters of Ovcharitsa Dam water, 2016 – 2017. *Legend:* \* R - Recommended norms; \*\*Maximum permissible concentrations.

Parameters	Years	C <sub>x</sub> ±SD (n=3)	$C_{min}$	C <sub>max</sub>	#Regulation No. 4 R*	Regulation No. 18/2009 MPC**	
Aerobic mesophilic	2016	$22000\pm3606$	19000	26000			
microorganisms, CFU/ml	2017	$23000 \pm 3606$	20000	27000	-	-	
California CEU/100 ml	2016	$3300 \pm 361$	2900	3600	10.000		
Coliforms, CFU/100 ml	2017	$3400 \pm 300$	3100	3700	10 000	-	
Total coli titor ml	2016	-	0.01	0.01		< 0.1	
Total con-thei, Illi	2017	-	0.01	0.01	-	< 0.1	
Eccharichia coli CEU/100 ml	2016	$130 \pm 52$	100	190			
	2017	$150 \pm 40$	100	190	-	-	
Facharichia coli titor ml	2016	-	1	0.1		< 1.0	
Escherichia con-inter, ini	2017	-	1	0.1	-	< 1.0	
	2016	$1200 \pm 100$	1100	1300		NT / 11 1	
Saimonella spp., CFU/100 ml	2017	$1300 \pm 173$	1200	1500	-	Not allowed	

The values of the most monitored parameters were within the range of the norms (recommended and mandatory) for carp fish as followed: pH – between 7.20 and 9.02, with exception of two cases in April and June 2017,

when the values were slightly over the upper limit, pH = 9.02; DO levels were over the minimum permissible value (> 7 mg/l), from 1.07 to 1.6 times; BOD<sub>5</sub> values were lower than the permissible limit for recommended norm (< 6 mg/l, from 1.71 to 4.61 times; NO<sub>2</sub><sup>-</sup> and P-PO<sub>4</sub> concentrations were lower than the allowable limits for recommended norms (< 0.03 mg/l and 0.4 mg/l) up to 15 times and from 2.10 to 5.71 times, respectively; trichlorobenzene (1,2,3-; 1,2,4and 1,3,5-), tetrachloroethane, trichloromethane and hexachlorobutadiene levels were more than 4 times, 30 times and over 2.5 times, respectively lower than the relevant maximum allowable concentration; coliform counts were 2.70 to 3.45 times lower than the maximum permissible counts.

Deviations from the norm were demonstrated by temperature and unionized  $NH_3$ values. The water temperature exceeded the upper permissible limit in the summer months (June-August), by 1.0-2.0 °C in 2016 and by 0.3-3.0 °C in 2017 (Table 1). The elevation of water temperature above the upper limit was not high, except during the summer months and probably did not affect significantly the carp fish diversity and populations. On the other hand, attention should be paid on the fact that biota and some physicochemical processes (oxygen solubility, hydrophobic interactions) are particularly sensitive to temperature changes (MIHAYLOVA *et al.*, 2012).

The unionized ammonia content in water exceeded the recommended and mandatory norms in February, April and August in 2016 and in February, April, June (only the mandatory norm), August and November (only the mandatory norm) in 2017 (Table 1, Fig. 1).



**Fig. 1.** Content of unionized ammonia in Ovcharitsa Dam water by months and years.

The presence of unionized ammonia in levels exceeding the norm in combination with low and normal concentrations of nitrites are indicative of fresh fecal water pollution (most likely from both fish farms) well as for subsequent as rapid ammonification that reduces the ammonia concentration by transforming it in nitrite (possibly the warm water and the large number of the water microorganisms contribute to this rapid effect).

Water transparency is an indirect parameter characterizing the water quality for fish. It depends on the amount of particles (inorganic - sediment and organic algae, phytoplankton, zooplankton, etc.) in the water. The transmission of light through the body of water is extremely important since the sunlight is the primary source of energy for all biological phenomena. Water transparency directly is related to orthophosphates and chlorophyll-a content in water and it is used for determination of the trophic state. In our case, the parameter's values varied between 1.00 and 1.20 m, which determined the trophic state of dam as hypereutrophic (RMB, 2019).

Some studies revealed relationships between water transparency and aquatic organisms that depend on vision for foraging, intra-species mating, or communication (HEUBEL & SCHLUPP, 2006; KARACAOGLU et al., 2006; LJUNGGREN & SANDSTRÖM, 2007). DE MELO et al. (2009) established a positive and highly significant correlation between water transparency and abundance, and distribution of the Cynodontidae Bananal species in the floodplain, Mato Grosso, Brazil. These results give reason to carry out such studies in Ovcharitsa dam and other surface water bodies in the country. Perhaps it's time to include this parameter to the national standard of water quality for fish.

*Ovcharitsa Dam water quality as a resource for irrigation* 

The controlled parameters, characterizing the water quality for

irrigation of agricultural crops (total 12) fall into three of the five groups of indices according to Regulation No. 18 (2009): salinity (EC), sanitary indicator microorganisms (total coli-titer, *Escherichia coli* titer and enteric pathogens – *Salmonella* spp.) and miscellaneous (T °C, pH, DO, COD, BOD<sub>5</sub>, unionized NH<sub>3</sub>, NO<sub>3</sub><sup>-</sup> and P-PO<sub>4</sub>).

Salinity. EC. This is the basic parameter, characterizing the water salinity, respectively the total salt concentration of the water. By years of measurement EC values were very close; their fluctuation between both years of the investigation was on the average 1.04 times (Table 1). The results obtained (750.0-821.0 µS/cm) were similar to EC values of Sazliyka River water (654.3–912.2 µS/cm, ZHELEV et al., 2015) which through Ovcharitsa River receives water from Ovcharitsa Dam, and much higher than the EC values of Maritsa River water (at Mirovo village, Stara Zagora district) - 296.0-378.0 µS/cm (KOSTADINOVA et al., 2017), left tributary of which is Sazliyka River. A probable cause of reduced EC while the water passes from a smaller to a larger surface water body (as in our case) is likely associated with increased water amount in each successive water body while the amount of dissolved salts remains relatively constant.

On the basis of this parameter the Ovcharitsa Dam water quality meets the requirements for irrigation of agricultural crops as all values were 2.43 to 2.67 times lower than the MPC for irrigation water (<  $2000 \mu$ S/cm), (Table 1).

Sanitary indicator microorganisms. This group includes parameters stipulated in Regulation No. 18 (2009) - total coli-titer, Escherichia coli titer and enteric pathogens – Salmonella spp., as well as some additional parameters - aerobic mesophilic microorganism (AMO), total coliforms and E. coli (Table 3). The values of all investigated microbiological parameters varied in different ranges within years and between the two years of the surveyed period. By years the parameters values varied more significantly as followed: for AMO - 1.37 times in 2016 and 1.35 times in 2017; for coliforms – 1.24 and 1.19 times and for Salmonella spp. - 1.18 and 1.25 times, respectively; for E.coli - 1.9 times for both vears; for total coli-titer and E.coli titer without fluctuations. Significantly narrower value ranges demonstrated the parameters when the two years of the observed period were compared: for AMO - 1.05 times at minimum and 1.04 times at maximum values; for coliforms - 1.06 and 1.03 times, and for *Salmonella* spp. – 1.09 and 1.15 times, respectively; for *E. coli*, total coli-titer and E.coli titer the values did not change. Relative persistence in those microbiological parameters of the water was observed during the two years of monitored period. This gives reason to assume that the factors of the environment in the dam water that affect the microorganism's diversity and populations are also relatively constant over the years.

The content of microorganisms in the water of the final water intake of the water of the Ovcharitsa Dam (on the country's territory) - Maritsa River, was drastically lower than in the dam water: for AMO 18-138 CFU/ml vs. 19000-27000 CFU/ml; for coliforms 155-340 CFU/ml vs. 2900-3700 CFU/ml; for Salmonella spp. 21-66 CFU/ml vs. 1100-1500 CFU/ml (KOSTADINOVA et al., 2017). Three main reasons for these substantial differences in the counts of the different microorganisms between the Ovcharitsa Dam water and the Maritsa River identified. water can be First, the temperature of the dam's water was higher than the temperature of the river water (the dam's water is used for cooling of TPP); second, the amount of dam water was less than that of the river water, it creates conditions for enrichment of the dam water with organic substances/wastes to a greater extent than for the river water due to the two fish farms for intensive rearing of fish and from the other hydrobionts living in the dam); and third, the dam water remains in the dam for a longer period of time compared to the water in the river, which entails slower change in the environmental conditions in dam water in comparison to river water. All this creates more favorable conditions for the survival and increasing of the microbial populations in the dam water.

Quality assessment of Ovcharitsa Dam water as a source for irrigation showed that total coli-titer (0.01 ml) and E. coli titer (1 -0.1 ml) values did not meet the requirements of Regulation No. 18 (2009) - < 0.1 and < 1.0 ml, respectively. For the other parameter (Salmonella spp.) the water also did not meet the irrigation requirements because intestinal pathogens are not allowed in the water. The reason for this restriction is that these microorganisms remain viable for long periods of time - up to 6 months and can contaminate irrigated soil and plants (ROSEN, They can survive 2000). in aquatic environments by a number of mechanisms, entry viable including into but а nonculturable state and/or residing within free-living protozoa (LIU et al., 2018). The results of our study correspond to the data of different investigations in this area which confirm that the water samples from fish ponds and dams usually contain high concentrations of aerobic mesophilic bacteria, E. coli, coliform bacteria and intestinal pathogens (including Salmonella spp.), which necessitate the need to apply monitoring programs on the microbiological indicators (GULUMBE et al., 2016; VASILE et al., 2017). The standard permits the use of such irrigation water for only after decontamination which is not always possible and economically justified.

*Miscellaneous*. All pH, DO, COD, BOD<sub>5</sub>, unionized NH<sub>3</sub>, NO<sub>3</sub><sup>-</sup> and P-PO<sub>4</sub> values were within the permissible limits for irrigation water (Table 1). Only the water temperature in the June-August (the irrigation period) exceeded the maximum allowed limit of 28 °C, by 1-2 °C in 2016 and with 0.3-3.0 °C in 2017. Notwithstanding this, the elevation of the water temperature above the allowed limit was not great and according to Regulation No. 18 (2009), the water can be used for irrigation when it meets the requirements of the other parameters of this group as in our case.

# Conclusions

two-year study (2016 - 2017)А of by water 12 Ovcharitsa Dam physicochemical parameters (temperature, transparency, pH, EC, DO, COD, BOD<sub>5</sub>, unionized NH<sub>3</sub>, NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, total N and P-PO<sub>4</sub>), 1 biological parameter (chlorophyll-a), 9 pesticides and VOC (atrazine, simazine, diuron, 1,2,3-, 1,2,4and 1,3,5trichlorobenzene, tetrachloroethane, trichloromethane, hexachlorobutadiene) and microbiological parameters (aerobic 6 mesophilic microorganism, coliforms, total coli titer, E. coli, E. coli titer, Salmonella spp.), legislation stipulated in Bulgarian concluding that: a) the ecological assessment of the dam water as a natural source determined the water ecological status as "poor" by chlorophyll-a content and as "very poor" by orthophosphates content; b) the pesticides and VOC concentrations did not exceed the environmental quality standards and determined the water as water "in good chemical status"; c) the dam water quality met the requirements for carp fish water by all monitored parameters, with the exception of temperature and unionized NH<sub>3</sub> values, which exceeded the norms in some months; d) by transparency the dam water trophic state was determined as hypereutrophic; e) the dam water did not meet the irrigation requirements as the values of total coli titer and E. coli titer exceeded the norms as well as due to the presence of intestinal pathogens (Salmonella spp.), which are not allowed in the water for irrigation.

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# Analysis of Anthropophitic Flora on the Territory of Lozenska Mountain, Bulgaria

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Abstract. The aim of the study is to analyze the taxonomical structure, origin and distribution of the anthropophytic element on the territory of Lozenska Mts. and to assess the degree of synanthropization of the flora. The study was carried out in the period 2016-2018. During the field studies the transect method was applied for collection and identification of the plant material. In view of possible comparisons and calculations of the synanthropic indice the surveyed area was divided into 4 sub-regions. On the territory of the Lozenska Mts. 274 species and 8 subspecies from 196 genera and 57 families were found. The anthropophytes are predominantly therophytes. In phytogeographical terms the character of the anthropophytic flora can be described as European-Asiatic with a strong boreal and less sub-mediterranean influence. Four species of nature conservation status were identified among the anthropophytes. The antropophytes of Lozenska Mts. are 33.3% of its vascular flora and almost twice of the same group of plants in the flora of Bulgaria (14,0%). The north-eastern part of the mountain is most affected by the synanthropic processes and less affected is the north-western part. For the period 1961-2018 the percentage of the anthropophytes in the surveyed area is increased by 7.2%, while the participation of the autochthonous species is decreased by 3.8%. The high values of calculated indices of synanthropization show the intensification of this process on the territory of the mountain.

Key words: synanthropization, anthropophytes, autochthonous flora, ecological groups, Lozenska Mts.

#### Introduction

The Lozenska Mountain is a part of the Floristic Region Western Sredna Gora with a height of 1195 m and an approximate area of 90 m<sup>2</sup>. About 14.3% of its territory enter into NATURA 2000 areas. With its proximity to the capital, the mountain is a valuable site from a commercial and recreational point of view. At the same time, the negative human impacts associated with unregulated felling,

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg grazing and others contributing to the erosion processes and droughts are a major cause of the increased processes of xerophytisation and degradation of plant communities which was registered more than 50 years ago in the first large-scale mountain vegetation study by GANCHEV (1961). In spite of the constant and high anthropogenic pressure, the study of the impact and spread of anthropophytes on the

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territory of the Lozenska Mountain, the risk of synanthropization and the reduction of autochthonous species in its floristic complex has not been carried out until now. Collecting and updating of the information on these species is important for the genesis of its local flora and for the control and management of ecosystems (PETROVA & VLADIMIROV, 2001).

The aims of the present study are:

1. Analysis of the diversity of anthropogenic elements, their distribution and origin on the territory of the Lozenska Mountain;

2. An assessment of the degree of synanthropization of its flora.

# Material and Methods

Lozenska mountain is part of Sredna gora floristic region. It is situated in Southwest Bulgaria close to Sofia city and covers 80 sq.km area. The highest elevation mountain rises at 1190 m a.s.l. (Popov Del Peak). Variety of rocks are revealed on the slopes of the mountain: sandstone mainly on the norhtern slopes and metamorphyc rocks on the southern slopes. Limestones and dolomites form a part of the ridge of the mountain peak of Bachul, Rakovica mound, Lalina mogila and Polovrak. The predominant soil types in the mountain are Chromis Cambisols and Humic Cambisols. The climate is characterized by relatively mild winters, low annual temperature amplitudes and two periods of maximum rainfall over the year. The average annual temperature is 9.3 ° C and the annual precipitation is 590.7 mm.

The survey was conducted in the period 2016 - 2018. During the field studies, the transect method for species identification was used. Based upon the fieldwork previous experience in the floristic investigation of the Lozenska Mountain GLOGOV & DELKOV, 2016) and after the preliminary observation of the whole territory during the present study 19 transects (averaged from 3,6 to 4,8 km each) were set up (Fig. 1) are selected to

encompass a maximum area and representative areas of habitat diversity. The terrains in the height range of 600 m to 1190 m a. s. l. were inventoried. Various habitats have been investigated - forests of different vegetation types and stages of succession, wet meadows, places around streams, river banks, villas zones as well as areas affected by anthropogenic activity.

The principles and the methodical approach of the present research and analysis follow the generally accepted practice of floristic studies in foreign and Bulgarian literary sources (KAMELIN, 1973; TOLMACHOV, 1986; ĆWIKLIŃSKI, 1970; WOJCIKOWSKA & MOYSIYENKO, 2008; PAVLOVA & GEORGIEVA, 2015; etc.). The taxonomic nomenclature and determination of plant taxon follow KOJUHAROV (1992); DELIPAVLOV & CHESHMEZHDIEV (2003) and "Flora of Bulgaria" - Vol. I-XI. (1963-2012). Life forms follow the classification of RAUNKIAER (1934). Floristic elements are according ASYOV & PETROVA (2012). Anthropophytes list follows STEFANOV & KITANOV (1962), PETROVA & VLADIMIROV (2001), etc. The conservation status of species is in line with Directive 92/43/EEC (EC, 1992), CITES (2009), BDA (2007), Red Book of Bulgaria (PEEV, 2015), IUCN, Red List of Bulgarian Vascular Plants (PETROVA & VLADIMIROV, 2009). Ecological groups are according PAVLOV (1998). Invasive alien species follow PETROVA et al. (2013).

In view of possible comparisons and calculations of the synanthropization indexes, the studied area is divided into subareas geomorphological considering the characterization of the mountain and the physico-geographic distribution of two parts north and south-east (IVANOV et al., 1969). Due to the greater area of the northern part, the difference in slope exposure and the soil specificity, the northern part is conventionally divided into three sub-areas with approximately the same area as the main boundary passes along the ridge of the 2). The fourth subarea mountain (Fig. corresponds to the southeastern part according to the division of IVANOV *et al.*, (1969). These 4 subareas in the present study will be named as follows: Part I (northwestern subarea); Part II (northeastern subarea); Part III (southwestern subarea); Part IV (southeastern subarea) (Fig. 1).

The floristic synanthropization indexes (S1) are calculated using the formula by WYSOCKI & SIKORSKI (2002):

$$S1 = (Ap + A)/C \cdot 100, \%$$

where: Ap - number of apophytes;

A - number of anthropophytes;

C - total number of species in individual plots (or the entire territory of the mountain).

#### Results

*Taxonomical structure* 

As a result of the study, 274 species and 8 subspecies of 196 genera and 57 families

were established (Table 1). They represent 48.7% of the anthropophytes in the flora of 563 Bulgaria species (PETROVA -& VLADIMIROV, 2001). The relation number of autochthonous species: number of apophytes: number of anthropophytes in 1961 (GANCHEV, 1961) was 43.0: 30.9: 26.1, and in 2018, it is: 39.2: 27.5: 33.3. The most anthropophytes (76.6%) from their total number on the territory of the mountain are registered in its Southwestern part, and the smallest number (47.8%) is found in its Northeastern part. At the same time, the highest percentage of the flora of each part of the mountain is the anthropophytes in its Northeastern part (37%) and the lowest those in the Northwest (31.7%). 66.4% of the anthropophytes are present in all parts, 8% in only three of the parts and 4.7% in only two of the parts.



Fig. 1. Map of Lozenska Mts. with regions and transects.

							Magnoliophyta					Total			
Area	axonomic range	axonomic range Lycopodiophyti		Equisetophyta	•	Polypodiophyt		Pinophyta		Liliopsida		Magnoliopsida		Anthrophytes (% of the entire flora of the mountain and total number anthrophytes)	
	Ε	Num- ber	%	Num- ber	%	Num- ber	%	Num- ber	%	Num- ber	%	Num- ber	%	Num- ber	%
1	fam.	0	0	1	2	1	2	0	0	5	11	40	85	47	82.5
art	gen.	0	0	1	1	1	1	0	0	16	12	115	87	133	67.9
Ч	sp.	0	0	1	1	1	1	0	0	20	12	150	87	172	62.8
2	fam.	0	0	0	0	1	3	0	0	3	8.1	33	89	37	64.9
art	gen.	0	0	0	0	1	1	0	0	11	11	91	88	103	52.6
Ч	sp.	0	0	0	0	1	1	0	0	16	12	105	80	131	47.8
~	fam.	0	0	1	2	1	2	1	0	4	8.2	45	92	49	86.1
art 3	gen.	0	0	1	1	1	1	1	0	16	10	139	88.1	158	80.6
4	sp.	0	0	1	1	1	1	1	0	23	11	184	88.3	209	76.3
	fam.	0	0	1	2	1	2	1	0	4	8.9	38	84	45	78.9
art 4	gen.	0	0	1	1	1	1	1	0	15	12	111	86	129	65.8
Ч	sp.	0	0	2	1	1	1	1	0	21	12	146	85	171	62.4
ka ain	fam.	0	0	1	2	1	2	1	1.8	5	7	49	54	57	62.6
zens unta 2018	gen.	0	0	1	1	1	1	1	0.5	21	11	172	88	196	48.3
Los mo	sp.	0	0	2	1	1	1	1	0.7	30	11	240	88	274	33.3
ska ain	fam.	0	0	1	3	1	3	0	0	4	11	30	83	36	50.7
zen: unt 1961	gen.	0	0	1	1	1	1	0	0	13	13	86	85	101	34.1
Lo.	sp.	0	0	2	1	1	1	0	0	23	15	128	83	154	26.1

**Table 1** Taxonomic structure of anthropophyte flora of the Lozenska Mountain.

Seven of the richest anthropophyte families determined on the territory of the mountain (Asteraceae, Poaceae, Fabaceae, Brassicaceae, Chenopodiaceae, Caryophyllaceae and Scrophulariaceae) match with the list of leading families from territory of all country (PETROVA & VLADIMIROV, 2001). The most numerous are families Asteraceae (42 species), Fabaceae (25 species) and Poaceae (20 species), and the most riches are genera: Vicia (9 species), Geranium (6 species), Bromus (6 species) and Veronica (5 species) (Table 2).

### Biological spectrum

The distribution of the two largest groups in biological spectrum shows

generally weak prevailing of therophytes above hemicriptophytes. In comparison with 1961 geophytes have decreased in half, and phanerophytes have increased with 77% because of introducing of exotes (Fig. 2).

# Distribution and origin

In phytogeographical terms (Table 3), the highest percentage is the Eurasian (29.2%) and the European species (24.1%), followed by the boreal (16.1%). In the period 1961-2018, the number of most of the floristic elements increased proportionally, with the exception of the Sub-Mediterranean, which declined by 41% and the Adventive species, which increased almost 8 times. Among anthropophytes are established two Balkan endemics (*Dianthus corymbosus* Boiss. and *Scabiosa triniifolia* Friv.) and 3 sub-endemics (*Orlaya grandiflora* (L.) Hoffm., *Verbascum blattaria* L. and *Verbascum chaixii* subsp. *austriacum* (Roem. & Schult.) Hayek.

**Table 2.** List of the richest with anthropophytes genera on the territory of the Lozenska Mountain.

	Territory of the Lozenska mountain								
Family	20	18	1961						
2	Number	Number	Number	Number					
	species	genera	species	genera					
Asteraceae	42	35	22	13					
Fabaceae	25	10	19	8					
Poaceae	20	15	12	9					
Brassicaceae	17	16	6	6					
Scrophulariaceae	16	7	9	6					
Caryophyllaceae	14	12	10	7					
Lamiaceae	12	9	6	6					
Boraginaceae	11	10	5	4					
Apiaceae	10	9	7	6					
Ranunculaceae	7	5	4	4					

Relict anthropophyte flora is presented by 8 species (*Hedera helix* L., *Lonicera xylosteum* L., *Equisetum palustre* L., *Pteridium aquilinum* (L.) Kuhn, *Juglans regia* L., *Rumex acetosa* L., *Salix alba* L. M *Salix fragilis* L.), tertiary relicts, most of which are distributed in the Southwestern part of the mountain.

# Ecological groups

The distribution of ecological groups in terms of light- factor shows a prevalent presence of over 75% of heliophyte species in each of the mountain parts. The participation of the sciophytes in the anthropophytic element is negligible (less than 2%).

With respect to the other main factor soil humidity (Fig. 4), anthropophytes with highest presence in all parts (with prevailing participation above 50%) are the mesophytes, following by mesoxerophytes (over 20%) and xeromesophytes (over 15%). The lowest percentage (below 2%) are the representatives of over-moistened and aquatic habitats. The strongest change for the period 1961-2018 is observed in xerophytes, whose participation did decrease with 47%.

*Conservation status* 

Among anthropophytes on the territory of Lozenska Mountain are determined species with conservation (environmentalprotective) status as 1 of them - Geranium bohemicum L. is included in Red list of Bulgarian vascular plants with category "endangered", 2 species (Bupleurum rotundifolium L., Smyrnium perfoliatum L. are included in Annex 4 of the Low of biodiversity (3EP?) and one species Opuntia humifusa (Raf.)Raf is in the list of CITES (2009). Habitats of these species been established only in the two southern parts of the mountain.

# Ivasive alien species (IAS)

IAS constitute 9,9% (27 species) from the anthropophytes of Lozenska Mountain (Table 5) and 44,3% from IAS in the flora of Bulgaria (PETROVA et al., 2013). In supplement, on the territory of the mountain are found habitats of 4 potentially IAS - Opuntia tortispina Engelm., Opuntia fragilis Nutt., Impatiense baulfourii Hook.f. и *Lupinus polyphylus* Lindl. Seven from the established IAS: Ailanthus altissima, Acer negundo, Robinia pseudoacacia, Amorpha fruticosa, Opuntia humifusa, Bidens frondosus and Fallopia × bohemica are strongly aggressive represent the biggest threat and to biodiversity, nature and humans (PYŠEK et al., 2009; PETROVA et al., 2013). Predominates the percentage of deliberately introduced species (PETROVA et al. 2013) invasive alien species (64,5 %) above unintentialy introduced (38,7%). Their average presence in separate parts of the mountain is 56,5%, as the biggest presented IAS there is in Part 11 (87,1%), and the lowest - in Part 2 (25,8%). Dominant IAS quantitative participation (their in in communities is above 50%) are 14 (45,2%) from species: Amaranthus hybridus L., Amaranthus retroflexus L, Elodea canadensis Michx., Erigeron annuus (L.) Pers., Erigeron canadensis (L.) Cronquist, Fallopia X bohemica (Chrtek&Chrtkova) J.P. Balley, Impatiense glandulifera Royle, Oenothera biennis L., Opuntia humifusa (Raf.) Raf., Robinia pseudoaccacia L.,

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Solidago gigantea Ait, Sorghum halepense (L.) Pers., Impatiense baulfourii Hook.f., Lupinus polyphylus Lindl.

Indices of anthropogenic changes of vascular flora

The comparison of indexes of synanthropization shows the highest anthropophytes impact in Northeastern part (67,5%) and the lowest – in Northwestern part (61,8%) of the mountain (Table 6).



**Fig. 2**. Biological spectrum of the anthropophytes on the territory of the Lozenska Mountain.

**Table 3.** Distribution of anthropophytes on the territory of Lozenska Mountain by floral elements.

Type floral element	Part 1	Part 2	Part 3	Part 4	Lozenska mountain 2018	Lozenska mountain 1961
	%	%	%	%	%	%
1. European	23.8	22.9	24.4	24.6	24.1	24.2
2. Euroasian	29.6	29.8	30.1	31.0	29.2	24.7
3. Sub-Mediterranean	4.7	6.1	6.7	5.3	6.9	11.7
4. Mediterranean	4.1	2.3	1.9	3.5	4	3.8
5. Pontic	5.8	6.1	6.2	4.7	5.1	5
6. Boreal	16.9	14.5	15.8	15.2	16.1	17.5
7. Alpine	0.6	0	0.5	0	0.4	1.3
8. Balkan Subendemic	1.2	0.8	1.0	0.6	1.1	1.9
9. Endemic	0.6	0.8	1	1.2	0.7	1.9
10. Cosmopolitan	9.3	13.7	7.7	10.5	7.7	6.5
11. Adventive	3.5	3.1	4.8	3.5	4.7	0.6
Total	100	100	100	100	100	100



Fig. 3. Distribution of the ecological groups in terms of light-factor.



Fig. 4. Distribution of ecological groups in terms of soil moisture.

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Species	Category of endangerment	Part 1	Part 2	Part 3	Part 4
<i>Opuntia humifusa</i> (Raf.)Raf	CITES	V			
Geranium bohemicum L.	Endangered /EN A4c according to IUCN and Red list			V	
Bupleurum rotundifolium L.	BDA (Annex 4)	V		V	
Smyrnium perfoliatum L.	BDA (Annex 4)			V	

**Table 4.** Distribution of the anthopophytes with conservation status.

**Table 5.** Distribution of invasive alien species on the territory of Lozenska Mountain.

Spe	cies	Part 1	Part 2	Part 3	Part 4
1.	Acer negundo L.	V		V	V
2.	Ailanthus altissima Swingle	V		V	
3.	Amaranthus hybridus L.	V	V	V	V
4.	Amaranthus retroflexus L.	V	V	V	V
5.	Amorpha fruticosa L.	V		V	
6.	Bidens frondosus L.				V
7.	Buddleja davidii Franch.	V			
8.	Chenopodium ambrosioides L.	V			
9.	Datura stramonium L.	V		V	
10.	Elodea canadensis Michx.				V
11.	Erigeron annuus (L.) Pers.	V	V	V	V
12.	Erigeron canadensis (L.) Cronquist	V	V	V	V
13.	Fallopia X bohemica (Chrtek&Chrtkova) J.P. Balley		V	V	
14.	Gleditsia triacanthos L.	V			
15.	Helianthus tuberosus L.	V			
16.	Impatiense glandulifera Royle	V		V	
17.	Koelreuteria paniculata Laxm.	V			
18.	Laburnum anagyroides Medik.	V			
19.	Oenothera biennis L.	V		V	
20.	Opuntia humifusa (Raf.) Raf.	V			
21.	Oxalis corniculata L.	V			
22.	Parthenocissus quinquefolia (L.) Planch.	V			
23.	Robinia pseudoaccacia L.	V	V	V	V
24.	Solidago gigantea Ait.			V	
25.	Sorghum halepense (L.) Pers.	V	V		V
26.	Xanthium italicum Moretti	V	V	V	V
27.	Xanthium spinosum L.	V		V	
28.	Opuntia fragilis Nutt.	V			
29.	Impatiense baulfourii Hook.f.	V			
30.	Lupinus polyphylus Lindl.	V		V	
31.	Opuntia tortispina Engelm.	V			

Group	Part 1	Part 2	Part 3	Part 4	Lozenska Mountain 2018	Lozenska Mountain 1961
Anthropophytes	172	131	209	171	274	154
Apophytes	163	108	167	156	227	182
Total number species	542	354	599	496	823	589
Index of synanthropization	61.8%	67.5%	62.8%	65.9%	60.9%	57.0%

**Table 6.** Distribution of synanthropization groups and indexes of synanthropization.

It is observed an increase with 3,9 % of the total index of synanthropizatin (S1) of the flora during the 2017 (S1=60,9%) in comparison with established in 1961 (S1=57,0%).

#### Discussion

Data on the increased participation of the anthropophytes and the decrease in the share of autochthonous species in the last 50 vears are evidence of gradual anthropophytisation of the mountain. This process is due to a certain extent, to the biological characteristics of anthropophytes and, above all, to their mobility or their ability to disperse rapidly in all directions and to retain themselves in a variety of conditions of existence, due to their high vegetative and seminal production, the high germination of their seeds preserved for a long time, as well as the conveniences that have their diasporas for rapid disperse at a greater distance from different transport agents. The high percentage of therophytes common phenomenon is а for the anthropophyte element of Bulgaria. According to STEFANOV & KITANOV (1962) the representatives of this group of plants have the ability to spread at great speed. The authors note that out of the group of anthropophytes the number of annual plants in Bulgaria is comparatively limited, which means that humans is the main factor for the distribution of annual anthropophytes and creation of conditions for their the accommodation and the retention of their new habitats. The presence of high rates of

hemicryptophytes can be explained by their prevalence in the biological spectrum of our flora as well as by the strategy of spreading of a large part of them associated with a variety of vegetative propagation conveniences.

Due to their wide spread throughout the country, their migration routes are difficult to understand and their origins in our country are varied. The predominance of species with European participation among the anthropophytes corresponds to the established by GLOGOV & DELKOV (2016) distribution of the floral elements for the whole flora of the mountain. typical for Lozenska Mountain as well as for other Bulgarian mountains is typical formation of anthropophyte communities at places where animals are raised, for example the species Verbascum lychnitis L. is accompanied by Thymus species, Trifolium repens L., Agrostis capillaris L., Chenopodium bonus - henricus L., Pastinaca hirsuta Pancic and others.

The phytogeographic analysis of anthropophytes shows their relationship to the climate change. The de Martonne aridity index calculated on a base of the average temperature and annual the annual precipitation is 30,6. According to the de Marton Classification Scheme, at such an IDM value, the area under study belongs to a humid climat type. (MITKOV & TOPLIYSKI, 2019). That explains the high presence of European and Boreal species related to the Mountain phytogeographic center (STEFANOV, 1943). On the other hand, the prevailing percentage of Eurasian species is an indicator of steppe climate-influenced xerophytic processes, whose varieties can be found in territories adjacent to the study area (MITKOV & TOPLIYSKI, 2019). The two precipitation maximums recorded on the territory of the Lozenska mountain are a sign of Mediterranean influence, as evidenced by the notable participation of Mediterranean and sub-Mediterranean species.

With regard to the distribution of the anthropophytes in the separated parts of the mountainous territory, it should be noted that among them predominates therophytes in Part 2 and Part 4. In these parts the process of ruderalization is most enhanced. A share of Part 4 covered by the open-cast quarries of mine "Chukurovo" is strongly anthropogenic and is a prerequisite for the invasive settlement of ruderal vegetation with the participation of Urtica dioica L., Datura stramonium L., Solanum nigrum L., Hyoscyamus niger L., Sambucus ebulus L., Amaranthus retroflexus L., species of the genus Chaenopodium, Cirsium, etc. The other high reason for percentage of anthropophytes is the deserted and nontillage agricultural lands agricultural lands, which covered larger areas of the territory of these two parts of the mountain.

The analysis of the distribution of populations of IAS on the territory of the mountain shows their predominant presence in the areas around rivers with constant water regime - Iskar, Rakita and Gabra, and on the lands of the village of Dolni Lozen and Chukurovo mine. For the most part, these species form populations that enter as asectators in the composition of the plant communities. Considering that IAS are mentioned as the second most important reason for the extinction of species after habitat destruction (PETROVA et al., 2013), there is a special interest in the ways of their entering the mountain. The high percentage of IAS in the western part of the Lozenska Mountains are considered to be related to the tourist flow and the existence of villa areas and settlements, where after cultivation of species for different purposes (mostly as ornamental plants) they

"go out" and "get wild". The proximity of the Iskar River should also be considered as the main road for the penetration and distribution in the mountain of many species, both invasive and synantropic, evidenced by their high concentration in the western part of the mountain.

The influence of both factors- climatic and anthropogenic on the distribution of anthropophytes on the Lozenska Mountain is complex. Their weight varying according to the distance of time. In the short and local terms, the human factor has a stronger influence, as evidenced by the presence of a significant number of Invasive alien species. But their naturalization, as well as the high percentage of Eurasian and Mediterranean species, are an indicator of gradual and irreversible long-term climate change.

# Conclusion

Antropophytes of Lozenska Mountain occupy 33.3% of its vascular flora, which is almost twice the percentage of the same group of plants in the flora of Bulgaria (14%). This fact and the high values of the indexes of synanthropization show the intensification of this process on the territory of the mountain. For the period 1961-2018. the percentage share of anthropophytes in the area surveyed increased by 7.2%, while the participation of autochthonous species decreased by 3.8%. The Northeastern part of the mountain is most strongly influenced by the processes of synanthropization, and the weakest influenced part is the Northwestern part of the mountain. The predominant life forms among the anthropophytes of the Lozenska Mountains is the therophytes, and in phytogeographical terms of view the character of the anthropophytes flora of the mountain can be described as European-Asian with a strong Boreal and less Sub-Mediterranean influence.

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# In vitro Plant Regeneration of Two Cucumis melo L. Genotypes Using Different Explant Types and Culture Medium

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**Abstract.** Development of an efficient *in vitro* plant regeneration system plays an important role for applying of biotechnological approaches for crop improvement. Therefore, the effect of added of BAP in germination phase on organogenesis of cotyledon and hypocotyl explants of two melon lines was investigated. The seeds germination medium contained three different concentrations of BAP (0.5, 1.0 and 1.5 mg L<sup>-1</sup>). The rate of plant regeneration was found to depend on genotype, explant type and culture medium. The cotyledons were more effective as explants for organogenesis and subsequent plant elongation than hypocotyls. Combination of 1.0 mg L<sup>-1</sup> of BAP in germination medium and 0.5 mg L<sup>-1</sup> BAP + 0.5 mg L<sup>-1</sup> IAA in next regeneration medium gives the better regeneration answer in two explant types of line 11/9, while in line AGY the most effective was the combination of 1.5 mg L<sup>-1</sup> of BAP and 0.5 mg L<sup>-1</sup> BAP + 0.5 mg L<sup>-1</sup> IAA. The experimental results indicated that pre-treatment with cytokinin BAP (1.0 mg L<sup>-1</sup>) in germination stage stimulates regeneration process in cotyledons and hypocotyls of melon line 11/9.

Key words: melon, cotyledons, hypocotyls, pre-treatment, cytokinin.

#### Introduction

Melon is one of the most important crops from Cucurbitaceous family which is described with great diversity of groups (ROBINSON & DECKER-WALTERS, 1997). Three varieties of Cucumis melo L. (var. reticulatus cantalupensis, var. and var. *inodorus*) are involved in breeding program in the Maritsa Vegetable Crops Research Institute - Plovdiv, Bulgaria (VELKOV & Traditional Petkova. 2014). breeding methods in melon have led to a considerable varietal improvement (NUÑEZ-PALENIUS et al. 2008). However, strong sexual incompatibility barriers at the interspecific

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg and intergeneric levels have restricted the use of that genetic potential to develop new enhanced melon and cultivars (NIEMIROWICZSZCZYTT & KUBICKI, 1979; ROBINSON & DECKER-WALTERS, 1999). Somaclonal variation is another important source of genetic diversity to obtain new variations useful hereditary in plant breeding (JAIN, 2001; LESTARI, 2006).

*In vitro* plant regeneration in melon has been achieved via direct and indirect organogenesis by use of diverse explant types: hypocotyls, cotyledons, leaves, cotyledonary nodes and petioles (CURUK *et al.*, 2003; SOUZA *et al.*, 2006; ZHANG *et al.*,

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2011; NADERI et al., 2013; IVANOVA et al., 2017). Shoot induction and regeneration have been obtained in culture medium supplemented with different combinations and concentrations of growth regulators (MELARA & ARIAS, 2009; MENDI et al., 2010a; AWATEF & MOHAMED, 2013; NADERI et al., effects 6-2016). Beneficial of Benzylaminopurine (BAP) or kinetin in combination with Indolil-3-acetic acid (IAA) on shoot induction have been observed in melon by several authors (LIBORIO et al., 2001; CHOI et al., 2012; SEBASTIANI & FICCADENTI, 2013). However, auxins are not always a prerequisite to achieve that goal, and cytokinins alone are able to induce bud formation (KENG & HOONG, 2005; REN et al., 2013). NADERI & MAHMOUDI (2017) reported that the application of 1.5 mg L<sup>-1</sup> BAP plus 250 mg L<sup>-1</sup> cefotaxim and 1 mg L<sup>-1</sup> BAP with 1000 mg L<sup>-1</sup> cefotaxim formed the most efficient media for plant regeneration. On the other hand, MENDI et al., (2010a) obtained more regenerants per explant on medium supplemented with combination of 0.5 mgL<sup>-1</sup> BAP and 0.5 mg L<sup>-1</sup> IAA (88%), compared to the use of 1.0 mg L<sup>-1</sup> BAP (75%) alone. KISS-BABA et al., (2010) also underlined the positive effect of IAA adding to BAP on shoot induction and regeneration in melon. However, some of the genotypes are recalcitrant and do not react with normal shoot-buts and plantlets formation (STIPP et *al.*, 2001).

Several experiments have been carried out to optimize culture conditions and to overcome the difficulties for shoot elongation and differentiation. Pre-treatment with phytohormones, explant source and position are used, but the success of these trails is with different effect (KISS-BABA et al., 2010; DO AMARAL et al., 2014). According to CHOVELON et al. (2011) environmental and requirement for hormonal melon regeneration are poorly understood, which makes difficult development of routine procedure for plant regeneration. Appropriate combining of explant type and culture conditions could significantly

increased the regeneration frequency of melon (GUIS *et al.*, 1998).

The aim of this experimental work was to study regeneration potential of different explant types and culture medium of two melon lines.

#### Materials and Methods

#### Plant material

The experimental work was carried out during the period 2016-2017 with two melon lines 11/9 and AGY, which is part of the Maritsa Vegetable Crops Research Institute's collection. Melon line 11/9 belongs to var. cantalupensis which is described with monoecious type of flowering, male sterility (ms-4), fruits possess elliptical shape, yellow ground colour of skin, orange colour of the flesh. Breeding line AGY belongs to var. *reticulatus* (Ananas type) which is described with gynoecious type of flowering, fruits possess oval shape, netted, yellow ground colour of skin, white colour of the flesh. Both lines are elite that have been inbred five times.

# *Explant types and treatment*

Seeds of the melon lines were surface sterilized in 5% calcium hypochlorite solution for 1 hour and rinsed three-times in sterile dH<sub>2</sub>O. For germination the seeds were sown on three variants of basal culture containing medium macroand micronutrients by MURASHIGE & SKOOG (1962), vitamins by GAMBORG et al. (1968), 30 g  $L^{-1}$  sucrose and 0.7% agar (MS0), differ by cytokinin concentration of 6-Benzylaminopurine (BAP) (0.5, 1.0 and 1.5 mg L<sup>-1</sup>), named MS1, MS2 and MS3, respectively. The pH of the medium was adjusted to 5.8 before autoclaving. All culture media were autoclaved at 121 °C for 20 minutes.

Explants of cotyledons (0.5 cm<sup>2</sup>) and hypocotyls (1.0 cm) were excised from 5-7 days old *in vitro* grown seedlings and cultivated in Petri dishes on MS0 medium. The next three combinations of BAP and IAA (Indolil-3-acetic acid) were studied: 1. 0.5 mg L<sup>-1</sup> BAP + 0.5 mg L<sup>-1</sup> IAA 2. 1.0 mg L<sup>-1</sup> BAP + 0.5 mg L<sup>-1</sup> IAA 3. 1.5 mg L<sup>-1</sup> BAP + 0.5 mg L<sup>-1</sup> IAA

When the regenerants were 2-3 cm long, they were transferred into 250 ml glass jars containing 25 ml  $\frac{1}{2}$  MS0 medium (half strength medium), 30 g L<sup>-1</sup> Sucrose, 0.7% Agar (rooting medium). For adaptation the plantlets 5-6 cm long were transferred in mixture of perlite:peat (1:1) (v/v), for a period of 10-12 days. Subsequently, the plantlets were transplanted to 5 L plastic pots with peat moss and perlite in ratio 1:1 (v/v) in greenhouse conditions.

The explants and regenerants were incubated in growth chamber at  $25^{\circ}C \pm 1^{\circ}C$  temperature, a photosynthetic proton flux density (PPFD) of 200 µmol m<sup>-2</sup> s<sup>-1</sup>, 16/8 h photoperiod and subcultured at intervals of 20 days to the same medium variants.

The experiment was carried out in three replications with 20 explants in each for the different genotypes, medium variants and explant types. The callusogenesis, organogenesis, regeneration frequency (% explants with regeneration) and number of regenerants per explant were examined for a period of 90 days.

# Statistical analysis

All data were statistically analyzed using the SPSS (SPSS INC., CHICAGO, USA, 2008) and Excel (MICROSOFT CO., 2016). Four-way analysis of variance and LSD test were performed at P = 0.05 on each of the significant variables measured.

# **Results and Discussion**

Melon cotyledon and hypocotyl explants from two studied lines reacted with callusogenesis in all studied medium variants. Depending of the explant type the callus morphology respond was different. In cotyledons callus was friable and whitish with leaf structures and elongated shoots, while in hypocotyls callus was transparent and watery with sporadic regeneration answer. Regeneration through callus phase is a precondition for induction of genetically stable changes in obtained plant-regenerants with higher frequency (NUNEZ-PALENIUS *et al.*, 2008).

The frequency of organogenesis in the studied melon lines depending on explant types and culture medium variants which varied from 0.0% to 100%. These results corresponded with other studies conducted under different genotypes and concentration of plant-growth regulators (CHEE, 1991; CURUK *et al.*, 2003; TEKDAL & CETINER, 2013). This could be explained by lack of correlations between explants type and components of cultural media and empiric investigations are reasonable.

Organogenic response was better expressed in cotyledons than hypocotyls. Organogenic process in hypocotyls of line 11/9 cultivated in culture medium variant MS3 containing 1.5 mg L<sup>-1</sup> BAP was not established (Fig. 1).

Induction of organogenesis in explants of line AGY was lower than line 11/9. The response of cotyledons of line AGY cultivated in germination medium MS2 was not observed whereas in the other medium variants it ranged within 15.0% - 100%. In hypocotyls organogenic structures was observed only in control germination medium (MS0) and combination MS3 + 2 variant of regeneration medium (Fig. 2). The genotype is the most important factor determining organogenic and regeneration potential. FICCADENTI & ROTINO (1995) have observed different reaction among the eleven cultivars belonging to the reticulatus and *inodorus* genotypes. The authors have found that C. melo var inodorus had a uniformly high regeneration rate whereas varieties exhibited reticulatus wide differences in their organogenesis. MOLINA & NUEZ (1995) have found genotypic variability of the *in vitro* answer in individual melon seeds. GALPERIN et al. (2003) has screened 30 different commercial melon cultivars for shoot de novo regeneration, but only one BU-21, had profuse regeneration of multiple shoots. The effect of the genotype on regeneration process in melon was demonstrated in current study, but other factors as explant types, hormonal regulation under *in vitro* condition enhance or limit melon regeneration.

Appropriate choice of the explant significantly influences the morphogenic ability. In melons, younger, smaller leaves and very young cotyledons were found to be most responsive (SOUZA et al., 2006). Differences observed in organogenesis in the both explant types may be due to also the influence of endogenous growth regulators and combination of these added in culture medium. For example, LESHEM (1989) showed shoot formation mainly proceeds from basal edge of the cotyledons and suggesting that this probably due to the accumulation of an endogenous growth regulator such as auxins. TORELLI et al. (2004) reported that the regeneration in hypocotyls tomato position-dependent of is and

associated with the movement and different concentration of hormones in the tissues.

The regeneration process of melon is limited by many factors. The main four factors that were studied Germination medium (Factor A), Regeneration medium (Factor B), Genotype (Factor C) and Expant type (Factor D) have proven influences on regeneration answer of melon (Table 1). Statistical data that effect of explant indicated type  $(\eta=32.65\%)$  dominated over other factors, followed bv Genotype (n=9.37%). Regeneration process strongly influenced also of interaction between Germination medium and Explant type ( $\eta$ =13.30%). Statistical significant differences were not demonstrated in interaction of factors B x D and B x C x D, which shows the important role of pretreatment on regeneration in melon and specifically requirement of the genotype established by other authors.



**Fig. 1.** Organogenic reaction in cotyledons and hypocotyls of line 11/9 in different culture medium variants. Values in columns followed by different letters are significantly different at P≤0.05 based on LSD Test (n=3).

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Fig. 2. Organogenic reaction in cotyledons and hypocotyls of AGY in different culture medium variants. Values in columns followed by different letters are significantly different at P≤0.05 based on LSD Test (n=3).

**Table 1.** Four-way analysis of variance and power of influence of variation factors on the regeneration depend on germination medium, regeneration medium, line and explant type. *Legend*: \* - P  $\leq$  0,05; \*\* - P  $\leq$  0,01; \*\*\* - P  $\leq$  0,001; n.s. – no significant.

Source	Type III Sum of Squares	df	Mean Square	Power of influence (ŋ%)	Sig.
A (Germination medium)	6269.531	3	2089.844	7.53	***
B (Regeneration medium)	916.146	2	458.073	1.10	**
C (Genotype)	7794.010	1	7794.010	9.37	***
D (Explant type)	27169.010	1	27169.010	32.65	***
A * B	2160.938	6	360.156	2.60	**
A * C	11065.365	3	3688.455	13.30	***
B * C	1109.896	2	554.948	1.33	**
A * B * C	5833.854	6	972.309	7.01	***
A * D	3840.365	3	1280.122	4.61	***
B * D	109.896	2	54.948	0.13	n.s.
A * B * D	2558.854	6	426.476	3.07	***
C * D	4469.010	1	4469.010	5.37	***
A * C * D	2690.365	3	896.788	3.23	***
B * C * D	178.646	2	89.323	0.21	n.s.
A * B * C * D	2990.104	6	498.351	3.59	***
Error	4062.500	48	84.635	4.88	
Total	83218.490	95			

There are a few studies of pretreatment with cytokinin BAP in germination stage on regeneration in melon. DO AMARAL et al. (2014) established a positive influence on the regeneration rate after adding of BAP in germination stage of recalcitrant cultivar 'Gaucho'. Histological analyses confirmed the development of more adventitious shoots when explants were cut of plants germinated of culture medium with low BAP concentration. Therefore, the addition of BAP in the culture medium during the phases of seed germination and regeneration must have affected the internal hormonal balance of the explants (WANG et al., 2011). In contrast, PINHO et al. (2010) in the same cultivar have achieved 29.66% regeneration frequency, even when the explants were removed from the plants grown on medium supplemented with BAP. According to KINTZIOS et al. (2002) the positive effect of pre-treatment before regeneration stage with growth regulators in embryogenesis of melon may be help cells to enter organogenic differentiation pathways.

Data presented in Table 2 shows that adding of BAP in germination medium stimulates regeneration answer of studied melon lines. However, increasing of BAP concentration during germination and levels this hormone higher of in regeneration medium leads to reduction of development. shoot The highest percentage of cotyledons and hypocotyls reacted with regeneration from line 11/9obtained combination was in of germination culture medium variants MS2 and regeneration medium variant 1 (100% and 50%, respectively), followed by MS0 + 1 (85% and 40%, respectively). In these combinations the number of regenerants per explant was high (1.65 and 1.50, respectively), but the highest regenerants was obtained in combination MS2 + 2 (1.75) (Fig. 3 a, b, c). The similar results were reported by DO AMARAL et al. (2014).

In line AGY regeneration process was registered only in cotyledons in eight of the twelve studied culture medium combinations. The highest value of regenerated explants was measured in combination of culture medium MS3 and 1 (75%), followed by MS0 + 2 (50%).

*In vitro* organogenesis was stimulated usually of combination of cytokinin and auxin in a certain ration depending on the genotype. Better regeneration answer is observed when explants were transferred on regeneration medium containing lower BAP (0.5 and 1.0 mg  $L^{-1}$ ) and IAA (0.5 mg L<sup>-1</sup>) concentrations. Higher levels of BAP decrease regeneration rate. Ficcadeni & Rotino (1995) have concluded that BAP was able to induce shoot formation, but the combination with Abscisic acid significantly increased the number of shoots per explant. According to Keng & (2005) addition of auxin in Hoong regeneration medium was not contributed multiple shoot formation, but was induced formation of friable callus. These results confirmed the data obtained by REN et al. (2013). On the other hand, the highest shoot formation was obtained from culture medium containing 0.5 mg  $L^{-1}$  BAP and 0.5 mg  $L^{-1}$  IAA (88%), while the lowest one – 1.0 mg L<sup>-1</sup> BAP (75%) alone or in combination with 0.1 mg  $L^{-1}$  IAA (60%) (MENDI et al., 2010a). The influence of combination of BAP with auxins (IAA, NAA, 2,4-D) on regeneration in melon was reported by many authors and the effect depends on genotype, explant type and additional culture conditions (MENDI et al., 2010b; ZHANG et al., 2011; IVANOVA et al., 2017). The regenerants from line 11/9 (20 plants) and line AGY (7 plants) were successful adapted and acclimatized (Fig. 3 d, f). Significant differences in e, morphology characters between obtained regenerants and initial lines were not observed. During the growth season, the plants were self-pollinated and seeds were received for subsequent studies.



**Fig. 3.** Regeneration of cotyledons (a), hypocotyls (b), plant-regenerants (c-d) and adapted and acclimatized plants (e-f).

**Table 2.** Regeneration frequency in cotyledon and hypocotyl explants of two melon lines. Values are means  $\pm$  standard deviation. Values in columns followed by different letters are significantly different at P≤0.05 based on LSD Test (n=3).

Lines			11/	9		AGY				
Mediur	n	Rege	eneration	Reg	g./exp.	Rege	neration	Re	g./exp.	
		%	±SD	No.	±SD	%	±SD	No.	±SD	
				Cotyle	dons					
MS0	1	85.0	$\pm 21.21^{ab}$	1.65	$\pm 0.35^{ab}$	15.0	$\pm 7.07^{\text{ef}}$	0.35	$\pm 0.07^{bc}$	
	2	60.0	$\pm 14.14^{abc}$	1.10	$\pm 0.14^{bc}$	50.0	$\pm 7.07^{bc}$	0.55	$\pm 0.14^{a}$	
	3	85.0	±21.21 <sup>ab</sup>	1.25	$\pm 0.49^{bc}$	40.0	$\pm 14.14^{cd}$	0.40	$\pm 0.14^{b}$	
MS1	1	15.0	±7.07 <sup>c</sup>	0.25	$\pm 0.07^{\rm e}$	30.0	$\pm 7.07^{de}$	0.30	$\pm 0.07^{bc}$	
	2	55.0	$\pm 7.07^{bc}$	0.35	$\pm 0.21^{de}$	15.0	$\pm 7.07^{\text{ef}}$	0.20	$\pm 0.07^{\circ}$	
	3	70.0	$\pm 14.14^{ab}$	0.90	$\pm 0.14^{cd}$	0.0	$\pm 0.00^{f}$	0.00	$\pm 0.00^{d}$	
MS2	1	100	$\pm 0.00^{a}$	1.50	$\pm 0.14^{b}$	0.0	$\pm 0.00^{f}$	0.00	$\pm 0.00^{d}$	
	2	55.0	$\pm 14.14^{bc}$	1.75	$\pm 0.35^{a}$	0.0	$\pm 0.00^{f}$	0.00	$\pm 0.00^{d}$	
	3	45.0	$\pm 21.21^{bc}$	0.85	$\pm 0.21^{cd}$	0.0	$\pm 0.00^{f}$	0.00	$\pm 0.00^{d}$	
MS3	1	70.0	$\pm 14.14^{b}$	1.00	$\pm 0.28^{cd}$	75.0	$\pm 14.14^{a}$	0.75	$\pm 0.07^{a}$	
	2	45.0	$\pm 7.07^{bc}$	0.25	$\pm 0.07^{\rm e}$	45.0	±7.07 <sup>cd</sup>	0.00	$\pm 0.00^{d}$	
	3	30.0	±14.14 <sup>c</sup>	0.40	$\pm 0.28^{de}$	65.0	$\pm 14.14^{ab}$	0.30	$\pm 0.07^{bc}$	

Hypocotyls											
MS0	1	40.0	$\pm 14.14^{ab}$	0.35	±0.07 <sup>b</sup>	0.0	0.00				
	2	0.0	$\pm 0.00^{d}$	0.00	$\pm 0.00^{\rm e}$	0.0	0.00				
	3	5.0	$\pm 0.00^{d}$	0.00	$\pm 0.00^{\rm e}$	0.0	0.00				
MS1	1	0.0	$\pm 0.00^{d}$	0.00	$\pm 0.00^{\rm e}$	0.0	0.00				
	2	0.0	$\pm 0.00^{d}$	0.00	$\pm 0.00^{\rm e}$	0.0	0.00				
	3	0.0	$\pm 0.00^{d}$	0.00	$\pm 0.00^{\rm e}$	0.0	0.00				
MS2	1	50.0	$\pm 0.00^{a}$	0.75	$\pm 0.07^{a}$	0.0	0.00				
	2	35.0	$\pm 7.07^{b}$	0.25	$\pm 0.07^{\circ}$	0.0	0.00				
	3	17.5	±3.54°	0.10	$\pm 0.00^{d}$	0.0	0.00				
MS3	1	0.0	$\pm 0.00^{d}$	0.00	$\pm 0.00^{\rm e}$	0.0	0.00				
	2	0.0	$\pm 0.00^{d}$	0.00	$\pm 0.00^{\rm e}$	0.0	0.00				
	3	0.0	$\pm 0.00^{d}$	0.00	$\pm 0.00^{\rm e}$	0.0	0.00				

# Conclusions

To develop an effective and appropriate regeneration system for melon is important to determine both composition of the germination and the regeneration media. The results of this experimental work demonstrated positive influence of pretreatment with 1 mg L-1 BAP on regeneration in cotyledon and hypocotyl explants of melon line 11/9. In line AGY the positive answer was observe only in cotyledons in the highest concentration of BAP. The effect of genotype is difficult to overcome, but combining of suitable explants with culture condition could significantly increase the regeneration frequency. Development of regeneration protocol applicable to a large number of genotypes leads to reducing time and efforts for creation of genetic diversity for breeding purposes. It is also important to conduct more studies of genetic control of the process and requirements of plant growth regulation in different phases of regeneration.

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# Physico-chemical, Agrochemical and Eco-chemical Characteristics of Biochar-treated Fluvisol

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**Abstract.** Recently there has been considerable interest in the use of biochar to improve soil quality and mitigate climate change. The aim of this study was to investigate the effect of two types of biochar on physico-chemical, agro-chemical and eco-chemical characteristics of a Fluvisol. The study site is the experimental field of Tsalapitsa village (Plovdiv). Wheat was cultivated with maize in crop rotation. The following treatments were included in the study: control with no biochar addition, biochar applied in 2 t.ha<sup>-1</sup> in 2016, produced by pyrolysis of rice straw; biochar applied in 3 t.ha<sup>-1</sup> in 2017, obtained by pyrolysis of oak barks at 500 °C. Biochar effect on the pH, EC, CEC and OM was negligible. The concentrations of the studied anions in water and 0.01M CaCl<sub>2</sub> extracts do not exceed the maximum permissible levels (MPCL) for drinking water except for nitrates in biochar (2017) in both extracts, where biochar from oak barks was applied. Dissolved organic carbon (DOC) content in water and 0.01M CaCl<sub>2</sub> decreased in variants with BC addition compared to the controls, most likely due to the ability of biochar to sorb DOC. Manganese and zinc in the studied soils are represented by free aqua-ions ( $M^{2+}$ ), especially high is the percentage of free manganese ions (96.3%), while for aluminium and iron, fulvic complexes are the predominant species, but no biochar effect on metal speciation was observed.

Key words: biochar, physico-chemical properties; soil solution composition, geochemical modeling, DOC.

#### Introduction

Intensification of agricultural production and anthropogenic impact are the cause for significant deterioration of soil fertility. The need to implement effective measures to improve soil quality and mitigate climate change requires the use of soil ameliorants (LAL, 2004). Recently there has been considerable interest in the use of biochar (MIKOVA, 2014; LEHMANN & JOSEPH 2009; OLIVEIRA *et al.*, 2017). Studies of (ATKINSON *et al.*, 2010; SUKARTONO *et al.*, 2011; LIU *et al.* 2012; OUYANG *et al.*, 2013; KHAN *et al.*, 2017, WU *et al.*, 2018) have shown that biochar increases water retention in soils, improves their physical properties, reduces leaching and export of nutrients, increases cation exchange capacity, carbon sequestration, immobilises soil pollutants, increases yields, reduces gas emissions in agriculture, etc.

Depending on the feedstock materials and the pyrolysis conditions, biochar acquires different physical and chemical characteristics that can modify the physical and chemical properties of soil. The large surface area of biochar increases ion exchange capacity and nutrient sorption (LEHMANN & JOSEPH, 2009). Application of

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg Union of Scientists in Bulgaria – Plovdiv University of Plovdiv Publishing House biochar modifies the composition of dissolved organic matter in soil (SMEBYE *et al.*, 2016) which directly or indirectly controls the mobility of metal ions and their bioaccumulation (LI *et al.*, 2018). All that makes biochar an efficient, cost-effective and environmentally friendly material for application and management of agroecosystems in the future (VERHEIJEN *et al.*, 2010).

There is insufficient information on the chemical processes which control the effect of biochar and its adsorption potential to retain and immobilize nutrients in soil solution, as well as the mechanisms of interaction with solution components, therefore development of research in this direction is necessary.

The aim of this study was to investigate the effect of two types of biochar on physico- chemical, agro-chemical and eco-chemical characteristics of a Fluvisol upon treatment with biochar.

# Materials and Methods

The study site is the experimental field of Tsalapitsa Village, Plovdiv District,  $(24^{\circ}35'E; 42^{\circ}14'N)$ , located 4 km off the left bank of the Maritsa River at an altitude of 160 m. The topography is flat, with a slight slope to the river  $(1 - 2^{\circ})$ . The soil type is Alluvial-meadow soil (Fluvisol). The surface A<sup>1</sup>p Horizon (0 - 20 cm) is brown (10YR5/3/) with sandy loam texture.

The soil samples from a field experiment with wheat "Sadovo 1" cultivar were analyzed. The  $N_{100} P_{120} K_{100}$  fertilization was applied: N in the form of urea -100 kg.ha<sup>-1</sup>, P as triple superphosphate -120 kg.ha<sup>-1</sup> and K as potassium sulphate -100 kg.ha<sup>-1</sup>. The P and K fertilizers were introduced prior to the main soil tillage in autumn and N fertilizer before the presowing treatment in spring (March). The biochar was applied before sowing of the The following treatments crop. were included in the study: 1) Control, with no biochar addition; 2) Biochar applied in 2 t.ha<sup>-1</sup> in 2016, produced by pyrolysis of rice

straw at 500 °C; 3) Biochar applied in 3 t.ha<sup>-1</sup> in 2017, obtained by pyrolysis of oak bark at 500 °C (pH = 7.9, CEC = 10.9 cmol.kg<sup>-1</sup>, C 29.8%). Pooled soil samples were taken to a depth at 0 - 10 cm and 10 - 20 cm, using a grid  $\Delta$  2 m, ~ 40 m<sup>2</sup> with a 3 cm wide and 25 cm long soil sampler. Soil samples were taken in three replications in spring. Lack of water repellency was noticed (water drop penetration time, WDPT was 1 - 5 s) according to the scale of DE BANO (1981).

Cation exchange capacity (CEC) was assessed as sum of titratable acidity (pH 8.2) and extractable Ca, by saturation with K malate at pH 8.2 (GANEV & ARSOVA, 1980). Electrical conductivity was determined in water (1: 5), ISO 11265 : 2002. Soil soil: pH/Eh were measured in a soil: water slurry of 1: 2.5. Total organic carbon (TOC) was determined by oxidation with  $K_2Cr_2O_7/$ H<sub>2</sub>SO<sub>4</sub> and fractionated TOC into humic organic carbon (HOC) and fulvic organic carbon (FOC) upon treatment with 0.1M Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> and 0.1M NaOH by the methods of KONONOVA (1966). Mineral nitrogen was assessed by the method of Kjeldahl (after PAGE et al., 1982) and available P and K by the method of IVANOV (1984).

Soluble heavy metals in soils were determined by the following methods: 0.01M  $CaCl_2$  extractable forms in soil: sollution ratio (1: 5) and shaking for 2 hours (VAN RANST *et al.*, 1999); water soluble forms in soil:water ratio 1: 5, by shaking for 1 hour, centrifuging and filtering (0.45 µm acetate cellulose filter), after KATOH *et al.*, (2012). Anions in the soil solution (Cl<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, phosphates, including dissolved organic carbon (DOC) were analysed with Spectroquant tests, Merck Millipore (PHARO 100). The ions speciation in soil solution was performed by Visual Minteq V. 3.1.

Statistical processing of the data was performed using One-way Anova from the statistical program Statgraphics Centurion 18.

# **Results and Discussion**

*Physicochemical characteristics* 

According to the physico-chemical analysis of the Fluvisol in the Tsalapitsa

experimental field, it can be classified as slightly acidic (pH 5.8 - 6.1), Table 1. The pH values of the control soils are very similar to those of the variants with biochar addition. The soil acidity in the biochar treated variants increased significantly  $(p \le 0.05)$  by 0.2 - 0.4 units in both layers. Similar results for increasing soil pH with biochar applied, produced by rice husks and mixed wood residues have been observed (LAIRD et al., 2010). The decrease of pH in the top layer of the soil compared to the deeper soil layer is also significant in all the variants and reaches 0.2 - 0.5 pH units, which is related to the long-term nitrogen fertilization. There is a low decrease of the electrical conductivity, ranging from 0.04 - 0.01 ms.cm<sup>-1</sup> in the upper soil layer of biochar treated soil compared to the control. In a field experiment, JONES et al, (2012) indicate that the electrical conductivity of the soil (46 to 43  $\mu$ S.cm<sup>-1</sup>) was not affected significantly after a 3-year addition of biochar. The level of the colloidal activity based on the cationexchange capacity (CEC 15.4 cmol.kg-1) in the control variants is defined as weak. There is a slight decrease of exchange capacity in the variants with biochar addition. Total acidity (full hydrolytic acidity at pH 8.2) is in the range of 4.0 - 4.7 cmol.kg-1 in the control variants while in the variants with biochar addition slightly but significantly decreases (exch.H 3.2 -3.6). The total carbon content in the studied soil ranges between 0.89 - 0.92% in controls without biochar (Table 2). It is characterized by low humus content (1.5%) of the humic type (Cx/Cf 2.80) according to the classification of Orlov and Grishina (ORLOV, 1985). The humic acids are 100% bound to Ca and Mg ions. Treating the soil with biochar does not significantly change the total carbon content. There is a slight decrease in organic carbon in biochar addition in depth. The humic type of humus has not changed (Ch/Cf > 2.0) in

biochar addition variants. At the same time in all variants the humic acids are bound with alkaline ions, which is a precondition for the more favorable hydro-physical properties of the soil.

The agrochemical analysis showed that the mineral nitrogen content in the control soils in both layers ranges between 10.9 - 21.9 mg.kg<sup>-1</sup> (Fig. 1). In the soil samples taken after soil treatment with biochar, mineral nitrogen content in the upper layer is higher than the control. Especially in variant BC<sub>2017</sub> in the layer 0-10 cm a significant amount of mineral nitrogen is formed. In the soil treated with BC<sub>2016</sub>, low nitrogen level is observed, indicating that the effect of the applied biochar is reduced over time. The data obtained for mobile phosphorus did not show large changes compared to the control variant. It can be seen (Fig. 1) that the applied biochar has an effect on the available K, increasing its soil storage by variants.

Anions contents in the soil solution

CHENG et al,. (2008); MAO et al., (2012); CUI et al., (2016); Li et al., (2017) found that, depending on the source materials used and the pyrolysis conditions of plant biomass, biochars with different surface chemical and functional groups are obtained, which specifically interact with ions in the soil solution. The content of Cl<sup>-</sup>anions in the soil solution varies within a narrow range of 7.5 - 24 mg.1<sup>-1</sup> (Fig. 2). It was found that in the variants treated with biochar the chloride concentration was lower than in the controls. It is known that chloride ions maintain nearly constant concentration in the arable soils which varies in narrow range. Chlorides are weakly adsorbed by the soil adsorption complex and have a transitional behavior in soil, which is assessed by the higher levels noted at the lower depths (Fig. 2). In a study conducted by NOVAIS et al. (2018) with contaminated wastewater, biochar was significantly less successful in adsorbing chloride and nitrate anions.

Variants	pH/H <sub>2</sub> O	EC	CEC	exch.H <sub>8,2</sub>	exch.Ca	exch.Mg	V
depth, cm		ms.cm <sup>-1</sup>		cmol.	%		
Control 0-10	5.8±0.1	0.09±0.02	15.4±0.2	4.7±0.2	9.0±0.1	1.8±0	69.7±1.0
Control 10-20	6.1±0.2	$0.06 \pm 0.02$	15.1±0.1	4.0±0.1	9.2±0.2	$1.9\pm0.1$	73.5±0.6
$BC_{2016}$ 0-10	6.2±0.1	$0.05 \pm 0.01$	$14.9 \pm 0.6$	3.2±0.1	9.2±0.1	$1.9\pm0.1$	78.3±0.4
$BC_{2016}$ 10-20	6.4±0	$0.04 \pm 0.01$	$14.8 \pm 0.1$	3.1±0.1	9.4±0.1	1.9±0	79.3±0.4
$BC_{2017}$ 0-10	6.0±0	$0.08 \pm 0.01$	$14.9 \pm 0.1$	3.6±0.1	9.0±0.1	$1.9\pm0.1$	76.3±0.4
BC <sub>2017</sub> 10-20	6.3±0.6	$0.06 \pm 0.01$	15.0±0.1	3.6±0.1	9.3±0.1	$1.9\pm0.1$	75.8±0
LSD p≤0.05	0.2	0.02	0.2	0.2	0.2	0.1	1.0

**Table 1.** Physico-chemical characteristics of the soil from the study site.

**Table 2.** Content and composition of soil organic matter in the control and biochar-treated soil. *Designation:* a - % of soil sample, b - % of total C, humic to fulvic acids ratio (Ch/Cf).

Soil sample, depth, cm	Total C %	SOM %	Organic C %			
			Extacted with 0.1M Na <sub>4</sub> P <sub>2</sub> O <sub>7</sub> + 0.1M NaOH			Ch/Cf
			Total	Humic acid	Fulvic acid	
Control 0-10	0.89	1.53	<u>0.19<sup>a</sup></u>	<u>0.14</u>	<u>0.05</u>	2.80
			21.35 <sup>b</sup>	15.73	5.62	
Control 10-20	0.92	1.58	<u>0.20</u>	<u>0.16</u>	<u>0.04</u>	4.00
			21.74	17.39	4.35	
BC <sub>2016</sub> 0-10	1.10	1.89	<u>0.21</u>	<u>0.16</u>	<u>0.05</u>	3.20
			19.09	0.21	4.55	
BC <sub>2016</sub> 10-20	0.82	1.41	<u>0.19</u>	<u>0.19</u>	0.00	-
			23.17	23.17		
BC <sub>2017</sub> 0-10	0.99	1.70	<u>0.21</u>	<u>0.18</u>	<u>0.03</u>	6.00
			21.21	18.18	3.03	
BC <sub>2017</sub> 10-20	0.89	1.53	<u>0.18</u>	0.14	<u>0.04</u>	3.50
			20.22	15.73	4.49	



**Fig. 1.** Content of mineral nitrogen (mg.kg<sup>-1</sup>), available phosphorus and potassium (mg.100<sup>-1</sup>g) in the Fluvisol.



**Fig. 2.** Content of chlorides, nitrates, sulphates and phosphates (mg.l<sup>-1</sup>) in biochar-treated Fluvisol.

The loss of nitrates through the soil profile and their reaching groundwater level, as well as eutrophication of surface waters is a major concern of anthropogenic soils and agricultural production. The application of significant amount of nitrogen and other biogenic elements is the for accumulation of residual reason mineral nitrogen in the soil profile after cultivation of crops, which is a potential source of deterioration of the quality of solid and liquid phase of soil (STOICHEVA et al., 2003; BORDOLOI et al., 2013; WANG et al., 2013; SIMEONOVA et al., 2017). The most vulnerable nitrate loadings to are territories where there is a combination of soils with light texture (such as the Alluvial-meadow soils, Fluvisols) and low water holding capacity, shallow aquifers and intensive farming (often vegetable production), leading to high levels of nitrates in the liquid phase, exceeding MPCL 50 mg.l<sup>-1</sup> as a the standard for drinking water. Many studies (LAIRD et al., 2010; KNOWLES et al., 2011; LIBUTTI et al.,

2016; BORCHARD et al., 2019) show that addition of biochar to arable soils can reduce nitrate and phosphate leaching. This could be explained with an increase in the anion exchange capacity of biochar, although the mechanisms are not yet well studied. Addition of biochar in soils also improves the biological fixation of nitrogen from nitrogen-fixing bacteria (RONDON et al., 2007). In other studies (YAO et al., 2012; HOLLISTER *et al.*, 2013; BORCHARD *et al.*, 2019) limited or no ability of biochar to retain nitrates from the soil solution was found.

soil solution ranges from 11.8 to 55.2 mg.l<sup>-1</sup> (Fig.2). The data indicate that the concentration of nitrates in solution in the variants with biochar is higher for the 0 -10 cm layer compared to the controls, while in the lower soil layers (10 - 20 cm) they decrease. The nitrate values do not exceed the MPCL (50 mg.l<sup>-1</sup>) in the standard for drinking water with one exception for the 0 - 10 cm layer of 55.2 mg.l<sup>-1</sup> in 2017 biochar-treated soil.

In our study, the nitrate content in the

The increase of solution nitrates in the  $BC_{2016}$  and  $BC_{2017}$  variants coincides with the higher levels of mineral nitrogen (Fig. 1) and might be due to the fertilizer applied.

It is known that phosphates can be prevented from leaching by chemisorption, as a result of which difficult or poorly soluble compounds are formed, in contrast to soluble nitrates. The phosphate content of the soil solution is low and the variation is slight from 2.05 to 4.7 mg.l<sup>-1</sup> (Fig. 2). LEHMANN & JOSEPH (2009) reported that soil treatment with biochar leads to higher capacity to sorb phosphorus compared to soil where only manure has been applied. According to a number of authors (CHINTALA et al., 2014; XU et al., 2014; TAKAYA et al., 2016; BORNØ et al., 2018) the sorption efficiency of biochar is determined by different mechanisms which control the phosphorus sorption capacity in soil, and hence the availability of P. As they themselves noticed, depending on the soil type and pH, the calcium and magnesium concentrations in the soil solution, the used plant material and other factors, sorption varies. In our study in the variants with BC<sub>2016</sub> addition the phosphate content in solution is slightly higher compared to BC<sub>2017</sub> variants in which lower than control concentrations were analyzed in the surface layers (Fig. 2).

For the sulphates, the trends are the following: the concentration in the solution ranges from 28.0 to 54.0 mg.l<sup>-1</sup> and higher concentration in the control variants are observed (Fig. 2). A decrease of sulphate content in both layers in the variants with biochar is found, confirming a sorption effect for the biochar.

Anion contents in the 0.01M  $CaCl_2$  soil extracts

The nitrate content in the  $0.01M \text{ CaCl}_2$ solution varies significantly (Fig. 3) in biochar variants (from 3.4 to 87.9 mg.l<sup>-1</sup>). In the control variants the variation is in narrower limits (17.7 - 26.8 mg.l<sup>-1</sup>). In all the variants a decrease in NO<sub>3</sub><sup>-1</sup> concentrations in the lower depth was found. In BC<sub>2017</sub> treated soil similarly to the H<sub>2</sub>O extracts, the concentration of nitrates is also higher in the 0 - 10 cm layer, exceeding the maximum permissible contaminant level of 50 mg.l<sup>-1</sup> for drinking mentioned water. As above. the introduction of nitrogen fertilizers in spring months possibly coincide with a period of drought, resulting in higher nitrogen content in the surface soil layer. For the sulfates smaller variation in the range of 41 - 52 mg.l<sup>-1</sup>, was observed and the concentration differences between the controls and the biochar variants are insignificant (Fig. 3). The data shows slightly higher sulphate content of the biochar variants which may be related with the effect of the chloride ion on displacing sulphate.

For the phosphates, lower values for the CaCl<sub>2</sub> extracts, than the H<sub>2</sub>O extracts were obtained, which might be due to the immobilizing effect of Ca ions on phosphate desorbed. In respect to the phosphate anions in the solution of 0.01M CaCl<sub>2</sub>, the highest content is found in the control for 0 - 10 cm soil layer - 6.5 mg.l<sup>-1</sup> and about four times lower content in the biochar variants (1.5 - 1.6 mg.l<sup>-1</sup>), which proves the effectiveness of biochar towards phosphate adsorption displayed in this extractant. Significant differences in the CEC and pH between the biochar-treated and the control variants were not observed, however studies of (KUZYAKOV et al., 2009; MAO et al., 2012) have shown that although the biochar is relatively stable, it can be partially mineralized by biological and chemical reactions in the soil, resulting in change of its surface characteristics and functional groups, making it more difficult to study the effects of adding it.

Dissolved organic carbon in water and 0.01M CaCl<sub>2</sub> extracts

The data show (Fig. 4) that in the control variants the concentrations of dissolved organic carbon (DOC) in water
and 0.01M CaCl<sub>2</sub> extracts (13.5 mg.l<sup>-1</sup> and 15.6 mg.l<sup>-1</sup>) are higher than those of biochar treated soil (5.2 - 12 mg.l<sup>-1</sup>). With respect to both extracts, the DOC concentration of the biochar variants is higher in CaCl<sub>2</sub> extraction, which can be attributed to the capacity of chloride to desorb specifically adsorbed organic ligands.

Studies of DUME et al., (2015) show that biochar produced by different raw materials at low or high temperatures leads to an increase in the dissolved organic matter in soil. When biochar is mixed in soil, it causes changes in soil properties (pH and EC) that affect the potential release of DOC, for instance the natural content of DOC from biochar will directly increase the total stock of organic matter in the soil. SMEBYE et al., (2016) found higher DOC values in soil mixtures (containing 10% biochar) than the sum of the dissolved organic carbon values in the soil and biochar. In our experiment the addition of biochar leads to a decrease in DOC in the soil solution. This is consistent with the study of (MUKHERJEE *et al.*, 2011), which established a decreasing in DOC in similar experiment using biochar, а suggesting that this was due to the ability of biochar to sorb DOC. Our results are also similar with studies of the (JONES et *al*, 2012), which added 0.25 and 50 t.ha<sup>-1</sup> biochar in three long field experiments and cultivated corn (in the first year) and grass (in the second and third). They found that biochar had no effect on the dissolved organic matter. On the other hand, biochar changes the composition of soil dissolved organic matter by adsorbing into its porous structures (KASOZI et al., 2010).

Metal speciation and geochemical modeling in 0.01 MCaCl<sub>2</sub>

Geochemical modelling *Visual Minteq* (Tables 3 and 4) was carried out on the speciation interactions in the soil solution of biochar-treated Fluvisol and the control soil without biochar addition.

In the surface layer (0 - 10 cm) of the control soil the following trends in the solution of 0.01M CaCl<sub>2</sub> were established. Aluminum cations are presented ~10% as aqua non-complexed free ions and other charged hydroxy-complexes, but the largest share (~76%) belonged to organic fulvic - aluminum complexes and hydroxyfulvic complexes at pH~ 6. Manganese and zinc are represented mainly by free uncomplexed ions (M<sup>2+</sup>), especially high is the percentage of free manganese ions (96.3%).

The following trends in variants with  $BC_{2016}$  addition are observed. The free and hydroxy complexes of Al are ~ 12.5%, 84% are fulvic complexes, for Mn - 98% are free aqua complexes, and for Zn 91% are free ions. In the  $BC_{2017}$  treated soil the share of free and hydroxy complexes of aluminium is increased, ~ 25%, ~70% are aluminium and hydroxy-complexes of aluminium. Manganese (98%) and zinc (93%) are represented as free ions.

Chemical speciation of aluminium in sub-surface layer indicated that 33.5% are free and hydroxy-complexes. For Zn and Mn, over 90% of the species in solutions are represented by the free aqua complexes which are most reactive and bioavailable.

Metal speciation (geochemical modeling) in  $H_2O$ 

Geochemical modeling of the ionic forms in the soil solution (water) was also performed. The obtained specific interactions are presented in the example in Table 4. When evaluating the speciation in water and 0.01M CaCl<sub>2</sub>, changes in the ratios between different ionic forms were outlined. Dissolved iron was found in water solution at concentrations of 0.125 -1.085 mg/L and the  $Fe^{3+}$  ionic forms are mainly represented by soluble fulvic complexes - 99% of the total forms. The percentage of Mn<sup>+2</sup> is slightly lower than in 0.01M CaCl<sub>2</sub> and a certain share (3.5%) has complexes in the gel phase of the organic matter.



**Fig. 3.** Concentration of nitrates, sulphates and phosphates (mg.l<sup>-1</sup>) in 0.01M CaCl<sub>2</sub> of biochar-treated Fluvisol.



**Fig. 4.** Dissolved organic carbon (mg.l<sup>-1</sup>) in water and 0.01M CaCl<sub>2</sub> extracts in controls and BC-treated soil by variants.

Table 3. Metal ions speciation in the surface layer (0-10 cm) of the studied soil in 0.01M CaCl<sub>2</sub>.

Compo- nent	Con	trol 0-10 cm	BC <sub>2</sub>	2016 <b>0-10</b> cm	BC <sub>2017</sub> 0-10 cm						
	% of		% of		% of						
	total	total Ion/complex/		Ion/complex/	total	Ion/complex/					
	concen-	oncen- form		concen- form		form					
	tration		tration		tration						
$Al^{+3}$	0.256	$Al^{+3}$	0.429	$Al^{+3}$	0.831	A1+3					
	1.534	AlOH <sup>+2</sup>	2.02	AlOH <sup>+2</sup>	3.89	AlOH <sup>+2</sup>					
	5.676	$Al(OH)_2^+$	6.458	$Al(OH)_2^+$	12.392	$Al(OH)_2^+$					
	2.046	Al(OH) <sub>3</sub> (aq)	2.21	Al(OH) <sub>3</sub> (aq)	4.235	$Al(OH)_3$ (aq)					
	1.116	Al(OH) <sub>4</sub>	1.27	Al(OH) <sub>4</sub>	2.437	$Al(OH)_4$					

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	13.22	$AlHPO_4^+$	3.639 AlHPO <sub>4</sub> <sup>+</sup>		6.386	$AlHPO_4^+$	
	57.401	/FA2AlOH(aq)	63.11	/FA2AlOH(aq)	52.261	/FA2AlOH(aq)	
	18.533	/FA2Al+(aq)	20.705	/FA2Al+(aq)	17.189	/FA2Al <sup>+</sup> (aq)	
$Mn^{+2}$	96.31	$Mn^{+2}$	97.575	$Mn^{+2}$	97.522	Mn <sup>+2</sup>	
	0.627	$MnCl^+$	0.524	MnCl <sup>+</sup>	0.521	MnCl <sup>+</sup>	
	2.073	MnSO4 (aq)	$4 (aq) 1.321 MnSO_4$		1.474	MnSO <sub>4</sub> (aq)	
$Zn^{+2}$	88.481	$Zn^{+2}$	90.566	$Zn^{+2}$	92.761	$Zn^{+2}$	
	1.661	ZnCl <sup>+</sup>	1.403	ZnCl <sup>+</sup>	1.431	ZnCl <sup>+</sup>	
	2.344	ZnSO <sub>4</sub> (aq)	0.059 ZnNO <sub>3</sub> <sup>+</sup>		1.725	ZnSO <sub>4</sub> (aq)	
	0.33	ZnHPO <sub>4</sub> (aq)	6.134	/FAZn⁺(aq)	0.063	ZnHPO <sub>4</sub> (aq)	
	6.83	/FAZn⁺(aq)	) 0.103 /FA2Zn(aq)		3.671	/FAZn⁺(aq)	
Ca <sup>+2</sup>	95.183	Ca <sup>+2</sup>	96.541	Ca <sup>+2</sup>	96.346	Ca <sup>+2</sup>	
	1.556	CaCl <sup>1+</sup>	1.303	CaCl <sup>+</sup>	1.294	CaCl <sup>+</sup>	
2.64		CaSO <sub>4</sub> (aq)	1.684	CaSO <sub>4</sub> (aq)	1.876	CaSO <sub>4</sub> (aq)	

Table 4. Metal ions speciation in the surface soil layer (0-10 cm) in water (BC<sub>2016</sub>).

BC <sub>2016</sub> 0-10 cm										
Compo-	% of total	Ion / complex/	Compo-	% of total	Ion / complex/					
nent	concentration	form	nent	concentration	form					
Fe <sup>+3</sup>	0.424	Fe(OH) <sup>2+</sup>	$Zn^{+2}$	70.891	$Zn^{+2}$					
	99.401	/FA2FeOH(aq)		0.986	/FA-Zn+2G(aq)					
$Mn^{+2}$	92.389	Mn <sup>+2</sup>		3.449	ZnSO <sub>4</sub> (aq)					
	3.653	MnSO <sub>4</sub> (aq)		24.05	/FAZn⁺(aq)					
	1.285	/FA-Mn+2G(aq)		0.231	/FA2Zn(aq)					
	2.228	/FAMn <sup>+</sup> (aq)	Ca <sup>2+</sup>	92.004	Ca <sup>2+</sup>					
A1+3	0.061	A1 <sup>+3</sup>		4.687	CaSO <sub>4</sub> (aq)					
	0.452	AlOH <sup>+2</sup>		1.279	/FA-Ca+2G(aq)					
	1.908	Al(OH) <sup>2+</sup>		1.763	/FACa <sup>+</sup> (aq)					
	0.72	Al(OH) <sub>3</sub> (aq)								
	0.375	Al(OH)4-								
	62.275	/FA2AlOH(aq)								
	31.007	/FA2Al <sup>+</sup> (aq)								
-										

With manganese and zinc there was a decrease in the proportion of aqua complexes and an increase in the fulvic complexes due to the fact that the higher ionic strength of the CaCl<sub>2</sub> solution caused some complexation with chlorides (Table 3). On the other hand, the competitive interactions of Mn and Zn with Ca for free ligands, lead to an increase in the proportion of free ions in solution with higher ionic strength of CaCl<sub>2</sub>.

No noticeable differences between control samples and biochar-treated soils in metal speciation in 0.01M CaCl<sub>2</sub> and water were found.

## Conclusions

The study shows that biochar effect on general soil properties pH, EC, CEC and OM was negligible, however effect of low doses of biochar on soil solution composition was observed. The chloride anions reduce their content in the soil solution at the variants with biochar compared to the control. The concentrations of nitrate anions in the soil solution are higher in the surface layer of the variants treated with biochar compared to the control and decreased in depth in all the studied variants. A significant nitrate variation in CaCl<sub>2</sub> extracts was established. The

concentrations of the studied anions in the water extract and in the 0.01M CaCl<sub>2</sub> do not exceed the maximum permissible levels MPCL for drinking water except for nitrates in BC<sub>2017</sub> in both extracts, where biochar from oak barks was applied. Sulfates have lower values in the variants with biochar compared to the controls, while for the phosphate anions there significant difference in their was no concentration in the solution compared to the controls. Dissolved organic carbon in water and 0.01M CaCl<sub>2</sub> decreased in variants with biochar compared to the controls, most likely due to the ability of biochar to sorb DOC. Manganese and zinc in the studied soils are represented by free aqua ions (M<sup>2+</sup>), especially high is the percentage of free manganese ions, while for aluminium and iron, fulvic complexes are the predominant species.

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# Accumulation of Heavy Metals in the Urban Soils of Natural Monument "Bunardzhik" (Plovdiv, Bulgaria)

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Abstract. Progressing urbanization makes soils structurally altered and that results in their degradation, so we aimed to analyze the content of some heavy metals in urban soils of the park zone of Natural monument "Bunardzhik", situated in the real center of the city of Plovdiv (Bulgaria). Soil samples were collected from the surface layers of urban soils located to the Western and Eastern park zones of the Bunardzhik Hill, according to transects starting from the main communication routes. Soil content of As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sr, V, U, Zn was determined by inductively coupled plasma-mass spectrometry (ICP-MS). The content of V, Mn, Cr, Cu, Zn and Pb is noticeably reduced in the Western park zone (p<0.05), leaving from the route and reaching the middle of the park zone. Urban soils from the Eastern park zone are more polluted and there no significant decreases in their content with increasing the distance from the main road artery have been found. All studied heavy metals and toxic elements could be arranged as follows: Mn (436 mg/kg) > Zn (81 mg/kg) > V (40 mg/kg) > Pb (39.7 mg/kg) > Sr (38.3 mg/kg) > Cu (31.3 mg/kg) > Cr (23.7 mg/kg) > Ni (22.0 mg/kg) > As (3.3 mg/kg) > U (1.9 mg/kg) > Cd (0.36 mg/kg) in the Western park zone and Mn (552 mg/kg) > Zn (84 mg/kg) > V (62 mg/kg) > Pb  $(52.3 \text{ mg/kg}) > Cr (36.3 \text{ mg/kg}) > Sr (33.7 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg}) > Ni (28.3 \text{ mg/kg}) > As (6.0 \text{ mg/kg}) > Cu (28.7 \text{ mg/kg$ mg/kg > U (3.1 mg/kg) > Se (1.6 mg/kg) > Cd (0.55 mg/kg) > Hg (0.23 mg/kg) in the Eastern park zone, respectively. The results obtained for the two studied park zones allow for their use for recreational purposes but some exceedance of the precautionary limits should be adressed.

Key words: urban soils, heavy metals, toxic elements, pollution.

#### Introduction

Heavy metals are natural constituents of the Earth's crust. Anthropogenic activities have drastically disturbed the biochemical and geochemical cycles of some heavy metals. Therefore, the assessment of the environmental risk due to soil pollution is of particular importance for agricultural

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg and urban areas, where heavy metals, which are potentially harmful to human health, persist in soils for a very long time.

Contamination of soil surface layers with heavy metals is mainly associated with urban areas which are intensively managed by humans (WALCZAK *et al.*, 2011). Heavy metals in urban soils may come from

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various human activities, such as industrial and energy production, construction, vehicle exhaust, waste disposal, as well as coal and fuel combustion (KOMAI, 1981; IKEDA & YODA, 1982; RITTER & RINEFIERD, 1983; CHON et al., 1995; WONG & MAK, 1997; MARTIN et al., 1998; LI et al., 2001). These activities send heavy metals into the air and the metals subsequently are deposited into urban soil as the metal containing dust falls. SAKAGAMI et al. (1982) reported that there was a close relationship between heavy metal concentrations in soils and those in the dust falls.

Human health in towns and cities is strongly dependent on the status of urban soils (SIMPSON, 1996). Strong compaction, contamination by wastes and atmospheric depositions, loss of organic matter, changes in soil reaction, structural degradation and infection by pathogenic microorganisms are only few of the many adverse processes that affect and modify the ecological functions of soils in urban areas (BULLOCK & GREGORY, 1991; JIM, 1993; BEYER et al., 1995; BLUM, 1998). Heavy metals in the soils can also generate airborne particles and dusts, which may affect the air environmental quality (CHEN et al., 1997; BANDHU et al., 2000; CYRYS et al., 2003; GRAY et al., 2003). In contrast to agricultural soils, urban soils, especially that in parks and residential areas which are not used for food crops, may also have a direct influence on public health since it can be easily transferred into human bodies (DE MIGUEL et al., 2007; MIELKE et al., 1999; MADRID et al., 2002). In particular, the ingestion of dust and soil has been widely regarded as one of the key pathways by which children are exposed to the heavy metals and metalloids from paint, leaded gasoline, vehicles and local industry (MEYER *et al.*, 1999; RASMUSSEN *et al.*, 2001).

Previous studies indicated that the extent of heavy metal pollution in urban areas varied across time (PFEIFFER *et al.*, 1991) and location (ALBASEL & COTTENIE, 1985), and that increased levels of heavy metals in urban soil was related to the

intensity of human activities and traffic volume (ZHENG et al., 2002). The investigation of soil heavy metal concentrations in parks and green areas in indicated Seville, Spain, that the concentrations of Pb, Zn and particularly Cu in the soil often exceeded the acceptable limits for residential, recreational and institutional sites (MADRID et al., 2002). The study of large parks in Stara Zagora (Bulgaria) reveals technogenic loads on soils with heavy metals on the bases of technogenic coefficients and coefficient of abnormality but without risks for ecosystems (PETKOV et al., 2010). Links between soil pollution with heavy metals and age of the park are determined also (LI et al., 2001; HOU et al., 2015; KACHOVA & ATANASSOVA, 2017). Recent research in some of the oldest urban parks of Sofia revealed that lead was imported to the soils mainly with dust particles (particulate matter) and accumulated in the surface layers of soil from urban parks (KACHOVA & ATANASSOVA, 2017). The data on the chemical variability in the total Cu and Cd concentrations confirmed that Cd and Pb are inferred as markers of anthropogenic pollution, Cu contents are attributed to litho(pedo)genic sources, while Zn is both of anthropogenic and litho(pedo)genic origin.

However, differences among cities including population density and industrial activities could have a large impact on the findings of individual studies. Moreover, little information is available on heavy metal concentrations in soils of urban parks located in older cities with large populations.

The aim of this study is to identify the soil environment in terms of the content of selected heavy metals, as well as to identify some of physical and chemical properties of urban soils of Natural monument "Bunardzhik" (Plovdiv, Bulgaria). Bv analysing the spatial distribution of heavy metal concentrations in relation to the correlations among these elements and distance from the routes, it may be possible to assess the contamination of the soils, the potential hazardous risk both for human health and for protected area.

# **Materials and Methods**

#### Study area and sampling sites description

Plovdiv (42°9'N, 24°45'E) is situated in south-central part of Bulgaria, in the southern part of the Plain of Plovdiv, on the two banks of the Maritsa River. It is the second-largest city after the capital Sofia with a population of over 347 000 inhabitants (NSI, 2018). Inside the city proper are six syenite hills (including Bunardzhik Hill), several industrial zones, densely populated central area, some moderately populated areas around it, wide network of busy streets and train tracks, big parks and other green yards. Air quality in Plovdiv is quite worsened due to the motor vehicle emissions and residential heating, then the industrial sector, which has a minor impact (ATANASSOV et al., 2006; PETROVA, 2011; PETROVA et al., 2013; 2014a; b; 2015; RIOSV, 2017).

The climate is temperate with mild influence from the Mediterranean Sea and a large temperature range between summers and winters. The average annual temperature is 12.3 °C with maximum in July (32.3 °C) and minimum in January (6.5 °C). The average relative humidity is 73%. It is highest in December (86%) and lowest in August (62%). The total precipitation is 540 mm - the wettest months of the year are May and June with an average precipitation of 66.2 mm, while the driest is August with an average of 31 mm. Gentle winds with speed of up to 1 m s-1 represent 95% of all winds during the year. The prevailing wind direction is from west, rarely from east. Mists are common in the cooler months, especially along the banks of the Maritsa River. On average there are 33 days with mist during the year (National Institute of Meteorology and Hydrology, 2018).

Bunardzhik Hill, also known as the Hill of the Liberators, has an outstanding value in the urban ecosystem as one of the major city-forming factors and it is: *i*) symbol of the city of Plovdiv; ii) basic element of his identity; *iii*) unique geomorphological entity; iiii) habitat of rare and protected plant species. Functionally, the territory of the hill is assigned to the green system of the city for wide public use according to the General Plan of the Territory of Plovdiv (2007) and the Program for development, maintenance and protection of urban green system (2013). In 1990 Bunardzhik Hill was declared for a Monument of garden-park art, and in 1995 the Ministry of Environment and Waters declared it as a Natural monument (Ordinance RD-466/22.12.1995, MOEW) according to Protected Areas Act (1996) in Bulgaria. The natural character of the territory (including ecosystems and the landscape as a whole) is significantly influenced by the human factor, with most of the habitats characterized by a low degree of naturalness (Management Plan of Natural Monument "Bunardzhik", 2017).

Bunardzhik Hill is situated in the real center of Plovdiv, Bulgaria, along the two of the main road arteries in Plovdiv (Ruski Blvd. and Vassil Aprilov Blvd.) (Fig. 1). Its territory covers an area of 22 ha and is divided to the four functional zones (Table 1) according to the Management Plan of Natural Monument "Bunardzhik" (2017).

## Soil sampling

As the park zone is the most influenced area, both from the traffic emissions and from anthropogenic pressure, this study focused on the park soils' investigation. Soil samples are taken in June 2017 from the Eastern and the Western park zones of the hill. These two park zones abut upon Ruski Blvd. and Vassil Aprilov Blvd., respectively, soil sampling was made along a so pollution gradient using the transect method (Fig. 1). Transects started from the road and were directed towards the hill in order to allow the effect of traffic on soil properties be assessed. Soil samples were collected at the 7.5 m, 25 m, and 50 m distance from the route (Table 2) on the

depth of 0-20 cm (ISO 10381). Each sample was formed by 5 subsamples. The topsoil horizon was chosen for the analysis because it undergoes the biggest impact of versatile origin – mechanical, physical, chemical and biological. The preparation of soil samples for analysis was carried out, according to ISO 11464.

# Soil analyses

Soil pН was measured using pHotoFlex Set, 2512000, WTW-Germany (ISO 10390), and the soil conductivity was measured using Multiset, F340, WTW-Germany (ISO 11265). Soil texture (pipette method by Wigner), humus content (by Turin) has been analyzed in the Laboratory of the Department of Agroecology and Evironmental Protection, Agricultural University of Plovdiv.

Total soil content of As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sr, V, U, Zn was determined by inductively coupled plasmamass spectrometry (ICP–MS) (Agilent 7700). ICP–MS system was calibrated with international standards. Quality control was performed using the standard reference plant material (NCS DC73348): the percent recovery ranged from 91 to 99%, depending on each analyzed element. The extraction of trace elements and analyzes have been made in the Laboratory of Analitical chemistry and Computer chemistry of the University of Plovdiv "Paisii Hilendarski".

## Statistical evaluation

Raw data from all analyses were processed using statistical software package Statistica 7.0. for Windows (Stat Soft Inc., 2004). Multi ANOVA (MANOVA) and Student/Fisher test were used for testing the differences of elemental concentrations, both between the soils samples from different road distance and also between the studied sampling sites (p<0.05). Relationships between the contents of individual elements in collected soil samples were tested using Pearson correlation coefficients (p<0.05). A cluster analysis was used for grouping the studied chemical elements on the basis of their content in studied urban soils from both park zones (Complete linkage, 1-Pearson r).

# **Results and Discussion**

General characteristic of the soils in the studied park zones

Urban soils are composed largely of a mixture of materials differing from those in adjacent agricultural and forest areas, and transformed deeply by human activity through mixing, importing and exporting material, and by contamination (CRAUL, 1992). Thus human activities modify urban soils thereby setting off anthropogenic lithogenesis and pedogenesis, particularly by imposing very rapid transformation cycles (BLUME, 1996; DE KIMPE & MOREL, 2000).

Based on their genesis, the soils in the Plovdiv area are classified as Fluvisol according to the FAO World Reference Base for Soil Resources (2014). Due to the prolonged human presence on the studied territory (more than 8000 years) and the increasing rate of urbanization in the last decades, soils properties are significantly influenced and now it is more appropriate to discuss them as Technosols and Anthrosol.

Soils in the low and relatively flattened peripheral zones of the Bunardzhik Hill are heterogeneous. They typically have a profile that is disturbed as a result of constructions, ameliorative and reinforcing activities. The terrain surface is typically artificially modeled in terms of alignment, inclination and exposure so as to form a kind of landscape for public service (Fig. 2).

Physical and chemical characteristics of studied urban soils from two park zones of the Natural Monument "Bunardzhik"

The pH values of the studied soil samples from the surface layer (0-20 cm) varied between 6.22 and 6.98 (from light acidic to neutral) in the Western park zone and between 5.87 and 6.22 (from medium acidic to light acidic) in the Eastern park zone (Table 3). Urban soils from the parks of the city of Sofia also showed slightly acidic to neutral reaction (KACHOVA & ATANASSOVA, 2017). DOICHINOVA & ZHIYANSKI (2013) reported similar pH values of urban soils from park zone in Sandanski (Bulgaria). They confirmed that pH intopsoil horizon was greater and tended to decrease in deeper layers which was found as a diagnostic feature for urbanized soils by GENCHEVA (2000). Studies in different environments (agricultural, urban and transition land-use zones) demonstrate that in the acid soils, heavy metals are more mobile than in the alkaline one, and the alkaline environment better sustains metals (KAZLAUSKAITĖ-JADZEVIČĖA et al., 2014).

In an urban environment soil organic matter (SOM) has manifold functions and it is with considerable ecological significance. In the present study, we found that soil organic matter varied between 1.55% (WPZ-50) and 3.41% (EPZ-7.5) (Table 3). So, the studied soils could be characterized as relatively fertile and able to meet the needs of plants. Higher values were measured close to the road and tended to decrease to the inner of two park zones (p<0.05). Urban soils of studied parks in Sofia were characterized by significantly higher soil organic matter content - from 5.4% to 8.3% (KACHOVA & ATANASSOVA, 2017), but our findings were close to data from Doichinova & Zhiyanski (2013) concerning the SOM content of urban soils from park zone in Sandanski (Bulgaria). As many authors have shown, soil organic matter may be affected by mixing of carbonaceous material like debris, ash, slag, garbage and sewage sludge, as well as from deposition of airborne particles (SCHLEUSS et al., 1998; BEYER et al., 2001). Therefore, the transformed chemistry and structure of soils may lead to serious disturbances in their biological activity and impoverishment of vegetation cover (MIRELES et al., 2012; HU et al., 2013).

Soil texture of urban soils from Bunardzhik Hill varied from light sandy to sandy loam (Table 3). The light soil texture respectively, with the higher sand content, could be related to a potentially higher soil vulnerability to processes of degradation in the presence of pollution, which is characteristic of the urban environment (WU, 2014; MAO *et al.*, 2014). We found that the soils from the Western park zone were with smaller clay fraction than soils from the Eastern park zone (p<0.05) (Table 3). As the both studied park soils originated from the same rocks, the variations in the soil texture could be due to the different type of evolution processes, managed by different exposure.

Content of heavy metals and toxic elements in the studied park soils from Natural Monument "Bunardzhik"

In urban areas, a wide range of substances being produced through combustion of fuels, industrial processes, road de-icing, and abrasion of vehicle exploitation materials (mainly tyres) are emitted to the environment. These are toxic gases and dust enriched with heavy metals. Such contamination undergoes deposition and penetrates into soils, damaging the main soil properties (SHANG *et al.*, 2012; MCBRIDE *et al.*, 2014).

The highest content of all the studied heavy metals and toxic elements was found in the sampling plots, situated along the route arteries – at 7.5 meters (Fig. 3 and Fig. 4). This content differed significantly with the concentrations of the same elements measured at the distance of 25 meters and 50 meters (p<0.05). So, the traffic inputs could be considered as the leading factor for soil contamination in the periphery of park zones. Our previous studies in Plovdiv revealed that the content of some trace elements in soils along the road network was in direct relationships both with the distance from road and the road location itself according to the wind rose (PETROVA et al., 2018). This situation reflected also on the soil microbial community structure, leading not only to the damages in the physico-chemical properties of soils but to the decrease into their quality and functions.

As a whole, park soils in the east side of Natural Monument "Bunardzhik" are more

Accumulation of heavy metals in the urban soils of Natural monument "Bunardzhik"...

contaminated then the park soils from the west side. Exceptions were found only for Cu and Sr (Fig. 3 and Fig 4). Heavy metals (mainly Cu, Pb, Zn, Cd, Ni, V) are derived by many anthropogenic processes and activities, such as domestic activities, residential heating, petrol and diesel vehicles (JOHNSON et al., 2011). Copper (Cu) is yet another lithogenous trace element found in soil, but the increased levels of it, as they are observed in the topsoil, can be put down on anthropogenic activity. is usually linked to Strontium (Sr) carbonaceous soil material of large scale broken rock, and so in this case, as its

content do not vary very much (38 mg/kg average in soils from Western park zone and 34 mg/kg average in those from Eastern park zone). Soil content of Hg and Se in the Western park zone is under the detection limit of 0.1 mg/kg but in soils from the other park these elements are more abundant (p<0.001) (Fig. 3 and Fig. 4). Compared to data from park zones in Sofia, the studied urban soils from Plovdiv (Bunardzhik Hill) have about 40% lower content of Cu, but are significantly more polluted with Pb (up to 3 fold), Cd (up to 2 fold) and Zn (up to 2 fold) (KACHOVA & ATANASSOVA, 2017).

Table 1. Functional zoning of the Bunardzhik Hill's area.

Zone	Area, ha	%	Functional purpose
Park zone (Zone 1)	4.55	20.5	Recreation, public services, sports and cultural events
Equat park zona			Recreation in semi-natural (forest-park) natural
(Zono 2)	10.81	49	environment, conservation of plant and animal species
(Zone 2)			and typical landscapes
High zone	E (1	25 F	Maintaining natural vegetation and endangered, rare
(Žone 3)	5.61	23.5	and endemic species
Memorial zone	1.07	ΕO	Dressention of the memoriale
(Zone 4)	1.07	5.0	Preservation of the memorials
Total:	22.04	100	-



Fig. 1. Functional zoning of Natural Monument "Bunardzhik" and sampling plots location.

# Table 2. Sampling plots description.

Park zone	Along Boulevard	Distance from the road	Soil sample symbol
Western park zone		7.5 m	WPZ-7.5
	Vassil Aprilov	25 m	WPZ-25
		50 m	WPZ-50
Eastern park zone		7.5 m	EPZ-7.5
	Ruski	25 m	EPZ-25
		50 m	EPZ-50



Fig. 2. Landscape in Western park zone (A) and Eastern park zone (B).

Table 3. Physical and chemical characteristics of studied urban soils from two park zones.

Soil sample	pН	Organic C, %	SOM, %	Soil texture
WPZ-7.5	6.41	1.75	3.02	
WPZ-25	6.98	1.35	2.33	Sandy soil
WPZ-50	6.22	0.90	1.55	•
EPZ-7.5	5.87	1.98	3.41	
EPZ-25	5.90	1.81	3.11	Sandy loam soil
EPZ-50	6.22	1.28	2.21	-



**Fig 2.** Content of heavy metals and toxic elements in soils from Western park zone of Natural Monument "Bunardzhik" (mg/kg, logarithmic scale).

Accumulation of heavy metals in the urban soils of Natural monument "Bunardzhik"...



**Fig 3.** Content of heavy metals and toxic elements in soils from Eastern park zone of Natural Monument "Bunardzhik" (mg/kg, logarithmic scale).



Fig 4. Comparison of our results with the precautionary limits (Regulation Norm №3/2008) about Cd, Pb and Zn content in soils of Natural Monument "Bunardzhik".

A comparison has been made with the national precautionary limits and threshold values accepted in Bulgarian standards for soils from public areas (Regulation Norm No.3, 2008). Although our results are quite below the maximal limits some problems were found with the precautionary values (Fig. 4). Lead in soils from the Eastern park zone exceeds these values up to 170% at distance of 7.5 m, and to a lesser extend at 25 m and 50 m, while in soils from Western park zone an increment of 155% have been found only at 7.5 m distance from road (p<0.05). Cadmium content in Western park zone is below the limits but the closest sampling plot

to the road in Eastern park zone was 125% contaminated up to above the precautionary values (p<0.05). Zink content in both sampling plots at 7.5 m distance was found to be 1% below the precautionary limits so some attention should be paid. Our findings are in good agreement with the statement of KACHOVA & ATANASSOVA (2017) that Cd and Pb are inferred as markers of pollution, variables. Anthropogenic Cu contents are attributed to litho(pedo)genic sources, while Zn is both of anthropogenic and litho(pedo)genic origin.

These results give us a reason to assume that in the area of our research, these

elements have a common origin, namely as waste products from road transport. This includes both exhausted gases and waste products from brake systems, tire wear, etc. A number of publications indicate that Fe is emitted from vehicle braking systems, Pb and Cd - from the exhaust gases, V from different metal composites (MAHER et al., 2008). Cr and are considered indicative of vehicle V emissions (GARTY et al., 1996) and partly associated with tire and brake abrasion, as we have shown in our previous studies (PETROVA, 2011; PETROVA et al., 2013; PETROVA *et al.*, 2014a, b; PETROVA *et al.*, 2015). So, the significant correlation with the elevated content of other studied elements in soil samples from 7.5 m distance from road lets to conclude that the traffic is the main emitter of toxic substances in the central city part of Plovdiv.

The results achieved in the cluster analysis of soil contamination showed some different relationships between heavy metals and toxic elements in the two investigated park zones (Fig. 5). Two main groups have been formed in the Western park zone, first of which included 10 of studied elements, and the second - only two (Mn and Sr) (Fig. 5A). According to BURAU & ZASOSKI (2002), it is possible to explain a stronger relationship between lithogenous elements as strontium (Sr) and manganese (Mn). When regarding the other elements, the strongest accumulation associations were formed by As-Cr-Cu, Cd-Pb-Zn and Fe-Ni-V, among which a strong linear interdependence is observed and it could be related to the pollution levels in the city. The weakest genetic links of these metals connect them with uranium (U) that is found in their cluster. Two main groups were observed in the cluster analysis of soils from Eastern park zone too (Fig. 5B), but they significantly differ from data obtained by the Western park zone. Similar accumulation tendency was found only for associations Cd-Pb-Zn and Fe-Ni (p<0.05).



**Fig 5.** Cluster analysis of heavy metals and toxic elements in soils from Western park zone (A) and Eastern park zone (B) of Natural Monument "Bunardzhik".

Our previous studies have shown significant differences in the trace elements content both in some herbs (PETROVA *et al.*, 2013, 2014a) and in the leaves of some ornamental trees, growing in these two park zones (PETROVA *et al.*, 2014b). For example, average Pb content in tree leaves from the

Western park zone was 6.7 mg/kg while in the Eastern park zone was 8.9 mg/kg, average content of Cu was 9.1 mg/kg and 12.4 mg/kg, respectively, while average content of Ni was 2.2 mg/kg and 3.0 mg/kg, respectively (PETROVA *et al.*, 2014b). Thereby, some activities in collecting and removing the fallen autumn leaves in park zones could be recommended as a practical measure for limiting the entry of pollutants in urban soils by the decomposition of organic matter.

#### Conclusions

The studied soils from the Eastern and Western park areas of the Natural monument "Bunardzhik" have different characteristics that are probably due to the complex impact of natural and anthropogenic factors. In the soils from the western side of the hill, the content of vanadium, manganese, chromium, copper, zinc and lead is noticeably reduced, leaving from Vassil Aprilov Blvd. and reaching the middle of the park zone. In the soils on the eastern side of the hill, as a whole, higher amounts of the majority of the studied elements are reported, and there is a lack of significant decreases in their content, increasing the distance from the main road artery - Ruski Blvd.

Based on the results obtained, all studied heavy metals and toxic elements could be arranged as follows: Mn (436 mg/kg) > Zn (81 mg/kg) > V (40 mg/kg) > Pb (39.7 mg/kg) > Sr (38.3 mg/kg) > Cu (31.3 mg/kg) > Cr (23.7 mg/kg) > Ni (22.0 mg/kg) > As (3.3 mg/kg) > U (1.9 mg/kg) > Cd (0.36 mg/kg) in the Western park zone and Mn (552 mg/kg) > Zn (84 mg/kg) > V (62 mg/kg) > Pb (52.3 mg/kg) > Cr (36.3 mg/kg) > Sr (33.7 mg/kg) > Cu (28.7 mg/kg) > Ni (28.3 mg/kg) > As (6.0 mg/ kg) > U (3.1 mg/kg) > Se (1.6 mg/kg) > Cd (0.55 mg/kg) > Hg (0.23 mg/kg) in the Eastern park zone, respectively.

The comparison with the normative base showed that the precautionary limits of zinc, cadmium and lead in the roadside sections of the Eastern and Western Park zone and of lead in the middle part of the Eastern park zone of Natural monument "Bunardzhik" were exceeded. The main source of these pollutants is urban traffic, and a possible reason for the higher concentrations in the Eastern park zone is the lack of wind, dust recirculation, canyon street effect, etc.

Elevated concentrations of heavy metals in the topsoil negatively affect soil biota and plant biodiversity and conditions are created for disturbances in mineral nutrition, changes of metabolic and physiological processes in green plants. The deteriorated status of green systems directly reflects on the quality of the city environment, and especially of the protected area Natural monument "Bunardzhik". Some adequate measure for future prevention of soil pollution should be implemented, i.e. removing of the fallen tree leaves in the autumn season, traffic optimization and restriction, etc.

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# Association Between the Environmental Parameters and Biological Indicators in Reservoirs with Intensive Net-Cage Aquaculture: A Case Study in Kardzhali Reservoir, Bulgaria

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Abstract. The present study analyzed the interactions between the environmental parameters, microbiological water quality indicators, and phytoplankton in Kardzhali reservoir, which is one of the largest and economically significant water bodies in Bulgaria, with highly developed cageaquaculture. Data sets of eighteen parameters from 5 monitoring sites during 2016-2018 were used for analysis. We have applied multivariate methods, aiming to identify the key parameters affecting the communities, including the impact of the net-cage farms. The ANOSIM (analysis of similarities), showed significant differences in the values of physicochemical factors between the control site and the area for aquaculture, with higher nitrate, total nitrogen, and COD (chemical oxygen demand) content near the net-cages. The results were confirmed by the high R-value (R=0.87; p<0.01). The conducted PCA (principal component analysis) showed that only three principal components (PCs) are need to group the physicochemical parameters, explaining 90.5% of the total data variation. PC1 was formed by nitrogen forms and COD, PC2 represents the physical source of the variability (pH and dissolved oxygen) and PC3 was loaded with total phosphorus (0.537) and ammonium nitrogen (0.764) concertation. The parameters with the highest impact on the abundance of heterotrophic bacteria (TVC) include temperature, TN, and COD, while the phytoplankton community was negatively correlated with Secchi depth and COD. The redundancy analysis confirmed that the location of the sampling station significantly affects the studied variables and that net-cage aquaculture is a major anthropogenic factor in Kardzhali reservoir.

**Key words:** Microbiological indicators, environmental factors, PCA, RDA, phytoplankton, Kardzhali Reservoir.

#### Introduction

Freshwater resources are an important component of the biosphere, ensuring the sustenance of aquatic beings (JACKSON *et al.*, 2001). Worldwide they constitute only 3% of the total water resources. The latest Eurostat information on water statistics shows that a significant proportion of freshwater in Bulgaria

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg is received as an external surface water inflow. In fact, Bulgaria had the highest volume of water received (85.1 billion m<sup>3</sup>) among the EU Member States, forming 84% of the total available annual water resources (Eurostat, 2017). This makes ensuring the best water quality of lakes, rivers, and reservoirs a sensitive issue and a great environmental concern.

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The economically significant reservoirs in Bulgaria are multipurpose water bodies. They are often used for irrigation, power production, aquaculture, recreational activities, etc. The agricultural, urban and industrial activities are considered to be major factors for eutrophication as a result of pollution with excess nutrients and toxic chemicals (ISCEN et al., 2008; LENART-BORON et al., 2016). The large Bulgarian reservoirs are often used for intensive aquaculture production. The accession of the country to the European Union in 2006, lead to an increase of the aquaculture production and construction of new farms (HADJINIKOLOVA, 2010). These processes can result in significant changes in major environmental factors. Such changes in the trophic state of the water bodies can lead to a number of problems such as algal blooms, anoxia, fish mortalities and reduction of the biodiversity (OUYANG et al., 2007). More these waters support microbiological so pathogenic communities including microorganisms (HONG et al., 2009). They are an indispensable component of the nutrient cycles and they are extremely sensitive to changes in the physicochemical parameters of the environment.

The effective program for the prevention of water pollution is based on the physical, chemical and biological parameters in order to sustain a balanced ecosystem (ISCEN et al., 2008; MISHRA, 2010). One of the major problems in the analysis of surface water quality has always been the complexity of the system itself and the uncertainty what environmental parameters to measure. This makes it harder to establish suitable indicators (YU et al., 1998). The data sets usually contain vast information regarding the studied environmental indicators and the classification of the data is crucial for the correct interpretation of the results. The problem can be approached through the application of multivariate statistical analysis cited in a number of papers (ISCEN et al., 2008; MISHRA, 2010; GU et al., 2016; LENART-BORON et al., 2016).

We have conducted the present study in the Kardzhali reservoir which is one of the oldest in Bulgaria. It has been an object of a number of previous papers focused mainly on the composition of the phytoplankton (BESHKOVA *et al.*, 2008; DOCHIN & STOYNEVA, 2014) and the physicochemical properties of the waters (TRAYKOV, 2005; ILIEV, 2015). However, there is no sufficient data concerning the lasting effect of the intensive net-cage aquaculture on ecosystem stability. The object of the present study was to assess the connection between the environmental factors and main biological indicators and to identify the key parameters affecting the communities in the reservoir.

#### Materials and Methods

Site description and sampling

The research was carried out in the aquatory of the Kardzhali reservoir for the period June 2016 - October 2018. The reservoir is situated in the central part of Southern Bulgaria. It was designed as a heavily modified water body and was classified as a large, deep reservoir (L11) according to the national classification lakes and reservoirs of (Regulation H-4/2012 on characterization of surface waters. Ministry of Environment and Water, Bulgaria. State Gazette, 22). Kardzhali reservoir is fed by the Arda River and has one outlet, where it drains into the same river. It is used for intensive aquaculture for over 30 years. At present 6 net-cage farms operate in the aquatory of the reservoir, used primarily for sturgeon farming and are concentrated in the area of 300 ha with allowed capacity 2610 t directorate, (East aegean river basin Fishfarming report, 2016).

Sampling was conducted at five sampling stations monthly from April to October each year, with the exception of 2016 (the study started in June), in order to assess the period of active feeding in the aquaculture farms and the active tourist season. The stations include one control site and four net-cage farms (NC), situated in the aquatory of the reservoir as follows: Control site (41.648541 N, 25.291899 E), NC1 (41.641229 N, 25.310090 E); NC2 (41.642802 N, 25.314325 E); NC3(41.639326 N, 25.319163 E); NC4(41.641198 N, 25.320919 E). The control site was situated upstream from the area for aquaculture in the open aquatory

equidistant from the net-cages and the riverine part of the reservoir, while the NC-sites were positioned in close vicinity of the four largest net-cage farms.

Water samples with an analytical volume of 500 ml for each site for microbiological analysis were collected with MICROS water sampler (Hydro-Bios Apparatebau GmbH, Germany) from the superficial water layer (0.5-1.0 m). The samples were kept in the dark at 4°C until analysis for no longer than 24 h (ISO 5667-1, ISO 5667-2, ISO 5667-3.). For the phytoplankton and physicochemical analysis mixed water, samples with an analytical volume of one liter were collected with Niskin-Type water sampler (Hydro-Bios Apparatebau GmbH, Germany) from the water column at each station. The sampling depths were calculated based on the Secchi depth. The samples were processed by the standard method of fixation with formalin to final concentration 4% and further sedimentation 5667-1:2006/AC:2007; (ISO ISO 5667-3:2003/AC:2007). For the physicochemical analysis, samples were stored in dark (4°C) until laboratory processing no longer than 24 h. Ninety-five samples for each of the analyzed parameters were collected in the period of study.

## Physicochemical analysis

Temperature and dissolved oxygen were measured in situ with WTW oxygen meter (OXY 1970i). Transparency was established by the Secchi disk method. The water pH was measured in the laboratory using WTW model pH-meter (315/SET). Chemical analyses, including Manganese III COD, ammonium (NH4-N) and nitrate (NO3-N) nitrogen, nitrite (NO2-N) nitrogen, and total nitrogen, were performed using standard analytical methods (ISO 8467:1993; ISO 5664:1984; ISO 26777:1984; ISO 7890-1:1986). Total phosphorus concentration (TP) was determined by Phosphate Cell Test (114543, Merck Millipore) on Spectroquant Nova 400 photometer (Merck Millipore). Chlorophyll-a concentration was determined spectrophotometrically (ISO 10260:1992).

#### Microbiological analysis

Microbiological indicators were analyzed by standard analytical procedures for waters. The enumeration of culturable microorganisms (total viable count) was conducted by colony count after inoculation in a nutrient agar culture medium (ISO 6222:2002). *E. coli* and fecal coliforms (FC) were enumerated by membrane filtration method for waters (ISO 9308-1:2014) after supplementation of the nutrient medium with novobiocin (5 mg.L<sup>-1</sup>) for the suppression of the bacterial background flora. Detection and enumeration of intestinal enterococci (FS) were done according to ISO 7899-2:2000.

# Phytoplankton analysis

The species composition was determined by light microscopy on Axioscope 2 plus (Carl Zeiss) with magnification 200x and 400x using standard taxonomic literature with the critical use of AlgaeBase (GUIRY & GUIRY, 2018). Diatoms were identified according to the methodology of COX (1996). The main counting unit was the cell and biomass was estimated by the method of stereometric approximations (ROTT, 1981, DEISINGER, 1984). The counting was carried out individually (cell, filament or colony). Assessed by the total biomass of each sample, defined as the amount of biomass of all species, summarized in separate taxonomic groups.

## **Statistics**

Statistical analyses were completed using Primer 6 (Primer-E, Ltd) and Microsoft Excel with the XLSTAT software package (Addinsoft). All parameters were standardized prior to the analysis. Principle Component Analysis (PCA) was used to detect environmental parameters variations between samples. Permutational multivariate analysis of variance (perMANOVA) and analysis of similarities (ANOSIM) was used to test spatial and temporal variations of physicochemical biological parameters. Redundancv and analysis applied to establish the was relationships between biological communities, and environmental factors. The plots were

constructed to visualize the similarity in the biological communities between the different sites. Correlation analysis (Pearson) was used to analyze the correlation between parameters. The significance of all statistical tests was assessed at  $\alpha = 0.05$ .

#### **Results and Discussion**

The average values for the analyzed environmental parameters are presented in Table 1. The results indicate seasonal changes in the water status with the highest water temperature, transparency and nutrient (NO3-N, TN, and COD) loadings in summer and lowest in the spring period. The ANOSIM, based on the physicochemical parameters, categorized the control site (Ctr) and the netcage area (NC) into two distinct groups, which were confirmed by the high R-value (R=0.87; p<0.01). Both, sampling year, the inter-annual sampling and site location affected the rate of change in water quality (p < 0.05). Permutational MANOVA design test was applied to further evaluate the spatial and temporal variations and to confirm the differences between the Ctr and NC sites. It substantiated the significant seasonal and spatial variation of the parameters established by the ANOSIM. After 5 permutations of random subsampling each the control site was consistently found to be significantly distinct from NC4 and NC5. In general, there were no differences in water temperature (F = 1.0287; p = 0.612), transparency (F = 2.0865; p = 0.136), pH (F = 0.699; p = 0.502) and phosphorus concentration (F = 0.3651; p = 0.6961). However, the nitrate (F = 6.0623; p = 0.0045), nitrite (F = 72.6801; p = 0.0000), total nitrogen (F = 14.8769; p = 0.00001), and COD (F = 20.1018; p =0.00000) content were higher around the netcages compared to the control station, and varied significantly between the three years of study (p > 0.001). A Principal Component Analysis (PCA) was used to reveal the association between different variables and to establish a new basis of factors. The initial set of 11 parameters was reduced to a smaller number of factors summarizing the correlating factors and separating the non-correlating parameters. In the present study, a scree plot is used to detect the number of principal components (Fig. 1A). It shows a pronounced change of slope after the third eigenvalue, so only three principal components (PCs) are need to group the water samples, explaining 90.5% of the total data variation. The adequacy of the model was confirmed by Kaiser-Meyer-Olkin and Bartlett tests. The factor biplot is shown in Fig. 1B. The quantitative analysis of the microbiological indicators for water quality and

the microbiological indicators for water quality and phytoplankton communities revealed seasonal and annual variation patterns following the variation in the environmental parameters. The total number of heterotrophic microorganisms was used as an indicator of the trophic status of a body of water. The spatial analysis demonstrated evident variation between the control site and the net-cage areas (ANOSIM R=0.325, p=0.003), with significant elevation in near the largest farms NC4 and NC5 (Fig. 2A). The abundance of heterotrophic bacteria in the aquaculture area remained high for the whole period, coinciding with intensive nutrition. This suggests that intensive aquaculture, accompanied by the release of untreated food and feces released by fish, is a major driver for bacterial communities in Kardzhali reservoir. Sanitary state indicators including fecal coliforms (FC), E. coli and fecal enterococci (FS) ware analyzed in order to separate the environmental pressure from the cages and from domestic wastewater discharges. For the whole period, the values for FC do not exceed 1000 CFU.100 ml<sup>-1</sup>, and for *E. coli* and FS - 100 CFU.100 ml<sup>-1</sup>. The highest values for all three years were established in samples from the NC1, NC2 and NC3 (p < 0.05). The persistent detection of E. coli and FC (albeit low in number) and their absence in the control zone in the reservoir indicates the presence of secondary sources of pollution other than cage aquaculture, possibly in the form of permanent discharge or fecal filtration - domestic wastewater in this area. Despite that, during the three-year monitoring campaigns, the reservoir was in excellent sanitary condition in terms of indicators (Fig. 2B), based on the criteria written in the sub-program for conducting research/own monitoring in connection with the assessment of the pressure and impact of intensive cage fish farming of the East Aegean RBMP 2017-2021. The results are evident for annual dynamics, with high levels of variation especially in 2017, indicative of a need for the development of a monitoring program.

The lowest phytoplankton numbers  $(0.09 \times 10^{6} \text{ cells.L}^{-1})$  and biomass  $(0.004 \text{ mg.L}^{-1})$  in the three years of study were established in the early spring period followed by an increase in the abundance, reaching its peak in the summer of 2016 and 2018, and in the autumn in 2017. In the peak period, the PHn and PHb averaged 33.3×10<sup>6</sup> cells.L-1 and 2.85 mg.L-1, which is 10-15 times higher than in the other months (Fig. 2C and Fig. 2D). The 2017 results stand out as outliers, compared to the other samples, reaching values of 291×10<sup>6</sup> cells.L<sup>-1</sup> and 4.16 mg.L<sup>-1</sup> respectively for abundance and biomass decrease along the longitudinal axis of the reservoir in the direction of the water current, with a secondary increase near the net-cage areas NC3 and NC5. However, the study found no significant differences between stations (Fig. 2C and 2D).

Pearson correlation matrix was constructed for the assessment of the relationship between microbiological indicators and phytoplankton communities in the studied aquatory. Only a few parameters exhibited significant correlation relationships (Table 2). The parameters with the highest impact on the abundance of heterotrophic bacteria (TVC) include Temp, TN, and organic matter content (COD). A negative correlation was established for all factors with the exception of *Temp*. Both phytoplankton abundance and biomass are negatively correlated with Secchi depth and COD. Redundancy analysis was applied to visualize the correlations of environmental factors with biological parameters in the control site and net-cage area. The resulting model could explain 59.97% of the total variation of biological parameters (Fig. 3). The findings highlighted the differences between reservoir sampling sites. The first RDA axis (explaining 38.79%) was significant (p < 0.001) and strongly correlated with the developed intensive cageaquaculture area (NC2, NC3, and NC4), situated in close proximity to Glavatartsi village. This gradient separated the zone with high anthropogenic pleasure from the lacustrine zone of the reservoir. The results indicated that differences in microbiological indicators were significantly correlated with water quality parameters. The first axis was negatively correlated with Secchi depth, pH, NO3-N, COD and dissolved oxygen, but positively correlated with TN concentration. The second RDA axis (explaining 21.18%) was also significant (p <0.001) and was related to Ctr and NC1 sites. It was positively correlated with water pH, NH4-N and COD.

**Table 1.** Physicochemical parameters (±SD) in the Kardzhali reservoir for the studied period (2016-2018). Legend: <sup>\*</sup>Ctr – control station; <sup>\*</sup>NC– average values for the net-cage area.

Station <sup>*</sup>	Secchi	ъU	Temp	Oxi	NH4-N	NO3-N	NO2-N	TN	ТР	COD
	(m)	рп	(°C)	$(mg.L^{-1})$	(µg.L <sup>-1</sup> )	(µg.L <sup>-1</sup> )	(µg.L <sup>-1</sup> )	(µg.L <sup>-1</sup> )	(µg.L <sup>-1</sup> )	(mg.L <sup>-1</sup> )
Ctr <sub>2016</sub> **	$3.3 \pm 1.4$	8.2±0.6	23.3±3.3	7.9±0.6	66±11	240±30	55±23	362±23	100±30	$4.69 \pm 0.1$
NC <sub>2016</sub>	$3.2 \pm 1.2$	$8.1\pm0.5$	23.5±3.1	6.9±0.8	74±12	268±56	63±61	418±46	123±51	$4.66 \pm 0.2$
$Ctr_{2017}$	$3.8 \pm 1.5$	$8.0\pm0.5$	20.4±4.1	9.1±2.3	83±10	380±102	78±9	$518 \pm 140$	87±61	$4.30 \pm 2.1$
NC <sub>2017</sub>	$3.5 \pm 1.4$	$8.0\pm0.5$	21.2±5.2	8.9±2.0	84±10	365±153	84±20	533±153	115±42	$3.98 \pm 1.8$
$Ctr_{2018}$	$3.0\pm0.6$	$8.3\pm0.8$	23.5±2.5	9.1±4.22	70±9	280±166	10±0	351±165	116±45	$1.67 \pm 0.2$
NC <sub>2018</sub>	2.7±0.7	8.2±0.7	23.8±2.3	7.9±2.35	66±17	240±113	2.5±0	310±105	99±30	2.09±0.6

<sup>\*\*</sup>Number of samples for each parameter: Ctr<sub>2016</sub> (n=5); NC<sub>2016</sub> (n=20); Ctr<sub>2017</sub> (n=7); NC<sub>2017</sub> (n=28); Ctr<sub>2018</sub> (n=7); NC<sub>2018</sub> (n=28)

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**Fig. 1**. Scree plot of the eigenvalues (A) and PCA biplot (B), based on the environmental parameters sampled from the control station and the four net-cage farms in the aquatory of Kardzhali reservoir.



**Fig. 2.** Variability plots, based on the annual data for TVC (A), sanitary indicators (B) phytoplankton abundance (C) and phytoplankton biomass (D).

Kardzhali reservoir is one of the most extensively studied water bodies in Bulgaria. The interest in its ecological status is driven by the high economic interest, as it harbors the largest net-cage farms in the country. The present study is focused on the changes in the physicochemical parameters and their association with some of the biological indicators in conditions of intensive aquaculture during the period of active feeding. The results from the physicochemical analysis point out that the water quality of the reservoir has not changed significantly over the last 18 years period of intensive study in 2001-2018. The established temperature regime and water transparency have been observed in earlier studies of the reservoir (TRAYKOV, 2005; ILIEV, 2015; DOCHIN, 2015). The correlation analysis (Pearson) showed no relationship between temperature and dissolved oxygen, which was generally close to saturation in summer and spring. This could also be due to the high level of PHn and PHb during these periods (BRAHIM ERRAHMANI et al., 2015). A principal component analysis was applied to further elucidate the intricate relationships between the environmental

parameters. The PCA allows transforming the original dataset into new, unrelated to each indicator (WU et al., 2014). Based on the correlation analysis, factors that were most significantly impacted by the cage aquaculture are Secchi depth and nutrients concentrations (NO3-N, TN, COD), while pH and dissolved oxygen concertation were not affected. During that period a large amount of uneaten feed remains in the water column (WESTON et al., 1996; BUREAU & CHO, 1999), which affects the water transparency and nutrient content in close vicinity of the farms (PEREIRA et al., 2004; GORLACH-LIRA et al., 2012). The uneaten feed and fish feces are a major source of phosphorus and nitrogen load in the intensive aquaculture, which explains the positive correlation between the parameters (PODEMSKI & BLANCHFIELD, 2006). The negative correlation between pH and COD is a result of the high organic load, which in combination with a high water temperature in the summer period in terms elevate the concentration of ammonia and organic acids (SHRESTHA & KAZAMA, 2007). This makes pH a suitable indicator of the nutrient load in the water (PARINET et al., 2004).

**Table 2.** Pearson correlation matrix of the environmental parameters and biological indicators in Kardzhali Reservoir 2016-2018. \*correlations are significant at  $\alpha$ = 0.05 (2-tailed).

	SD	Chl-a	pН	Temp	Oxi	$\mathrm{NH_4^+}$	NO <sub>3</sub> -	$NO_2^-$	TN	TP	COD	Phn	Phb	TVC	FC	E. coli	FS	
SD	1																	•
Chl-a	-0,669	1																
pН	-0,476	0,544	1															
Temp	-0,640	0,677	0,577	1														
Oxi	0,100	0,058	0,678	0,200	1													
$\mathrm{NH_4}^+$	-0,019	0,130	-0,114	-0,019	0,099	1												
NO <sub>3</sub> -	0,610	-0,623	-0,416	-0,767	-0,052	-0,047	1											
$NO_2^-$	0,310	-0,305	-0,286	-0,426	0,000	0,523	0,347	1										
TN	0,607	-0,577	-0,436	-0,766	-0,044	0,188	0,939	0,598	1									
TP	-0,206	-0,106	-0,028	0,124	-0,092	0,053	-0,313	0,220	-0,218	1								
COD	0,423	-0,261	-0,771	-0,511	0,001	-0,061	0,565	0,549	0,643	-0,244	1							
Phn	-0,227	0,114	-0,408	-0,391	-0,329	0,352	-0,010	0,299	0,099	0,274	-0,265	1						
Phb	-0,386	0,206	-0,169	0,054	-0,192	0,454	-0,247	-0,127	-0,213	-0,007	-0,497	0,874	1					
TVC	-0,141	-0,017	-0,040	0,491	0,127	0,179	-0,287	0,217	-0,398	0,544	-0,427	0,272	0,066	1				
FC	-0,202	0,298	0,050	0,096	-0,250	0,138	-0,085	0,181	-0,005	0,445	0,152	0,019	-0,045	-0,047	1			
E. coli	-0,173	-0,032	-0,235	-0,086	-0,287	-0,020	0,003	-0,141	0,000	0,143	-0,168	0,137	0,164	0,082	0,651	1		
FS	-0,147	0,074	-0,216	-0,016	-0,298	-0,013	-0,069	-0,211	-0,076	0,063	-0,136	0,015	0,147	-0,012	0,833	0,693	1	

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**Fig. 3.** Biplot in the redundancy analysis (RDA) of microbiological indicators and phytoplankton abundance in relation to environmental parameters.

The microbiological indicators and the phytoplankton seasonal variation limits established in the present study coincide with other observations in Kardzhali Reservoir for the period 2009-2012 (ILIEV, 2015; DOCHIN & STOYNEVA, 2014; DOCHIN, 2015). In the summer, PHn and PHb exceed significantly the values observed in the lacustrine zone of the reservoir in previous periods (TRAYKOV, 2005). The spatial distribution of both biological parameters did not differ significantly between stations, which is untypical for lake-like reservoirs. Under a slow water flow, typical for Kardzhali reservoir, it is expected to observe reduction in the phytoplankton and TVC abundance in the direction form riverine to lacustrine zone, in result of self-purification mechanisms such as sedimentation, dilution, sunlight inactivation, predation and starvation (MEDEMA et al., 2003, NAHURSKA &

DEPTULA, 2004: ILIEV, 2015). Such tendencies are more similar for eutrophic reservoirs (DOCHIN et al., 2017b), and differ from the earlier established patterns of distribution (TRAYKOV, 2005). Moreover, in the summer period have been established a significant increase in the abundance of potential producers of cyanoprokaryotic toxins, such as representatives of the genera Anabaena, Aphanizomenon, and Microcystis. A bloom of the potential toxin producers of the blue-green Pseudanabaena c.f. mucicola and Gloeotrichia echinulata have also been reported in the autumn of 2017 at all stations (DOCHIN & ILIEV, 2019). Multivariate statistical methods used

in the present study could help interpret the complex data matrices and to better understand the relationship between water quality and its impact on the biological communities (SHRESTHA & KAZAMA, 2007). The results confirmed that the location of

the sampling station significantly affects the studied variables and that net-cage aquaculture is a major anthropogenic factor in Kardzhali reservoir. The redundancy analysis corroborated that the environmental factors, such as water temperature and nutrient concentration have a profound impact on the freshwater microbial community and influence their density (LINDSTRÖM et al., 2005; CALIZ & CASAMAYOR 2014; ILIEV, 2015; LEW et al., 2016). Temporal differences in water column bacterial indicators, due to variations, been temperature have previously reported for reservoirs and agricultural watersheds, with greatest abundances in July and August (SIMON & MAKAREWICZ, 2009; NORTH et al., 2014). The increase of microbial population could affect the number of bacteria in the fish processing during handling and (GORLACH-LIRA et al., 2012). Our research that selected environmental confirms factors regulate the microbial community Kardzhali reservoir ecosystem. in However, a significant part of the parameters as pH, well-known to regulate the microbial communities (ZMISLOVSKA et al., 2001; LIN et al., 2012; LEW et al., 2016), had no visible impact in the current conditions of the ecosystem. In this regard, the observation of a secondary increase in the TVC at station NC4 and NC5 is most likely caused by additional input of organic matter in the area. The constant presence of E.coli and FC indicates the possible sewage and fecal contamination from the adjacent villages (Mishra, 2010). It showed that cage-farms are not the only source of anthropogenic pressure.

RDA confirmed that the distribution of the phytoplankton was highly related to the parameters Secchi depth and organic content (COD), which is agreement with the result of BRAHIM ERRAHMANI *et al.* (2015) and O'FARELL *et al.* (2002) who pointed out that the phytoplankton growth is strongly influenced by suspended matter content, which reflects on the water

transparency. WU et al. (2014) also state that the nutrient composition and concentration is the driving force for phytoplankton development. The elevated concertation of phosphates during summer and early autumn and the low during the winter and spring periods are often observed in other lakes and reservoirs (TAMMEORG et al., 2014). As a result, TP does not correlate with the development of phytoplankton communities, and due to its excess, it is no longer a limiting factor. The pH, dissolved oxygen, and temperature described, as basic parameters for phytoplankton development (DOCHIN, 2015; BRAHIM ERRAHMANI et al., 2015) had no effect in the present study, due to the close proximity of the sampling stations to the cage farms.

# Conclusions

The present work demonstrates that the environments in reservoirs with intensive aquaculture are characterized by fluctuations of the physicochemical factors, biological and microbiological indicators for water quality, caused by the nutrient loads. The organic inputs affect food-web interactions and appear to be one of the primary mechanisms that influence microbial phytoplankton communities. There was no correlation between TP and the phytoplankton parameters which may indicate that phosphorus was not a limiting factor despite its low natural bioavailability and rapid mineralization suggesting regular rates, phosphorus inputs from the feed and fish feces. The study confirms the reliability of the PCA and other multivariate analysis for the interpretation of the intricate relationships between the parameters in the complex data sets in heavily modified water bodies. RDA clearly revealed that the long-term exploitation is related to changes in physicochemical water quality, which in alterations terms lead to in the phytoplankton and bacterial communities. During the three-year monitoring campaigns, the reservoir was in an excellent sanitary condition. The persistent detection of low number E. coli and FC on the other side, in the vicinity of the net-cages and their absence in the control zone, suggests that there are nonpoint sources of pollution other than aquaculture activities, such as wastewater discharges from the nearby villages. Our findings point out the necessity of constant control and more frequent monitoring, using sensitive indicators such as phytoplankton and microbiological parameters.

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# Biosorption of Pb (II), Cd (II) and Hg (II) Ions from Model Solutions on Pretreated Waste Bacillus thuringiensis Biomass

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**Abstract.** In the present study waste pretreated *Bacillus thuringiensis* biomass was applied as an ecofriendly biosorbent for Pb (II), Cd (II) and Hg (II) from model aqueous solutions. Heat inactivated and alkali treated biomass showed ability for removal of metal ions from single solutions in the following order Pb(II) > Cd(II) > Hg(II). It was proved that the major groups involved in biosorption are hydroxyl/amino, alkyl, carbonyl and phosphoryl groups. The influence of different factors as pH, initial sorbate concentration, biosorbent concentration, contact time was evaluated.

Key words: biosorption, toxic metals, Bacillus.

## Introduction

Heavy metals are one of the major environmental pollutants, which according to their toxicity occupy the second place after the pesticides. Heavy metals originate mainly from industrial activities such as ferrous and nonferrous metallurgy, ore-mining, ceramic and glass enterprises, as well as those for oil production and processing, thermal power plants using solid and liquid fuels, transport etc. (AHMED & AHMARUZZAMAN, 2016). Some heavy metals such as Cu, Fe, Zn, Mo and other are essential to human life but even at slightly higher concentrations the same metals are toxic. (SMITH et al., 2015). Cd, Hg and Pb are characterized by high toxicity, mutagenic and cancerogenic action (GOYER, 2004; GOYER &

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg CLARCSON, 2001). The excessive intake of these metals leads to serious health problems. Lead damages central nervous and reproductive systems and also causes psychiatric disorders (MASON et al., 2014). Cadmium causes atrophic rhinopharyngitis, chronic bronchitis, nefropathological damages, osteoporosis and osteomalacia (BERNHOFT, 2013). Mercury causes anemia, toxic hepatitis, tremor, vegetovessel distony, polyneuropathy and encephalopathy (PATRICK, 2002).

According to the United State Environmental Protection Agency (US EPA, 2009), the maximum contaminant level of these metals in drinking water is as follows: cadmium - 0.005 ppm; lead - 0.015 ppm and mercury - 0.002 ppm.

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The conventional methods of removing heavy metals (filtration, precipitation, oxidation, reduction, ion exchange and membrane technologies) are costly and ineffective in case of effluents with low metal concentration, particularly in the range of 1-100 mg dm<sup>-3</sup> (ZOUBOULIS *et al.* 2004). Due to this fact, the biosorption has focused as an alternative for heavy metal removal (GADD, 2009; SALAM, 2019).

The aim of this study was to investigate the ability of waste pretreated dead biomass of *Bacillus thuringiensis* to remove Pb (II), Cd (II) and Hg (II) from model solutions. The factors that influence biosorption efficiency such as pH, contact time, metal ion concentration and biosorbent dosage were evaluated. Competitive biosorption from binary and ternary solutions was also examined.

#### Materials and Methods

#### Biomass

Waste biomass of *Bacillus thuringiensis* used for microbiological production of lipases at laboratory conditions was separated from culture broth by filtration and was washed three times with distilled water.

## Pretreatment procedures

The washed biomass was dried a hot air oven at 80 °C for 12 h. The inactivated biomass was stored at 4 °C until further use and referred as a heat treated biomass.

Alkali pretreated biomass was prepared as follows: 5 grams of biomass were suspended in 100 cm<sup>3</sup> 1 M solution of NaOH and then the obtained mixture was sterilized for 15 minutes at 121<sup>o</sup> C (GÖKSUNGUR *et al.*, 2005). Bacterial cells were collected by centrifugation at 3000 rpm for 20 minutes. The NaOH treated cells were washed several times with deionized water to remove excess of sodium hydroxide until the pH reached the near neutral range. The pretreated biomasses were dried at 80<sup>o</sup> C to a constant weight, then were powdered in a mortar and pestle and stored at 4<sup>o</sup> C until use and referred as a caustic treated biomass.

#### **Biosorption studies**

The stock solutions (1000 mg dm<sup>-3</sup>) of Pb (II), Cd (II) and Hg (II) were prepared by a weighed

quantity of analytical grade  $Pb(NO_3)_2$  (Aldrich), Cd(NO<sub>3</sub>)<sub>2</sub>.4H<sub>2</sub>O (Aldrich) and Hg(NO<sub>3</sub>)<sub>2</sub>.H<sub>2</sub>O (Aldrich) in deionized water. Working solutions of different concentrations of each heavy metal were prepared as single solutions by adequate dilution of the initial stock solution. The pH of working solutions were adjusted to required values with 0.1 M HNO<sub>3</sub> or 0.1 M NaOH.

Biosorption experiments were carried out in batch mode using 250 cm<sup>3</sup> Erlenmeyer flasks containing 100 cm<sup>3</sup> single metal solution with desired initial concentration and biomass dose. They were agitated on a shaker for 120 minutes at 250 rpm. Samples were taken in intervals, centrifuged at 3000 rpm for 20 minutes and the liquid supernatant was analyzed for Pb (II), Cd (II) and Hg (II) ions concentration. The batch studies were performed a different experimental condition such as pH, initial metal concentrations (5 – 75 mg dm<sup>-3</sup>), contact time (5 – 120 min), biomass concentration (1.0 – 4.0 g dm<sup>-3</sup>). The temperature was maintained constant at 25  $\pm 0.2^{\circ}$  C in all studies.

#### Biosorption from ternary solution

Biosorption from ternary system was studied using a solutions containing 10 mg dm<sup>-3</sup> of each metal ion. The experiments were carried out at pH 5.0, biosorbent dosage 1 g dm<sup>-3</sup> and contact time 90 min.

#### Date analysis

The amount of metal ion sorbed per unit mass of the biosorbent was evaluated by using the following equation:

 $q = (C_0 - C_f) \times V / m, mg g^{-1}$ 

where  $C_0$  and  $C_f$  are the initial and final metal ion concentrations, respectively in mg dm<sup>-3</sup>; V – the volume of metal solution, dm<sup>3</sup>; m – the biomass concentration, g dm<sup>-3</sup>

The biosorption removal was calculated as follows:

$$R = (C_0 - C_f) \times 100 / C_0, \%$$

All biosorption experiments were done in triplicate. For all graphical representations, the mean values considered and standard deviations within triolicate were to small to be plotted as error bars (< 1 %).

### Analytical Methods

The metal ion concentration in the solution was determined by atomic
absorption spectroscopy (AAS) using an atomic absorption spectrometer PinAAcle 900 T (THGA/FLAME) Perkin Elmer.

The main functional groups present on the cell wall of pretreated bacterial biomass *Bacillus thuringiensis* were recorded in a FTIR spectrometer Nicolet iS10 (Thermo Fisher Scientific, USA), equipped with total reflectance sampling accessory (smart iTR). The analysis was carried out at following conditions: 64 scanning, 4 nm resolution, spectral range 4000 – 400 cm<sup>-1</sup>.

### **Results and Discussion**

Effect of biomass modification

It has been found in a number a studies, that the pretreatment of different kinds of biomass causes an increasing in their removal efficiency towards metal cations. This fact may be due to an elimination the impurities from cell wall surface, an exposure of activemetal binding sites embedded in the cell wall or chemical modifications of the cell wall components (DAS *et al.*, 2007; GÖKSUNGUR *et al.*, 2003; İLHAN *et al.*, 2004; KIROVA *et al.*, 2012; KIROVA *et al.*, 2015a; YAN & VIRARAGHAVAN, 2000).

In our study, as seen on Fig. 1, heat inactivated and alkali treated biomass showed ability for removal of metal ions from single solutions in the order Pb(II) > Cd(II) > Hg(II).

The calculated biosorption capacity for alkali treated cells was 8.35, 7.28 and 6.85 mg g<sup>-1</sup> respectively. In the case of thermally inactivated biomass, the biosorption capacity decreases with 24.62 % for Pb (II), 27.03 % for Cd (II) and 27.81 % for Hg (II) ions. The calculated values are for 10 mg dm<sup>3</sup> initial metal concentrations, pH 5.0, biomass concentration 1 g dm<sup>3</sup> and contact time 120 min.

#### FTIR analysis

FTIR spectroscopy has been used to identify the functional groups responsible for the biosorption of metal ions and other pollutants (inorganic and organic compounds) on the cell surface of the biomass/ biosorbent. The FTIR spectra of unloaded and Pb (II)- loaded alkali treated biomass were obtained and presented (Fig. 2).

From the Fig. 2A it can be seen that the FTIR spectra present distinct peaks, confirming the complex structure of the cell wall. The broad band situated in the range 3500 и 3100 cm<sup>-1</sup> does not allow a clear indication of the functional groups, because overlapping of -OH and -NH stretching is possible (MALEKI & MASHINCHIAN, 2011; SIMONESCU, 2012; PAN et al., 2007). The peak at 2929 cm<sup>-1</sup> is attributed to -CH stretching vibration from alkyl chains. Overlapping of -OH stretching from carboxylic acids with C-H stretching vibrations in the range from 3400 to 2400 cm<sup>-1</sup> have been reported (VENEAU et al., 2012). The amide I band (stretching vibrations in carboxyl or amide groups) appears at 1644 cm<sup>-1</sup> and the amide II band (N-H bending vibrations and C-N stretching vibration superposition) - at 1539 cm<sup>-1</sup>. Sometimes, the  $C(=O)-O^{-}$  anti-symmetric stretching vibration in carboxylate so-called  $v_{C=O}$  (I) appears around 1544.20 cm<sup>-1</sup> wavenumbers (PAN et al., 2007). The peak at 1402 cm<sup>-1</sup> can be attributed to N-H bending in amine group. The peak at 1080 cm<sup>-1</sup> is caused by P=O symmetric stretching vibrations (YUAN et al., 2009). For the lead loaded biomass, the peaks at 3277 and 2929 cm<sup>-1</sup> almost disappeared, indicating that amine and hydroxyl groups may be involved in the biosorption process. The carboxyl peak observed for unloaded biomass at 1644 cm<sup>-1</sup> is shifted to 1650 cm<sup>-1</sup>. The peak at 1539 cm<sup>-1</sup> (amid II) was weaker and shifted to 1533 cm<sup>-</sup>. The peak at 1080 cm<sup>-1</sup> was also weaker and shifted to 1076 cm<sup>-1</sup>. The peak at 1402 cm<sup>-1</sup> disappears. From the obtained results it can be concluded that the major groups involved in biosorption are hydroxyl/amino, alkyl, carbonyl and phosphoryl.

# Effect of pH

The initial pH of the aqueous solution is the key factor influencing the biosorption process, the surface charge of the biomass and the solution chemistry of metal ions. At low pH values the binding sites of the cell surface are in protonated form resulting in a net positive surface charge, which could in turn, decrease the biosorption of the available positive charged metal ions. In contrast, an increase in pH an opposite effect is observed (FAROOQ *et al.*, 2010).

The effect of initial pH of the solution on the Pb (II) recovery was studied at pH ranging from 2.0 to 5.0 to avoid Pb(OH)<sub>2</sub> precipitation, because at pH values higher than 5.0 several low soluble hydroxide species were observed (OZDEZ *et al.*, 2009); for Cd (II) and Hg (II) – from 2.0 to 6.0 (Fig. 3).

The results indicated that the recovery efficiency in all cases is pH dependent. There was an increase in Pb (II), Cd (II) and Hg (II) removal with increasing pH, a plateau was reached in the pH range 5.0 – 6.0 for cadmium

and mercury. The calculated removal efficiency at pH 5.0 was 83.5 % for Pb (II); 77.2 % and 68.50 % for Cd (II) and Hg (II) ions respectively, at pH 6.0. Therefore, pH 5.0 was selected for all further experiments. These results were in good agreement with data for the effect of pH on Pb(II), Cd (II) and Hg (II) biosorption reported by other authors (KIROVA *et al.*, 2015a; GÖKSUNGUR *et al.*, 2005; SVECOVA *et al.*, 2006; PAN *et al.*, 2007).

# Effect of biomass concentration

Biomass concentration is another important factor affecting biosorption efficiency and the sorption capacity. Efficiency of Pb (II), Cd (II) and Hg (II) removal was examined as a function of biomass dosage (from 1.0 to 4.0 g dm<sup>3</sup>).



**Fig. 1.** Pb(II), Cd(II) and Hg(II) uptake of *Bacillus thuringiensis* biomass (1 – heat inactivated, 2 – alkali treated).

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Fig. 2. FTIR spectra of unloaded (2.A) and Pb (II)-loaded (2.B) pretreated biomass.



**Fig. 3.** Effect of pH on the removal efficiency of Pb (II), Cd (II) and Hg (II) ions (initial concentration 10 mg dm<sup>-3</sup>, biomass dosage 1 g dm<sup>-3</sup>, contact time 120 min).



**Fig. 4.** Effect of biomass dosage on Pb (II), Cd (II) an Hg (II) removal efficiency (initial concentration 25 mg dm<sup>-3</sup>, pH 5.0, contact time 120 min).

It was observed (Fig. 4, that increasing biosorbent concentration resulted in an increase in removal efficiency for metal ions onto waste pretreated *Bacillus thuringiensis* biomass. The removal efficiency increases from 71.04 % to 85.21 % for Pb (II), from 59 to 71.5 % for Cd (II) and from 53.8 to 67.80 % for Hg (II) ions with the rise of biomass concentration from 1.0 to 4.0 g dm<sup>3</sup>. The calculated metal uptakes showed a reverse trend. For Pb (II), the biosorption uptake decreased from 17.76 to 5.33 mg g<sup>-1</sup>.

A high biomass concentration increases the specific surface area and the number of active binding sites leading to increased removal efficiencies, but the metal uptake per gram of biomass decrease (BAI & ABRAHAM, 2001; FRAILE *et al.*, 2005; KIROVA *et al.*, 2015b). A biomass concentration 1 g dm<sup>3</sup> is used in subsequent experiments.

#### Effect of initial concentration of metal ions

Biosorption studies with waste pretreated biomass  $(1 \text{ g dm}^3)$  were conducted using

solutions containing from 5 to 75 mg dm<sup>-3</sup> metal ions at pH 5.0 and contact time 120 min. The results are shown on Fig. 5.

The biosorption capacity of the waste pretreated biomass increased with increasing initial concentration of metal ions. A saturation value was observed around 50 mg dm<sup>-3</sup> for Pb (II), Cd (II) and Hg (II) ions and the calculated uptakes were 19.45, 16.28 and 15.39 mg g<sup>-1</sup> respectively. Increasing initial metal concentration generally caused a decrease in removal efficiency and this fact could be attributed to the gradual blocking of the free active sites for metal ion binding on the surface of the biomass. At 5 mg dm<sup>-3</sup> initial metal concentration 95. 6 % Pb (II), 81 % Cd (II) and 65 % Hg (II) were removed. The waste pretreated biomass showed a higher affinity for Pb (II) than for Cd (II) and Hg (II) ions. Similar results have been reported in other studies (KIROVA et al., 2015a; TODOROVA et al., 2019; TÜZÜN *et al.*, 2005).

### Effect of contact time

Contact time is one of the important factors affecting the efficiency of biosorption, but contact time depends on the experimental conditions –

type of biomass, particle size, type of metals, etc. Biosorption of Pb (II), Cd (II) and Hg (II) ions by waste alkali pretreated biomass was studied with different contact times (5 – 120 min).



Fig. 5. Effect of initial metal concentration of metal uptake (1 - Pb (II), 2 - Cd (II), 3 - Hg (II)).



**Fig. 6.** Effect of contact time on the biosorption (pH 5.0, biosorbent dose 1.0 g dm<sup>-3</sup>, initial concentration 25 mg dm<sup>-3</sup>).

As seen on Fig. 6, the biosorption for each of the studied metal ions can be divided into two stages. The first stage was shorter (about 30 min) and characterized with higher biosorption speed and faster increase in the metal uptake. For second stage, a slower increase in metal uptake were observed. Equilibrium was reached at 90 min. At the end of the first stage the removal efficiency was 66.48, 52.44 and 45.32 % for Pb (II), Cd (II) and Hg (II) ions.

WANG (2012) found that the biosorption of Cd (II), Zn (II) and Cu (II) on *Saccharomyces cerevisiae* biomass was rapid for first 15 min and equilibrium was nearly reached after 90 min. OVES *et al.*, (2013) studied the biosorption of five heavy metals (Ni, Pb, Cu, Cd and Cr) by *Bacillus thuringiensis* strain OSM29. They observed that the initial sorption rate was highest at the beginning and moved to equilibrium within half hour.

#### Biosorption from ternary solutions

Industrial effluents are usually composed of several different metal ions and for this reason biosorption from ternary metal system was studied. The biosorption uptakes of the caustic pretreated biomass were 8.02, 5.31 and 4.51 mg g<sup>-1</sup> for Pb (II), Cd (II) and Hg (II) respectively. In the ternary system, the metal uptake was reduced for Pb (II) with only 3.95 %, for Cd (II) and Hg (II) - with 27.08 and 34.16 %, respectively, compared to the single metal solutions. The caustic pretreated biomass demonstrated the highest affinity to Pb (II) ions. Similar results were reported in other studies (KIROVA et al., 2015a; TÜZÜN et al., 2005). This fact can be explained with the larger ionic radius (1.19 Å), electronegativity (2.33) and covalent index (6.46) for Pb (II) ions, comparing to Cd (II) and Hg (II) ions (KIROVA et al., 2015a; PEREIRA et al., 2011; SALAM, 2019; SENGIL & OZACAR, 2009).

DUTTA *et al.* (2016) studied the competitive biosorption of Cd(II), Pb(II), and Cu(II) ions on EDTA-treated baker's yeast cells was studied by using artificially prepared wastewater containing 100 mg dm<sup>-3</sup> of each metal at pH 5. A significant decrease in Pb(II), Cd(II) and Cu(II) uptake was observed (from 98.93, 21.12 and 13.28 mg g<sup>-1</sup> to

20.40, 1.20 and 6.55 mg  $g^{-1}$ , respectively). GÖKSUNGUR et al., (2003) observed also reduction in the uptake using ethanol - treated yeast cells. PARASZKIEWICZ et al. (2009) studied the removal efficiency of Pb(II), Cd(II) and Zn(II) ions on pretreated waste biomass Curvularia lunata, from single, binary and ternary solutions. They observed that the presence of Pb(II) significantly interfered in the sorption of zinc and cadmium ions. About 3-fold decrease in zinc and cadmium removal was detected in binary and ternary systems as compared with single solutions of Zn(II) and Cd(II). The removal efficiency of Pb(II) from single, binary (Pb +Zn, Pb + Cd) and ternary solutions was calculated as follow: 81.9, 79.2, 72.1 and 80.6 %. As seen the studied bisorbent of pretreated waste biomass of B. thuringiensis exceeded other microbial biosorbents as effective sorbent for competitive heavy metals removal from ternary solutions.

#### Conclusions

Alkali treated waste biomass from Bacillus thuringiensis can be applied as effective biosorbent for heavy metals removal from aqueous solutions. The waste pretreated biomass showed a higher affinity for Pb (II), followed by Cd (II) and Hg (II) ions. The highest removal capacity for Pb (II) was reached at optimal process parameters pH 5.0, biomass dosage 1 g dm<sup>-3</sup> and contact time 90 min. The studied biosorbent is prospective because it successfully removes heavy metals not only from single but even from ternary solutions, which means that is is effective at model conditions closer to real waste waters. Additional studies for application of waste B. thuringiensis biomass in immobilized form for heavy metals removal are in progress.

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# Mollusc Records from the Area of Manyas Lake (Bird Paradise Lake) in Western Anatolia, Turkey

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**Abstract.** This study was carried out simultaneously with a fish parasitological studies of the first author. Six gastropod species (5 freshwater and 1 hygrophilous terrestrial one) and three mussel species were identified in this case study. Five of the gastropods and two mussel species are first records from Manyas Lake. Although Manyas Lake is a Ramsar area and a National Park, this wetland has not been investigated as detailed in terms of mollusc fauna.

Keywords: Turkey, Manyas Lake, Gastropoda, Bivalvia, biodiversity.

# Introduction

Manyas Lake is also known as Bird Paradise Lake (Kuş Cenneti in Turkish). This wetland is situated in western Turkey, located in Marmara Region. This nutrientrich lake has international importance point of biodiversity, because it is on the bird migration route between Asia, Europe and Africa. More than 270 species of birds have been recorded at the lake. The surface area of the lake is 160 km<sup>2</sup> and shallow lake with an average depth of 3 meters. The lake is fed by underground water sources and the Kocaçay and Mürüvvetler Stream in the south and Sığırcı Stream in the north of the lake (WWF, 2008; EFE *et al.*, 2008).

The importance of this ornithological site was highlighted by Ordinarius Prof. Curt Koswig being German hydrologist and zoologist in 1 April 1938. The 64 ha sanctuary area near Sigirci Stream to the northeast of the lake was accepted as a National Park by the government in 1959. This area was awarded class a status by the European council in 1976. 10,200 hectare of the lake in 1994 was placed on the Ramsar convention list and whole lake was given protected status in 1998 (WWF, 2008; EFE *et al.*, 2008).

The waste from animal farms (poultry, cattle and sheep etc), discharge of domestic and industrial enterprises including acid, fertilizer etc., paddy waters used in agricultural production, waste water containing pesticides causes pollution in Manyas Lake. As a result of this, various studies have shown that limnological parameters in the lake have changed.

Freshwater molluscs are one of the important animal groups that make up

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg Union of Scientists in Bulgaria – Plovdiv University of Plovdiv Publishing House biodiversity. They form an important ring of the food chain in the aquatic ecosystems. Manyas Lake is one of the most important wetlands in Turkey. Although Manyas Lake is a Ramsar area and a National Park, this wetland has not been investigated as detailed in terms of mollusc fauna to date. (1973) found Anodonta Bilgin cygnea waterstoni on the southern shores of the manyas lake. BILGIN (1987) reported two bivalv species such as Unio pictorum ascanicus, Unio elongatulus eucirrus in the karadere stream that spilled into manyas lake. KINZELBACH (1989) found Anodonta palustris gravida from manyas lake. BALIK et al. (2005) reported six mollusc species (Radix ovata, R. peregra, Lymnea stagnalis, Planorbis planorbis, Anodonta palustris gravida, Unio cf. crassus) in the Sigirci Stream that spilled into Manyas Lake.

The aim of this preliminary study is to determine mollusc diversity which is an important food source of fish and waterfowl in lake and mollusc species which are intermediate hosts to parasites.

# **Materials and Methods**

Mollusc samples including gastropods and mussels were collected from littoral zones by hand dredge, spatula and oar during parasitological surveys of first author on July 2018-2019. The vegetation areas, aquatic plants, stones and rocks were selected for the material collection randomly. The molluscs found were preserved in 70 % ethanol and stored in glass vials with plastic screw-caps. The data to related their biotopes were recorded on these glass vials and the molluscs cards. The photos of gastropods were performed using Leica 1500 stereomicroscope; Canon EOS 1100D for bivalvs. Identifications and comparisons were performed according to ZHADIN (1952), GLÖER (2002).

# **Results and Discussion**

Six gastropod species (*Lymnaea stagnalis, Physella acuta, Radix auricularia, R. labiata, Oxyloma elegans, Viviparus viviparus*) and three bivalv species (Unio mancus, Anodonta anatina, A. cygnea) are reported in this study. Physella acuta, Radix auricularia, R. labiata, Oxyloma elegans, Viviparus viviparus, Unio mancus, Anodonta anatina are new records for the mollusc fauna of Manyas Lake.

Phylum Mollusca Class Gastropoda Cuvier, 1795 Subclass Pulmonata Cuvier, 1814 Ordo Basommatophora Family Lymnaeidae Rafinesque, 1815 Genus Lymnaea Lamarck, 1799 Lymnaea stagnalis (Linnaeus, 1758) (Fig.

1a). Stations: 40° 07' 53" N 28° 02' 57" E,

Dasklyeion. *Habitat:* Gravel bottom and sandy bottom.

Records in turkey: YILDIRIM & SCHUTT (1996), YILDIRIM (1998), ÇAMUR ELIPEK (2003), ÖZBEK et al. (2004), YILDIRIM (2004), KOŞAL ŞAHIN & YILDIRIM (2007), KOŞAL ŞAHIN (2012), KOŞAL ŞAHIN (2013), AKBABA & BOYACI (2015), ARSLAN et al. (2017).

*Remarks:* the species was reported from the lake by BALIK *et al.* (2005).

Family Physidae Fitzinger, 1833

Genus Physella Haldeman, 1843

*Physella acuta* (Draparnaud, 1805) (Fig. 1b).

*Stations:* 40° 07' 53" N 28° 02' 57" E, Dasklyeion; 40° 14' 39" N 27° 57' 18" E Bereketli.

*Habitat:* Gravel bottom and sandy bottom.

Records in turkey: BILGIN (1973), SCHUTT & ŞEŞEN (1989), ERTAN et al. (1996), YILDIRIM & SCHUTT (1996), YILDIRIM et al. (1996), YILDIRIM (1998), YILDIRIM & KARAŞAHIN (2000), ÇAMUR ELIPEK (2003), USTAOĞLU et al. (2003), ÖZBEK et al. (2004), YILDIRIM (2004), ÖKTENER (2004), AKBULUT et al. (2009), KALYONCU & ZEYBEK (2009), KILIÇASLAN & ÖZBEK (2010), ZEYBEK et al. (2012), GÜRELLI & ÖZBEK (2012), KOŞAL ŞAHIN (2012), KOŞAL ŞAHIN (2013), ZEYBEK et al. (2014), GÜRLEK et al. (2016), KOŞAL ŞAHIN & ZEYBEK (2016), KOŞAL ŞAHIN et al. (2017), KOŞAL ŞAHIN & ALBAYRAK (2017), ARSLAN et al. (2017), GÜRLEK (2019). *Remarks:* The species is new record for the lake.

Family Lymnaeidae Rafinesque, 1815 Genus Radix Montfort, 1810

Radix auricularia (Linnaeus, 1758) (Fig. 1c).

*Stations:* 40° 07' 53" N 28° 02' 57" E, Dasklyeion.

#### Habitat: Gravel bottom and sandy bottom.

Records in turkey: BILGIN (1967), GELDIAY & BILGIN (1969), BILGIN (1973), SCHUTT (1988), SCHUTT & ŞEŞEN (1989), SOYLU (1990), ERTAN *et al.* (1996), YILDIRIM (1998), YILDIRIM & KARAŞAHIN (2000), YILDIRIM (2004), USTAOĞLU *et al.* (2003), BALIK *et al.* (2003), ÖKTENER (2004), ÖZBEK *et al.* (2004), YILDIRIM *et al.* (2005), KOŞAL ŞAHIN & YILDIRIM (2007), KALYONCU *et al.* (2008), ŞEREFLIŞAN *et al.* (2009), AKBULUT *et al.* (2008), ŞEREFLIŞAN *et al.* (2009), AKBULUT *et al.* (2009), KILIÇASLAN & ÖZBEK (2010), GÜRELLI & ÖZBEK (2012), KOŞAL ŞAHIN (2012), KOŞAL ŞAHIN (2013), AKBABA & BOYACI (2015), GÜRLEK *et al.* (2016), KOŞAL ŞAHIN & ZEYBEK (2016), KOŞAL ŞAHIN *et al.* (2017).

*Remarks:* The species is new record for the lake.

Order Stylommatophora Family Succineidae H. Beck, 1837 Genus Oxyloma Westerlund, 1885 *Oxyloma elegans* (Risso, 1826) (Fig. 1d). *Station:* 40°13' 55" N 28° 02' 55" E, Kuş Cenneti Natural Park.

Habitat: Gravel bottom and sandy bottom.

*Records in turkey:* SOYLU (1990), SCHUTT & ŞEŞEN (1993), USTAOĞLU *et al.* (2003), KALYONCU *et al.* (2008), GÜRLEK *et al.* (2016), KOŞAL ŞAHIN *et al.* (2017), GÜRLEK (2019).

*Remarks:* The species is new record for the lake.

Subclass Caenogastropoda Cox, 1960 Order Architaenioglossa Haller, 1890 Family Viviparidae Gray, 1847 Genus Viviparus Montfort, 1810 *Viviparus viviparus* (Linnaeus, 1758) (Fig. 1e).

*Stations:* 40° 07′ 53″ N 28° 02′ 57″ E, Dasklyeion; 40° 14′ 39″ N 27° 57′ 18″ E Bereketli.

Habitat: Gravel bottom and sandy bottom.

Records in turkey: ÇAMUR ELIPEK (2003), ÖKTENER (2004), KOŞAL ŞAHIN & YILDIRIM (2007), KOŞAL ŞAHIN (2012), KOŞAL ŞAHIN (2013), AKBABA & BOYACI (2015), ARSLAN *et al.* (2017).

*Remarks:* The species is new record for the lake.

Family Lymnaeidae Rafinesque, 1815 Genus Radix Montfort, 1810 *Radix labiata* Rossmässler,1835 (Fig. 1f). *Stations:* 40° 07' 53" N 28° 02' 57" E, Dasklyeion.

# Habitat: Gravel bottom and sandy bottom.

Records in turkey: BILGIN (1967), GELDIAY & BILGIN (1969), BILGIN (1973), SCHUTT (1988), SOYLU (1990), ERTAN *et al.* (1996), YILDIRIM (1998), USTAOĞLU *et al.* (2003), BALIK *et al.* (2003), ÖZBEK *et al.* (2004), YILDIRIM (2004), YILDIRIM *et al.* (2005), KOŞAL ŞAHIN & YILDIRIM (2007), KALYONCU *et al.* (2008), ŞEREFLIŞAN *et al.* (2009), AKBULUT *et al.* (2009), KILIÇASLAN & ÖZBEK (2010), GÜRELLI & ÖZBEK (2012), ZEYBEK *et al.* (2012), KOŞAL ŞAHIN (2012), KOŞAL ŞAHIN (2013), ZEYBEK *et al.* (2014), AKBABA & BOYACI (2015), GÜRLEK *et al.* (2016), KOŞAL ŞAHIN & ZEYBEK (2016), KOŞAL ŞAHIN *et al.* (2017), GÜRLEK (2019).

*Remarks:* The species is new record for the lake.

Bivalvia Linnaeus, 1758

Unionida Stoliczka, 1871

Unionidae Rafinesque, 1820

Unio Philipsson, 1788

Unio mancus Lamarck, 1819 (Fig. 1g).

*Stations:* 40° 07' 53" N 28° 02' 57" E, Dasklyeion; 40° 10' 56" N 27° 52' 14" E Gölyaka

Habitat: Gravel bottom and sandy bottom. Records in turkey: SCHUTT & SESEN (1993),

ÖKTENER (2004), EKIN & BAŞHAN (2010), EKIN & BAŞHAN (2011).

*Remarks:* The species is new record for the lake.

#### Anodonta Lamarck, 1799

Anodonta anatina (Linnaeus, 1758) (Fig. 1h).

*Stations:* 40° 07' 53" N 28° 02' 57" E, Dasklyeion; 40° 10' 56" N 27° 52' 14" E Gölyaka; 40° 14' 39" N 27° 57' 18" E Bereketli.

Habitat: Gravel bottom and sandy bottom.

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Records in Turkey: ERCAN et al. (2013).

*Remarks:* The species is new record for the lake.

Anodonta cygnea (Linnaeus, 1758) (Fig. 1i).

*Stations:* 40° 07' 53" N 28° 02' 57" E, Dasklyeion; 40° 10' 56" N 27° 52' 14" E Gölyaka; 40° 14' 39" N 27° 57' 18" E Bereketli.

*Habitat:* Gravel bottom and sandy bottom.

Records in turkey: BILGIN (1980), KINZELBACH (1989), SOYLU (1990), ÇAMUR ELIPEK (2003), BAŞÇINAR (2003), ÖKTENER (2004), ÖZBEK et al. (2004), KOŞAL ŞAHIN & YILDIRIM (2007), AKBULUT et al. (2009), BAŞÇINAR et al. (2009), KOŞAL ŞAHIN (2012), KOŞAL ŞAHIN (2013), ERCAN et al. (2013), KOŞAL ŞAHIN et al. (2017).

*Remarks:* this species was reported as *anodonta palustris gravida* drouet, 1879 in manyas lake by KINZELBACH (1989).



Fig. 1. a. Lymnaea stagnalis (2.5 cm), b. Physella acuta (2.5 mm), c. Radix auricularia (1.25 cm), d. Oxyloma elegans (1.5 cm), e. Viviparus viviparus (3.5 cm), f. Radix labiata (3 mm), g. Unio mancus (3 cm), h. Anodonta anatina (2 cm), i. Anodonta cygnea (4.5 cm). This contributes researches to determine of molluscs with 7 new mollusc record in Manyas Lake.

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# Inventory of Genus Primula (Familly Primulaceae) in the Herbarium of Agricultural University – Plovdiv (SOA), Bulgaria

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**Abstract.** Genus *Primula* L. (Primulaceae) is represented in Bulgaria by 8 species. The study is an observation of the whole recent collection of the genus, stored in the Herbarium of Agricultural University – Plovdiv (SOA). The collection consists of 352 herbarium sheets (185 of them from Bulgarian localities), representing 36 species (8 from Bulgaria). The chorological information from the literature and from the herbarium specimens is databased and mapped. Inspite of the relatively low representativeness, the *Primula* collection of SOA is an important resource for the taxonomical and chorological investigations in this group.

Key words: Primula, herbaria collection, SOA.

#### Introduction

The typical genus Primula consists about 500 species, with highiest diversity level in the mountain regions of Eastern Asia (HU & KELSO, 1996), Himalayas and West China (KOVTONIUK & GONCHAROV, 2009). A few species are distributed in the mountains of Africa, tropical Asia and South America. In one of the newest classifications of genus Primula is divided to 37 sections (RICHARDS, 2003). The genus is represented in Europe with 34 species, grouped in 4 sectionsand in North America - 20 species, grouped in 5 sections. The current data for the frora of Bulgaria describes 8 species (PEEV, 1982; DELIPAVLOV, 2003; ASSYOV & PETROVA, 2012).

A comprehensive work on the genus *Primula* is that of SMITH & FLATCHER (1949), dealing mainly withs species of Nepal and Himalayas. A center with a great biodiversity in the world is Western China,

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg where about 300 species of *Primula* known, and the process of describing new taxa continues (YUAN XU *et al.*, 2016).

Historically, the knowledge of the genus in our flora can be tracked back to the first edition of VELENOVSKY (1891) and the later Supplementum (VELENOVSKY, 1898), with included 4 species: P. vulgaris Huds., P. elatior (L.) Hill, P. veris L. and P. frondosa Janka. The number of species and their treatment in different floristic editions can be traced in "Flora of Bulgaria". The first edition of the "Flora" (Stojanov STEFANOV, 1925) lists 7 species. In the second edition (STOJANOV & STEFANOV, 1933) the authors consider a hybrid combination P. acaulis (L.) Hill × P. officinalis (L.) Hill. In the third edition (STOJANOV & STEFANOV, 1948) are mentioned 8 species. Variability is considered - Only one variety of of P. officinalis is indicated - var. suaveolens Bert. For P. elatior Jacq. is considered var. icarnata

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(Godr. et Gren.) Pax. In the fourth edition (STOJANOV *et al.*, 1966) is added the new variety of *P. officinalis* var. *columnae* Ten. The authors also include 3 widely cultivated ornamental species – P. sinensis Lindl., *P. obconica* Hance and *P. malacoides* Franch. In the taxonomical work for vol. 8 of Flora Reipublicae Bulgaricae" (PEEV, 1982) are represented the investigations on the genus till the beginning of the 80's of 20'th century. The species count remains the same. The author also describes a cultivated hybrid *P. hortensis* Host (*P. elatior* × *veris* × *vulgaris*) and mentions also 4 rarely cultivated species.

A review of the available literature on the genus *Primula* indicates that the species have not been a subject of targeted in-depth research for the last 35 years in Bulgaria. The available materials date from the period 1880-1980, and in the recent decades there is a lack of modern collections from the country.

The number of species cited by DELIPAVLOV (2003) for our country is 9 – the decorative species *Primula auricula* L. is included. In one of the recent generalizations on the chorology of higher plants in Bulgaria, 8 wild species of needles are mentioned (ASSYOV & PETROVA, 2012).

The primary purpose of the study is inventory and evaluation of *Primula* collections in SOA herbarium.

# Materials and Methods

The classification scheme for the Bulgarian species follows the main taxa adopted by DUBY (1828), with few exceptions uniquely accepted in the modern taxonomy of the genus *Primula*.

The available samples of genus *Primula*, stored in the collections of Agricultural University Plovdiv - SOA (according to Index Herbariorum - Tiers, 2016) are inventoried.

The progres of the taxonomic perceptions of the *Primula* genus over the years has created great synonymy, which is also present in the SOA collection. In order

to avoid synonymous errors, a synonymous list of the species is drawn up.

from The data the supporting information are stored in a relational database. The chorological information is organized according the recent floristic regions (KOZUHAROV et al., 1983) The floristic regions are databased and described in the maps and the text using the codes: 1s -Black Sea Coast (south), 1n – Black Sea Coast (north), 2 - North-Eastern Bulgaria, 3 -Danubian Plain, 4w - Balkan Foothill Region (west), 4e – Balkan Foothill Region (east), 5w - Balkan Range (west), 5c - Balkan Range (central), 5e – Balkan Range (east), 6 – Sofia Region, 7 - Znepole Region, 8 - Vitosha Region, 9 - West Frontier Mountains, 10s -Struma Valley (south), 10n – Struma Valley (north), 11 - Belasitsa, 12 - Slavianka, 13 -Mesta Valley, 14s – Pirin (south), 14n – Pirin (north), 15 - Rila, 16w - Sredna Gora (west), 16e - Sredna Gora (east), 17w - Rhodopes (west), 17c - Rhodopes (central), 17e -Rhodopes (east), 18 - Tracian Lowland, 19 -Toundja Hilly Plain, 20 - Strandja (Fig. 1).

The countries are listed with their ISO 3166-1 Alpha 2 codes: AT- Austria, AZ-Azerbaijan, BA- Bosnia and Hertzegovina, CH- Switzerland, DE- Germany, DK-Denmark, ES- Spain, FI- Finland, FR- France, GB- United Kingdom, GE- Georgia, HR-Croatia, IT- Italy, KZ- Kazakhstan, MK-North Macedonia, NO- Norway, PL- Poland, RO- Romania, RS- Serbia, RU- Russian Federation, SE- Sweden, SI- Slovenia, SK-Slovakia, TR- Turkey, US- United States of America, UZ- Uzbekistan, ?- unknown.

The information from the herbarium sheets is stored in a relational database using the software application dSOA, following an approved model (STOYANOV, 2009). Each record contains the sample number, taxon name, floristic region, WGS84 coordinates, UTN (MGRS) code, altitude, nearest toponym, date, authors, and memo field with the text from the label. The maps are generated by the same software using the UTM coordinates.

The status of the taxa is referred in the

database International Plant Name Index (IPNI). The reference for the Bulgarian taxa, their status and chorology is done from the Bulgarian "Floras" and "Keys", as well as the known floristic records cited in the text. The available information in SOA is compared to the data from the Conspectus of Higher Plants in Bulgaria, which also the reference to the floristic elements (ASSYOV & PETROVA, 2012).

The results are presented in a taxonomic scheme, with their names accepted and their synonyms noted. Each inventory taxon is presented with a heading in a comparable, uniform pattern.

The recent data about genus *Primula* in Bulgaria (horyzontal and vertical distribution, chorology, ecology, and phenology) are reviewed from the literature and from the herbarium sheets.

#### **Results and Discussion**

Genus Primula L.

Sect. Primula

Primula vulgaris Huds. Syn. Primula acaulis (L.) Hill., Veg. Syst. 8: (1765) 25; P. officinalis Hill; Primula veris var. acaulis L. Sp. Pl. 143 (1753).

European floristic element, indicated for the whole territory of Bulgaria, on 0 - 900 m a.s.l. Available materials from P. vulgaris show poor representation in the SOA collection. 26 specimens are deposited in the herbarium, of which 11 are from Bulgarian localities (Fig. 1-H), the rest are comparative materials (Table 1). The Bulgarian specimens from SOA confirm the regions: 1n, 5, 7, 8, 11, 16.1, 18 and 20. There are two subspecies within the species: subsp. vulgaris with the same distrubution, and subsp. *sibtorpii* (Hoffman) Sm. & Forest found in the regions 1s, 5e and 20 (5e and 20 confirmed in SOA). The hybrid combination *P. vulgaris* × *P. veris* subsp. canescens is not confirmed in wild, because these materials are revised by Peev (1974r.) as *P. veris* subsp. *canescens*.

Primula elatior (L.) Hill., Veg. Syst. 8: 25 (1765). Syn. Primula veris var. elatior L., Sp. Pl. 143 (1753).

European floristic element, indicated for the regions 5w, 5c, 8, 9, 14n, 15, 17w, 17c, in the alpine zone, on 2000 - 2800 m a.s.l. The Bulgarian localities are represented by 14 herbarium sheets from 5c, 14n, 15, 17w and 17c (Fig. 1-A). The comparative materials are 13 (Table 1).

*Primula veris* L., Sp. Pl. 142 (1753). Syn. *Primula suaveolens* Bertol., J. Bot. (Desvaux) 2: 76 1813; *Primula veris* var. *officinalis* L., Sp. Pl. 142 1753.

European-Mediterranean floristic element, indicated for the whole country, from the sea level up to 2500 m. The deposited specimens are 72, and 17 are from abroad (incl. *Primula clolumnae* Ten., Table 1). The materials from Bulgaria localities represent the regions: 4w, 5c, 5e, 6, 7, 8, 9, 10s, 11, 16w, 16e, 17w, 17c, 18, 19 and 20, in altitudes 130 - 2200 m a.s.l. (Fig. 1-G).

Sect. Auricula Duby

subgen. *Auriculastrum* Schott; sect. *Cyanopsis* Schott, Sippen Österr. Primeln 14. 1851.

*Primula deorum* Velen., Syn. *Auricula-ursi deorum* (Velen.) Soják; *Primula bulgarica* Georgieff ex. Degen.

Bulgarian endemic species, local for Rila Mts, on altitude 1900-2800 m a.s.l., around along streams, glacial lakes, peatlands and snow drifts. Listed in the Bulgarian Law of Biological Diversity (Appendix 3) and the Red Data Book of Bulgaria (PEEV & TSONEVA, 2011) – in the category "Vulnerable", in the IUCN Red List and in the Bern Convention.

In SOA are deposited 23 specimens. The earliest collections are done by Urumov (SOA 8530, 1907), or marshy places in the Rhodopes, but without exact locality or toponym. The data to the specimen is probably referring to the Rila-Rhodope mountain range without exact location. The rest of the specimens are collected in the 60s and 70s of the 20<sup>th</sup> century. They represent some alpestric localities from Rila Mts (fig. 1-B), with altitudes between 1990 and 2660 m a.s.l. Two exsiccates signed as *Primula deorum* Velen. f. *alba* Delip. & Cheschm.,

Inventory of Genus Primula (Familly Primulaceae) in the Herbarium of Agricultural University

(1972) are deposited in the herbarium. This variability is not accepted in the taxonomical review of the genus (PEEV, 1982).

Primula minima L., Sp. Pl. ed. 1 (1753) 143.

Alpine-Carpatic-Balcan floristic element, indicated for altitudes 2000-2900 m a.s.l., in the regions 5w, 5c, 8, 14, 15, 17w  $\mu$  17c. The specimens in SOA are 24 (Fig. 1-C) from Bulgaria and 1 from abroad. The Bulgarian exsiccates are from the regions 5c, 14n  $\mu$  15. The specimens deposited in 5c, showed lower limit of altitude range – up to 1530 m.

Sect. Aleuritia Duby

*Primula farinosa* L., Sp. Pl. 143 (1753). 1768. Syn. *Primula farinosa* subsp. *exigua* (Velen.) Hayek, Repert. Spec. Nov. Regni Veg. Beih. 30(2): 25 1928; *Primula exigua* Velen., Sitzungsber. Königl. Böhm. Ges. Wiss., Math.-Naturwiss. Cl. 1888: 32 1889.

European floristic element, indicated in 5w, 5c, 8, 9, 14n, 15, 17w, 17c, between 1600-2600 m a.s.l. The deposited herbarium sheets are 38 образци, and 9 of them are from abroad (Tab. 1). The Bulgarian specimens are from the regions 5w, 5c, 8, 14n and 15, collected from altitudes 980 to 2700 m a.s.l. (Fig. 1-E).

*Primula frondosa* Janka, Syn. *Primula farinosa* subsp. *frondosa* (Janka) Stoj. et Stef., Fl. Bulg., ed 1, vol. 2 (1925) 861.

Bulgarian endemic, local for 5c, on altitudes 900-2000 m. This species is listed in the Red Data Book (PEEV & TSONEVA, 2011) in the category "endangered", and in the IUCN Red List. The exsiccates are from altitudes between 970 and 2200 m a.s.l. (Fig. 1-E).

Primula halleri G. F. Gmel., Syn. Aleuritia halleri (J.F.Gmel.) Soják

Alpine-Carpatic-Balcan floristic element, indicated only for the regions 14 and 15, on altitudes between 2250 and 2800 m a.s.l. The 12 specimens in SOA are from the both regions (Fig. 1-F). One specimen is from Austria. Specimens with numbers 8525 (Arnautski Peak, Stojanov & Stefanov) and 48110 (Rila Monastery, Stojanov) decrease the lower limit of vertical distribution to 1100 m.

Table 1. Comparative specimen	s of	genus
Primula in SOA.		

Species	Count	Origin
	10	AT, AZ, DE, HU,
P. auricula L.	12	PL, SK, UZ, ?
P austriaca Wetsst.	1	?
P. balbisii Lehm.	1	?
P. bicolor Raf.	1	?
P. carnathica Fuss.	1	?
P. cortusoides L.	1	?
P. clusiana Tausch	3	АТ.?
P carniolica Jaca	2	IT.
P. daonensis (Levb)	_	
Levh	2	AT
<i>P. discolor</i> Schur	1	?
P. elatior (L.) Hill.	13	BA, DE, FR, PL, SE, SI
P. farinosa L.	9	AT, BA, DE, SE, SK
<i>P. druadifolia</i> Franch.	1	?
<i>P. glutinosa</i> Wulfen	5	AT, DE
P. orandiflora Lam	1	?
P. halleri G.F.Gmel.	1	AT
P. hirsuta All.	_	
(P. viscosa Vill.)	5	AT, FR, ?
P. integrifolia L.	1	СН
P. kitaibeliana Schott	1	HR
P. latifolia Lapeyr.	2	DE, FR
P. longipes Freyn & Sint	. 1	TR
<i>P. malacoides</i> Franch.	1	?
P. minima L.	1	RO
P. marginata Curtis	3	?
P. mistassinica Michx.	1	US
P. nivalis Pall.	1	UZ
<i>P. nutans</i> Georgi	4	FI, SE
<i>P. palinuri</i> Petagna	1	IT
<i>P. pedemontana</i> Thomas ex Caudin	1	RS
P scotica Hook	5	$NO_2$
P snectahilis Tratt	3	INC, : IT
D stricta Hornom	1	NO SE
D willow Wulfon	2	NO, JE AT
r. oniosa vvunen	5	AT EC ED CE
P. veris L.	17	MK, RO, RU,
		SE, SK
P. vulgaris Huds.	15	BA, DK, GB, DE,
P znulfeniana Schott	1	IT, KZ, MK, UZ SI
	1	

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**Fig. 1.** Specimens of genus *Primula* deposited in SOA: A- *P. elatior;* B- *P. deorum;* C- *P. minima;* D- *P. frondosa;* E- *P. farinosa;* F- *P. halleri;* G- *P. veris;* H- *P. vulgaris.* 

The collection of genus *Primula* in SOA consists 352 herbarium sheets. 185 of them (54%) are from Bulgarian localities, 159 (45%) - from abroad, 8 - without geographic data. Four samples are not determinated as species. The phenologic data is noted on the labels of 290 herbarium labels.

The phase with flowers is the most suitable for determination. Thats why, the prefered period for the collections is May-July. Fewer materials are collected in April and August. Single plants are collected from March and September.

This corresponds to the altitude and the months of flowering. The earliest flowering species are *P. veris* and *P. vulgaris*, but they are poorly represented as number of specimens and this is the reason for the

small harvests during these months. The prevalence of collections from the summer months corresponds to the high altitudes, typical of highland and alpine species.

Most specimens are collected from the 5c, 14n, 15 and 17w regions. In general, the fact that the materials deposited in the SOA collection are locally mainly in southern Bulgaria (Fig. 1).

The analysis of the materials studied shows a reduced scientific interest in the genus as a whole. This is evidenced by the small number of herbarium specimens for a period of more than 100 years - 184 sheets of Bulgarian collections are deposited during 1887 - 2012. It is also noteworthy that the authors of the collections are more than 20 botanists, the largest number of them being collected by: D. Delipavlov (49

records); Art. Dimitrov (42 records); N. Stojanov (38 records); M. Popova (20 records); B. Stefanov (17 records); I. Cheshmedzhiev (12 records); and other botanists, authors of single collections. About 20 specimens have not data about the authors. The most intensive collection period is 1894 - 1980. The oldest bulgarian specimens of Primula are collected since 1856. All this points to the lack of targeted studies within the genus, which led to a backlog of up-to-date information on the number of species and their distribution on the territory of our country. The major revision notes of the genus in SOA have been made by Peev since 1974 and are the basis for the development in Flora of Bulgaria item 2 (PEEV, 1982). Subsequent to this period, the intake of materials into the herbarium was of low intensity, both by region and by the author of the collections, carried out sporadically, on various botanical expeditions. The new literary chorological data for the *Primula* species are few.

The SOA collection contains a considerable number of comparative materials of Primula species - 93 herbarium sheets (about 40% of the collection). Along with the species found in Bulgaria, 28 other species that are not found in Bulgaria are presented (Table 1). This is the heritage in result of the intensive interinstitutonal exchange during the period of the active inventory work of Bulgarian flora. The bigger part of the materials are collected between 1864 and 1926, from various phytogeographic areas from whole Europe.

Materials of some natural hybrids of *Primula* are stored too (Table 2).

Table 2. Natural hybrids of *Primula* stored in SOA.

Hybrid	Count	Countries
<i>P.</i> × <i>biflora</i> Hutter ex A.Kern.	1	?
<i>P.</i> × <i>bosniaca</i> Beck ex Fiala	1	?
P. × brevistyla DC.	1	?
P. columnae × P. vulgaris	1	BA
<i>P. × digenea</i> A.Kern.	1	?
P. × intermedia Port.	1	?
P. × media	1	FR
<i>P. × pannonica</i> A.Kern.	4	AT, RS, SK
<i>P. × ternovania</i> A.Kern.	1	?
<i>P.</i> × <i>travnicensis</i> Wiesb.	1	BA

#### Conclusions

The herbarium SOA represents all species of *Primula* in Bulgaria – *P. veris* (67); *P. farinosa* (38); *P. minima* (33); *P. elatior* (30); *P. vulgaris* (25); *P. deorum* (22); *P. frondosa* (13) and *P. halleri* (12). Although *P. veris*, *P. vulgaris* and *P. elatior* are indicated throughout the country the materials in SOA do not represent the actual distribution by floristic regions.

On the basis of the literature and the deposited specimens of *Primula* in SOA, it is visible that the studies of this genus doesn't cover all floristic regions. Displayed by floristic regions, the region with highest count of specimens is 15 (48 records) followed by 17 (32 records), 5 (29 records), 14 (27 records) and 11 (6 records). Few, often single, materials are from the regions 1, 6, 9, 16, 18 and 20, which considers a lower biodiversity level. Materials from the

regions 3 and 12 are not found.

The vertical distribution of the species in the country displays a highest diversity in the mountain and alpine belt (1600 – 2900 m a.s.l.). As habitat features, these are mountain and alpine meadows, peatlands, streams and moist soils, as well as rocky crevices, etc., generally localities of natural character and high humidity. This group includes local and regional endemic species such as Primula frondosa, P. deorum and P. halleri, as well as the alpine species P. minima and P. farinosa with specific edaphic preferences. The widest range of vertical distribution (0-2200 m a.s.l.) is established for P. veris, followed by P. frondosa (900 - 2000 m a.s.l.). Part of the samples, with inaccurate or incomplete topographic information about locality and altitude, were not included in the analysis (about 25 exsiccates).

As a drawback of the collection, it can be noted that, despite the taxonomic revisions of part of the materials, no intraspecific taxa were identified in the polymorphic groups of *P*. *officinalis* and *P. elatior*; there is material with incomplete chorology and inaccurate data regarding the localities; unspecified taxa have been imported. Over 20 species and natural hybrids are stored in the herbarium, including local endemics from the Balkan Peninsula and protected taxa from different parts of Europe.

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# Influence of Urbanization on the Populations of the European pond Turtle Emys orbicularis (L., 1758) in the City of Plovdiv (Bulgaria)

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Abstract. Landscape change associated with urbanization presents a great challenge for conservation, because of the way it affects biodiversity. Turtles represent a very important component of the urban ecosystems. The European pond turtle, Emys orbicularis (L., 1758) is one of the two native aquatic turtle species, occurring in Bulgaria, including the city of Plovdiv, which is the second largest city in the country and its territory covers mainly the urbanized environment and adjacent terrains. According to the data for the last 100 years, the European pond turtle in the studied region is increasing its distribution range within the city, due to the development of dense system of irrigation canals. The presence of the invasive species Trachemys scripta elegans was registered in the region of Plovdiv City. The abundances and average density of the populations are relatively low and the age structure of the population of *E. orbicularis* from Maritsa River has the highest percentage of adults and juvenile individuals, indicating that the population of this species in the city is unstable. The main threat to European pond turtle in Plovdiv City is the anthropogenic factor, which is mainly through the loss of suitable habitats on the one hand and the direct destruction of specimens on the other.

Key words: Emys orbicularis, urban populations, urbanization, Plovdiv City, Bulgaria.

# Introduction

Urbanization is recognized to be one of the main factors for habitat loss leading to local biodiversity extinction in urban areas (MCKINNEY, 2008). Landscape change associated with urbanization, particularly sprawl, has been significant during the last

© Ecologia Balkanica http://eb.bio.uni-plovdiv.bg half century and is expected to continue through the next decades (ALBERTI *et al.*, 2003). Effects of landscape change on stream ecosystems have been extensively documented (PAUL & MEYER, 2001). Modifications of the land surface during urbanization produce changes in both the

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type and the magnitude of runoff processes. These changes result from vegetation clearing, soil compaction, ditching and draining, and covering the land surface with impervious roofs and roads.

Turtles face an increasingly serious set of threats to their survival throughout the world (KLEMENS, 2000), making them one of the most severely threatened vertebrate groups. They also represent a very important component of urban ecosystems, occurring in a variety of aquatic habitats (and some terrestrial surroundings) and therefore tolerate the impact of human activity to varying degrees. This leads to a reduction in compared their diversity to natural conditions and changes in the structure of populations. In their addition to urbanization can result in changes in distribution, movement, home range and activity of native species (FORMANN et al., 2002) and thus understanding anthropogenic effects on activity and habitat use is essential for developing sound conservation and management plans in urban areas. However, this problem is still poorly studied in Europe, and especially in Bulgaria.

In Bulgaria, the European pond turtle (E. orbicularis) is distributed along rivers, streams, irrigation canals and in marshes, ponds, dams and fisheries throughout the country up to 1100 m a.s.l. (BESHKOV & NANEV, 2002) and it can be registered even in cities and urban areas. The city of Plovdiv is the second largest city in Bulgaria and its territory covers mainly the urbanized environment and adjacent terrains. The favorable geographic location of the city, the presence of the Plovdiv hills and the influence of the Maritsa River determine the existence of unique natural areas with rich biodiversity. As stated by MOLLOV et al. (2013) there is still a huge gap of knowledge about the species' ecology, population structure, reproductive biology and ethology in the country, especially in urban areas. The objectives of this study were to estimate the distribution, population size, density, age structure of the populations of E. orbicularis

in the city of Plovdiv and to evaluate the main conservation problems and threats for the urban populations.

### Material and Methods

The data presented in the current study was obtained in the period from March 2007 to October 2010 in the territory of Plovdiv City and its surroundings.

The general data for each individual observed includes: date, time, place of observation and altitude (marked with the GPS receiver "Garmin eTrex Vista"); age group (adult, subadult and juvenile) established on the basis of the dimensional characteristics and coloration of the individuals (ARNOLD & OVENDEN, 2002); the dominant vegetation in the habitat.

Zoogeographic classification was done after BEŠKOV & BERON (1964). The chorotype classification follow PETROV (2007).

The index of occurrence (A) was calculated, using the following Eq. (1) (after PETROV & MICHEV (1985)):

$$A = \frac{N}{S} .100 (1),$$

where A – index of occurrence; N – the number of UTM (1x1 km) squares in which the species is found; and S – the total number of squares in the surveyed area.

The abundance (Ab) for each studied population was calculated. According to SUTHERLAND (2000), abundance is defined as the total number of individuals of a given species found in a given territory. In the present work, due to differences in transect length, we used a number of individuals per 1000 linear meters (Eq. 2). Data obtained in this way gives a better opportunity for comparison and analysis.

Ab=
$$\frac{n}{L}$$
.1000 (2),

where Ab – abundance (number of individuals per 1000 linear meters); n – number of observed individuals; and L – area studied in linear meters.

For the calculation of the average density of amphibians and reptiles populations ( $D_s$ ) in the standing water bodies, we divided the number of all reported specimens by the total area of the water basin. In the present work the population density is presented as the number of individuals per m<sup>2</sup>, calculated according to Eq. (3) (after SUTHERLAND, 2000):

$$D_s = \frac{n}{A} (3),$$

where  $D_s$  – density (number of individuals per m<sup>2</sup>); *n* – total number of all observed specimens; and *A* – the area of the water basins in m<sup>2</sup>.

Description of line transects used

Maritsa River passes through the whole territory of the city of Plovdiv in a west-east direction. Within the boundary of the studied region falls a 13 km section of the river, which was divided into 13 one-kilometer transects. From the irrigation canals passing through the study area, transect was marked along one of them (where the presence of *E. orbicularis* was recorded) – from the road to the village of Tsaratsovo to Plovdiv State Fishery (4400 m). These flowing basins, as well as two large (over 1 km<sup>2</sup>) standing water basins – Rowing Base, Plovdiv and Plovdiv State Fishery, were also surveyed.

Environmental factors

In this study, some basic abiotic and biotic factors (air and water temperature, water pH and predominating vegetation) affecting the distribution, and some population properties of the European pond turtle populations, were measured by standard methods. Air and water temperature were measured with digital thermometer "TCM", minimal capacity 0.1°C and water pH was measured using digital pH-meter "WTW", minimal capacity 0.01.

#### Natural environment of the city of Plovdiv

Plovdiv Municipality is located within the Plovdiv Field in the middle of the Thracian Lowland (south Bulgaria) at 160 m a.s.l. The city of Plovdiv takes up about 53 km<sup>2</sup> of the Plovdiv municipality and represents an urbanized area with a population of 375,580 inhabitants. The study area covered 127 km<sup>2</sup>, calculated from the UTM map of Bulgaria (10x10 km). The borders of the research area were identified based on a 1-kilometer UTM grid (10x10 km standard quadrats were divided into 100 smaller quadrats of 1x1 km). Thus, the study area includes the administrative boundaries of the city (Fig. 1) and the surrounding areas, excluding other urban areas (MOLLOV & VELCHEVA, 2010).

# Statistical analyses and software used

The processing and mapping of GPS data was accomplished with the "MapSource" v.6.12 software by Garmin Ltd. on an electronic topographic map, and the visual presentation utilized ArcGIS v.10.0 (ESRI, 2011).

The statistical processing of the data was done with the software package "Statistica" v.7.0 (StatSoft Inc., 2004). Data was analyzed for distribution normality by the Shapiro-Wilk test. When comparing or looking for correlations between individual variables, non-parametric  $(x^2-test,$ Mann-Whitney U-test tests for independent variables, Spearman correlation index) were used when the data did not have a normal distribution. These differences were statistically valid with  $p \le 0.05$  [ $\alpha = 5\%$ ]. Principal Component Analysis (PCA) was applied, and values greater than 0.7 were accepted as statistically valid for this test (FOWLER et al., 1998).

### **Results and Discussion**

#### Distribution in the city of Plovdiv

Based on literary data (1910-2001), *Emys* orbicularis is registered in total of 3 localities in 3 UTM quadrats (Distribution index – 2.36), the contemporary number of localities (after MOLLOV & VELCHEVA, 2010) is 11 in 9 UTM quadrats (Distribution index – 7.09).

The European pond turtle has been reported for the "Maritsa River in Plovdiv City" by KOVACHEV (1910), "near the "Ostrova" Area in Plovdiv City" by ANGELOV (1960) and State Fisheries – Plovdiv by KIRIN (2001). In the current study it was recorded with a total of 11 localities in 9 UTM quadrats (LG1710, LG1720, LG1762, LG1783, LG1607, LG1608, LG1619, LG1629, LG1649). All recorded localities are presented in Fig. 2.



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Fig. 2. Distribution of the European Pond Turtle (*Emys orbicularis*) in the city of Plovdiv.

Within the city limits we also recorded the invasive Red-eared Slider - Trachemys scripta elegans (Wied-Neuwied, 1839). The red-eared slider is a decorative freshwater turtle species, sold in pet shops as a pet. Unfortunately, when the turtles become too large to be kept in captivity, their owners release them in different places in the country. Because of the high ecological plasticity of the species, it successfully survives in natural conditions and that is why it has become a potentially dangerous invasive species in Europe (CADI & JOLY, 2004). So far there are two records of redeared sliders in Plovdiv City. The first one is from 2004, when a specimen was spotted on the banks of the Maritsa River near the bridge of the international fair - LG1649 (Dulev, Plovdiv, pers. comm., 2004) and the second was registered in this study on 14.VII.2008 - a couple (male and female), released in a fountain in the garden behind the Regional Natural History Museum -Plovdiv (LG1648).

# Habitat distribution

The urban landscape of Plovdiv City is a complex matrix of residential and busyness buildings, roads, open, wooded, and other terrestrial habitats, as well as dense network of rivers and irrigation canals. According to MOLLOV (2011) the European pond turtle occurs in 6 urban habitat types in the city of Plovdiv - constant standing, freshwater ponds; rivers and streams; irrigation canals; riparian willow formations; riparian reed formations and floodplain crops (rice fields). It is a typically aquatic species, that can be rarely observed far away from the water basins.

Ecological classification and level of synanthropy

Following the classification given by MOLLOV & VELCHEVA (2015) according to the humidity and temperature regime, the European pond turtle can be classified as hydrophylic and mesothermic species. The species usually inhabit aquatic, humid and midhumid habitats with moderate temperatures. The species was recorded in all three zones along the urban-rural gradient in the city and occurs in a wide range of urban habitats and can be classified as "hemerodiaphoric", according to Klausnitzer's classification (KLAUSNITZER, 1990).

# Zoogeographic classification

According to the Chorotype classification after PETROV (2007), *Emys orbicularis* is classified as Turanian-Euro-Mediterranean chorotype. Another classification used in the current study is by faunistic complexes and elements (after BEŠKOV & BERON, 1964). According to that classification, the European Pond Turtle belongs to the Mediterranean faunistic complex, Holo-Mediterranean faunistic element.

According to PETROV (2007), the presence (and even predominance in some areas, including the city of Plovdiv) of Mediterranean chorotypes in the reptiles in Bulgaria is not a surprise, given the geographic location of the country and its relatively warm climate. The predominance of the Mediterranean chorotypes in Plovdiv City could also be explained by the theory of the "heat island effect" (OKE, 1982).

# Ecological properties of the populations

The specie's populations were studied along urban-rural gradient along Maritsa River in 2007 (a 13 kilometer stretch within the city limits), an irrigation canal in the north part of the city, the Rowing Canal – Plovdiv and Plovdiv State Fishery.

Along Maritsa River of the 13 onekilometer transects surveyed, all turtles were found between the fourth and the eighth kilometer (center of the city). The abundance (individuals per 1000 linear meters) of the species's population in Maritsa River, calculated by us, using line transect method was 0.692, and the abundance in the studied irrigation canal – 0.454. This species adheres to such coastal areas where there is preserved tree vegetation on the very waterfront. Such plots are almost absent in most of the studied water basins and this is Influence of Urbanization on the Populations of the European pond Turtle Emys orbicularis...

the reason for the limited distribution of the species in the studied area.

The Principal Component Analysis (PCA) and Spearman correlation rank were performed to trace the impact of the four factors studied on the abundance of species's populations. Both tests were done, following the effect of air (Factor 1) and water temperature (Factor 2), water pH (Factor 3) and the type of coastal vegetation (Factor 4) on the size of their populations (Table 1 and 2).

For *E. orbicularis*, a statistically significant value was found for the second factor (Table 1), which explains about 26% of the results. An average positive, statistically significant correlation with the water pH and a weak one with no statistical significance with the coastal vegetation type were also recorded for this species (Table 2).

Turtles from the Emydidae family are characterized as long-lived organisms with delayed sexual maturity and a long reproductive lifespan. These traits, together with high adult survivorship and low egg and hatchling survivorship, result in a stable population age structure that should be dominated by juveniles and subadults (CONGDON *et al.*, 1994). As a result, the ratio of non-reproductives to reproductives should greatly exceed one, and adults should represent only a small fraction of the population (GIBBS & AMATO, 2000). The age structure of the population of *E. orbicularis* was recorded only at the Maritsa River. The ratio between the three age groups is as follows: 1:0.75:0.5, with the largest percentage being the adults (44.44%), followed by the subadults (33.33%) and the juveniles (22.22%). There was no statistically significant difference found for this species from the 1:1:1 ratio ( $\chi^2$ =0.67; df=2; p=0.72). This age structure shows that this urban population of the species is unstable and is likely to decrease.

The only large standing water reservoirs (over 1 km<sup>2</sup>) in the studied area are the Rowing Canal and Plovdiv State Fishery. The area of the Rowing Canal is 1.25 km<sup>2</sup> and the total area of the fishponds is 1.46 km<sup>2</sup>. The average density of the *E. orbicularis* populations in the two basins are presented in Table 3.

In Plovdiv State Fishery, there are more species of amphibians and reptiles compared to the Rowing Canal due to the great variety of coastal and aquatic vegetation and micro-habitats in the fishponds compared to the total absence of such conditions in the canal. In addition, the Rowing Canal is used much more intensively and is visited by thousands of people every day, which is the main reason for the low species richness and lower population density (Fig. 3).

**Table 1.** Factorial coordinates of the variables based on the PCA correlations based on the abundance of *Emys orbicularis* in Maritsa River in the studied region. *Legend:* Values marked with \* – statistically significant values (p>0.70).

	Factor 1	Factor 2	Factor 3	Factor 4
Emys orbicularis	0.35	0.84*	0.31	-0.30

**Table 2.** Spearman correlation rank of the four major abiotic factors and the abundance of the population of *Emys orbicularis* in the Maritsa River in the studied region. *Legend:* Values marked with \* – the correlation is statistically significant (p<0.05).

Abiotic factor	Population abundance (Ab)
Air temperature, °C	0.074
Water temperature, °C	0.063
Water pH	0.606*
Type of vegetation	0.353

**Table 3.** Average density (ind./ $m^2$ ) of the European pond turtle's populations in the two large standing water basins in the study area.



**Fig. 3.** European Pond Turtle (*Emys orbicularis*) – subadult specimen from the Rowing Canal in the city of Plovdiv (15.05.2008, photo: I. Mollov).

Population densities of *E. orbicularis* may vary considerably, depending on the region. In general the population density of European pond turtle in Plovdiv City is extremely low. Extensive review on the population densities of this species in various regions throughout its range is given by AYAZ *et al.* (2008) and other authors, unfortunately data comparisons are impossible, due to the different methods used.

*Conservation significance, problems and threats* 

According to the contemporary Bulgarian and international legislation the European pond turtle is included in Appendices II and III of the Bulgarian Biodiversity Act (BDA, 2002); Appendix II of the Convention for the Conservation of Wild European Flora and Fauna and Natural Habitats, (Bern Convention, 1979); Appendices II and IV of Council Directive 92/43 of the Council of the European Economic Community of 21.05.1992 on the Conservation of Natural Habitats and Wild Fauna and Flora (EC, 2006); listed as "near threatened" (NT) category in the Europe's Red List (COX & TEMPLE, 2009) and the Red List of Endangered Species to IUCN (2019). *Emys orbicularis* is characterized by high conservation significance at national and international levels. Although it is common species in the country, its conservation is necessary in order to preserve the native herpetofauna (as part of European and world fauna) and to preserve biodiversity in cities.

The main threat for the species in the city of Plovdiv os the direct destruction by man and partly the loss of habitats. A negative influence on the populations comes from the collecting of live specimens by private collectors. According to BESHKOV and corroborated bv (1993),our observations, occasionally E. orbicularis are caught by accident on the hooks of fishermen, and most of them are often killed afterward. The two species of aquatic snakes are also killed by fishermen because they rarely "attack" the caught fish.

Keeping the red-eyed slider (*Trachemys scripta elegans*) as a pet and releasing it into various water basins in the city can lead to the potential distribution of this highly invasive species, which is a competitor to *E. orbicularis*.

On the basis of the results of the present study, we can make the following recommendations for the conservation of the *Emys orbicularis* populations in Plovdiv City.

A particularly negative influence on the species' populations is the so-called "cleaning" of the Maritsa River bed, which is carried out periodically by the municipality. This cleaning does not only remove the trees, branches and debris that fall in the river bed, but also all the tree and shrub vegetation along the shore. This activity not only directly destroys the habitats, but also increases water erosion. Our recommendation is that river clearing is limited only to the cleaning of the branches and debris that have entered the water and the bed of the river.

Another very negative impact is caused by the collection of sand and inert materials from the banks of the Maritsa River. This activity in some places has led to unrecognizable river banks, as some species are directly affected, including *E. orbicularis*. A positive role for the distribution of the European pond turtle in the city has been the construction of a dense network of irrigation canals on the outskirts of Plovdiv City. In order for this network to continue to function, it is necessary to maintain and repair it in many places.

With regard to the pollution of water basins, where *E. orbicularis* occur in the city, we recommend detailed future research on and monitoring of the impact of the most common pollutants in these ponds, as well as on the reproduction and survival rates of the species in these areas.

# Conclusions

According to the data for the last 100 years, the European pond turtle in the studied region is increasing its distribution within the range city, due to the development of dense system of irrigation canals. The presence of the invasive species Trachemys scripta elegans was registered in the region of Plovdiv. The abundances and average density of the populations are relatively low and the age structure of the population of E. orbicularis from Maritsa River has the highest percentage of adults and juvenile individuals, indicating that the population of this species in the city is unstable. The main threat to European pond turtle in Plovdiv City is the anthropogenic factor, which is mainly through the loss of suitable habitats on the one hand and the direct destruction of specimens on the other.

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# Production of Extracellular Phospholipase C by Species of Genus Bacillus with Potential for Bioremediation

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**Abstract.** The application of phospholipases produced by species of genus *Bacillus* in different industries reduces the negative impact on the environment by reducing the need of toxic chemicals, consumed energy, and produced carbon emissions. In the current study one hundred sixty-six bacterial strains belonging to the genus *Bacillus* were tested for phospholipase C production. Eighty-seven percent of the studied strains demonstrated phospholipase C activity on egg-yolk agar. Strain *Bacillus thuringiensis* was selected as the most promising for the phospholipase production with initial activity of 19.61 U/ml. The nutrient medium composition and cultivating conditions were optimized for achieving higher enzyme yields. The highest phospholipase production was achieved on the following conditions of the liquid medium: 1% of yeast extract as a source of nitrogen; 0.5% NaCl; 0.4% of glucose as a carbon source; NaHCO<sub>3</sub> – 3 g/l; Na<sub>2</sub>HPO<sub>4</sub> – 0.4 g/l; 1 mM ZnCl<sub>2</sub>; pH 7; inoculated with 3% (1.4 x 10<sup>9</sup> cfu/ml) subculture and 8 hours duration of the cultivation. The production of phospholipase C by the selected strain was scaled up in a bioreactor with a volume of 2l.

Key words: phospholipase C, Bacillus, bioremediation.

#### Introduction

The human civilization is facing a global environmental crisis, that could change the lives of everyone. A lot of different measures being taken are to avoid further environmental problems, but the main responsibility lies on the global industry. At the moment some of the older industrial production technologies are being replaced with green alternatives, based on green chemistry and green biotechnology. The main goal is the reduction of used dangerous chemicals and materials, reduction of the used water and energy, eliminating the produced dangerous byproducts and to reduce the production of greenhouse gases.

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One of the possible solutions to these problems is the use of biocatalysts enzymes, in different kinds of chemical processes. They can effectively increase the speed of the occurring chemical reactions, they are active at a lower temperature, which leads to a reduction in the consumed energy for heating, they reduce the quantity of the used toxic chemical compounds and they can be produced from by-products and wastes of other industries. For the production of enzymes, bacterial species are often preferred. Of them Bacillus spp. have many favorable properties. Some of the species have GRAS status - they are considered as safe for food and food

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additives production. They have short division cycles, they can be cultured in short periods, they can secrete the desired proteins in the medium, which makes the enzyme easier for purification. All these properties are making production cost-effective (SEWALT *et al.*, 2016).

In the food and beverage production industries enzymes also find broad field off application. For example, phospholipases can be used in the refining process of vegetable oils. The biological catalyzers can be used as a replacement to the classical refining technologies, which are relying on a caustic process using strong alkaline and phosphoric acid solutions for the removal of phospholipids, which reduce the quality of the oils. Data form industrial experiments at Bunge's oil refining plants (USA) show, that the used enzymes for degumming have reduced the amount of consumed energy and the produced greenhouse gases. For the refining of 266 000 tons of soy oil the consumed energy is reduced with 112 000 GJ, the carbon emissions are reduced with 12 000 tons, SO<sub>2</sub> emissions are reduced with 140 tons, PO<sub>4</sub> emissions are reduced with 100 tons, and the produced ethylene is with 4 tons less (DE MARIA et al., 2007). On an annual basis, the food industry throws away tons of materials rich in keratin in the form of hair, fur, skin, nails, and other byproducts which are considered dangerous waste, but they could be a good source of amino acids. It is possible these waste materials to be used as a substrate for process enzymatic hydrolysis using proteolytic enzymes. The final products will be rich in single amino acids and small peptides, which could be used as a valuable nutrition source for animals (SINGH & BAJAJ, 2017). Another valuable application is in the baking industry. The quality of the produced bread and similar products is declining in time with the crystallization of the starch in the products. In this case, amylase enzymes can be used to increase the shelf life and the quality of the products. Careful calculations show that the financial loses of producing

bread with short shelf life are bigger than the cost of the treatment of the drought with amylases (JEGANNATHAN & NIELSEN, 2013).

But enzymes can be used not only in manufacturing processes. They can be used for the treatment of chemical contaminations in the environment. CHRISTOVA et al. (2019) have identified a strain of Bacillus cereus, which has the ability to degrade up to 93% of the oil hydrocarbons in the examined medium for about 48 hours. The research group has immobilized the isolated strain and their results show that the preparation remains almost fully active for 47 days, at 28°C up to 20 cycles of usage. Other authors have reported similar results. also SAKTHIPRIYA et al. (2015) have isolated strain of Bacillus cereus, which can degrade up to of the oil hydrocarbons in the 80% contaminated environment. Enzymes can be also for the removal of toxic used agrochemical contaminants in the soil, like pesticides, herbicides, and fungicides. Some strains of Bacillus amyloliquefaciens have hy-1 gene with a length of 858 bp, which is coding protein, which can hydrolyze the fungicide carbendazim to 2-aminobenzimidazole. The isolated protein is identified as a type of phospholipase, which successfully can remove the fungicide off the surface of contaminated cucumbers. This experiment proves that enzymes can be used as an effective tool in the bioremediation processes (LI *et al.*, 2019). There are already developed technologies that use Bacillus subtilis and other species that produce highly active lipase and phospholipase enzymes that hydrolyze contaminated with fats and oils waste waters. The produced free fatty acids and alcohols can then be easily digest by the active sludge in the water treatment plant (Patent No. PCT/JP2013/055504).

Although lipases and phospholipases are hydrolytic enzymes in nature, they could be used in valuable biosynthesis industrial processes. Their ability to carry on transesterification reactions can be used for the biosynthesis of fatty acids alkyl esters, which commercially are better known as
biodiesels – an important source of renewable fuels (ANOBOM *et al.*, 2014).

The aim of the current work is to examine the extracellular phospholipase C production capabilities of strains of genus *Bacillus* and to optimize the condition for the enzyme production.

#### Materials and Methods

#### Bacterial strains

Strains of genus *Bacillus* (166 strains) from the microbial collection of the Department of "Biochemistry and microbiology", Faculty of Biology, University of Plovdiv, Bulgaria were examined for phospholipase C activity. Cultures of the organisms were maintained on nutrient agar medium at 4°C for routine laboratory use. For long-term use, the strains were maintained in nutrient agar under paraffin layer at 4°C.

The initial screening was based on the ability of the strains to hydrolyze phospholipids in egg yolk agar medium.

#### Hydrolysis of phospholipids

Each strain was inoculated on egg yolk agar (one egg yolk; nutrient broth – 3 g; yeast extract – 0.5 g; glucose – 0.5 g; 5 ml 0.1M CaCl<sub>2</sub>.2H<sub>2</sub>O; 95 ml H<sub>2</sub>O; pH 7.2) and incubated for 24 to 48 hours at 37°C. The formed halo around the colony was measured. Strains that have formed halo larger than 5 mm were taken for further analyses.

# *Fermentation conditions and separation of culture filtrates*

A subculture was prepared prior to the fermentation by inoculation of the strain in nutrient broth and incubation for 8h on a rotary shaker at 37°C, 120 rpm. The cell density of the culture was then modified to 6.0 McF units.

Erlenmeyer flasks with volume of 300 ml, containing 30 ml growth medium (casein hydrolysate – 10 g/l; Bacto Peptone – 10 g/l; NaCl – 5 g/l; glucose – 4 g/l; NaHCO<sub>3</sub> – 3 g/l; Na<sub>2</sub>HPO<sub>4</sub> – 0.4 g/l; ZnCl<sub>2</sub> – 0.01 g/l; pH 7; sterilized for 15 minutes at 121°C) were inoculated with 3% (6.0 McF) subculture (GERASIMENE *et al.*, 1980). The inoculated

flasks were set on a rotary shaker at 37°C, 120 rpm for 12 - 48 hours. For the separation of the cells, 10 ml culture medium were centrifuged at 4°C, 14 000 rpm for 20 minutes. The supernatant was collected for further analysis.

*Optimization of the culture conditions for enzyme production* 

Optimum phospholipase production was studied at different incubation periods (0 - 36 h), with different volumes of the inoculum between 1 – 10%, carbon sources (mannose, fructose, maltose and ribose), concentrations of the carbon source (0.2 – 0.8%), different sources of nitrogen (bactopeptone, casein hydrolysate, yeast extract, beef extract, KNO<sub>3</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), and different metal ions (Mg<sup>2+</sup>, Mn<sup>2+</sup>, Zn<sup>2+</sup>, Ca<sup>2+</sup>).

Fermentation in bioreactor

Fed-batch cultivation was performed in a 2 L bench-top bioreactor (Minifors, Infors HT, Switzerland) equipped with turbine impeller and connected to a digital control unit. The set points for temperature was 37°C, constant pH was not maintained, no compressed air was supplied, the agitation speed was maintained to 100 rpm, no antifoam agent was used. The composition of the medium was identical to the established optimal medium composition in flasks experiments. Every 2 hours, 20 ml sample was taken from the vessel for the determination of the phospholipase C activity, cell density, and pH of the culture.

Enzyme assays

The determination of phospholipase C activity is based on the enzymatic digestion of L- $\alpha$ -Phosphatidylcholine, extraction of the produced phosphate compounds, and determination of their concentration according to the method described by TAKAHASHI *et al.* (1981).

#### **Results and Discussion**

One hundred and sixty-six *Bacillus* strains were tested for production of PLC in a medium with phosphatidylcholine as e substrate. The number of strains by species is presented in Fig. 1a. Production of Extracellular Phospholipase C by Species of Genus Bacillus with Potential for Bioremediation



**Fig. 1.** Enzyme activities of strains of genus *Bacillus* – qualitative analysis. 166 examined strains (a.). Phospholipase C activity of strains of genus *Bacillus* (b.).

After the qualitative assays strains, which formed hydrolysis zones or formed haloes larger than 5 mm, were selected for the quantitative determination of enzyme activity. Of all examined strains thirteen were selected for quantitative determination of their extracellular phospholipase С activity. Bacillus thuringiensis №17 had highest phospholipase C activity reaching 19.61 U/ml (Fig. 2). In previous research we have shown that these strains can produce also extracellular amylase and protease enzymes with activity within the range of 2.80 - 9.20 U/ml (STEFANOV et al., 2018a; STEFANOV et al., 2018b).



**Fig. 2.** Quantitative determination of phospholipase C activity of strains of genus *Bacillus* 

The present work and in previous studies on protease (STEFANOV et al., 2018a) and amylase (STEFANOV et al., 2018b) activity showed that the examined strains are characterized by a diverse extracellular enzyme production. From all Bacillus cereus strains, 94% produced phospholipase C, and 93% produced proteases. Only 29% could produce lipases, 27% amylase enzymes, and 3% had cellulolytic activity. Eighty-seven percent of all examined strains of Bacillus thuringiensis had phospholipase C activity, 88% had protease activity, 16% had amylase activity, 15% had lipase activity, and only 2% exhibited cellulolytic activity. All examined strains of Bacillus sphaericus exhibited weak extracellular enzyme activity.

Bacillus species are known for their ability to secrete a vast variety of proteins in the culture medium. They can produce more than 40 different extracellular enzymes. Transcriptomic analyses of the species during different stages of cultivation show that they produce more than 3800 coding transcripts but in varying quantity. It has been shown that *Bacillus pumilus* transcribes genes that are coding two lysophospholipases and three phospholipases. Their initiated expression is during the exponential phase of growth, but its maximum is reached during the early stationary phase of growth. Some species of Bacillus have several genes that code different types of extracellular proteases, which are form bio-technical interest. It is shown that the expression of epr and subE begins at the exponential phase of growth, while aprE, aprX, and wprA are highly expressed during the stationary phase of growth. The major proteolytic activity of the isolated supernatant is contributed to the products of these 3 genes. All this shows that the expression of the different proteases is controlled different bv regulatory mechanisms and may even have different secretion paths (HAN et al., 2017).

The production of a vast variety of extracellular enzymes makes some strains of genus Bacillus valuable tools for saving the environment and avoiding ecological disasters. It has been shown that strains that proteolytic, produce lipolytic, and/or amylolytic enzymes could efficiently reduce the biological oxidation demand (BOD), chemical oxidation demand (BOD), nitrates and, phosphates by up to 56.25% after 72 hours (SONUNE & GARODE, 2018). This could prevent the depletion of oxygen in the water and the suffocation of its main consumers.

*Chromatographic analysis of the products from the hydrolysis reactions* 

The identification of the products of the hydrolysis reaction of L- $\alpha$ - phosphatidylcholine (Sigma-Aldrich, USA), mediated by phospholipase C, isolated from *Bacillus thuringiensis* No17, was done using thin-layer chromatography (Fig. 3). The reduced amount of the substrate (Fig. 3b) correlates with the appearance of the reaction products (Fig. 3a). It can be seen from the chromatogram that phospholipase C from *B. thuringiensis* No17 hydrolyzed the highest amount of the substrate.

The used substrate for the hydrolysis reaction was sonicated as a pretreatment to form lipid vesicles. Phospholipase enzymes prefer phospholipid substrates in the form of small vesicles with a diameter of 20-100 nm instead of single molecules. It was established by other researchers that the produced phospholipases from species of genus *Bacillus* bind strongly to vesicles with

a high content of phosphatidylcholine. Although the strong binding between the substrate and the enzymes, which lasts for an average of  $378 \pm 49$  ms, the high phosphatidylcholine concentration reduces the enzyme activity. Phospholipases also prefer smaller vesicles with higher surface tension (YANG *et al.*, 2015).



Fig. 3. Thin-layer chromatography of the substrate and the products of the enzyme hydrolysis with phospholipase C from *B. thuringiensis №17.* (1) L-α- phosphatidylcholine (99%, Sigma-Aldrich, USA); (2) the substrate after hydrolysis with PLC from *B. thuringiensis №17.* System (a) - petroleum ether:diethyl ether:acetic acid (6:4:0.2); System (b) - chloroform:methanol:water (65:25:4).

Dynamics of the phospholipase production during growth

The production of phospholipase enzymes started after the second hour of

cultivation and it slowly increased, reaching its maximum at the 8-th hour with an activity of 20.69 U/ml. After twelve hours of cultivation, the enzyme production slowly dropped reaching 2.42 U/ml at the 24-th hour. The pH of the culture medium doesn't change dramatically during the cultivation. In the beginning, the medium was weakly acidified to pH 6.75 at the fourth hour and then the medium gradually was alkalized to pH 7.90 at the 24-th hour. The analysis of the cell density showed that the exponential phase of growth begins nearly at the begging of the cultivation and it is over at the 8-th hour (Fig. 4).

The maximum enzyme production was reached between the late exponential phase of growth and the beginning of the stationary phase. The rate of phospholipase production correlates with cell density. This

type of fermentation, in which phospholipase C is produced from species of genus Bacillus is classified as growth associates (SHILOACH et al., 1973). This phenomenon may be explained by the fact that many (primary) enzymes, produced from *Bacillus*, are needed for the survival and development of the bacterial culture especially in the early stages of the fermentation (BLANCO et al., 2016). It has been discovered that the synthesis of phospholipases is under the control of the quorum-sensing system of the bacterial community (ELLEBOUDY et al., 2011; DONG et al., 2002), but the presence of these enzymes is not due to cell lysis or sporulation. Thus, higher phospholipase production is achieved at certain cell density. The enzymes are secreted in the medium (ELLEBOUDY et al., 2011).



**Fig. 4.** Growth and phospholipase C activity of *Bacillus thuringiensis* strain 17 in a liquid medium of GERASIMENE *et al.* (1980).

#### Effects of different carbon sources

To determine the effects of different carbon sources on the phospholipase C production, glucose, fructose, ribose, inulin, or maltose, respectively, at a concentration of 0.4%. were added to the base medium. The control medium contained glucose as a source of carbon (Fig. 5). Our results showed that maximum enzyme production was achieved when the medium was supplemented with glucose. High enzyme activity was also achieved when the strain was incubated in medium, containing maltose. The production was reduced when

the medium contained ribose or inulin (Fig. 5). The results of THAYUMANAVAN & BOOPATHY (2005) also show that glucose is a good choice as a source of carbon for the production of phospholipase C from B. thuringiensis. Bacillus thuringiensis can utilize other carbohydrates like fructose, sucrose, and lactose, but more complex carbon sources (like starch) are less preferable. This can reduce the cell density and the production of extracellular enzymes respectively (BULLA et al., 1980). It has been discovered that the use of molasses as a cheaper source of carbon results in lower phospholipase production (THAYUMANAVAN & BOOPATHY, 2005).



## Fig. 5. Effect of the carbon source on the phospholipase C production. \*Control – glucose.

According to ZHAO et al. (2018), research high concentration of the glucose, fructose or maltose in the liquid medium may reduce or even inhibit the synthesis of phospholipase enzymes. Our results showed that a gradual increase in the concentration of glucose to a maximum amount of 0.4% leads to a rise in phospholipase C production. Further increase in the concentration results in a decrease and inhibition of the enzyme synthesis. The results of THAYUMANAVAN & BOOPATHY (2005), show that maximum phospholipase production is achieved when the medium contained 0.65% of glucose. For many microorganisms, glucose is the preferred carbon source. When it is present in the medium in excess, it may repress the catabolism of other substrates and the synthesis of the needed enzymes. This phenomenon is called catabolic repression and it is an evolutionary mechanism, which allows the organisms to utilize first the substrate, which guarantees them the fastest growth in the competitive environment (SINGH & BAJAJ, 2004).



**Fig. 6.** Effect of glucose concentrations on the phospholipase C production. \*Control - 0.4% glucose.

#### Effects of different nitrogen sources

The source of nitrogen is another important factor for the growth of the bacterial culture and for the production of phospholipase C. In the current study the influence of six nitrogen sources was studied - peptone, bactopeptone, casein hydrolysate, potassium and veast extract, nitrate, ammonium sulfate. The results showed that the production of phospholipase C is slightly higher when the growth medium was supplemented with yeast extract. It has been proven that *Bacillus thuringiensis* №17 cannot utilize inorganic sources of nitrogen (Fig. 7). Although some strain can utilize inorganic nitrogen compounds, the majority of Bacillus thuringiensis strains prefer organic nitrogen (Thayumanavan & Boopathy, 2005; EL-BENDARY, 2006). To ensure the growth of the bacterial culture the medium must be supplemented with source of at least one of the following amino acids - glutamate,

aspartate, valine, leucine, serine or threonine, but the addition of cysteine or cystine will lead to full growth inhibition and the formation of toxic compounds (EL-Bendary, 2006), but complex organic sources of nitrogen are preferred - peptone, casein hydrolysate, yeast extract and others (ICGEN et al., 2002). When the medium is rich in free proteins and protein hydrolyzates the added free amino acids are used as a source of carbon (EL-BENDARY, 2006).





#### Effects of different metal ions

More than half of the discovered enzymes are metalloenzymes, including the phospholipases, but according to other researchers, only 30% of the enzymes require metal ions to carry out their normal functions (WALDRON & ROBINSON, 2009). Phospholipases, produced by species of genus Bacillus contain two zinc atoms in their protein structure. The removal of one of the zinc atoms results in partial reduction of the enzyme activity. The removal of the second atom results in the full inhibition of the activity. This effect is reversible if zinc ions are added to the enzyme solution (LITTLE & OTNASS, 1975). Some metal ions can enhance the enzyme activity and others can inhibit it. In the current study, the influence of 4 sources of metal ions in 1 mM

concentration (CaCl<sub>2</sub>, ZnCl<sub>2</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>) on the phospholipase C production was studied. As a control was used a medium that didn't contain any metal ions. The results showed that the enzyme production is reduced when the medium contained manganese ions. The production of phospholipase C is greatly enhanced when the medium contained zinc ions. Other researchers also report that the presence of metal ions in the medium is of great importance for the growth of the bacterial producer and for the enzyme production (THAYUMANAVAN & BOOPATHY, 2005). Zinc ions can directly participate in the enzyme reaction or they may maintain the protein structure stable. They also function in all catalytic sites as Lewis acid - chemical compound, containing free orbital, which is capable of accepting electron pairs.





#### Effects of the volume of inoculum

To determine the effects of the volume of the inoculum over the production of phospholipase C, Erlenmeyer flasks with a sterile medium where inoculated with varying volumes (between 1 – 10%) of 8-th hour bacterial culture with a cell density of  $1.4 \times 10^9$  cfu/ml. It was found that maximum enzyme production occurs when the medium is inoculated with 7.5% of

inoculum. The quantity of the produced enzyme between the different examined volumes of inoculum is not significant, but the tendency is clearly visible. The rate of enzyme production rises with the larger volumes of inoculum. The reduction of the enzyme production when using volumes larger than 7.5% may be explained with the more intensive consumption of nutrients in the medium. The bacterial culture also produces compounds that are inhibiting the growth and development of the culture. (HAYES & LOW, 2009).





Production of phospholipase C in bioreactor

The dynamics phospholipase of production was studied in a scale-up experiment using a bioreactor with a volume of 2 liters. Bacillus thuringiensis №17 was inoculated in a medium with the determined optimum composition. The production of phospholipase C enzymes began at the second hour of cultivation, slowly increased and reached its maximum at the 8th hour of cultivation with activity of 14.87 U/ml after which the production dropped down. During the cultivation, the medium was slowly acidifying reaching pH 6.21 at the twelve hours of cultivation. The bacterial culture entered almost immediately in the exponential phase of growth, which ended after the 6-th hour of cultivation. In our scale-up experiment, maximum phospholipase production was achieved in the early stationary phase of cultivation with pH 6.17 of the medium.





Although during the cultivation in bioreactor and Erlenmeyer flasks the production of phospholipase C was in identical time intervals, the cell density, pH of the medium and enzyme activity varied. During the cultivation in flasks, the medium first slowly acidified to pH 6.75, but later the medium was alkalized to pH 8. During the cultivation in a bioreactor, the medium was steadily acidified to pH 6. The difference in the cell density of the two cultures was quite significant. In flask maximum enzyme production was achieved when the culture had a cell density of 7.4 McF, but in bioreactor only 5.9 McF. The difference equals 0.5 x  $10^9$  cfu/ml. Even the enzyme activity was lower when the strain was cultivated in a bioreactor. This can be explained with many factors that are important during scale-up fermentation the form of the vessel, the speed of agitation, the supplementation with oxygen, production of foam, the influence of antifoaming agents and others. Many researchers also describe similar effects although using similar medium composition and fermentation conditions the results

between flask and bioreactor fermentation differ from one another (BEG et al., 2003). The lower cell density may correlate with the lower enzyme production. It is discovered that cell communication in the form of quorum sensing is regulating the gene expression in the bacterial community. In gram-positive bacteria, cell communication is using cytoplasmic secreted sensors, regulated by and reimported signal peptides. Quorum sensors in genus Bacillus include Rap, NprR, and PlcR, which are part of the RNPP protein family. Besides Rap these RNPP proteins are transcriptional factors, which directly regulate the gene expression. In this quorum-sensing regulates many way, important functions in the Bacillus cereus group. In this situation, quorum-sensing regulates the expression of phospholipase enzymes (which expression is regulated by PlcR) (SLAMTI et al., 2014). If the cell density is not high enough, the cell to cell communication is hampered and the expression of some key enzymes is reduced.

#### Conclusion

It was determined that *B. thuringiensis* No17 was the best producer of phospholipases between the studied species of genus Bacillus, with an activity of 19.61 U/ml. The strain showed consistent extracellular phospholipase C activity. The production of phospholipase C was detected at the second hour of cultivation and it reached its maximum at the 8-th hour of cultivation during the end of the exponential and the beginning of the stationary phase of growth. Such early production of extracellular lipolytic enzymes is a feature of economic phospholipase value. The highest С production was achieved when the medium was supplemented with 0.4% of glucose, yeast extract as a source of nitrogen and 1 mM ZnCl<sub>2</sub> and inoculated with 7.5% of inoculum. After the optimization of the medium, the duration of the cultivation, and the volume of used inoculum, the activity reached 31 U/ml. Scale-up study with a

benchtop bioreactor with a volume of 2 liters achieved the highest exoenzyme production at the 8 hours of cultivation, which correlated with the flask experiments. The selected strain and studied phospholipase enzyme have a significant potential for application in the field of bioremediation cleaning contaminated soils, digesting of spilled oils and fats in wastewaters and even reducing the energy consumption and the generated greenhouse gas emissions in some industries. Replacing the old chemical technology of refining oils with enzyme degumming using phospholipases results in reduced production of carbon emissions, reduced consumption of dangerous chemical compounds and product with higher quality. Phospholipases can be a sustainable and effective alternative to some old and inefficient technologies.

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Short note

### Successful Wintering of the Noctule Nyctalus noctula on a Balcony in Warsaw (Central Poland)

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**Abstract.** Wintering of the noctule *Nyctalus noctula* was recorded on a balcony in Warsaw (central Poland) in winter 2018/2019. About 40 individuals of this species inhabited a shopping bag closed on top. At the end of February and the beginning of March 2019 bats ended hibernation and flew out of this provisional roost. Three dead individuals remained there. Recent climate change resulting in milder winters makes noctules able to overwinter in central Poland even in shelters poorly isolated from external conditions.

Key words: bats, Chiroptera, behaviour, hibernation, climate warming, urban habitats, Poland.

#### Introduction

Several decades ago, in the second half of the 20<sup>th</sup> century, the noctule Nyctalus noctula (Schreber, 1774) from central and eastern Europe was considered a species performing regular seasonal migrations at distances as long as 1500 km or more (STRELKOV, 1969; HUTTERER et al., 2005). Polish noctules were found during hibernation in areas located south and south-west from Poland (HUTTERER et al., 2005). Its natural roosts are known mostly from tree holes (GEBHARD & BOGDANOWICZ, 2004) which makes difficult hibernation in colder parts of Europe. Recently, the cases of overwintering of this species, especially in urban areas, have been more and more often recorded in central part of the continent (GAISLER, 1999; KAŇUCH & 2000; DZIEGIELEWSKA Celuch, DZIEGIELEWSKI, 2002; BIHARI, 2004; CEĽUCH et al., 2006; CICHOCKI et al., 2015) as well as in

eastern part of the continent (GODLEVSKA, 2015). The number of hibernating noctules also markedly increased in various regions of Poland, including eastern part of the country (LUPICKI *et al.*, 2007). This species is common in the whole territory of the country and belongs to the most abundant bats (SACHANOWICZ *et al.*, 2006).

#### **Results and Discussion**

Wintering noctules were found on a balcony on the second storey of a residential block (Fig. 1) in Warsaw, central Poland (52.28 N, 20.98 E). The distance of this site to central point of a city is 6 km. Owners of the apartment found bats in a shopping bag made of thick linen and closed atop (Fig. 2). On 29<sup>th</sup> January 2019 the place was searched and it appeared that noctules winter there in an aggregation of about 40 individuals (Fig. 2). After raising the bag most bats slightly moved,

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Fig. 1. Residential building where noctules hibernated (Photo: K. Janus).



Fig. 2. Wintering shelter of a group of the noctule on a balcony (Photos: K. Janus).

which evidenced that animals were alive. In the end of February and the beginning of March the bats left their wintering place. Three dead individuals remained there.

Winter 2018/2019 was relatively mild in central Poland. Mean air temperature of the coldest month (January 2019) was -1.6°C. Minimum recorded temperatures were: -7.3°C (November 2018), -10.5°C (December 2018), -11.9°C (January 2019) and -7.3°C (February 2019). The longest periods when the lowest daily air temperatures fell below -5°C occurred in January and lasted five and three days (data of IMGW-PIB). According to information provided by the owners, bats were seen in previous years in late autumn and early winter entering a crack in the wall just above the balcony. This roost was used by wintering individuals. In 2018 the crack was sealed when the wall was insulated. Hibernation site selected by noctules close to previous roost indicates a high level of site fidelity of this species.

Regular and frequent wintering of the noctule in Warsaw is a relatively new phenomenon. Two decades ago, winter findings of this species were still rare (LESIŃSKI et al., 2001), while in the last years the bats have been frequently noted (unpublished data of authors). In this part of Europe bat hibernation in poorly isolated sites is difficult. The noctule belongs to species resistant to periodical temperature declines below 0°C. It was found that winter aggregations are able to survive severalday-long frosts even fifteen degrees below zero (SLUITER et al., 1973). This feature may explain successful overwintering of most individuals that chose shelters poorly protected against frosts in Warsaw. Attempts of wintering on a balcony were also noted in Wrocław in south-western Poland (CICHOCKI et al., 2015) where winters are definitely warmer than those in Warsaw. Circumstances of that finding were similar - bats occupied the balcony when they lost shelters on attic due to renovation of the whole building.

Due to recent climate change and offer of alternative roosts in urban areas, the noctules have probably adjusted their migratory behaviour from long-distance seasonal migrations towards south hibernacula to occasional hibernation in the area of summer residence. This change is beneficial because migrations pose numerous risks and increase biological exhaustion of an organism. One may expect that the sedentary individuals of the noctule will achieve a slightly greater longevity. The longest lifetime of the species recorded so far is about 12 years (HEISE & BLOHM, 2004). If similarly mild winters will happen more regularly, one may expect that the number of noctules wintering in Poland will increase, also in eastern part of the country, which is characterised by a colder climate.

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