ECOLOGIA BALKANICA

International Scientific Research Journal of Ecology

Volume 13, Issue 2

December 2021

UNION OF SCIENTISTS IN BULGARIA – PLOVDIV

UNIVERSITY OF PLOVDIV PUBLISHING HOUSE

International Standard Serial Number
Online ISSN 1313-9940; Print ISSN 1314-0213 (from 2009-2015)

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- Scopus (SJR_{2020} = 0.144; $SiteScore_{2020}$ = 0.2; $SNIP_{2020}$ = 0.207)
- SCImago Journal Rank (SJR, 2016-2020 - Q4)
- Thomson Reuters Master Journal List - Zoological Record (Web Of Science)
- Thomson Reuters BIOSIS Reviews (Web Of Science)
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- All-Russian Institute of Scientific and Technical Information (VINITI) (Referativnyi zhurnal)
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Union of Scientists in Bulgaria – Plovdiv
6 Metropolit Paisii Str., 4000 Plovdiv, BULGARIA
E-mail: ecologia_balkanica@abv.bg

University of Plovdiv Publishing House
24 Tsar Assen Str., 4000 Plovdiv, BULGARIA

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ECOLOGIA BALKANICA – Volume 13, Issue 2 / 2021

CONTENTS

Research Articles

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 1-21

Influence of Landscape Organization on Surface-water Quality Forming on an Example of Ustya River Basin (Ukraine)

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Abstract. River ecosystems of Polissya are in a state of deep degradation. Agricultural lands, close to watercourses, are localized on the terraces of river valleys, in some places - floodplain. Such placement of lands in case of improper operation poses an ecological threat not only to the Ustya River, but also to the entire river system of the Goryn River, a tributary of which it is. With this in mind, the aim of our research was to assess the current hydrological, hydrochemical and hydrobiological condition of water bodies in the Ustya River basin, to identify the causes of possible deterioration and to justify measures to improve them. The ecological condition of the aquatic ecosystems of the Ustya River, in particular their soil cover, the granulometric composition of the basin and its contribution to the pollution of surface waters of the territory are determined in article. The ecological condition of the Ustya River basin is assessed and the main factors of anthropogenic changes of aquatic ecosystems within the Ustya River basin are determined, as well as the scale and consequences of their impact on water quality. The analysis of the processes of water quality formation within the water bodies of the Ustya River basin is carried out, the main directions of dynamics on three blocks of indicators are estimated and the integrated ecological indices of water quality in the observation points are determined. Solving the set tasks allowed us to comprehensively assess the ecological situation of landscapes in the Ustya River basin.

Key words: aquatic ecosystems, floodplain soil, water quality, sustainability of landscapes.

Introduction

The formation and sustainable development of the ecosystem is based on the ecological conditions of the respective landscapes, on the basis of which their individual morphological features are formed (Fedonyuk et al., 2019). One of the priority areas of sustainable ecosystems and solving environmental problems that are formed at the local, regional and global levels is the optimal organization and land use of state territory on the land, which gives the greatest effect of environmental measures (Jungwirth et al., 2002). This approach is important for the use of river ecosystems, which are characterized by complex terrain and intense erosion processes.

River ecosystems of Polissya are in a state of deep degradation, which consists of siltation, lowering of water levels and total pollution (Berge et al., 2010). The degradation of Polissyan small rivers has been intensive during the last 60-70 years, which is due to the significant plowing of landscapes up to the water's edge. As a result of such management, the streams that replenish the rivers began to disappear. This, in turn, has led to siltation of the river, overgrowing it with wetland vegetation, waterlogging, reducing runoff and deteriorating water quality (Gupalo et al., 2020).

Agricultural lands close to streams are located on the terraces of river valleys, in some places - floodplain, especially in the delta arm (lower) part. A significant part of them is covered by drainage and irrigation melioration systems, from which water is discharged into watercourses, streams, etc. Such placement of lands in case of improper exploitation and lack of constant ecological monitoring poses an ecological threat not only for the Ustya River, but also to the entire river system of the Goryn River, a tributary of which it is.

According to various sources of information, today in Ukraine the natural landscapes of rivers are almost destroyed, or are at different stages of degradation. And water protection zones, if any, are reduced to small coastal plantations that do not properly perform the function of sewage filtration (Hryb & Voytyshyna, 2009; Vystavna et al., 2018; Reva et al., 2014).

The main hydro-chemical parameters of the Ustya River tributaries have been at a consistently low level for the last five years. Some of the tributaries are contaminated with organic matter (1.1–1.7 MPC) and total iron (1–2.8 MPC) (Shelyuk & Shcherbak, 2018).

In the Pripyat basin, where the Goryn River and its tributaries belong, the ecological condition of small rivers has deteriorated in recent years due to the inefficient operation of existing treatment facilities of both large enterprises and production departments of housing and communal services. The level of mineral nitrogen, which reaches 1.4 mg / l (1 - 7 MPC for various water bodies of the basin), has increased especially (Fedoniuk et al., 2019).

Proof of the unfavorable state of the hydro-ecological network of Ukraine is a comprehensive assessment of the ecological condition of small river basins located in different natural areas, conducted by the Ukrainian Research Institute of Water Management and Environmental Problems under the guidance of Professor AV Jacyk (Jacyk et al., 2007). Of the 62 pools studied, only one is characterized as "minor changes". The ecological condition of the six-river basins (10%) was assessed as satisfactory, 25 (40%) as bad, 19 (31%) as very bad and 11 (18% of all studied basins) as catastrophic.

The Ustya River basin with all its constituent components - riverbed, floodplain, terrace, slopes is a sufficiently self-regulating system that is able to function independently of the influence of external factors (Romanchuk et al., 2017; Klymenko et al., 2018; 2019; Fedoniuk et al., 2019). The geosystem of the basin is quite complex both in terms of the number of factors that affect its functioning and the peculiarities of interaction between them. Given the basin approach provided by the EU Water Framework Directive, economic entities, influencing certain natural components, make their adjustments to the entire ecological state of the studied hydro system (Fedoniuk et al., 2019b). In this way, the river becomes an integral indicator of environmental quality, where the indicators of landscape structure, surface water quality and diversity of living organisms are closely interrelated. Many authors note the impact of water quality and landscape structure on biodiversity over the living conditions of organisms (Mollov et al., 2009; Yancheva et al., 2015; Fedoniuk et al., 2020). As a result, there is a need to outline a system of indicators and tools for accurate forecasting of possible changes in the qualitative and quantitative state of individual components of landscapes and their possible impact on surface water quality. It should become a tool to optimize river basin management, formation, use and protection of the landscape.

The aim of this study was to assess the current hydrological, hydro-chemical and hydro-biological status of water bodies in the Ustya River basin, to identify the causes of possible deterioration and to find relationships between deterioration factors and water quality indicators.

Material and Methods

To analyze the ecological situation in the basin of the Ustya River, water, coastal soil and bottom sediments were taken. Sampling of soil, bottom sediments and water was carried out at points confined to large settlements and places of increased anthropogenic pressure from the source to the mouth of the Ustya River. A total of 10 permanent observation points were laid, as well as soil samples at a distance of 5-50 m from the water's edge. The observation points assessed the relief, the rate of land transformation, soil and vegetation, fauna, sources and volumes of anthropogenic pressure and the main causes of landscape degradation (Table 1).

The research scheme was divided into three main blocks:

1. Description of the river basin: hydrographic and water management zoning, climate, relief, geology, hydrogeology, soils, vegetation, hydrological regime, features of the river basin.

2. Identification and classification of water bodies (WM), as well as analysis of their initial state - the territory of the basin was characterized by indicators: ecoregion; category of surface waters; typology; geographical and hydromorphological differences; change of ecological condition; protected natural areas.

The study of particle size distribution was carried out on the basis of sampling from 220 points. The particle size distribution was determined in accordance with Kaczynski's classification (Kaczynski, 1953).

The ecotonization index was applied to assess the ecological stability of the landscape (Timofeeva, 2014):

$$Y = Li / S;$$

where: Y - index of species diversity;
L - the length of the ecotone, m;
S - area of the agro-landscape, ha.

Estimation of the index of species diversity: less than 5 m / ha - very weak ecotonization; 5-10 m / ha - weak ecotonization; 10-20 m / ha - average ecotonization; more than 20 m / ha - high ecotonization.

3. Analysis of the structure of the landscapes of the Ustya River basin according to the indicators of coefficient of ecological stabilization of the landscape (KESL1) and the coefficient of ecological stabilization of biotechnical elements (KESL2) and their influence on the formation of surface water quality.

To assess the ecological condition of landscapes within the location of observation points used the method of E. Klementova and V. Heinige (Klementova & Heinige, 1995), according to which we calculated the KESL1 and KESL2. The calculation of the anthropogenic transformation index was carried out according to the method of Hoffman KG (Hoffman, 1977). Estimation of the degree of anthropogenic transformation of the territory and calculation of anthropogenic transformation of the territory of the agro-landscape was carried out according to the method of Shishenko PG (Shishenko, 1999).

Water samples were taken for chemical and biological analysis, physical properties were determined in the field, other samples were preserved in accordance with the methods of sample preparation for analysis (Instructions for sampling, preparation of water and soil samples for chemical and hydro-biological analysis by hydro-meteorological stations and posts, 2016), approved by the Order of the SES of Ukraine dated January 19, 2016 № 30.

Water quality at the observation points was assessed in accordance with Romanenko & Zhukinskiy (1998), according to which all analyzes to determine water quality indicators were divided into three main blocks:

1. Block of water quality assessment according to the criteria of salt composition. This group includes indicators of water quality in terms of mineralization and electrical conductivity, sulfate and chloride content.

2. Water quality assessment unit according to chemical saprobological criteria. This group includes indicators of water quality by oxygen regime, suspended solids, transparency, pH, ammonium nitrogen content, nitrates and nitrites, phosphorus phosphates, permanganate oxidation and biochemical oxygen consumption.

3. Block of water quality assessment according to the criteria of content of specific substances of toxic and radiation action. This group includes indicators of water quality in terms of total iron, manganese, petroleum products, phenols, surfactants in water, cadmium, copper, zinc, total chromium, nickel in bottom sediments.

Each individual quality index was determined for each indicator and the average block index was calculated taking into account all these indicators. In accordance with the obtained data, the total ecological index of I_E water quality was calculated as the average value of the three block indices.

To determine the subcategories of water quality corresponding to the average values of group. In accordance with the same method, certain classes and categories were assigned according to ecological conditions: I class with one category (1) - excellent; Class II - good, with two categories: very good (2) and good (3); Class III - satisfactory, with two categories: satisfactory (4) and mediocre (5); IV class with one category (6) - bad; V class with one category (7) - very bad. We also compared the results of analyzes with SanPiN # 4630-88 Sanitary rules and standards for the protection of surface waters from pollution.

ArcGis Pro 2.5.0 software was used to map the project, and a digital terrain model was developed using the Geoprocessing: 3D Analyst toolkit. Tracing of watercourses, delineation of watercourses and their orders was carried out using the software product ArcGis Pro 2.5.0, ingesting the algorithm of hydrological modeling of the working module Spatial Analyst: Hydrology - "basin", "catchment area", "flow direction" and others hydrological parameters. The order of watercourses was determined by the Strahler method (Strahler, 1952) on the basis of a digital terrain model applying the same software product. The hydrological modeling was done on the basis of a digital elevation model.

Results and Discussion

According River passport the water body is the Ustya River, a tributary of the Goryn River, which in turn belongs to the basin of the Pripyat River. The total length of this river is 68 km. The catchment area is about 762 km^2. The Ustya River, which flows from south to north in Rivne and Zdolbuniv districts, is located in the southern part of Rivne region, or more precisely on the Volyn upland. The formation of a hill on a submerged structural basis is considered a kind of inversion of the terrain (Fig. 1). Ustya has 28 tributaries up to 10 km and three tributaries larger than 10 km: Bezodnya (length - 13 km, catchment area - 68.9 km^2) and Ustya (stream) (length - 24 km, total catchment area - 126 km^2).

All rivers of Rivne region belong to the basin of the Pripyat River, they are fed by meltwater, snow water. A smaller share of water is formed due to the inflow of groundwater and precipitation. Types of soils such as sod-podzolic

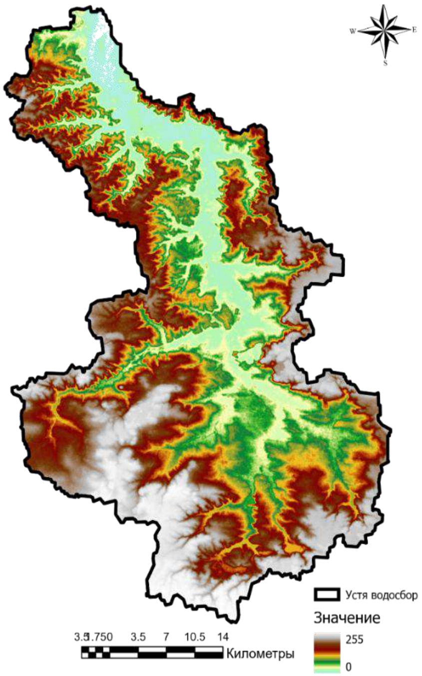


Fig. 1. Digital relief model of the Ustya River basin.

gleyed, light gray and gray podzolic, chernozems podzolic and peat soils are dominant in the catchment area of the Ustya River. Most of the soils inherent in this area are characterized by low natural fertility.

Water bodies of the study area belong to M5.1.4 - Sub-basin of the Pripyat River, water management area M5.1.4.46 - Goryn River from the border of Khmelnytsky and Rivne regions to the state border (excluding the river Sluch). A number of small rivers and streams, which are tributaries of the Ustya River, flow through the basin. Among the tributaries are the largest Kunin and Spasov.

According to agro-climatic zoning, the Ustya River basin belongs to the zone of sufficient moisture with SCC in the range from 1.3 to 1.6 with a moderately uncomfortable subzone for bioclimatic zoning. In accordance with the geobotanical zoning, the Usti basin lies entirely within the Central European Province of Deciduous Forests, South Poland-West Podolia Sub-Province of Deciduous Forests, Meadows, Meadow Steppes and Eutrophic Swamps of the Lublin-Volyn District of Hornbeam and Oak, Oak and Oak.

The surface waters of the region are defined as hydrocarbonate-calcium, with the sum of ions from 100 to 200 mg / l, and the total hardness of 1.5-2.5 mg-eq / l.

The Ustya River basin is located in the region of enhanced erosion processes, where the area of washed away soils is up to 70%. Erosion, contamination with fertilizers and pesticides and the use of heavy machinery should be singled out among the factors that determine the structural features of soils.

The soil cover of the Ustya River basin will mostly cover agricultural landscapes. Agro-landscapes occupy the largest part, but under natural landscapes such a large share remains. The analysis of particle size distribution allowed to classify soils at the level of varieties, and to assess their physical properties. In samples taken in floodplain soils in the Ustya River basin, 4 soil varieties were found: heavy loam, medium loam, light loam and light clay, with a predominance of medium and light loams 66 and 31%, respectively) (Fig. 2).

Analysis of the particle size distribution of floodplain soils in different areas of the Ustya River basin showed that the predominant soil fractions here are coarse-dusty - 43-67% and dusty - 2-30%. This fractional ratio is explained by the peculiarities of the hydrological regime, namely the position in the basin, periodic spills, depth and flow velocity. According to the results of the research, the content of physical clay (less than 0.01 mm) did not vary much depending on the zones of the basin, and for the upper, middle and lower parts of the basin ranged from 27 to 56%. At the same time, insignificant variation of the range of minimum and maximum values was noted only in the middle zone.

The soils of the Ustya River basin in different parts of the basin do not differ significantly in particle size distribution. in all zones the coarse-dusty fraction is predominant - 43-67% and dusty 12-31% (Fig. 3). However, the increased content of dusty fraction does not significantly affect the water-physical properties of the soil.

For a detailed landscape analysis, we divided the territory of the Ustya river basin into separate arrays, the precondition for the division was hydrographic features and natural and anthropogenic circumstances that have developed historically in some parts of the studied basin (Fig. 4).

Thus, separate arrays are tributaries on which settlements are located, which in one way or another make certain adjustments to the quality of water in the first sections of the river, as well as individual fragments of the river, in which changes in water composition may be influenced by certain natural or anthropogenic factors (urbanization, wastewater discharge, etc.) - see Table 2.

The study of the soil cover of the catchment area of the Ustya River showed the predominance of loamy soils. Such soils are favorable for water and physical properties, have sufficient gas exchange, loose and poorly compacted composition, have sufficient moisture permeability, but they are largely susceptible to water and wind erosion. The forest cover of the territory is extremely low. In the overall structure of the basin, it is 5.8% (Table 3).

Other important indicators that affect the quality of surface waters of the territory is the plowing degree of the territory. It was noted above that although the plowed area in the river basins of Rivne region reaches 70-80%, the Ustya river basin and its tributary plowed less - 55%, but in the basins of the tributaries Untitled (right tributaries, Kunin and Spasov) reaches 60%. Given the state of plowing of the territory, the ecological state can be defined in general as "intense", and the degree of plowing is "medium" (Table 4).

The basin of the Ustya River lies within the Ecoregion 16 Eastern Plains, the type of river is defined as UA_R_12_S_2_Si - a small river on a hill in silicate rocks (according to the Methodology for determining the arrays of surface and groundwater (paragraph 16 of section II)).

A great threat to the quality of surface waters of the region is a significant decrease in the piezometric level in the artesian horizon, which leads to the flow to it over the entire area of depression of substandard waters from adjacent waterproof strata containing high concentrations of sulfates, chlorides, sodium, fluorine, sodium, hydrogen sulfide, iron.

Table 1. Monitoring points within the Ustya River basin.

No	Research points
1	Ustya River, 0 km, place of confluence with Horyn river, below the village Orzhiv.
2	Ustya River, 5 km in relation to the confluence point in Horyn river, 3 km above the village. Orzhiv.
3	Ustya River, 20 km in relation to the Goryn River, 1 km below the city of Rivne, 4.5 km below the discharge of wastewater from the USC of the city of Rivne.
4	Ustya, Rivne, below the entrance of the reclamation canal, the area of st. Zolotiyivska.
5	Ustya, within the city of Rivne, near the pizzeria "LaRiva".
6	Ustya River, Rivne, below the storm sewer discharge in the area of Myru Avenue.
7	Ustya River, 25 km in relation to the confluence point in the Goryn River, 0.25 km below the wastewater discharge from the USC of Rivne.
8	Ustya, Rivne, near the cafe "Venice".
9	Ustya, Rivne, district street Soborna, transition to the Wild Market.
10	Exactly, lake. Hydropark, on the side of Zdolbunivska Street.
11	Ustya, Rivne, Avangard Stadium.
12	Ustya River, 30 km in relation to the confluence point in the Goryn River, 2 km above the city of Rivne, 0.2 km below the discharge of wastewater from the USC of the city of Rivne.
13	Ustya, Rivne, district street Botanical.
14	Lake Basiv Kut, within the city of Rivne, from the bridge, at the confluence with the Ustya River.
15	Ustya River, 35 km from the confluence point with the Horyn River, 1.2 km from the border of Rivne and Zdolbuniv districts and 2 km below the confluence of the tributary of the Spasiv, where the settlements of Hlynsk and Spasiv.
16	Ustya River, below Zdolbuniv, 0.5 km above the discharge from the USC of Kvasyliv village of the Rivne District Housing and Utilities Service.
17	ОСК смт. Kvasyliv of the VKH service of Rivne district, leaving the OS.
18	Ustya, Novy Dvir, street Priozernaya Dachna, from the bridge.
19	Ustya River, 38 km in relation to the confluence point in Horyn river, 20 km above Rivne, below the village of Branch-2.

20	Ustya River, 45 km in relation to the confluence point with the Goryn River, a reservoir below the village of Ivachkove, at this point a tributary is formed, without a name.
21	Ustya River, 60 km from the confluence point with the Goryn River, 1.8 km below the village of Derman-1.
22	Ustya, river source, point on the earth's surface, located above 2 km (southwest) s. Derman-1, 65 km in relation to the confluence point in the river Goryn.

Table 2. Soil cover of the Ustya River basin.

No	Soil name	Area, ha	Power of humus horizon, cm	Organic matter, %	Soil losses from water erosion from the open surface t/ha per year	Assessment of soil erosion resistance	Actual soil losses due to erosion t/ha per year	Maximum allowable erosion rate t/ha per year
1.	Gray podzolic weakly and moderately washed light loam soils on loess loam	25900	20-31	1.1-2.1	14-20	Weak	6-12	2-3
2.	Dark gray light loam on loess loams	14770	26-39	1.3-2.6	5-7	Medium good	4-6	3-4
3.	Slightly washed loamy chernozems on loess loams	11600	35-60	2.7-3.8	25-29	Average	12-15	4-5
4.	Loamy chernozems on loess loams	7900	45-85	3.5-5.0	6-9	Good	5-7	5-6
5.	Meadow-chernozem medium loamy on loam	5850	45-95	4.1-5.8	1-2	Good	1-2	5-6

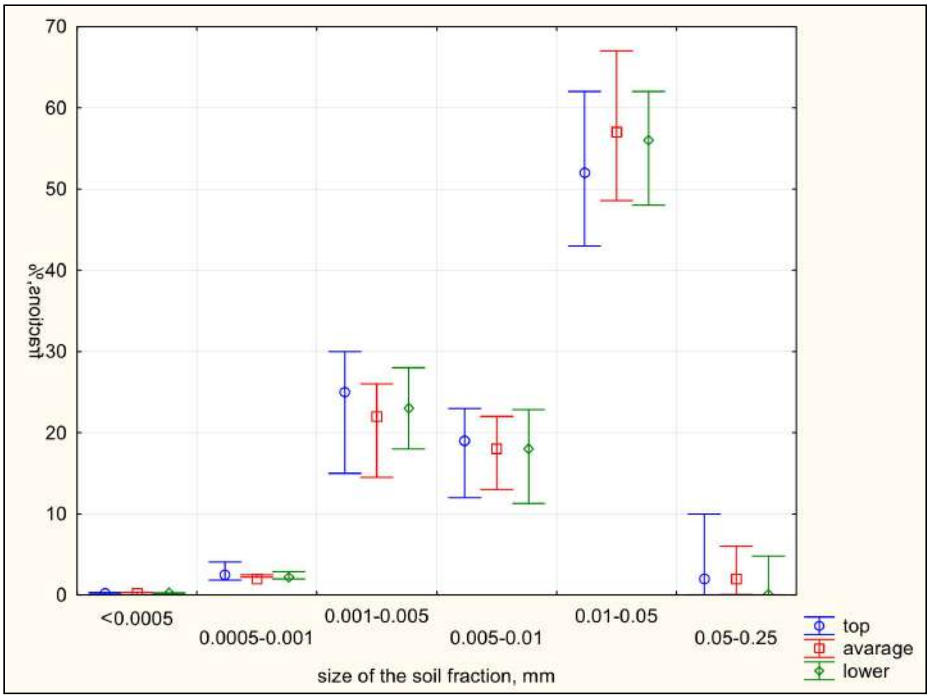


Fig. 2. Granulometric composition of floodplain soils of the Ustya River basin.

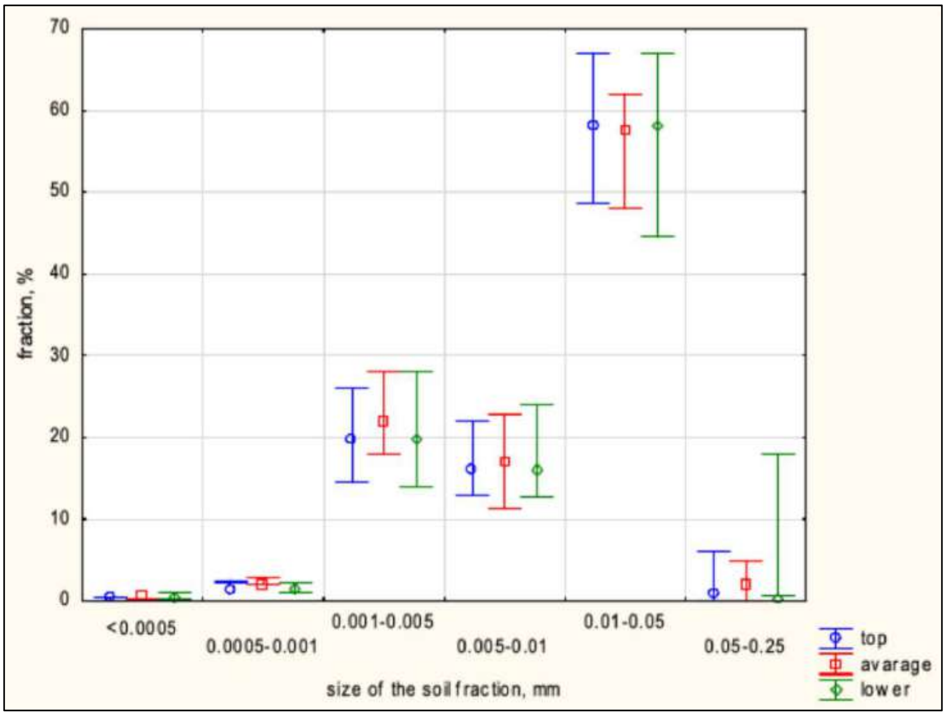


Fig. 3. Fractional composition of soils of different parts of the Ustya River basin, %.

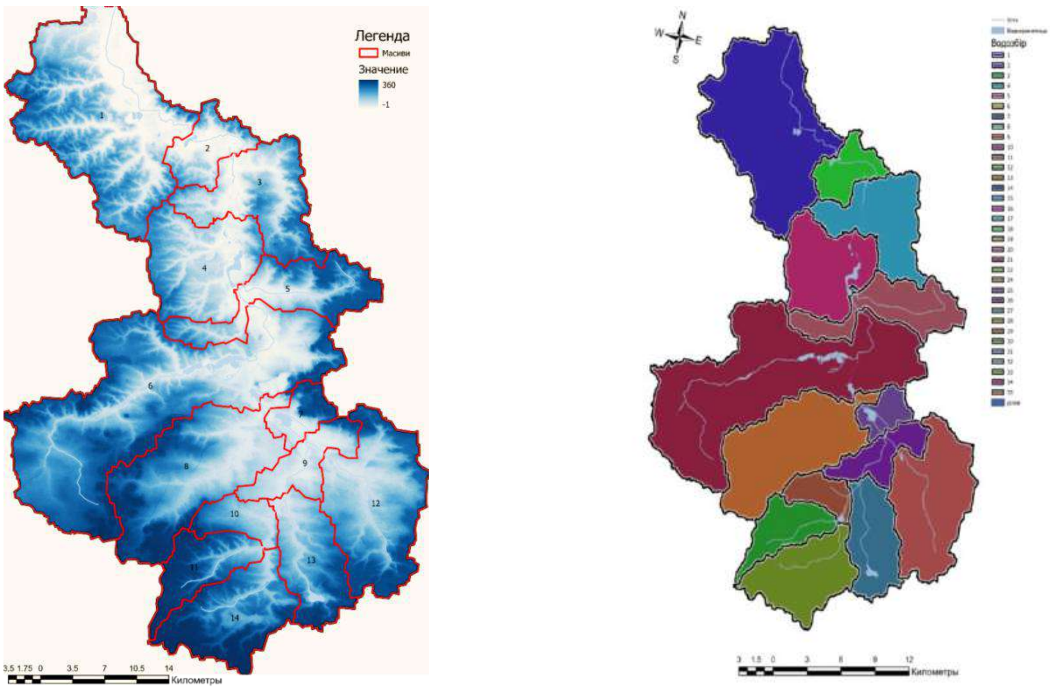


Fig. 4. Hydrographic network (left) and arrays of the Ustya river basin (right).

Table 3. Descriptions of the arrays of the Ustya River basin.

Array	Area, km^2	Description
1	120,69146	The river section from the village Orzhiv to the village Gorodok
2	21,30160	The river section from the village Zoziv to the village Velykyy Oleksin
3	49,28374	The river section from the village Maly Oleksyn to the Olimp Stadium in Rivne (section of the Ustya River below the wastewater discharge from the Rivne USC of the Rivne VKG service)
4	51,77316	Rivne (above the wastewater discharge) to the Basiv Kut reservoir
5	42,08506	village Novyi Dvir, a right-hand tributary from the village Kvasyliv
6	167,38465	Kvasylivsko-Zdolbunivska section, tributary Shvydivka
7	13,40319	village Novomilsk, pond Staromilsky
8	79,67950	A tributary of the Abyss from the village Kunin to the village Zdovbytsia
9	22,46611	village Ivachkiv, the confluence of the tributary, which originates near the village Ukrainske
10	14,97197	village Druga Gilcha, pond
11	26,13729	A tributary that originates near the village Klopit and flows near the village Druga Gilcha
12	69,31233	A tributary that originates near the village Ploske to the village Uvachkiv
13	36,51726	village Derman-1 of Zdolbuniv district
14	46,99269	Untitled tributary 2 (Verkhiv village)
Total area, km^2	762.215463	Untitled tributary 3 (Ivachkiv village)

Table 4. Ecological and landscape analysis of the Ustya River basin and its largest tributaries.

Characteristic	Ustya		Untitled (right tributary)		Kunin		Spasiv	
	km^2	%	km^2	%	km^2	%	km^2	%
Forest cover	55.7	5.8	5.7	8	9.9	13	16.1	13
Wetlands	6.3	0.7	1.8	2.5	6.08	8	1.9	1.5
Lakeiness	8.2	0.9	0.14	0.2	0.08	0.1	8.06	6.5
Protected areas	21	2.2	0.21	0.3	0.15	0.2	2.6	2.1
Plowing	529	55	43.7	61.5	45.6	60	73.2	59
Drainage	232.0	24	10.2	14.3	13.9	18.4	4.2	3.4
Urbanization	97.3	10.1	1.8	2.5	0.91	1.2	4.96	4.0
Others	32.7	3.4	6.03	8.5	36.2	0.1	14.3	11.5
Total	961.4	100	71	100	76	100	124	100

Table 5. Determining the degree of plowing of the territory.

	% of plowing	Degree of plowing	Assessment of ecological condition
1	73	strong	critical condition
2	62	strong	critical condition
3	43	average	tense state
4	33	moderate	tense state
5	50	average	tense state
6	47	average	tense state
7	66	strong	critical condition
8	70	strong	critical condition
9	72	strong	critical condition
10	55	average	tense state
11	49	average	tense state
12	53	average	tense state
13	49	average	tense state
14	66	strong	critical condition

The current hydro-chemical state of water bodies of the Ustya river basin has acquired a natural-technogenic character, and the analysis of the available hydro-chemical information allows to outline the specifics and characteristic features of the current state of river systems of Rivne region. Thus, as of 2016–2019, the water composition had a fairly high average annual level of mineralization (520–680 mg/l), and the relatively high proportion of chlorides (over 30 mg/l) in the structure of major ions indicates the intensity of the surface water pollution process. communal and livestock effluents. The nature of the annual amplitude of color of natural waters varies from the value of the quality scale "colorless" in the second half of autumn, in winter - to light gray and gray in the period of flooding and light green - in the summer period. This indicates significant volumes of terrigenous material in rivers during snow-melt and intensive development of phytoplankton (primarily green and blue-green algae) during the growing season, and thus, indirectly, the presence in the water of high concentrations of nutrients (nitrogen compounds and phosphorus). Thus, in the channel waters there was an increased content of nutrients, in particular, ammonium nitrogen (1.5-2, and sometimes more times higher than the ecological optimum), nitrites (1.5-2.5 times higher than the ecological optimum), nitrates (0.5-1.5 times higher than the ecological optimum). Unsatisfactory high values of biochemical oxygen demand (BOD_5)(2-3 times higher than the ecological optimum) indicate excessive pollution of channel waters with organic matter of autochthonous (products of extinction of aquatic organisms, especially planktonic) and allochthonous genesis (surface runoff). The presence of such specific products of technogenesis as petroleum products and heavy metals (chromium, zinc, copper, nickel) in concentrations below, at the limit and above the critical values of the MPC (maximum permissible concentrations) is noted in surface waters. The oxygen regime remains positive, which determines the predominance of oxidation processes in river waters and is an indicator of their high self-cleaning potential.

According to organoleptic parameters, surface waters have a completely satisfactory condition. The smell of water has a river character, without foreign odors of mustiness and the presence of putrefactive processes. It is characterized by a level of 2 within all investigated points.

The formation of the chemical composition of the waters in the Ustya River basin is influenced by the marl-Cretaceous deposits common here, the Upper Cretaceous and Tertiary systems, and carbonate-rich soils. This causes moderate mineralization and hydrocarbonate-calcium type of river waters (Kononenko, 1951). Water in rivers belongs to the hydrocarbonate class in the ratio of major ions, calcium group, the second type, its chemical composition is defined as $C_{Ca}II$ (Polishchuk et al., 1978).

Analysis of the dynamics of the salt composition block index of the Ustya River allows us to note that in general in 2016-2019 the water quality did not differ significantly depending on the seasons.

The natural state of the river is characterized by the values of the sum of ions, which correspond to 2 categories, for sulfates - 1, and for chlorides - 3, which is defined according to the ecological classification as "very good" and "good". At the source of the river near the Derman Village does not vary significantly during the year, so in the study period, water quality was determined within the second with the transition to the third category, ie in the range from "clean" to "fairly clean" class II quality ("good"). it can be described as "good" and "very good". Even the extreme values of the index water in this area is defined as "good". This quality condition is typical along the Kvasyliv Village.

Downstream of the Ustya River in the Kvasyliv village water quality in this unit deteriorates to the third with the transition to the second category of quality class II ("good"). Within the same category, water quality is maintained almost all year round, but in all seasons there were extremes of 3.67-4 categories, ie the transition from "good" to "satisfactory", which we associate with wastewater of Kvasyliv village, which is often insufficiently treated and contains increased concentrations of sulfates and chlorides. In addition of Kvasyliv village, such peaks of salt composition indices were observed within the Rivne city, especially in the areas below the wastewater outlet.

Analysis of the dynamics of the block index of salt composition of the Ustya River, at a distance of 35 km from the confluence point in the Goryn River, 2 km below the confluence of the Spasiv tributary, allows us to note that in general it can be described as "good". Although the chloride-sulphate composition of water causes variation of water quality within 3 subcategories of class II to 4 subcategories of class III, the mineral composition is generally favorable and is characterized by stable indicators within the entire basin of the Ustya River.

In general, the water quality in the region varies within 2-3 subcategories of class II water quality, only in the area of Kvasylivsko-Zdolbunivska area and in the area of Rivne the salt content is the highest, on the contrary, water quality was not significantly affected. This indicates the presence for the Ustya River of brines of sodium chloride composition, with a salinity of more than 250 g/dm^3, as well as the release of excessive amounts of wastewater with high content of sulfates and chlorides.

Analysis of the block of tropho-saprobological composition of surface waters of the Ustya River. According to the content of dissolved oxygen, the water of the Ustya River has an extremely wide range of variations - from 1.74 to 10.20 mgO_2 / dm^3 (from 1 to 7 categories of water quality), especially critical are the hot periods. category V class, which was visually noted in the intensification of water flowering processes and the appearance of a putrid odor.

Fluctuations in the content of suspended particles and water transparency often depend on the season: plain rivers are characterized by the maximum content of suspended solids in spring floods and minimum - in winter, mountain rivers are usually increased turbidity during rains and snowmelt in the mountains (Romanchuk et al., 2018, Fedoniuk et al., 2018). Control of these two indicators of water is very important from the toxicological point of view. Because the bulk of heavy metals and radionuclides are localized in suspended particles. Thus, according to Moore J.W. and Ramamurti S. (1987) for surface waters of moderate turbidity in suspended particles accumulate more than 90% of lead, 30–35% of arsenic and cadmium, more than 20% of mercury, etc. (Ramamurti, 1987).

Natural waters have the ability to maintain the pH during the year at about the same level, even with the receipt of alkalis or acids from external sources. Determine the ability to neutralize acids and alkalis when they enter natural waters due to dissolved carbon dioxide and bicarbonate ions (Fedoniuk et al., 2018). Most regulations set a standard for surface water in the range of 6.5 to 8.5.

In all studied periods, the transparency of water was very low, in particular it fluctuated in the range of 22… 36 cm, which corresponds to 6 categories of class IV, which indicates that certain vital processes of aquatic organisms can be suppressed. Decreased water transparency may indicate the presence of a clay fraction and a high content of iron and manganese compounds in the water.

The good transparency of water along the entire length of the river is also due to several reasons: high plowing of the coastal strip and a significant influx of humic substances due to the fragmentary cover of vegetation along the banks. Another reason is the composition of groundwater that feeds the main watercourse of the Ustya River, which is characterized, like most rivers in this region, by an increased amount of iron in almost all studied observation points. Significant areas of the river floodplain are swampy and wet, which determines the increased levels of BOD and permanganate oxidation due to the influx of hardly oxidizable humic substances, which determine a slight decrease in water transparency to 22-36 cm.

Significant variations within the basin of the Ustya River were observed for pH. In fact, this is the only indicator in this block, which sometimes recorded "excellent" water, although the worst values often reached 4 categories (slightly polluted) class III (mediocre).

Deterioration of indicators on hydrophysical and hydrochemical indicators was noted in the areas below the village. Kvasyliv, which we associate with the increased plowing of the floodplain by homesteads and more intensive agricultural production in the area.

Estimates of NDVI (normalized difference vegetation index) show that this area is devoid of vegetation and plowed most of the year, which causes constant leaching of soil fractions into the water of the Ustya River. This also explains many places with silt.

The inflow of substances from agricultural fields causes significant variations in pH towards alkalization, which can be explained by the inflow of mineral fertilizer residues into aquatic ecosystems. Thus, the range of pH variations was within 3-4 categories of II-III water quality classes (from excellent to mediocre).

Concentrations of suspended particles (turbidity) during the study period within the Basiv Kut reservoir above Rivne were relatively low, their values ranged from 16.80 to 32.93 mg / dm^3 (within 3–4 categories) (Fig. 5). The highest concentrations of suspended particles in river water were observed in summer and autumn, and the lowest - in winter.

In winter, content of suspended particles was the lowest, so, the average value for the winter was 7.5 ± 1.89 mg / dm^3 (category 3), the same values varied in the range from 6.4 mg / dm^3 (category 2) to 68.0 mg / dm^3 (6 category).

In the spring the turbidity of the water increased, the average value for the spring period was 20.47 ± 10.97 mg / dm^3 (category 4), the same values varied in the range from 12.00 mg / dm3 (category 3) to 22.4 mg / dm^3 (4 category).

In summer, the general values of the content of suspended particles increased, and in autumn reached a maximum. The average value in the summer was 25.10 ± 4.54 mg / dm^3 (category 3), the same values varied from 4.40 mg / dm^3 (category 1) to 44.00 mg / dm3 (category 5). In autumn, the average value was 24.36 ± 3.60 mg / dm^3 (category 4), the same values ranged from 4.79 mg / dm3 (category 2) to 44.0 mg / dm^3 (category 5).

Studies of water quality along the Ustya River and its tributaries over a 5-year period (2015–2019) showed that the highest turbidity was recorded within 25 km of the confluence point in the Goryn River, 0.25 km below the wastewater discharge from the USC. Rivne, VKH service, Rivne. The maximum values of average river values of 34.11 and 22.95 mg / dm^3, respectively, are recorded here, which corresponds to 4-5 categories of quality class III.

According to the analysis, the content of ammonium nitrogen reached critical divisions in all periods. The average values of ammonium nitrogen content were at the level of 1.02 mg / dm^3, which identifies this water as "bad" in both class and category. Seasonal fluctuations in ammonium ion content are usually characterized by a decrease in the spring and an increase in the summer, probably due to increased decomposition of organic matter. In the autumn-winter period, the increase in the content of ammonium ions is associated with continued decomposition of organic matter with a slight fixation of phytoplankton due to a decrease in the intensity of photosynthesis.

Extreme values of ammonium content were observed in almost all seasons. In winter, the average value was 1.39 mgN / dm^3. Although the variation of values was significant: from 0.09 to 7.60 mgN / dm^3. In the spring, the content of ammonia ions decreased slightly, which is clearly followed by a decrease in the average values (0.44 ± 0.27 mgN / dm^3), which correspond to the 4th category of class III, and the extreme values (2.70 mgN / dm^3), However, in the summer and autumn periods the content of ammonia ions increased to 0.86 and 0.93 mgN / dm^3, respectively, which generally corresponds to the 5th category of class III. The maximum values of ammonium ion content were recorded in the Ustya River, 25 km from the confluence point in the Goryn River, 0.25 km below the wastewater discharge in Rivne - 9.6 mgN / dm^3 (3.7 times higher than the MPC) on September 2, 2017, as well as in the area of treatment facilities of the Kvasyliv village – 7 mg / dm^3, (2.7 times higher than the MPC) – on February 19, 2016.

It is known that ammonium ions formed during biochemical transformations are oxidized by nitrite bacteria into nitrite ions (with subsequent formation of nitric acid). Therefore, the analysis of nitrogen metabolism was carried out comprehensively, taking into account the possible processes of transformation of forms of nitrogen content. Consequently, the content of nitrite forms of nitrogen showed a slightly worse situation in terms of water quality in the Ustya River. 7 category V class)) with average values within 0,083 mgN / dm^3 (bad, 6 category IV class), which does not exceed the established standards SanPiN # 4630-88 (3,3 mgN / dm^3), but in most cases exceeds the standards, set at (0.08 mgN / dm^3). The nitrite content in winter and spring was higher. Thus, the average values for the 4-year period reached 0.072 and 0.082, respectively, which were defined as 6 category IV class. In summer and autumn, their content decreased slightly due to faster oxidation processes in aquatic ecosystems, although the average values were defined as category 6 category IV - 0.068 mgN / dm^3. However, the maximum value was observed in autumn 2015 at the level of 3,450 mgN / dm^3 (September 2, 2015) in the area of Kvasyliv village (Rivne district). During the same period, the surface water in the area of the Rivne wastewater outlet was in the range of 1.33 mgN / dm^3. This confirms the fact that a significant contribution to the deterioration of surface water quality is made by anthropogenic activity, because in the source of water quality in terms of nitrite ions is 2-3 categories better than water quality in covered areas of wastewater outlets.

The nitrate form of nitrogen is formed in the final stages of nitrogen oxidation, and elevated concentrations of this compound indicate enhanced decomposition of organic matter under conditions of slow oxidation. At the end of the summer, an increase in the content of nitrates in the water was recorded due to the activity of phytoplankton. And even by mid-October 2019, these values did not fall. The average values of the summer period of 2019 were recorded at 2.90 mg / dm^3, which corresponds to category 7 (very dirty) V class (bad), at the same level recorded the worst values, the best indicators were recorded in winter at 0.43 mg / dm^3 (Category 3 (good) class II water quality). In winter, the situation worsened due to the slowdown of oxidation processes and the cessation of nitrogen consumption by autotrophs. Thus, in winter and spring, the content of nitrates in water averaged 0.78 and 0.83 mg/dm^3, respectively. However, even in winter, significant exceedances of the background content of nitrates were recorded - at the level of 8-9 mg / dm^3, but the extremum was still recorded in autumn (September 2, 2015) in the Kvasylivsko-Zdolbuniv section - within 12 mg / dm^3.

Deteriorated nitrogen regime is fully consistent with other data of the trophosaprobological block. This indicates an excessive anthropogenic component of the impact on water quality in the form of agricultural production in the river floodplain and the inflow of wastewater from settlements.

The term "total phosphorus" means all types of phosphates contained in water - soluble and insoluble, inorganic and organic phosphorus compounds. Significant variations were observed for this indicator in the water of the Ustya River. Thus, the average values were noted at the level of 0.396 mg/dm^3, which corresponds to 7 categories of water quality (dirty) V class (poor). Deterioration to category 6 (dirty) of the IV class (bad) was recorded at the lowest values. The ratio of N: P is not pleasant for the absence of significant "flowering" of reservoirs, as evidenced by the lack of oxygen supply to the water.

Water blooms were observed in August in all years in the central part of the river, where the floodplain is excessively plowed, which indicates a temporary imbalance of organogenic elements in the water.

In winter and spring, the average water quality index for phosphorus in the form of phosphates was 4.50-4.82, while in summer it increased to 6.59, and slightly decreased in autumn to 5.8. The maximum value of phosphorus content of phosphates was recorded on September 2, 2015 near the village of Kvasyliv, at the point of wastewater discharge - 8.3 mg / dm^3, which is 2.4 times higher than the requirements of **SanPiN** # 4630-88 Sanitary rules and standards for the protection of surface waters from pollution (3.5 mg / dm^3).

It is known that the degree of contamination of water with organic compounds is defined as the amount of oxygen required for their oxidation by microorganisms under aerobic conditions

(Maier & Gentry, 2015; Fedonyuk et al., 2020). Water oxidation is an extremely important indicator of water quality analysis, because this value characterizes the total content of organic matter and easily oxidizable inorganic impurities (hydrogen sulfide, sulfites, iron compounds II, etc.), which enter reservoirs under the condition of most natural processes, for example, with rain and melt water, due to the development of plant and animal organisms, erosion of drainage channels, wastewater discharge (Aristarkhova et al., 2021).

In addition, oxidation is an important indicator of the hygienic characteristics of water. According to its dynamics, it is possible to make decisions on the application of certain sanitary and hygienic measures. Increased oxidation in water bodies may indicate excessive discharge of wastewater or pollutants from other sources. In view of the above, this indicator can be a prerequisite for taking measures for sanitary protection of the reservoir. The determination of oxidation is based on the reaction of organic compounds with oxidants, as a result of which the amount of oxidant and its equivalent amount of oxygen spent on the oxidation of these compounds is determined.

To characterize the water in the experimental conditions, we also determined the dichromate oxidation. Because, in comparison with permanganate oxidation, it more accurately characterizes the content of organic pollutants, because potassium dichromate oxidizes about 90% of the organic substances present in the water, including difficult to oxidize.

In addition to the indicator of dichromate and permanganate oxidation, we determined the indicator that characterizes the degree of organic pollution of the reservoir - biochemical oxygen demand (BOD). This is an indicator of the amount of oxygen used to oxidize organic impurities in water, provided that it undergoes biochemical processes over a period of time (in our case, 5 days). It is known that the main oxidants of organic matter in water are bacteria, which use these compounds as a source of food and energy.

The water of the Ustya River is rather mediocre in terms of pollution by organic compounds. This is evidenced by all the studied indicators of water quality. Permanganate oxidation on average was recorded at the level of 5.48 mg O_2/dm^3, which defined it as the 5th category (moderately contaminated) of the III class. The worst values were recorded at the level of the 7th category (bad) of the V class - 15.0-15.7 mg O_2/dm^3 (February 19, 2018 and August 30, 2016). At the same time SanPiN # 4630-88 Sanitary rules and standards for the protection of surface waters from pollution requires no more than 4.51 mg O_2/dm^3, "Environmental safety standards of water bodies». " - not more than 3.0 mg O_2/dm^3.

In spring and winter, water quality in terms of biochemical oxygen consumption was between 4 and 5 categories (4.55-4.99), at the level of 4.4-5.6 mg O2 / dm^3. In summer, the quality of surface waters deteriorates to the transition to the sixth category, the average value of the summer season is 5.64, and the worst value - 6.136 (the sixth category with the transition to the seventh).

Data on dichromate oxidation confirmed the obtained data on permanganate oxidation, as the average values of this indicator characterized the water of the Ustya River as slightly polluted (category 4, class III), and the worst - bad (category 7, class V). Data on chemical oxygen demand also varied seasonally, in winter and spring the figures were slightly lower - on average at 26.8… 27.9 mg O_2/dm^3, slightly contaminated (category 4, class III), in summer and autumn deteriorated to 5 categories on average - 38.65 and 32.2 mg O_2/dm^3, respectively.

The worst value was recorded at 112 mg O_2/dm^3 on January 18, 2016. At the same time SanPiN №4630-88 requires a level of chemical oxygen demand not more than 30 mgO_2/dm^3, "Standards for environmental safety of water bodies». " - not more than 50 mgO_2/dm^3.

A separate point should be noted the concentration of iron in the water of the Ustya River, where inflated values are due to geochemical processes, as in the Ustya River basin unloaded groundwater with the highest concentration of iron ions in the Ukrainian Polissya, the content of which in the natural state reaches 8 μg / dm more (Lange, 1959). Therefore, on a general scale, the iron content in the water of the Ustya River varied within the 5th category (moderately polluted) of the third quality class (mediocre). However, according to the scale interpreted for northern Polissya, taking into account the background level of this element, the water of the Ustya River is defined as "very good" (2 category II class), with the worst values at level 3 category II class.

It should be noted the low content of heavy metals in the surface waters of the Ustya River, while the content of cadmium, zinc, chromium total, nickel water was determined at the level of category 1 quality class I or "excellent", with the worst indicators, as a rule, not recorded indicators below 3 categories of the II class ("good"). According to the content of mercury, copper and lead, the average values were fixed at the level of the 2nd category of the II class.

The manganese content was critical. It should be noted that this content is somewhat due to regional characteristics and the structure of the river basin. Its increased content is recorded almost everywhere in the water bodies of Polissya. This can be justified by leaching from forest litter, iron-manganese soil nodules, high content in peatlands. An additional imprint is caused by the high swampiness of the territory of Polissya and the proximity of the illuvial horizon of sod-podzolic soils typical for Polissya. As we suppose, extremely high concentrations of manganese at the level of category 5 of the III class of water quality were noted for the Ustya River, which cannot be explained only by its high content in the composition of humic substances, and therefore it should be considered with anthropogenic component.

The content of oil products in the water of the Ustya River was also insignificant, the average data on the content of this compound were at the level of category 1 of I class quality, slightly higher content was observed near the bridge over which the road runs. However, even at the worst values the water quality did not fall below the II class of water quality of the 2nd category (good). The presence of phenols and cyanides in the water was not noted. The presence of synthetic surfactants was recorded in small quantities, however, their content was recorded within the normative values and the worst values were identified as the 2nd category ("very good").

Similar values were confirmed by calculations of the coefficients of bottom accumulation of heavy metals and accumulation of pollutants in aquatic organisms. According to these two indicators, data were obtained, which once again confirmed the previously obtained data on the content of the main categories of heavy metals and toxins. Water is defined on average at the level of 2-3 categories of quality class II (good).

In general, the quality of water in the Ustya River is defined as favorable in terms of the content of specific substances of toxic and radiation action (Fig 5). The average values of water quality showed 2 subcategories with a transition to 1, with deterioration only in the content of total iron and manganese, which is characteristic of the geological, geomorphological and soil conditions of northern Polissya Ukraine. This causes the transition of water quality in the worst indicators to subcategory 2-3 (between the second and third).

In general, the quality of water in the Ustya River is defined as favorable in terms of the content of specific substances of toxic and radiation action. The average values of water quality showed the first with the transition to the second subcategory of water quality, with the worst indicators within 2 subcategories.

Ecological sustainability of the landscapes of the Ustya river basin. During the last decades, the landscapes of the Ustya river basin have changed significantly in the direction of increasing urban areas and areas set aside for agriculture. The creation of an integrated system of measures for the formation and maintenance of ecologically stable landscapes involves their detailed and diverse assessment.

Among the ecological problems that should be outlined in characterizing the ecological condition of the main tributaries of the Ustya basin can be distinguished as follows: for the rivers Untitled (which originates near the village of Mizotska and flows into the Ustya near the village of Zdovbytsia - Zdolbuniv) and tributaries Kunin sources of anthropogenic impact are primarily in the plowing of the territory - 43.7 and 45.6%, respectively, over-regulation of runoff, lack of coastal protection strips, etc. For the Kunin tributary there is a problem of the influence of the drainage system on the formation of runoff.

The tributary of the Spasov is generally characterized by the same problems, in particular the plowing of the territory is generally higher than the average in the basin (73.2%), runoff regulation and a significant number of ponds, the lack of coastal protection strips is complemented by the general lack of local treatment facilities .

To this end, we have used a number of coefficients that can best reflect the level of stability and anthropogenic transformation.

In order to assess the sustainability of landscapes and to prevent the fact that this area is characterized by the distribution of many Red Book species, we used to assess the indicator - the index of species diversity (ecotonization index). Which we calculated in the context of each massif, while assessing the edges of the plow, the edges of the beam and riparian strips, the edges of forests, the boundaries of plowing with other lands, boundaries and more.

In our previous work, we used this criterion to assess the stability of the landscapes of the rivers of Polissya (Romanchuk et al., 2018; Fedoniuk et al., 2018; 2019, Orlov et al., 2021) it is he who reflects the data on the ratio of areas, which are engaged in stabilizing and destabilizing components of landscapes – KESL 1, and the ratio of environmentally stable elements to the whole landscape – KESL 2.

As the landscape analysis showed, according to KESL1, the landscape has a pronounced unstable landscape.

To confirm the above data, we calculated the coefficients of ecological stability of the landscape (KESL2). In this case, the array № 14 (KESL2 - 0.51) can be considered stable, namely in the place of the tributary Untitled in the area of s. Upper, downstream the level of landscape instability increases, at the source of the Ustya River the level of instability defines the landscape as unstable (KESL2 - 0.48). A significant decrease in the level of landscape stability to "unstable" occurs in the "Kvasyliv-Zdolbuniv section" and is maintained at the same level until the village. Orzhiv, where the river Ustya flows into the river Goryn.

One of the reasons for the significant deterioration of water quality in the Ustya River basin is significant erosional dismemberment, as the river floodplain has an erosive-cumulative character. The analysis of the obtained data showed that within all arrays the average dismemberment and tense ecological condition were noted and strong dismemberment and extraordinary ecological condition were noted within the Kvasylisko-Zdolbunivska section (array 6).

Analysis of the stability and anthropogenic transformation of landscapes, we collaborated with the analysis of the quality of surface water ecotonization index (Table 5). Correlation analysis of the relationship between ecotonization indices and surface water quality indices has shown that as the integrated water quality index increases, the ecotonization index decreases. For almost all arrays with points where ecotonization indices were higher than 10, water quality was lower than 4 categories (Fig. 7A). At the same time, close correlations are observed for all water quality blocks, and especially for the blocks of salt composition and substances of toxic and radiation action.

In addition, we analyzed in detail the landscape structure of the Ustya River basin, for which we applied the criteria KESL1 and KESL2 (Fig. 7B-C). There are inverse correlations between KESL1 and KESL2 and indices of salt and trophosaprobological state, as well as integrated ecological indices of water quality, slightly weaker relationships were found with indices of toxic and radiation substances, obviously due to the fact that too high concentrations of substances in this category were not detected. This is evidenced by the fact that the presence of destabilizing components of the landscape mainly affects the flow of organic and organogenic substances to the surface waters of the territory (Table 6).

There are no significant relationships between the indices of erosional dismemberment of the terrain and indicators of water quality in certain areas.

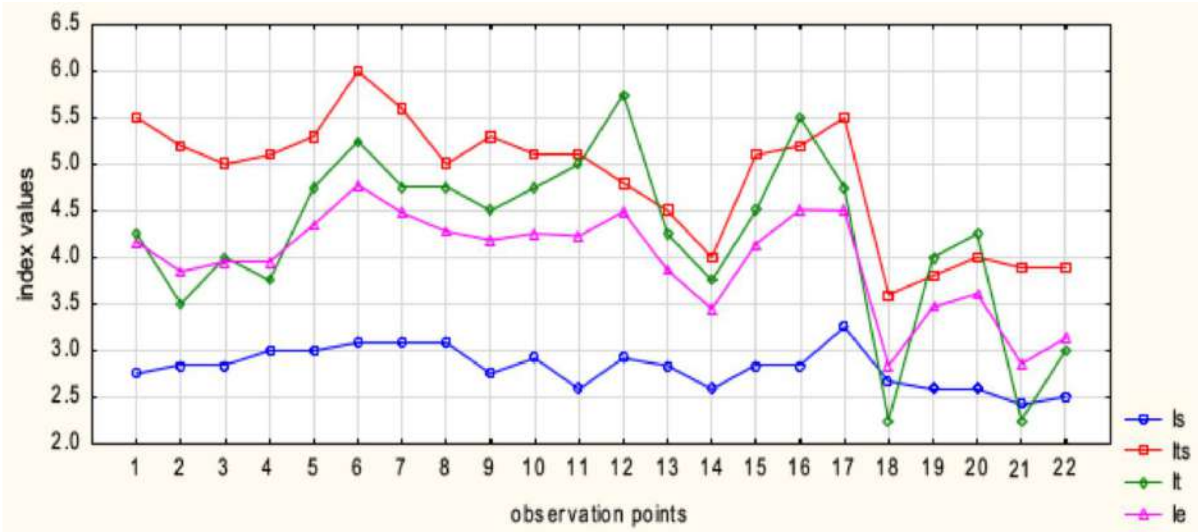
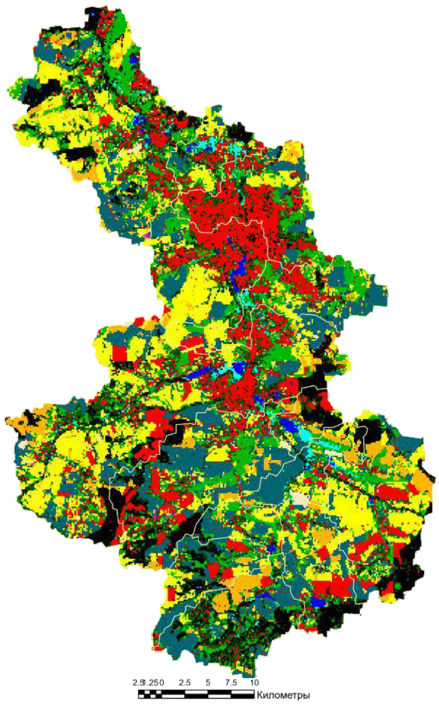


Fig. 5. Ecological classification of surface water quality of the Ustya river Obsrervation points according table 1. *Legend: Is* - salt composition index; *Its* – trofosaprobological index; *It* -toxicological composition index; *Ie* - general ecological index.

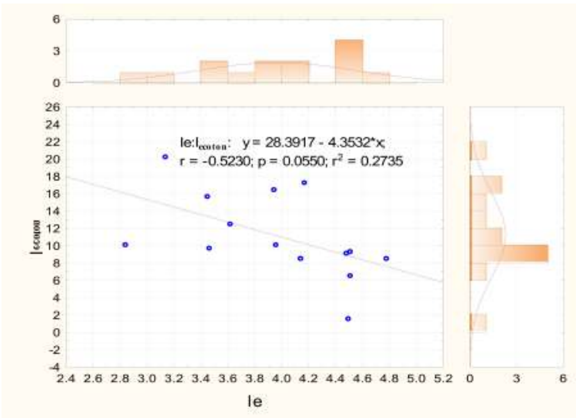


Building area and alienation for the road network
Arable land, hops
Orchards, acacias
Coniferous forests
Cities
Meadow
Pastures
Reservoirs and drains
Deciduous forests, natural spruces

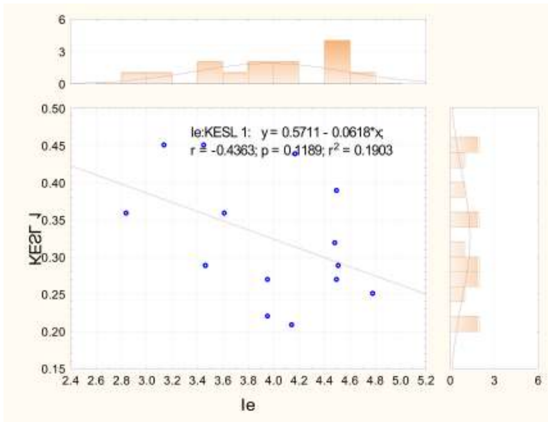
Fig. 6. Types of landscape cover (landscape biotechnical elements).

Table 6. Ecological assessment of the landscapes stability of the Ustya River catchment area.

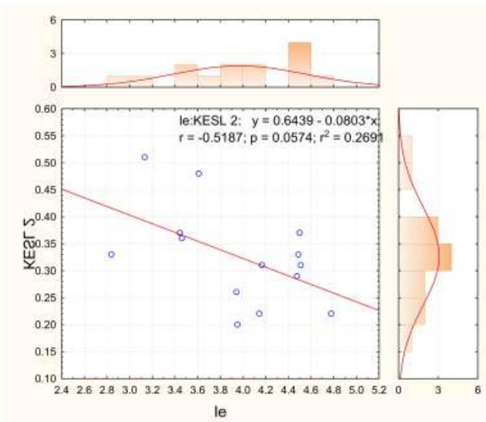
Array	I_{EKOTON}	KESL 1	K_{ER}	KESL 2	Iat	The degree of anthropogenic transformation of landscapes
1	17.3	0.44	0.41	0.31	846	Very high
2	16.5	0.27	0.59	0.26	796	Very high
3	10.2	0.22	0.47	0.20	524	Average
4	8.5	0.25	0.40	0.22	385	Moderate
5	9.2	0.32	0.53	0.29	499	Average
6	1.6	0.39	0.71	0.33	507	Average
7	15.6	0.45	0.50	0.37	576	High
8	8.6	0.21	0.38	0.22	765	Very high
9	9.4	0.29	0.43	0.31	815	Very high
10	6.5	0.27	0.36	0.37	545	High
11	10.1	0.36	0.32	0.33	465	High
12	9.7	0.29	0.46	0.36	489	High
13	12.6	0.36	0.43	0.48	502	High
14	20.3	0.45	0.46	0.51	685	Very high



A)



B)



C)

Fig. 7. Relationship of ecotonization indices, KESL 1 and KESL 2 with the qualitative composition of surface waters of the Ustya river basin.

Table 7. Correlation analysis of the relationship of the main landscape criteria and water quality indices.

Variable	Correlations (stattya nova) Marked correlations are significant at $p < .05000$ N=14 (Casewise deletion of missing data)					
	I_{ecoton}	KESL 1	K_{er}	KESL 2	% of plowed territory	The degree of anthropogenic transformation of landscapes
s	-0.60	-0.59	0.03	-0.59	-0.40	-0.20
ts	-0.29	-0.46	0.03	-0.64	-0.11	0.16
t	-0.59	-0.29	0.38	-0.30	-0.10	0.04
Ie	-0.52	-0.44	0.22	-0.52	-0.15	0.06

Conclusions

River ecosystems of Polissya are in a state of deep degradation, which consists of siltation, falling water levels and pollution. Analysis of the particle size distribution of floodplain soils in different zones of the Ustya River basin showed that the predominant soil fractions here are coarse-grained and dusty. This fractional ratio is explained by the peculiarities of the hydrological regime, namely the position in the basin, periodic spills, depth and flow velocity. In all studied periods the water transparency was very low, in particular it fluctuated in the range of 22… 36 cm, which corresponds to 6 category IV class, that is, it can be defined as poor in quality. This indicates that certain vital processes of aquatic organisms may be suppressed. Decreased water transparency may indicate the presence of a clay fraction and a high content of iron and manganese compounds in the water. Low indicators of the trophosaprobological block indicate an excessive anthropogenic component of the impact on water quality in the form of agricultural production in the river floodplain and the inflow of wastewater from settlements. The use of some indices to determine the characteristics of landscapes has shown close links with water quality indicators. For almost all massifs with points where ecotonization indices were higher than 10, water quality was lower than 4 categories, that is, it was identified as contaminated. Consequently, the peculiarities of hundreds of landscapes are decisive for the formation of water quality in the Ustya River basin.

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Received: 08.04.2021
Accepted: 11.11.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 23-36

Flora and Vegetation of "Elenova gora" Natural Forest Reserve, Central Balkan Range (Bulgaria)

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Abstract. The study aims to investigate the flora, medicinal plants and vegetation diversity of "Elenova Gora" natural forest reserve, situated on the southern slopes of Central Balkan Range. The reserve occupies an area of 53.88 ha. The flora without mosses comprises of 132 vascular plant species, classified into 50 families and 108 genera. The most species-rich families are *Asteraceae*, *Lamiaceae*, *Poaceae*, *Ranunculaceae*, *Apiaceae*, *Aspidiaceae*, *Rubiaceae*, *Rosaceae*. Sixty five medical plants and 8 species of conservation concern were found (2 Balkan endemics, 3 species included in the Red List of Bulgarian vascular plants and in the Biodiversity Act and 3 species included in Appendix II of CITES). Eight relevés were collected during 2014 following the Braun-Blanquet approach. The vegetation diversity is represented by 3 classes (*Carpino-Fagetea sylvaticae, Mulgedio-Aconitetea* and *Thlaspietea rotundifolii*), 3 alliances (*Fagion sylvaticae, Petasition officinalis* and *Stipion calamagrostis*), 4 associations (*Asperulo odoratae-Fagetum sylvaticae, Festuco drymejae-Fagetum sylvaticae, Petasitetum hybrido-kablikiani, Parietarietum officinalis*) and 1 plant community (*Abies alba-Fagus sylvatica*). On the territory of the reserve was established 1 habitat type (9130 *Asperulo-Fagetum* beech forests) protected by Directive 92/43/EEC and the Bulgarian Biodiversity Act. Although its small size "Elenova Gora" natural forest reserve harbors relict beech forests and related herbaceous riverside communities with significant plant diversity and conservation value for maintenance of the beech forests of Central Balkan mountain and of the country.

Key words: Central Balkan Range, beech forests, plant diversity, medicinal plants, vegetation diversity.

Introduction

"Elenova Gora" natural forest reserve occupies a small territory of only 53,88 ha and is located close to the eastern border of "Central Balkan" National park. It was established in 1961 (Order № 2200/30.08.1961 of General Department of Forestry at the Council of Ministers) to protect a rare natural formation of beech forests (*Fagus sylvatica* L.) with age between one and two hundred years. The reserve's territory is a part of the Bulgarian NATURA 2000 network and falls into Central Balkan – buffer site (BG0001493). Various natural as well as semi-natural and anthropogenic phytocoenoses surround the reserve.

The flora on the territory of the reserve has not been studied before. Some indirect, outdated or too general data about the diversity of vascular plants and in particular the group of medicinal plants in the region of the reserve are represented in the studies of Urumov (1929) and Bondev (1995). Also some general information about the floristic composition of the natural beech forests can be found in Radkov (1963), Garelkov (1967), Marinov et al. (1982, 1987), Bondev (1991) and Dimitrov (2015). The vegetation diversity in the reserve has been previously referred as part of the large-scale study of beech forests in Bulgaria by Tzonev et al. (2006).

The aim of this study was to investigate the floristical, medicinal plants' and the syntaxonomical diversity of "Elenova Gora" natural forest reserve and to assess their current condition.

Material and Methods

Study area

The study was conducted in "Elenova Gora" natural forest reserve, located near the village of Skobelevo (Pavel Banya municipality) and falling within the territory of Kalofer Mountain, which comprise the southern slopes of Central Balkan Range. It covers only 53.88 ha and is distributed between 845 and 1310 m a.s.l. (Fig. 1).

The northeast boundary of the reserve is limited by the valley of Gabrovnitsa River, a left tributary of Tundzha River and therefore northeast slopes prevail. The dominating slope inclination is 30-35°, but in some places it reaches 40-55°. The reserve is influenced by the mountain variant of the Temperate-Continental climate (Velev, 2002) with its typical summer maximum and winter minimum of precipitation (Stanev et al., 1991). The territory of the reserve is composed of Mesozoic rocks, represented by alternating sandstones, siltstones and marls with layers of clay limestone. The soils are dark and transitional Eutric Cambisols with varying mechanical composition from clay-sandy to medium sandy-clay (Zlatunova, 2017). The indigenous vegetation in the area consists of pure beech forests (Bondev et al., 1991).

Floristic composition sampling

The field studies were conducted in June-August 2014. The taxonomic identification and nomenclature of vascular plants as well as biological types and life forms follow the key reference sources for Bulgaria (Assyov & Petrova, 2012; Delipavlov & Cheshmedzhiev, 2003; Jordanov, 1963–1979; Kozhuharov, 1992, 1995; Peev, 2012; Velčev, 1982, 1989;). Floristic elements follow Assyov & Petrova (2012). The conservation status was determined according to *IUCN*, *CITES*, Biological Diversity Act (2002), Red List of Bulgarian vascular plants (Petrova & Vladimirov, 2009), the list of Balkan endemic species (Petrova & Vladimirov, 2010) and Red Data Book of Republic of Bulgaria (Peev (ed.), 2015). The list of medicinal plants is in accordance with Appendix 1 of Medicinal Plants Act (2000) and their special regimes of protection and regulated harvesting with Order №RD-203/02.03.2020 and Appendix 4 of Biological Diversity Act (2002).

Vegetation sampling

During the vegetation season 2014 a total of 8 relevés were collected following the Braun-Blanquet approach (Braun-Blanquet, 1965; Westhoff & van der Maarel, 1973). The sample plots were placed in the most homogenous parts of communities. The sample plots were square-shaped with size of 16 m^2 for grassy and 100 m^2 for woody vegetation (Chytrý & Otýpková, 2003). The total cover of vegetation and the abundance and cover of the species were estimated in percentages. Altitude, slope inclination and location were measured by Garmin eTrex Vista whereas the exposition was determined by a compass. Soil depth was classified as shallow (<10 cm depth), (2) moderately deep (10-20 cm) or deep (> 20 cm). All relevés were stored in TURBOVEG database (Hennekens & Schaminée, 2001) and included in the Balkan Vegetation Database (GIVD ID: EU-00-019) (Vassilev et al., 2016). The numerical classification was performed by PC-ORD (McCune & Mefford, 1999) and JUICE 7.0 (Tichý, 2002) software packages. Sørensen (Bray-Curtis) was used as distance measure and similarity was calculated by Ward's clustering method. The species values were square-root transformed.

Habitat types were determined according to *Council Directive 92/43/EEC* (EC, 1992), Annex

1 of the Biological Diversity Act (2002), Manual for Determination of Habitats with European Importance in Bulgaria (Kavrakova et al., 2009) and the Bulgarian Red Data Book, vol. 3 (Biserkov et al., 2015).

Results

Vascular flora

The flora of "Elenova Gora" natural forest reserve comprises of 50 families, 108 genera and 132 vascular plants, which account 31,4% of the family diversity in the country, 11,9% of the genera and 3,4% of the species. Most of the inventoried species are spermatophytes – 45 families (90%), 99 genera (91,7%) and 119 species (90,15%). On the territory of the reserve no representatives of *Lycopodiophyta* or *Equisetophyta* were found. The taxonomic structure of the flora is presented in Table 1. *Polypodiophyta* comprises 10% of the total number of families, 8,3% of the genera and 9,9% of the species in the reserve. *Pinophyta* is represented by the fewest taxa - 4% of all families, 1,9% of the genera and 1,5% of the species. *Magnoliophyta* dominates in taxonomic diversity with 86% of the families, 89,8% of the genera and 88,6% of the species, found in the reserve. The list of taxa is provided in Appendix 1.

Table 1. Taxonomic structure of the flora of "Elenova Gora" forest reserve.

Taxon	№ of species	№ of genera	№ of families
Polypodiophyta	13	9	5
Pinophyta	2	2	2
Magnoliophyta	117	97	43
Magnoliopsida	96	80	36
Liliopsida	22	2	2
Total number	**132**	**108**	**50**

The families with the highest number of species are *Lamiaceae* and *Asteraceae*, including 11 species each (8,3%), *Poaceae* – 10 (7,6%), *Ranunculaceae* – 7 (5,3%), *Aspidiaceae* and *Rubiaceae* – 6 each (4,5%), *Rosaceae* – 5 (3,8%).

The life forms of the flora of "Elenova Gora" natural forest reserve is presented in Fig. 2. The significant participation of phanerophytes and geophytes in the life spectrum of the community shows some specific features of the studied beech forest community. The group of phanerophytes is represented mainly by deciduous species with the small exception of *Abies alba* and *Taxus baccata*, which are presented with single individuals. The group of geophytes includes rhizomatous, bulbous and tubero-bulbous herbaceous species, which take part in the community-specific early spring ephemeroidal complex.

The phytogeographic composition of the flora of "Elenova Gora" natural forest reserve is presented in Table 3. The species with European, Euro-Asian, subBoreal and Boreal distribution constitute the typical core of the beech community in a floristic aspect. To this must be added the specific focus of sub-Mediterranean and Euro-Mediterranean species entering the north through the southern slopes of Stara Planina Mts.

Table 2. Floristic elements in the flora of "Elenova Gora" natural forest reserve.

Floristic element	№ of species	Share (%)
Eur-As	23	17,4
Boreal	17	12,9
Eur	16	12,1
subMed	14	10,6
Eur-Med	14	10,6
subBoreal	13	9.8
Eur-Sib	10	7.6
Kos	5	3.8
Eur-subMed	5	3,8
Eur-OT	3	2,3
Med	3	2,3
Bal	2	1.5
Pann-Bal	2	1,5
Eur-NAm	1	0,8
Med-CAs	1	0,8
Bal-Anat	1	0,8
Pont-OT	1	0,8
subMed-CAs	1	0,8
Total number	**132**	**100**

Two Balkan endemics – *Crocus veluchensis* and *Angelica pancicii* were found (Petrova & Vladimirov, 2010). In the

Bulgarian Red List of vascular plants (Petrova & al., 2009) are included *Angelica pancicii* (VU), *Atropa bella-donna* (VU) and *Taxus baccata* (EN). In Appendix 3 of the Biological Diversity Act (2002) are enlisted *Angelica pancicii* and *Taxus baccata* as the second species is also included in the Bulgarian Red Data Book (Peev et al., 2015) and assigned as "endangered". Three orchids – *Cephalanthera damasonium*, *Dactylorhiza saccifera* and *Neottia nidus-avis* are in Append. III of CITES. There are no species included in the *Convention on the Conservation of European Wildlife and Natural Habitats* (EC, 1979) and the *Council Directive 92/43/EEC* (EC, 1992).

Medicinal plants

Of the recorded vascular plants, 65 species are considered medicinal (marked with asterix (*) in Appendix 1), referring to 37 families and 58 genera. Their number equals half (49,2 %) of the total list of plants in the reserve and 8.4% of the medicinal plants in the country. The families with biggest number of medicinal plants on the investigated territory are *Ranunculaceae* (7 species), *Lamiaceae* (6 species) and *Apiaceae* (4 species).

Eight medicinal species are protected by the Medicinal Plants Act – *Phyllitis scolopendrium, Asplenium trichomanes, Angelica pancicii, Asarum europaeum, Taxus baccata, Atropa bella-donna, Galium odoratum*. They are under special regime of protection and regulated harvesting according to order issued annually by the Minister of Environment and Water (Order №RD-83/03.02.2014, Order №RD-115/13.02.2015, Order №RD-77/09.02.2016, Order №RD-89/03.02.2017, Order №RD-56/01.02.2018, Order №RD-88/30.01.2019, Order №RD-203/02.03.2020).

Fig. 1. Map of the studied area of "Elenova Gora" natural forest reserve.

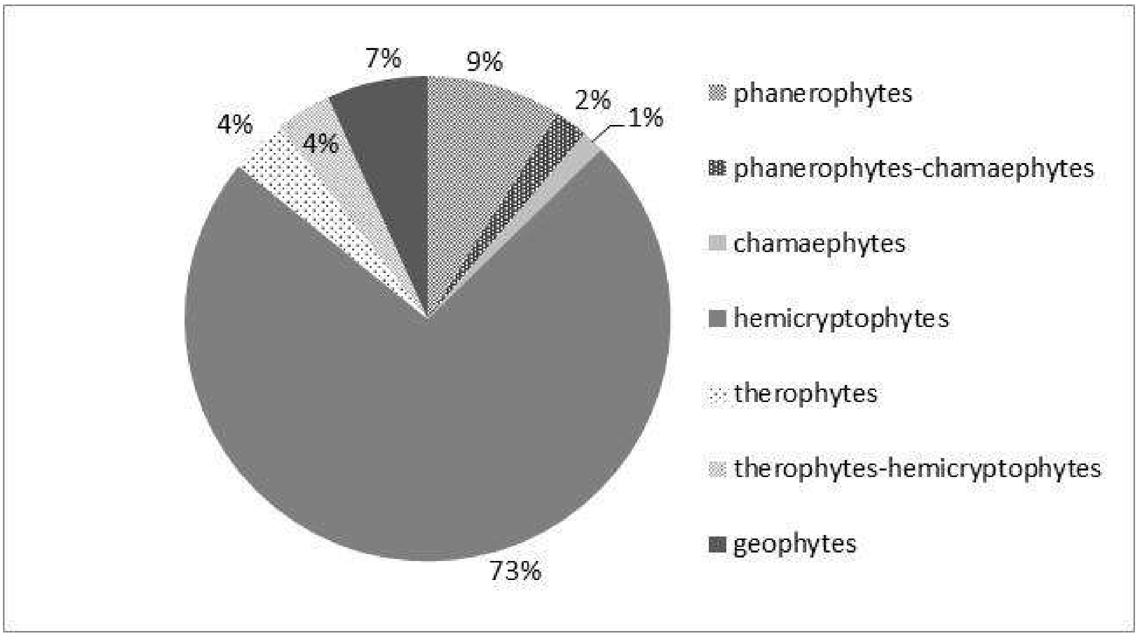

Fig. 2. Life form spectrum of the flora of the "Elenova Gora" natural forest reserve.

The first 7 are forbidden for commercial use throughout the country, while the species *Galium odoratum* is restricted for use only outside the territory of national parks. Five taxa are included in Annex 4 of the Biodiversity Act (*Dryopteris* sp. div., *Polystichum* sp. div., *Crocus* sp. div., *Polygonatum odoratum*, *Dactylorhiza* sp. div.) because of the vulnerability of their populations as a result of previously wider harvesting. The mentioned species are represented with single localities and low abundance of subpopulations on the territory of "Elenova Gora" natural forest reserve. According to the status of the protected area their collection, including for personal needs, is prohibited.

According to the degree of preference to specific habitats medicinal plants in the reserve can be divided into two main groups. The first group includes characteristic of mesophytic forest habitats widespread species with numerous populations, such as *Dryopteris filix-mas, Athyrium filix-femina, Polypodium vulgare, Acer platanoides, Sanicula europaea, Asarum europaeum, Pulmonaria officinalis, Cardamine bulbifera, Mercurialis perennis, Lamium maculatum, Oxalis acetosella, Anemone nemorosa, A. ranunculoides, Geum urbanum, Galium odoratum, Polygonatum odoratum, Arum maculatum, Asplenium trichomanes, A. ruta-muraria, Saxifraga rotundifolia*, etc. The second group includes species with limited distribution in the reserve. Some of them are widespread in the country, but due to some features in their biology and ecology in most cases they form small populations, such as *Polystichum lonchitis, Phyllitis scolopendrium, Atropa bella-donna*, etc. A special case is *Taxus baccata*, which is of limited distribution in the country and on the territory of the reserve there is only one individual in the southwestern part of the reserve. No significant natural resources of medicinal plants have been identified in "Elenova Gora" natural forest reserve. Populations of most medicinal species are represented by small groups (up to a dozen individuals) or small spots (up to several dozen individuals).

Vegetation

Based on numerical analysis, the syntaxonomical diversity of Elenova Gora reserve is represented by 3 classes, 3 orders, 3 alliances, 4 associations and 1 community.

The syntaxonomical scheme proposed:

Cl. *Carpino-Fagetea sylvaticae* Jakucs ex Passarge 1968

Ord. *Fagetalia sylvaticae* Pawlowski 1928

All. *Fagion sylvaticae* Luquet 1926

Ass. *Asperulo odoratae-Fagetum sylvaticae* Sougnez et Thill 1959

Ass. *Festuco drymejae-Fagetum sylvaticae* Reşmeriţă 1977

Abies alba-Fagus sylvatica plant community

Cl. *Mulgedio-Aconitetea* Hadač et Klika in Klika et Hadač 1944

Ord. *Petasito-Chaerophylletalia* Morariu 1967

All. *Petasition officinalis* Sillinger 1933

Ass. *Petasitetum hybrido-kablikiani* Sillinger 1933

Cl. *Thlaspietea rotundifolii* Br.-Bl. 1948

Ord. *Stipetalia calamagrostis* Oberd. et Seibert in Oberd. 1977

All. *Stipion calamagrostis* Jenny-Lips ex Br.-Bl. 1950

Ass. *Parietarietum officinalis* Csürös 1958

Table 3. Phytocoenological table of vegetation diversity on the territory of "Elenova Gora" reserve.

Ordinal number		**1**	**2**	**4**	**3**	**8**	**6**	**5**	**7**
Relevé No in Balkan Vegetation Database		13097	13098	13120	13119	13124	13122	13121	13123
Altitude (m)		788	891	1030	840	1089	1082	1056	1095
Exposition (degree)		-	360	360	270	360	360	270	360
Inclination (degree)		-	40	50	40	20	60	40	50
Plot size (m^2)		100	100	100	100	100	100	16	16
Total coverage (%)		90	90	90	90	95	90	95	90
Cover of tree layer (%)		80	90	90	90	95	85	0	0
Cover of shrub layer (%)		1	1	3	5	0	3	1	5
Cover of herb layer (%)		30	30	50	25	1	15	95	90
Cover of bryophytes (%)		20	20	10	70	0	15	50	0
Cover of lichens (%)		0	0	5	5	0	1	0	0
Maximum tree heigh (m)		30	25	35	30	25	30	0	0
Tree diameter (at 1.5 m heigh)		20	25	45	40	30	40	45	50
Latitude		42.73764	42.74303	42.73917	42.67225	42.74500	42.74194	42.74028	42.74278
Longitude		25.15594	25.14617	25.15055	25.37722	25.14639	25.57778	25.15917	25.15361
Diagnostic species of association *Asperulo odoratae-Fagetum sylvaticae*									
Galium odoratum	herb layer	10	15	5	.	.	.	.	1
Cardamine bulbifera	herb layer	2	0.1	1	.	.	.	.	.
Viola reichenbachiana	herb layer	0.1	.	.	.	.	.	.	.
Mycelis muralis	herb layer	0.5	.	2	.	.	.	.	.
Fagus sylvatica	tree layer	80	90	80	90	10	45	.	.
Fagus sylvatica	shrub layer	.	.	.	5	.	2	1	5
Fagus sylvatica (juvenile)	herb layer	0.1	0.1	.	.	.	.	.	.
Diagnostic species of association *Festuco drymejae-Fagetum sylvaticae*									
Festuca drymeja	herb layer	.	.	.	18	.	.	.	.
Prenanthes purpurea	herb layer	.	.	.	0.5	.	0.5	.	.
Rubus hirtus	shrub layer	0.5	0.1	1	0.5	.	.	.	.
Diagnostic species of *Abies alba-Fagus sylvatica* community									

Abies alba	tree layer	.	.	.	.	85	50	.	.
Abies alba	shrub layer	.	.	1	0.5	.	1	.	.
Diagnostic species of association *Petasitetum hybridi*									
Petasites hybridus[1]	herb layer	.	.	.	.	.	.	70	.
Diagnostic species of association *Parietarietum officinalis*									
Parietaria officinalis[2]	herb layer	.	.	.	.	.	.	.	80
Diagnostic species of class *Carpino-Fagetea sylvaticae*									
Dryopteris filix-mas	herb layer	10	4	3	.	.	.	.	.
Acer platanoides (juvenile)	herb layer	0.1	.	.	.	.	.	.	.
Acer pseudoplatanus	shrub layer	.	.	.	.	.	1	.	.
Sanicula europaea	herb layer	1	.	.	.	.	.	.	.
Arum maculatum	herb layer	0.1	.	.	.	.	.	.	.
Symphytum tuberosum	herb layer	0.1	.	.	.	.	.	.	.
Geranium robertianum	herb layer	0.5	0.1	5	.	.	.	2	.
Stachys sylvatica	herb layer	0.1	.	.	.	.	.	.	5
Neottia nidus-avis	herb layer	0.1	.	.	.	.	.	.	.
Oxalis acetosella	herb layer	0.5	1	1	.	0.5	1	.	.
Staphylea pinnata	shrub layer	1	.	.	.	.	.	.	.
Aremonia agrimonoides	herb layer	0.1	.	.	.	.	.	.	.
Mercurialis perennis	herb layer	.	0.1	35	.	.	.	.	.
Campanula rapunculoides	herb layer	.	.	.	1	.	.	.	.
Euonymus europaeus	shrub layer	.	.	.	1	.	.	.	.
Luzula luzuloides	herb layer	.	.	.	3	.	2	.	.
Brachypodium sylvaticum	herb layer	.	.	.	1	.	.	.	.
Poa nemoralis	herb layer	.	.	.	0.5	.	.	.	.
Galium rotundifolium	herb layer	.	.	.	3	.	2	.	.
Euphorbia amygdaloides	herb layer	.	.	1	.	.	.	.	.
Actaea spicata	herb layer	.	.	1	.	.	.	.	.
Polystichum setiferum	herb layer	.	.	.	.	.	.	8	.
Blechnum spicant	herb layer	.	.	.	.	.	.	3	.
Melica uniflora	herb layer	.	.	.	.	.	0.5	.	.
Stellaria nemorum	herb layer	.	.	.	.	0.5	0.1	.	1
Diagnostic species of class *Robinietea*									
Sambucus nigra	shrub layer	.	.	1	.	.	.	.	.
Diagnostic species of class *Thlaspietea rotundifolii*									
Epilobium lanceolatum	herb layer	.	.	.	.	.	.	2	.
Diagnostic species of class *Mulgedio-Aconitetea*									
Aegopodium podagraria	herb layer	0.1	0.5	.	.	.	.	10	.
Urtica dioica	herb layer	.	.	.	.	.	.	10	5
Senecio nemorensis	herb layer	.	.	.	.	.	1	1	.

Chrysosplenium alternifolium	herb layer	.	.	.	.	.	.	5	.
Diagnostic species of class *Epilobietea angustifolii*									
Lapsana communis	herb layer	2	0.1	.	.	.	0.5	.	.
Glechoma hederacea	herb layer	.	.	2	.	.	.	.	.
Stachys alpina	herb layer	.	.	2	.	.	.	.	.
Tanacetum macrophyllum	herb layer	.	.	.	.	.	.	2	5
Atropa belladonna	herb layer	.	.	.	.	.	.	1	.
Galeopsis speciosa	herb layer	.	.	.	.	.	.	.	3
Other species									
Lamiastrum galeobdolon	herb layer	0.5	.	.	.	.	.	.	.
Polygonatum officinalis	herb layer	0.5	.	.	.	.	.	.	.
Carex remota	herb layer	.	.	.	1	.	.	.	
Geranium macrorrhizum	herb layer	.	.	.	1	.	0.5	.	.
Orthilia secunda	herb layer	.	.	.	0.5	.	.	.	.
Hieracium racemosum gr.	herb layer	.	.	.	1	.	0.5	.	.
Brachythecium velutinum	moss layer	.	.	.	10	.	.	.	.
Dicranum scoparium	moss layer	.	.	.	10	.	.	.	.
Hylocomium splendens	moss layer	.	.	.	40	.	.	.	.
Neckera crispa	moss layer	.	.	.	5	.	.	.	.
Tortella tortuosa	moss layer	.	.	.	5	.	.	.	.
Lamium purpureum	herb layer	.	.	2	.	.			
Fraxinus excelsior	tree layer	.	.	15	.	.	.	.	.
Fraxinus excelsior	shrub layer	.	.	2	.	.	.	.	.
Hypnum cupressiforme	moss layer	.	.	5	.	.	.	.	.
Pterigynandrum filiforme	moss layer	.	.	2	.	.	.	.	.
Brachytheciastrum velutinum	moss layer	.	.	3	.	.	.	.	.
Salvia glutinosa	herb layer	.	.	.	.	.	.	5	.
Circaea lutetiana	herb layer	.	.	.	.	.	.	1	.
Galium aparine	herb layer	.	.	.	.	.	.	2	.
Solanum dulcamara	herb layer	.	.						
Calamagrostis arundinacea	herb layer	.	.	.	.	.	10	.	.
Polypodium vulgare	herb layer	0.5	.	.	1	.	.	.	.

[1]species also diagnostic for al. *Petasition officinalis*, ord. *Petasito-Chaerophylletalia* and cl. *Mulgedio-Aconitetea*; [2]species also diagnostic for al. *Stipion calamagrostis*, ord. *Stipetalia calamagrostis* and cl. *Thlaspietea rotundifolii.*

Association *Asperulo odoratae-Fagetum sylvaticae* (Table 3, rel. 1–3).

Vegetation description: Its communities have closed horizontal structure with total cover 95-100%. Dominant species is *Fagus sylvatica*. There are 4 well-developed layers – tree layer (cover 90-95%), shrub layer (cover 1-5%), herb layer (cover 25-30%) and moss layer (10-20%). Other species with higher cover and abundance, e.g. more than 10% are *Galium odoratum*, *Dryopteris filix-mas*, *Mercurialis perennis* and *Fraxinus excelsior*.

Ecology and distribution: This plant community type occurred on flat to moderately steep terrains with inclination up to 50° and northern exposition. Soils are moderately deep and bedrock is sediment. It is widest distributed vegetation type on the territory of reserve.

Syntaxonomy: This association is widespread on the territory of Bulgaria (Tzonev et al., 2006, Pedashenko et al., 2015, Vassilev et al., 2016). Species composition and vegetation structure of its communities on the territory of the reserve are similar to other phytocoenoses in the country.

Habitat types: According to EUNIS habitat classification are classified to G1.693 Balkan Range beech forests, whereas following Directive 92/43/EEC it is classified to 9130 *Asperulo-Fagetum* beech forests.

Association *Festuco drymejae-Fagetum sylvaticae* (Table 3, rel. 4)

Vegetation description: This association has closed horizontal structure and total cover of vegetation is 90%. It is characterized by poor species composition. Dominant species is *Fagus sylvatica* and subdominant species are *Festuca drymeja* and *Hylocomnium splendens*. Shrub layer is formed by young trees of *Fagus sylvatica* as well as *Euonymus europaeus*, *Rubus hirtus* and *Abies alba*. Herb layer has cover 25-30%. Moss layer is well-developed (about 70%) and is formed by *Hylocomnium splendens*, *Dicranium scoparium*, *Brachythecium velutinum*, *Neckera crispa*, *Tortella tortuosa*.

Ecology and distribution: This plant community type has limited distribution on the territory of reserve and occurred on moderately steep terrains with inclination up to 40° and north-western exposition. Soils are moderately deep and bedrock is sediment.

Syntaxonomy: Floristically and ecologically similar stands were studied from the territory of Osogovska Mts., Stara Planina Mts., Rui Mt., Rhodopi Mts., Sredna Gora Mt., Mikrenski Hills, Mt. (Tzonev et al. 2006).

Habitat types: According to EUNIS habitat classification are classified to G1.693 Balkan Range beech forests, whereas following Directive 92/43/EEC it is classified to 9130 *Asperulo-Fagetum* beech forests.

Abies alba-Fagus sylvatica community (Table 3, rel. 5-6)

Vegetation description: Species poor community with closed horizontal structure dominated by *Abies alba* and subdominant is *Fagus sylvatica*. Total vegetation cover is 90-95%. There are 4 layers – tree layer (90-95%), shrub layer (3-5%), herb layer (2-15%) and moss layer (up to 15%).

Ecology and distribution: On the territory of the reserve it is found on moderately steep to steep terrains with inclination up to 60° and northern exposition. Soils are shallow to moderately deep. Bedrock type is sediment.

Syntaxonomy: Floristically and ecologically similar stands were studied from the territory Stara Planina Mts., Western Rhodopi Mts., Rila Mts., Vitosha Mt., Rui Mt. and Belassitsa Mt (Tzonev et al., 2006).

Habitat types: According to EUNIS habitat classification are classified to G1.693 Balkan Range beech forests, whereas following Directive 92/43/EEC it is classified to 9130 *Asperulo-Fagetum* beech forests.

Association *Petasitetum hybrido-kablikiani* (Table 3, rel. 7)

Vegetation description: This community includes dense stands of *Petasites hybridus*, which is a dominant species with cover 90-95%. In vertical structure of phytocoenosis there are formed 2 layers – herb layer (90-95%) and moss layer (50%). Other species with higher cover and abundance are *Aegopodium podagraria* and *Urtica dioica*. Litter is weakly accumulated.

Ecology and distribution: It has very limited distribution on the territory of reserve, along tributaries of Gabrovnitsa river. This community covers an area of about 100 m^2. Soils are wet and rich of gravel and sediments. The stand is found on moderately steep terrain with inclination about 20° and western exposition. Bedrock type is sediment.

Syntaxonomy: This is a new association for the territory of the country. Generally, in Bulgaria, the *Petasites hybridus* phytocoenoses were poorly studied. Pedashenko et al. (2015) report association *Petasito hybridi-Platanetum orientalis* Kárpáti et Kárpáti 1961 from Kongura reserve, Mt. Belasitsa, where *Petasites hybridus* is a dominant species in the herb layer. The diversity and syntaxonomy of plant communities dominated by *Petsites* sp. have been investigated in regional context on the territory of Bulgaria and Romania from Nazarov et al. (2021, in press). They were classified to 3 associations - *Petasitetum albae* Dihoru ex Nazarov et al. 2021, *Petasitetum hybrido-kablikiani* Sillinger 1933 and *Telekio-Petasitetum hybridi* Morariu ex Resmeriță et Rațiu 1974.

Habitat types: According to EUNIS habitat classification are classified to E5.5722 Moesian butterbur tall herb communities.

Association *Parietarietum officinalis* (Table 3, rel. 8)

Vegetation description: Very species-poor community with closed horizontal structure and dominated by *Parietaria officinalis*. Total vegetation cover is 90-95%. Other species found in the species composition are *Stachys sylvatica, Urtica dioica, Tanacetum macrophyllum.*

Ecology and distribution: It has limited distribution on the territory of reserve on steep slopes with northern exposition above Gabrovnitsa river and covering an area about 80-120 m^2. Soils are moderately deep to shallow, skeletal and rocky.

Syntaxonomy: This is a new association for the territory of the country. *Parietaria officinalis* is well-known as a dominant or subdominant species in stands of *Platanus orientalis* and *Alnus glutinosa* woodlands and rarer in wetter *Fagus* forests in the country. It is also reported from Romania (Sanda et al., 2008).

Habitat types: According to EUNIS habitat classification are classified to H2.6G Eastern Carpathian calcareous thermophilous screes.

Discussion

The floristic composition and phytocoenotic structure of the beech forests, protected in "Elenova Gora" natural forest reserve are typical for the area of Central Balkan Range. The natural reserve of "Byala Krava", situated alongside the eastern border of Central Balkan Range mountain has similar characteristics. Sediment rocks and brown forest soils between 699 and 1071 m a.s.l., steep northern expositions and a boundary river are some of the similar features. "Byala Krava" reserve (93.4 ha) is almost two times bigger than "Elenova Gora" reserve and it is floristically richer, e.g. number of species (250), genera (171) and families are higher respectively with 89%, 58% and 16% (*Management plan of "Byala krava" natural forest reserve, 2014*). Three beech forest associations are also found in "Byala Krava" reserve: *Asperulo-Fagetum sylvaticae* Sougnez et Thill 1959, *Festuco drymejae-Fagetum sylvaticae* and *Umbilico erecti-Fagetum sylvaticae* Tzonev et al. 2006. The last one is also presented by two subassociations - *typicum* Tzonev et al. 2006 and *laurocerasetosum officinalis* Tzonev et al. 2006. The subassociation with the Euxine species *Laurocerasus officinalis* has refugial origin. The presence of the subAtlantic-subMediterranean species of *Taxus baccata* in "Elenova Gora" reserve, in a combination with high humidity and other ecological characteristics of the particular beech community bring it closer to the relict forests in "Byala Krava" reserve. Other common for both reserves Tertiary relict species are *Carpinus betulus, Acer campestre, A. pseudoplatanus, Hedera helix, Fraxinus excelsior, Asarum europaeum, Corylus avellana, Lamiastrum galeobdolon, Clematis vitalba, Isopyrum thalictroides, Sanicula europaea, Staphylea pinnata. Acer heldreichii, Daphne mezereum* and *Ruscus hypoglossum*. The presence of medicinal plants and their characteristics is almost the same within the two reserves with a prevalence in their numbers in "Byala Krava" reserve.

The associations of *Petasitetum hybrido-kablikiani* and *Parietarietum officinalis* are represented in "Elenova Gora" natural forest reserve. The investigation of the phytocoenoses of *Parietarietum officinalis* association adds a new data about syntaxonomy and ecology of *Thlaspietea rotundifolii* class in Bulgaria. Up to now the class *Thlaspietea rotundifolii* is represented by 3 orders, 3 alliances, 5 associations and 1 community in the vegetation of the country (Tzonev et al., 2009) and its syntaxonomical diversity is studied only on the territory of Rila Mts. and Pirin Mts. There is a scientific strive for a continuous research of the syntaxonomical diversity of this class on the territory of Bulgaria in order to reveal the present syntaxa diversity. The association in the reserve has a similar composition and structure with the phytoceonses of the association in Romania, studied by Sanda et al. (2008).

Conclusions

The beech forests in "Elenova Gora" reserve is a part of a refugium hot spot during the last Ice Age and nowadays it protects a specific floristic relict complex from the Tertiary Period. The floristic diversity is represented by 132 vascular plant species, referred to 50 families and 108 genera. Woody vegetation is represented by two associations: *Asperulo odoratae-Fagetum sylvaticae, Festuco drymejae-Fagetum sylvaticae* and 1 vegetation community (*Abies alba-Fagus sylvatica*). There is a limited area covered by the riverine hygrophilous vegetation of *Petasitetum hybrido-kablikiani* association. One order (*Stipetalia calamagrostis*), one alliance (*Stipion calamagrostis*) and one association (*Parietarietum officinalis)* were found for the first time for the vegetation diversity in Bulgaria.

Acknowledgements

The funding for the presented investigation was provided by "Fortis Facility" Ltd. and the results are included in chapter "Biological characteristics" of the Management plan of "Elenova Gora" strict reserve, prepared in accordance with Contract No. 97-CB5/13.05.2014 between the Fortis-NPM-IBER DZZD and the Central Balkan National Park Directorate.

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Received: 28.04.2021
Accepted: 27.10.2021

Appendix 1. List of recorded taxa in „Elenova Gora" natural forest reserve. Medicinal plants (Appendix 1 of Medicinal Plants Act 2000) are marked by asterix (*).

Polypodiophyta
Polypodiopsida
***Aspidiaceae**: Dryopteris carthusiana* (Vill.) H. P. Fuchs, **Dryopteris filix-mas* (L.) Schott, *Gymnocarpium dryopteris* (L.) Newman, **Polystichum aculeatum* (L.) Roth, *P. lonchitis* (L.) Roth, *P. setiferum* (Forskal) Moore; ***Aspleniaceae**: *Asplenium ruta-muraria* L., **A. trichomanes* L., **Phyllitis scolopendrium* (L.) Newman; ***Athyriaceae**: *Athyrium filix-femina* (L.) Roth, *Cystopteris fragilis* (L.) Bernh.; ***Blechnaceae**: Blechnum spicant* (L.) Roth; ***Polypodiaceae**: *Polypodium vulgare L.*
Pinophyta
Pinopsida
***Pinaceae**: *Abies alba* Miller; ***Taxaceae**: *Taxus baccata* L.
Magnoliophyta
Magnoliopsida
***Aceraceae**. Acer campestre* L., **A. platanoides* L., *A. pseudoplatanus* L.; ***Apiaceae**. Aegopodium podagraria* L., **Angelica pancicii* Vandas, **A. sylvestris* L., **Heracleum sibiricum* L., *Physospermum cornubiense* (L.) DC., **Sanicula europaea* L.; ***Araceae**.* **Arum maculatum* L.; ***Araliaceae**.* **Hedera helix* L.; ***Aristolochiaceae**.* **Asarum europaeum* L.; ***Asteraceae**. Achillea crithmifolia* Waldst. & Kit., *Hieracium gentile* Boreau, *H. racemosum* gr., *H. sabaudum* L., *Lapsana communis* L., *Mycelis muralis* (L.) Dumort., **Petasites hybridus* (L.) Gaertn., *Prenanthes purpurea* L., *Senecio nemorensis* L., *Tanacetum macrophyllum* (Waldst. et Kit.) Schultz-Bip., *T. parthenium* (L.) Schultz-Bip.; ***Balsaminaceae**. Impatiens noli-tangere* L.; ***Betulaceae**.* **Betula pendula* Roth, **Carpinus betulus* L., **Corylus avellana* L., ***Boraginaceae**. Myosotis sylvatica* Hoffm., **Pulmonaria officinalis* L., *Symphytum ottomanum* Friv., *S. tuberosum* L. ssp. *nodosum* (Schur) Soo; ***Brassicaceae**.* **Alliaria petiolata* (M. Bieb.) Cavara & Grande, **Cardamine bulbifera* (L.) Crantz, *C. impatiens* L., *Arabis procurrens* Waldst. & Kit.; ***Campanulaceae**. Campanula rapunculoides* L.; ***Caprifoliaceae**.* **Sambucus nigra* L.; ***Caryophyllaceae**. Moehringia trinervia* (L.) Clairv., *Stellaria alsine* Grimm, *S. nemorum* L.; ***Celastraceae**: *Euonymus europaeus* L.; ***Cornaceae**: *Cornus mas* L.; ***Euphorbiaceae**.* **Euphorbia amygdaloides* L., **Mercurialis perennis* L.; ***Fagaceae**.* **Fagus sylvatica* L.; ***Geraniaceae**.* **Geranium macrorrhizum* L., **G. robertianum* L.; ***Lamiaceae**. Ajuga reptans* L., *Calamintha grandiflora* (L.) Moench, **Galeopsis speciosa* Mill., **Glechoma hederacea* L., * *Gl. hirsuta* Waldst. & Kit., *Lamiastrum galeobdolon* (L.) Ehrend. et Polatschek, *Lamium garganicum* L., **L. maculatum* (L.) L., **L. purpureum* L., *Salvia glutinosa* L., **Stachys sylvatica* L.; ***Oleaceae**.* **Fraxinus excelsior* L.; ***Onagraceae**. Circaea lutetiana* L., *Epilobium lanceolatus* Sebast. et Mauri, *E. montanum* L.; ***Oxalidaceae**.* **Oxalis acetosella* L.; ***Papaveraceae**.* **Chelidonium majus* L.; ***Parnassiaceae**.* **Parnassia palustris* L.; ***Primulaceae**.* **Lysimachia nummularia* L.; ***Pyrolaceae**. Orthilia secunda* (L.) House; ***Ranunculaceae**.* **Actaea spicata* L., **Anemone nemorosa* L., **A. ranunculoides* L., **Clematis vitalba* L., **Helleborus odorus* Waldst. & Kit., **Isopyrum thalictroides* L., **Ranunculus ficaria* L.; ***Rosaceae**. Aremonia agrimonoides* (L.) DC., **Geum urbanum* L., **Fragaria vesca* L., *Rubus hirtus* Waldst. et Kit., **Sorbus aucuparia* L.; ***Rubiaceae**. Cruciata glabra* (L.) Ehrend., **Galium aparine* L., **G. odoratum* (L.) Scop., *G. pseudaristatum* Schur, *G. rotundifolium* L., *G. schultesii* Vest.; ***Saxifragaceae**.* **Chrysosplenium alternifolium* L., **Saxifraga rotundifolia* L.; ***Scrophulariaceae**.* **Veronica officinalis* L.; ***Solanaceae**.* **Atropa bella-donna* L., **Solanum dulcamara* L.; ***Staphyleaceae**. Staphylea pinnata* L.; ***Ulmaceae**.* **Ulmus glabra* Huds.; ***Urticaceae**.* **Parietaria officinalis* L., **Urtica dioica* L.; ***Violaceae**. Viola reichenbachiana* Boreau, *V. riviniana* Rchb.
Liliopsida
***Cyperaceae**. Carex echinata* Murr., *C. remota* L., *C. sylvatica* Huds.; ***Iridaceae**. Crocus veluchensis* Herbert; ***Juncaceae**. Luzula luzuloides* (Lam.) Dandy, *L. sylvatica* (Hudson) Gaudin; ***Liliaceae**. Polygonatum latifolium* (Jacq.) Desf., **P. odoratum* (Mill.) Druce; ***Orchidaceae**. Cephalanthera damasonium* (Mill.) Druce, *Dactylorhiza saccifera* (Brongn.) Sóo, *Neottia nidus-avis* (L.) Rich.; ***Poaceae**.* **Anthoxanthum odoratum* L., *Brachypodium sylvaticum* (Huds.) P. Beauv., *Calamagrostis arundinacea* (L.) Roth, *Dactylis glomerata* L., *Festuca drymeja* Mert & Koch, *F. gigantea* (L.) Vill., *Hordelymus europaeus* (L.) Harz, *Melica uniflora* Retz., *Milium effusum* L., *Poa nemoralis* L.

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 37-45

Phytoplankton Study in Pomorie Lake, Black Sea (Bulgaria)

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Abstract. Coastal wetlands, such as Pomorie Lake, are among the most dynamic and complex ecosystems in the world and their environmental safety requires special preservation. The aim of the present paper is to study the phytoplankton composition in Pomorie Lake, a protected area in Bulgaria. The development of a total of 49 phytoplankton species distributed in 8 classes was recorded and 6 phytoplankton species were recorded in „bloom" concentrations. The most abundant species were from class Dinophyceae (17 species, 34.69%) and class Bacillariophyceae (9 species, 18.37%). The research found a decrease in the proportion of diatoms (Bacillariophyceae class) and an increase in the number of green and euglena algae. This pilot study contributes to the current characterization of the ecological status of Pomorie Lake. According to the established phytoplankton biomass and part of the ecological assessment criteria for "mesotrophic" lake types the ecological status of Pomorie Lake in 2017 is between "moderate" - "good".

Key words: Pomorie Lake, Black Sea, phytoplankton, Dinophyceae, Bacillariophyceae.

Introduction

Pomorie Lake is part of the Burgas lake complex (Burgas, Atanasovsko, Mandra and Pomorie Lakes), which is one of the three most significant wetland complexes for congregations of waterfowl along the Bulgarian Black Sea Coast.

During recent years the urbanization and building activities around the lake have become more intensive and some of the small wetland habitats on the periphery of the lake are already extinct. Furthermore, a significant pollution of the surrounding land is caused by household and building waste. The natural water regime of the lake is disturbed, because the canal that connects it to the sea was obstructed by sediments.

As it is known phytoplankton is an indicator used for aquatic ecosystem assessments, because it consists of primary producers with extremely short lifecycles, which respond early to any change in the environment (Niemi & McDonald, 2004; Maialen et al., 2014; Pasztaleniec, 2016).

Historical data on the composition, distribution and dynamics of the phytoplankton in the lake has been described mainly by Vasilev et al. (1998), (documented 39 species (pyrophyta and diatoms)) and Stoyneva (2010) (63 algal taxa of 9 classes, 24 genera, mainly oligo- and mesohaline species).

The values of the quantitative parameters such as biomass and abundance have been described as high, characterized by high degree of eutrophication of the water.

Nowadays, the studies of the phytoplankton in Pomorie Lake as a component of the biota are limited and there`s a lack of systematic quantitative phytoplankton data. Therefore, in this pilot study we set out to investigate the dynamics and composition of phytoplankton in Pomorie Lake during 2017.

Material and Methods

Lake hydrology and hydrochemical characteristics

Pomorie Lake is a hypersaline lagoon area near Pomorie town and has a size of 814 ha. According to Raynova (2010) the freshwater catchment area of the lake is located between the Kamenar and Kableshkovo Villages. The total catchment area is 33.55 km^2. The water surface area is 6 km^2 and the water volume is 6 million km^3 on average (Varbanov, 2002). The average depth of the lake is 1 m and it reaches a maximum of about 1.6 m (Vasilev & Mitrophanova, 1998).

The lake water has mildly to moderately alkaline pH levels with oxygen saturation between 50 and 240% (Hiebaum, 2010). Surprisingly low average salinity levels of 19-19,9 ‰ (Popov, 2015, Burgas, pers. comm), similar to those of the Black Sea, have been measured due to non-functioning southern drainage system. The main reason for that salinity change was the different hydrological regimes and the inflow of fresh water from Kamenarska River. The river was first drained into the protective drainage channel near the lake, but then after the backfilling in the area of the inflow, its fresh water had been entering the lagoon for years.

Phytoplankton sampling and analysis

Six phytoplankton samples were collected with Ruttner bottle (1,8 L) from the surface water layer (0-0.20 m), from 6 different sites (Table 1, Fig. 1) during April, July and October in 2017. Sampling sites were selected so that they can provide a satisfactory geographical coverage of the area according to methodology of Moncheva & Parr (2010). They were fixed with formalin (up to 2% solution) and concentrated by sedimentation (Morozova-Vodianitskaya, 1954). The microalgae were analyzed with an Olympus BX41 light microscope (by light field microscopy and phase contrast; at 100x, 200x, 400x and 800x magnifications) and in Sedgwick Rafter counting chambers (1 ml) and Palmer - Maloney (0.05 ml) by standard methodology (Moncheva & Parr, 2010). Picoplanktonic cells (< 2 μm) were not examined.

The ecological status was assessed according to the evaluation criteria of Surface water characterization (2013), using 5 classes from excellent to very poor environmental condition as defined in Annex 2 of Ordinance № 4. Only a few of the metrics were analyzed due to the pilot character of this study.

A geometric method (Edler, 1979; Olenina et al., 2006) was applied to determine the biomass of microalgae and the individual sizes of several representatives of each species were measured. The cell volume was calculated using geometric formulas (Olenina et al., 2006). We assume that the cell density is 1.

The taxonomic classification of phylum and classes has been made in accordance with WoRMS (2019).

The Phytomar 2.0 (Klisarova, 2008) and Excel 12 (Microsoft Corporation, 2007) software were used for Shanon's index (calculated by abundance) and graph calculations.

Table 1. Sampling sites in Pomorie Lake.

Site №	GPS coordinates
1	42°36'26.2"N 27°37'31.3"E
2	42°33'56.5"N 27°37'57.0"E
3	42°33'58.0"N 27°37'40.4"E
4	42°34'05.5"N 27°37'32.2"E
5	42°34'17.0"N 27°38'07.2"E
6	42°34'04.3"N 27°38'12.7"E

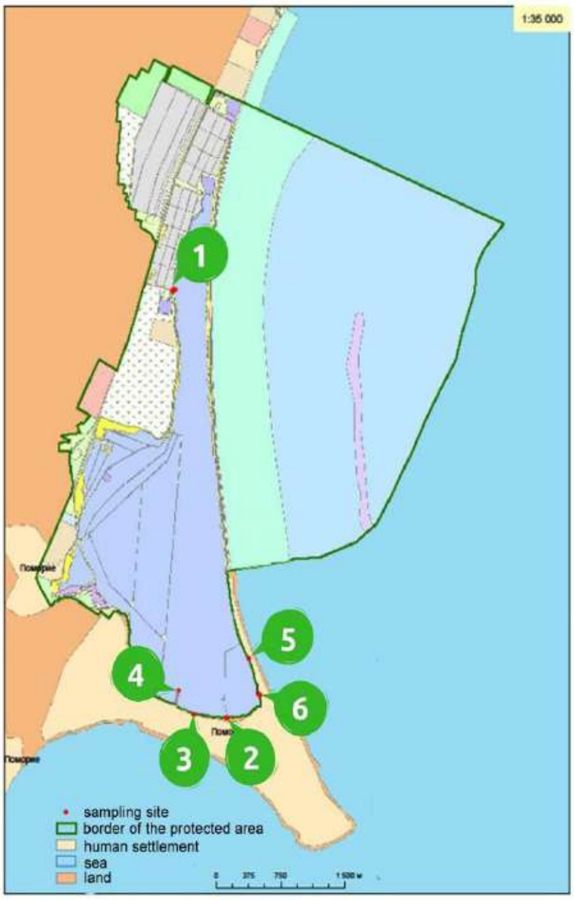

Fig. 1. Map of the study area with indications of the sampling sites.

Results and Discussion

Species composition of phytoplankton

The development of a total of 49 species distributed in 8 classes was recorded in the phytoplankton of Pomorie Lake during 2017. The most abundant species were from class Dinophyceae (17 species, 34.69%) and class Bacillariophyceae (9 species, 18.37%). The classes Chlorophyceae, Cyanophyceae and Euglenophyceae were registered with 6 species or 12.24%, and the rest 3 classes (Cryptophyceae, Trebouxiophyceae, Zygnematophyceae) were respectively registered with 2 and 1 species or 10.20% of the total taxonomic composition (Table 2, Fig. 2).

Compared to the earlier studies of Vasilev et al. (1998), a decrease in the share of diatoms (class Bacillariophyceae) and an increase in the number of green and euglena algae were found. The tendency of development of a large number of typically marine eurythermal and euryhaline microalgae species remains (Vasilev et al., 1998).

In April, a total of 20 species from 6 classes were found in the lake. (Table 2). The maximum annual biodiversity was observed for class Dinophyceae (11 species) and class Trebouxiophyceae (3 species) (Table 2, Fig. 3). No species of the class Bacillariophyceae were registered.

In July, 23 species from 7 classes were found. The number of species varied from 13 to 17 species per sampling site. The classes Dinophyceae (7 species), Bacillariophyceae (6 species) and Cyanophyceae (4 species) were presented with the highest number of species. The remaining groups of microalgae were registered with a small number of species (Table 2, Fig. 3).

Table 2. Taxonomic list of phytoplankton species existing in Pomorie Lake in 2017. *Legend:* 1-6 numbers of sampling points in combination with the month of sampling: A - April, J – July, O - October.

№	Taxa/Sampling site, Month	Site 6, A	Site 4, J	Site 2, J	Site 5, O	Site 1, O	Site 3, O
	BACILLARIOPHYCEAE						
1	*Chaetoceros socialis*				+		+
2	*Chaetoceros sp.*		+		+		

3	*Cocconeis scutellum*		+	+			
4	*Cyclotella caspia*				+	+	+
5	*Diatoma tenuis*		+				
6	*Navicula sp.*		+	+	+	+	+
7	*Nitzschia sp.*		+				
8	*Nitzschia tenuirostris*					+	+
9	*Pleurosigma elongatum*			+			
	DINOPHYCEAE						
10	*Akashiwo sanguinea*	+					
11	*Cochlodinium sp.*	+					
12	*Glenodinium danicum*	+					
13	*Glenodinium sp.*			+			
14	*Gonyaulax sp.*	+					
15	*Gonyaulax spinifera*		+				
16	*Gonyaulax turbynei*	+					
17	*Gymnodinium agiliforme*	+			+		
18	*Gymnodinium albulum*				+	+	+
19	*Gymnodinium sp.*	+	+	+	+	+	+
20	*Gyrodinium spirale*		+				
21	*Oblea rotunda*				+	+	+
22	*Peridinium pusillum*		+				
23	*Peridinium sp.*	+	+		+		+
24	*Polykrikos schwarzii*	+					
25	*Prorocentrum cordatum*	+					
26	*Prorocentrum micans*	+	+	+			
	CHLOROPHYCEAE						
27	*Ankistrodesmus sp.*				+		
28	*Chlamydomonas sp.*						+
29	*Chlorogonium sp.*					+	+
30	*Chlorophyceae indet.*		+		+	+	+
31	*Coelastrum sp.*	+					
32	*Monoraphidium convolutum*	+					
	EUGLENOPHYCEAE						
33	*Euglena sp.*				+		+
34	*Eutreptia lanowii*				+	+	+
35	*Eutreptia sp.*			+			
36	*Eutreptia viridis*				+		
37	*Eutreptiella sp.*					+	
38	*Phacus dangeardii*	+	+				
	CRYPTOPHYCEAE						
39	*Cryptomonas sp.*	+	+	+	+		+
40	*small Flagellates*		+	+	+	+	+
	CYANOPHYCEAE						
41	*Anabaena sp.*	+					
42	*Lyngbya sp.*		+				
43	*Merismopedia sp.*	+		+			
44	*Oscillatoria sp.*			+	+	+	+

45	*Spirulina sp.*					+
46	*Cyanophyceae indet.*	+		+		
		TREBOUXIOPHYCEAE				
47	*Chlorella pyrenoidosa*	+				
48	*Chlorella vulgaris*	+				
		ZYGNEMATOPHYCEAE				
49	*Penium sp.*		+	+		

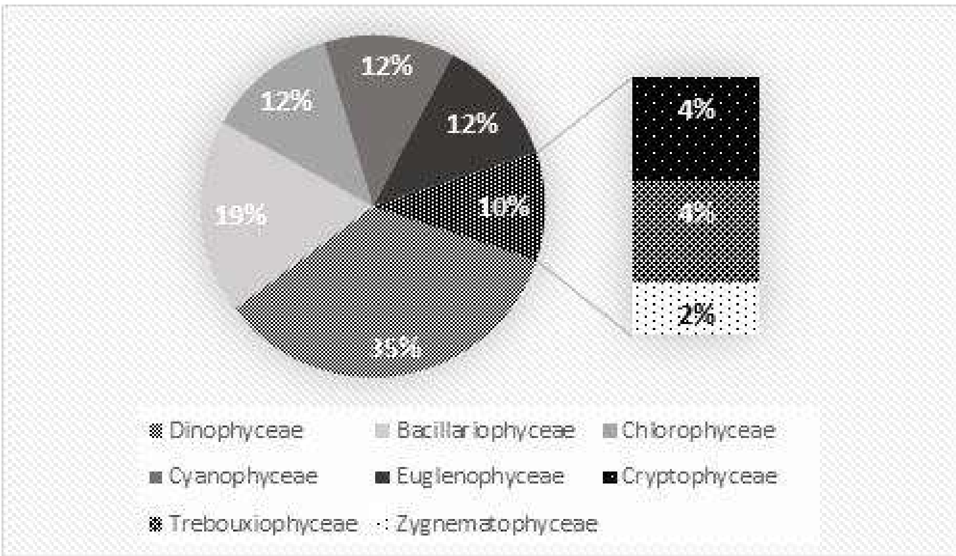

Fig. 2. Taxonomic composition (%) of the phytoplankton in Pomorie Lake by class in 2017.

In October, 22 species from 6 classes were found. We recorded a reduction in the numbers of peridinea and diatom species and an increase in green algae (Table 2, Fig. 3). The number of species in the different sampling sites varied from 13 to 16 species. This shows that the number of species in the study of this basin has slightly increased compared to the numbers (5-13 species) that was reported by Stoyneva (2010).

The Dinophyceae class constitutes the largest share of the taxonomic composition in the spring (55%, April). The share of peridinea species decreases toward the end of the year. In the autumn (October), the proportion of species of class Euglenophyceae increases, probably related to the accumulation of the organic matter in the water (as the Euglenophyceae are indicators of organic pollution according to Petrova & Gerdzhikov (2010). The classes Dinophyceae (7 species), Bacillariophyceae (6 species) and Cyanophyceae (4 species) were presented with the highest number of species during this warm month. The classes Chlorophyceae and Cryptophyceae were also increasing their share. The remaining groups of microalgae were registered with a small number of species (Table 2, Fig. 3). Species of Cyanophyceae are the most abundant in the summer and spring while the diatoms (Bacillariophyceae) - in the summer and autumn 2017 (Fig. 3).The total monthly average number of phytoplankton species had not changed significantly and had been registered between 20 and 23 phytoplankton species.

According to Stoyneva (2010) in 2007 various green and cryptophytic flagellates had a leading role in the composition of phytoplankton. Diatoms were also present.

The established taxonomic structure of phytoplankton in Pomorie Lake is comparable to that in marine coastal waters, characterized by the highest shares of the classes Dinophyceae and Bacillariophyceae (Petrova & Gerdzhikov, 2015). Perhaps the reason is in the decreasing salinity of the lake - from 52-55‰ (2007) (Hiebaum, 2010) to 19-19.9‰ (Popov, 2015, Burgas, pers.comm.) According to Rozhdestvenski (1986) the average salinity Black Sea coastal water is 16.47‰.

Quantitative dynamics of phytoplankton

A total of 6 phytoplankton species in „bloom" concentrations were recorded in Pomorie Lake. Although there are a lot of uncertainties of what constitutes a bloom (Smayda, 1997) usually concentrations exceeding > 1 x 10^6 cells.l^{-1} for a single species or > 5 x 10^6 cells.l^{-1} for 2-3 species or biomass > 10000 mg.m^{-3} are considered "bloom" (Moncheva & Parr, 2010). In the spring, the green algae *Chlorella pyrenoidosa* bloomed with the highest abundance - 41.06 x 10^6 cells.l^{-1}. High concentration of species from Cyanophyceae (2 µm) - 1.45 x 10^6 cells.l^{-1} was observed in the summer. Most "blooming" species (4) were found during the autumn period.

Phytoplankton "blooming" was recorded at all sites in the autumn, with the highest values occurring at the "Site 3": small Flagellates - 12.79 x 10^6 cells.l^{-1}; *Gymnodinium albulum* - 2.69 x 10^6 cells.l^{-1} and *Gymnodinium sp.* - 1.35 x 10^6 cells.l^{-1}. At the "Site 5", the blue-green *Oscillatoria sp.* 1.56 x 10^6 cells.l^{-1} was also "blooming". The distribution of algal groups by biomass was uneven between sampling sites. In October 2017, within three days and at three different stations phytoplankton values which reflected the current state of the cenosis were measured. These parameters were the result of regular ecological modulations formed under the influence of temporary or permanent factors. According to Uzunov & Kovachev (2002) biomass is always an instantaneous, residual, resultant value.The highest phytoplankton quantities were detected during the spring (Figure 4), then a second peak was recorded during the autumn. In the summer very low values of phytoplankton development were registered.

High values of the Shannon Index in Pomorie Lake - suggesting a "very good" ecological status of the lake water according to BQE (Biological Quality Element) Phytoplankton. The spring and autumn phytoplankton "blooms" present worse ecological status during these seasons. According to the average phytoplankton biomass levels established in the present study (Table 3) and the ecological assessment criteria for "mesotrophic" lake types (Surface water characterization, 2013) the ecological status of Pomorie Lake in 2017 is between "moderate" and "good".

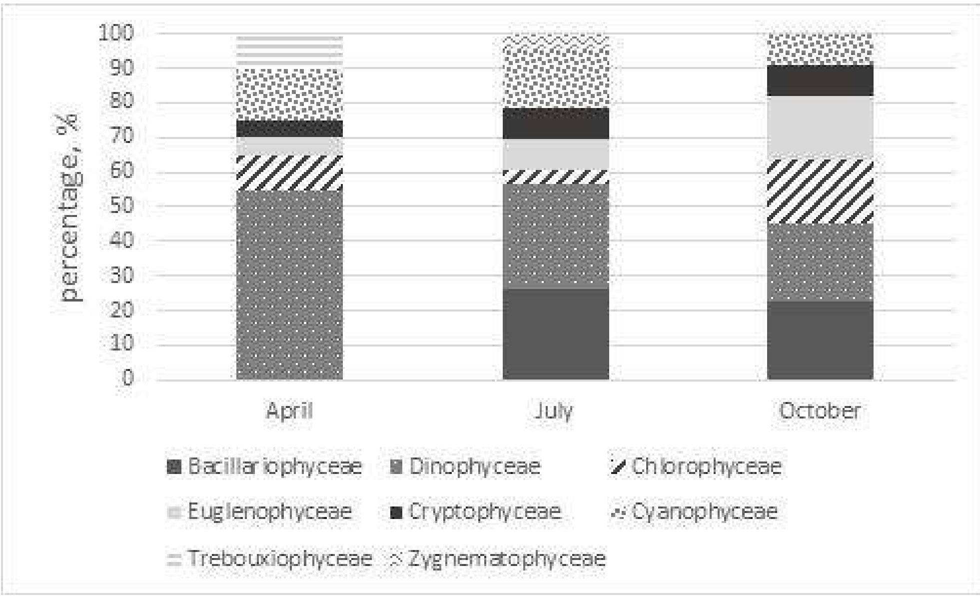

Fig. 3. Seasonal dynamics of the phytoplankton taxonomic composition.

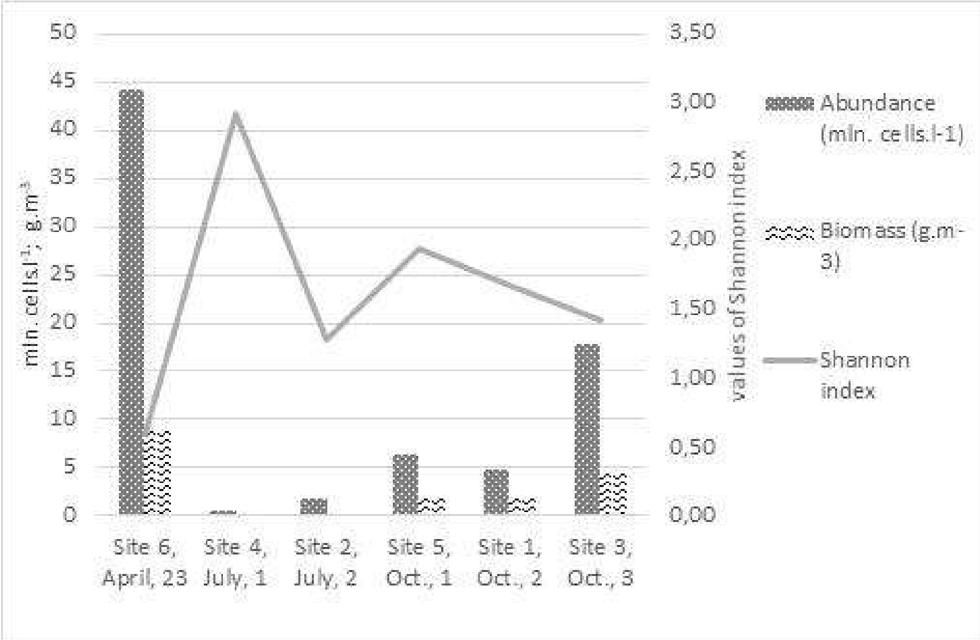

Fig. 4. Phytoplankton abundance (x 10^{-6} cells.l^{-1}), biomass (g.m^{-3}) and Shannon index, Pomorie Lake, values from all sampling sites.

Table 3. Seasonal averages of phytoplankton abundance, biomass and Shannon's diversity index from Pomorie Lake.

Season	Abundance (x 10^6cells.l-1)	Biomass (g.m^{-3})	Shannon index
Spring	44.20	8.94	0.59
Summer	1.18	0.43	2.10
Autumn	9.72	2.86	1.68
Average	18.37	4.07	1.46

Compared to Atanasovsko Lake the number of species is smaller than that of Pomorie Lake (Stoyneva, 2010), but the main groups forming the phytoplankton in the two wetlands are similar. In Black Sea coastal lakes such as Atanasovsko and Vaya (Burgas Lake), the Cyanoprokaryotes have a significantly higher qualitative and quantitative participation in the phytoplankton according to the results of Belkinova et al. (2020) and Dimitrova et al. (2014). The resulted data on the abundance (1.18 x 10^{-6} cells.l^{-1}) during summer is similar to those reported by the authors in Atanasovsko Lake during 2007 (1.15 x 10^{-6} cells.l^{-1}).

These comparisons can only be preliminary since both the studied periods and the number of analyzed samples differ.

Conclusions

The present study was an attempt to supplement the observations on the ecology of Pomorie Lake, as well as to make a qualitative and quantitative assessment of the phytoplankton. This study analyzed the current taxonomic structure and distribution of phytoplankton in 2017.

As a result of analyzed current taxonomic structure and distribution were established 49 species belonging to 8 classes. Dynophyceae is the dominant family, whereas Cyanophycea is species poorer. A probable reason for that state was the entry of Black Sea water into Pomorie Lake.

Were registered six species with "blooming" concentrations. The spring was characterized by the highest development of phytoplankton in the lake, while summer had the lowest values.

According to some of the criteria for ecological assessment and the established biomass of phytoplankton, the ecological condition of Pomorie Lake was between "moderate" and "good".

Acknowledgements

We wish to express our sincere gratitude to the Green Balkans Association, MASRI-IFR, RIEW-Burgas (Regional Inspectorate of Environment and Water) for their assistance during the fieldwork and to Trakia University which founded the survey as a part of PhD thesis (Ecological study of benthic molluscs in Pomorie Lake Protected Area).

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Received: 28.04.2021
Accepted: 01.11.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 47-55

Influence of pH, Organic Carbon and Total Nitrogen Content on the Amount and Distribution of Different Microbial Groups in the Organic Layers of Luvisols

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Abstract. The influence of altitude, pH, organic C and total nitrogen content on the Quantity and Distribution of soil microflora in the organic layers of Luvisols was investigated. The experimental plots include A horizon of 5 soil profiles. Aiming to determine the biogenity of analyzed plots, the total microbial number and the percentage share of different microbial groups has been examined. The samples were taken from forest soil according to microbiological requirements. The standard microbiological method for analyses was used. The obtained results showed different biogenity and percentage share of microbial groups according to the different environmental conditions – pH, org. C and total N. Strong correlation between total microbial number and org. C and total N has been found. Soil with the largest total microbial number (6.7 lg CFU/g dry soil) was the soil with the highest account of org. C (49.98 g.kg^{-1}) – tested plot (TP) 2. pH as a parameter of soil environment has been correlated with the distribution of microbial groups.

Key words: microorganisms, total microbial number, forest soil, pH, org. C, total N.

Introduction

Soil environment is one of most complex habitat for microorganisms due to the different condition (Horner - Devine et al., 2004). Microorganisms are one of most important biological components of the soil (Bhatt et al., 2015). They are the main agent responsible for decomposition of organic compounds (Schloter et al., 2018). Microorganisms have fundamental role in the transformation of substances in the soil according to their metabolism (Jacoby et al., 2017).

The development, diversity and abundance of microbial communities in the soil are influenced by the main characteristics of the soil - its acidity, organic carbon content and total nitrogen (Jha et al., 1992). A number of studies are focused on the change of the microbial community in the event of a change in the nutrient base and environmental conditions (Willey et al., 2008), but do not consider the overall effect that the individual parameters have on the soil microflora. The humus-accumulating soil horizon is characterized by a greater microbial abundance, as a result of the increased content of nutrients and the presence of more suitable water-air and temperature conditions of the environment

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(Fritze et al., 2000), which is the main prerequisite for its analysis in the present study.

Bhatia (2008) points out that the physical properties and the amount of humus is directly related to the abundance of microflora. In their studies, Farrell et al. (2014) and Khatoon et al. (2017) emphasize the fact that the content of organic carbon and total nitrogen are directly related to the activity of soil microflora.

Lauber et al. (2009), cite the soil reaction as one of the main soil factors that influence the growth and development of microorganisms. Many soil chemical and biological reactions are controlled by the pH of the soil (Hendershot et al, 1993). pH range for optimal microbial activity and nutrient availability has been considered to be between 5.0 and 7.0 (Vanmechelen et al., 1997). Differences in acidity suggest different conditions for the development of different microbial groups (Fierer & Jackson, 2006). Other authors have shown that pH cannot be used as a stand-alone indicator of the abundance and distribution of microbial biota (Cho et al., 2016).

However, there is only a few studies have attempted to analyze and verify the combinative connection between soil microbial community and pH, org. C and total N.

According to this, the main aim of the present study is to investigate the connection between the quantity and distribution of the microbial community on the one hand and pH, the content of organic C and total nitrogen on the other, in soils, including statistic relationship between the analyzed parameters.

Materials and Methods

The subject of the study is soils from part of the territory of the Western Stara Planina Mts. - lower belt with the northern slopes exposition. The studied territory covers the part of the Stara Planina Mts. between the western border of Bulgaria and the Iskar Gorge. According to the climatic zoning, the Western Stara Planina Mts. falls into two climatic regions: Fore mountain and mountain region. Both regions are part of the temperate continental climate subregion. The studied area is characterized by precipitation from 750 to 1000 mm (Velev, 2002; Koleva–Lizama, 2006). According to Bondev (2002) the studied part of the Western Stara Planina is part of the European region of deciduous forests and the Illyrian province.

The territory of the Western Stara Planina Mountain falls in the Moesian forest growing area, subregion Northern Bulgaria.

In the monograph Soils in Bulgaria (1960), the main type of soils for the territory of the Western Stara Planina Mountain in the lower forest belt are Luvisols. These forest soils are typical in the study area and they have a more intensive biological cycle, more pronounced processes of mineralization of organic matter, deeper leaching, textural differentiation between the surface horizon and the underlying agric horizon (Koinov et al., 1998)..

Collection of sampling. Soil samples for microbiological and soil analyses were taken from 5 soil profiles on the territory of Stara Planina Mountain. The soil is Luvisols. Soil analyses were performed by standard methods and includes determination of soil acidity in aqueous extract ($pH_{(H2O)}$), using a glass pH electrode in a 1:5 (V/V) suspension of soil in water (ISO 10390); determination the organic carbon were found according modification method of Turrin (Filcheva & Tsadilas, 2002) and total nitrogen content was determined by Kjeldahl digestion (ISO 11261).

Microbiological analyses included determination of total microbial number, determination of percentage distribution of different microbial groups, as bacilli, non-spore forming bacteria, actinomycetes and micromycetes. Total number of microorganisms was determined by Koch's method, involving successive dilutions and subsequent inoculation on appropriate elective agar medium. The count was made as colony forming units per gram dry soil mass (CFU/gram dry soil mass) under logarithm (lg). The statistical analyses of data were carried out by StatSoft Statistica 12 program under significance thresholds 95%.

Results

Five soil profiles were studied and analyzed. The morphological characteristics of the soil profiles of the studied soils define as Luvisols the soils from all Tested Plots (TP) – TP1, TP 2, TP 3, TP 4 and TP 5 according to the requirements of the Basic classification of soils in the country (Penkov et al., 1992).

The studied soils have a characteristic profile of the type 0ABtC. On the surface of some of the profiles there is accumulated litter. For the purpose of the present study, the soil characteristics of the A horizon were considered. As emphasized above, the humus-accumulating soil horizon (A horizon) is characterized by a greater abundance of microbial communities and is the main soil horizon in which the transformation of soil organic matter takes place.

The considered soil profiles are set at an altitude of 185 m to 651 m in order to cover the variety of specific environmental factors in the study of soil microflora. Table 1 presents the characteristics of the tested soils.

Table 1. Main characteristics of soil profiles.

TP №	Location	Altitude (m)	Exposure	Vegatat ion	pH	Humus (%)	org. C ($g.kg^{-1}$)	Total N ($g.kg^{-1}$)	C:N
1	N 43 23 09 E 23 14 51	185	NW	*Quercus frainetto* *Pinus nigra* *Fraxinus ornus*	5.10	2.42	14.03	2.06	7
2	N 43 31 26 E 22 41 56	390	NW	*Quercus cerris* *Carpinus betulus*	7.40	8.62	49.98	6.66	8
3	N 43 30 42 E 22 38 17	510	N	*Carpinus betulus* *Quercus robur*	4.90	5.49	31.87	3.60	9
4	N 43 36 41 E 22 31 54	610	NW	*Fraxinus ornus*	6.20	4.53	26.29	3.77	7
5	N 43 10 30 E 23 09 12	615	N	*Carpinus betulus* *Fagus sylvatica*	4.60	3.84	22.26	2.85	8

There is no relationship between the increase in altitude and the amount of humus and org. C in the A horizon of the studied soils, as evidenced by the low correlation coefficient (r=0.13). As a consequence, we have a low correlation between altitude and the amount of total nitrogen (r=0.093).

Obtained results for the studied soils show that the A horizon is characterized by a pH in the range of 4.6 at an altitude of 615m to 7.4 at an altitude of 390 m. These differences in acidity are due to the large diapason of altitude and influence of different vegetation. The acidity in the considered profiles varies from very strongly acidic in TP5 to alkaline in TP2. Profiles № 1 and № 3 are characterized by pH 5.1 for TP1 and 4.9 for TP3 in the humus-accumulative horizon, which in accordance with the adopted classification means that in this profiles soil has a strong acidic reaction. This acidity indicates that the aggressive fraction of humic acids is related. pH values at TP5 indicate very strong acidic reaction, for which is characterized by the presence of free organic acids - fulvic acids (Ganev, 1990). TP4 is characterized by pH 6.2 or the soil in this plot has a slightly acidic reaction. With neutral reaction of pH 7.4 has been determined TP2. There is no correlation between soil pH and altitude change (r=0.10).

The obtained data on the content of org. C in the studied soils show, that it varies from medium to very high (on the scale of Vanmechelen, 1997). In TP1 and TP5 its quantity is respectively 24.20 $g.kg^{-1}$ and 38.38 $g.kg^{-1}$. TP3 and TP4 are characterized by high org. C content. The highest content of org. C is reported at TP2 – 49.98 $g.kg^{-1}$.

Similar to org. C, the content of total N in the humus-accumulating horizon varies from medium to very high (on the Vanmechelen scale, 1997). The soil from TP1 is determined with average content of total nitrogen (2.06 $g.kg^{-1}$). According to the received data TP3, TP4 and TP5 have a high content of total nitrogen, and in TP2 it is very high (6.66 $g.kg^{-1}$).

The ratio org. C/total N in the studied soils is in the range of 7-9. These ratios are rated as very low on the Vanmechelen scale and show an advanced degree of transformation of organic matter to the formation of stable humic substances.

A large number of factors such as active reaction, stock of soil with org. C and nitrogen, altitude and others affect the number of microorganisms in the soil and the microbial activity. The quantitative characteristic of the general microflora is one of the microbiological indicators that can be used to assess the biogenicity of the soil.

Table 2 presents the reported microbiological indicators of the studied soils in colony forming units per gram dry soil mass under logarithm (lg CFU/g dry soil).

The total microbial number of the tested plots is reported, as well as the percentage of the individual microbial groups in the microbial community. The total microflora is presented as lg of the sum of CFU (colony forming units) of non-spore-forming bacteria, bacilli, actinomycetes and micromycetes in Table 2. The influence of the pH, org. C and the total nitrogen was statistically analyzed both in relation to the formation of the general microflora and in relation to the influence of the parameters on the distribution of the microbial groups.

Table 2. Total microbial number lg CFU/g dry soil.

TA	Total microbial number	*Bacillus* sp.	Non-spore forming bacteria	Actynomicetes	Micromycetes
1	6.12 ± 0.22	6.05 ± 0.38	4.25 ± 0.90	5.09 ± 0.34	4.90 ± 0.34
2	6.70 ± 0.22	5.01 ± 0.38	6.68 ± 0.90	5.28 ± 0.34	4.41 ± 0.34
3	6.45 ± 0.22	5.75 ± 0.38	6.15 ± 0.90	5.60 ± 0.34	5.71 ± 0.34
4	6.26 ± 0.22	5.41 ± 0.38	5.77 ± 0.90	5.97 ± 0.34	4.35 ± 0.34
5	6.24 ± 0.22	5.68 ± 0.38	5.64 ± 0.90	5.63 ± 0.34	5.59 ± 0.34

The total microbial number of microorganisms in the tested accumulative horizons –varied from 6.12 lg CFU/g dry soil to 6.70 lg CFU/g dry soil. The soil with the highest total microbial number was found in the TP2 on 390 m altitude and northwest exposure.

According to the "r" factor no correlation was found between altitude and total biogenicity (r= 0.06). In contrast, other studies prove a high correlation between soil biogenicity and altitude change, but at higher altitude gradient ranges (Grigorova-Pesheva, 2019).

Soil pH determines the solubility of many nutrients and their availability to plants and soil microflora (Mohammed & Zigau, 2016; Fierer & Jackson,2006). In contrast, other authors argue that the degree of development of soil microflora cannot be evaluated only on the basis of effect of acid reaction on the soil microbial biota (Cho et al., 2016).

Based on the obtained results, we believe that the process of soil acidification in the considered forest ecosystems is natural and the autochthonous soil microflora is adapted to it. This is proved by the performed correlation analysis of the two parameters. The measured active reaction of the soil and the amount of the total soil microflora show a relatively weak correlation (Fig. 1.).

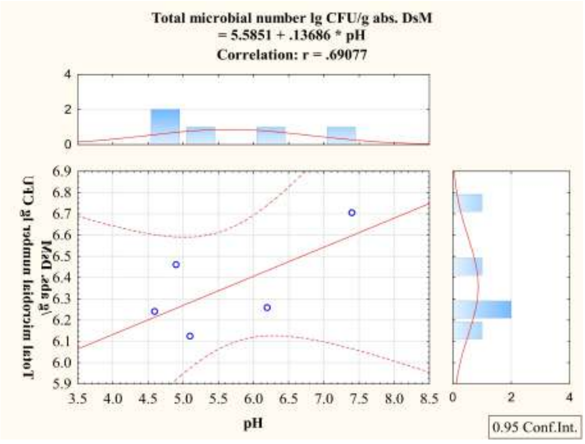

Fig. 1. Statistical relationship between total microbial number and pH.

The obtained correlation coefficient has a value of r = 0.69 at pH values in the range from 4.6 to 7.4. Other authors also come to similar conclusions, showing that soil pH is not a sufficient indicator for the development of the microbial biocenosis, but a complex approach is needed (Cho et al, 2016). However, the data show a much higher proportion of micromycetes compared to other microbial groups in the studied soils, where the acidity is lower, namely TP3 (5.71 lg lg CFU/g dry soil) and TP5 (5.59 lg CFU/g dry soil), which shows the influence of the soil reaction on the redistribution of microbial groups.

In contrast to pH, the amount of org. C significantly affects the biogenicity of the soil and this is evidenced by the high correlation coefficient between the two parameters: R= 0.98 (Fig. 2.).

The correlation between the content of org. C and the biogenicity of the soil has been proven and by other authors Khatoon et al. (2017). Org. C is a source of nutrients and plays a key role in maintaining the activity of soil microbial biota.

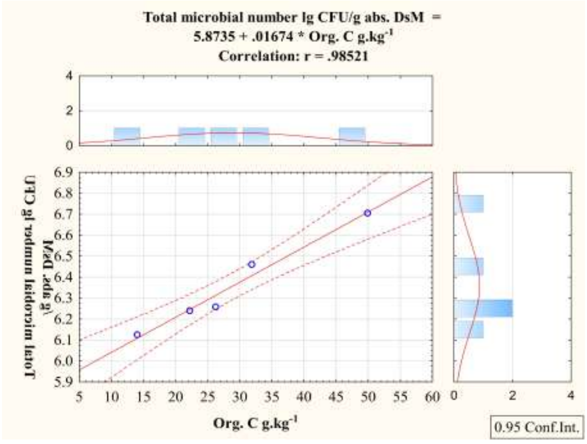

Fig. 2. Statistical relationship between total microbial number and Org. C.

The obtained results show a high value of the correlation coefficient when considering the statistical relationship between the amount of total nitrogen and the total microbial number (r=0.94), (Fig. 3).

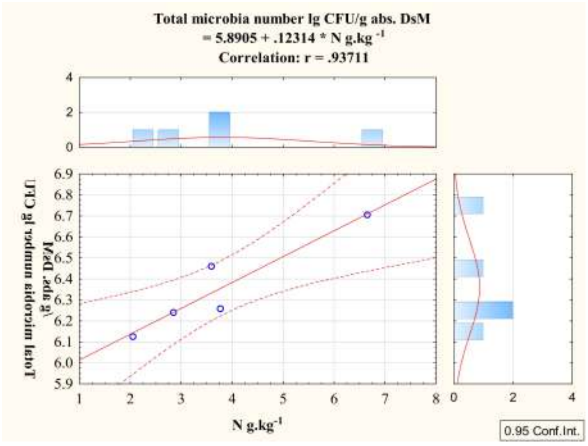

Fig. 3. Statistical relationship between total microbial number and total N.

This relationship is not surprising, given that soil microbial biota is involved in all aspects of the nitrogen cycle. The strong correlation between the two parameters is mainly related to the transformation of organic matter by microorganisms, i.e. the use of total nitrogen as a source of energy and as a material for building the bodies of microorganisms. With the increase of the content of total nitrogen in the soil, the exponential development of the soil microflora begins, which is responsible for the transformation of the various forms of nitrogen. Our results correspond to the results reported in other scientific studies (Li at al., 2018).

Fig. 4 shows the composition of the microbial communities by groups as a percentage of the total microflora for the individual TPs.

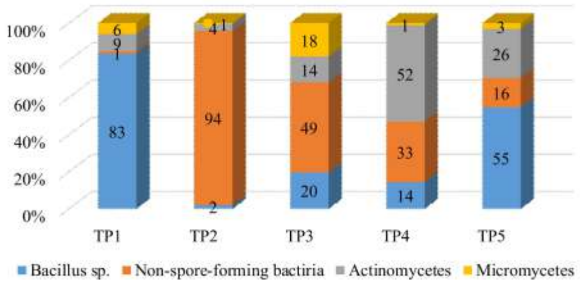

Fig. 4. Percentage share of microbial groups in the total microbial number, (%).

The percentage participation of different groups of microorganisms in the soil is an indicator of the course of the individual stages of transformation of soil organic matter (SOM). Various studies have focused on the relationship between the dominant group of microorganisms and the different soil characteristics (Zhang et al., 2013). As pointed out by Perfanova et al. (2015) the distribution of microbial groups in the soil microbial community is one of the main indicators of the degree and rate of transformation of SOM.

The graph clearly shows the differences in the dominant microbial group for the individual tested plots. In the tested plots with lower pH, the percentage of micromycetes is increased. This group of microorganisms prefers more acidic media than bacilli and non-spore-forming bacteria (Mohammed & Zigau, 2016). The obtained data show a strong statistical correlation between the percentage participation of the group of non-spore-forming bacteria and the amount of org. C (Fig.5).

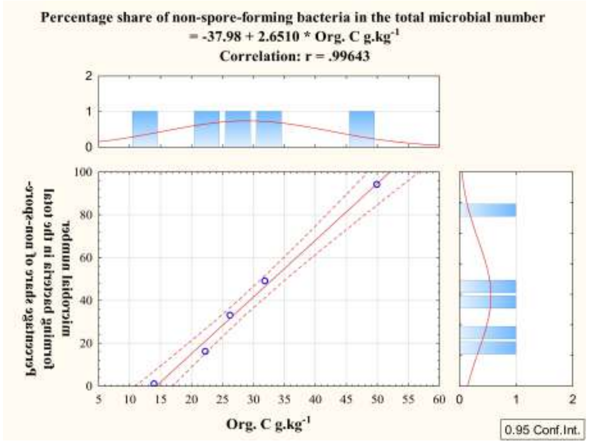

Fig. 5. Statistical relationship between % share of non-spore-forming bacteria and the quantity of Org. C.

With increasing content of org. C increases the percentage of non-spore-forming bacteria. Respectively in TP1, where the content of org. C is almost twice as low, the percentage of non-spore-forming microorganisms is only 1%.

The large percentage share of the group of bacilli in the A horizon of TP1 shows predominant processes of advanced transformation of SOM and the presence of more stable forms of organic matter. The situation is similar in TP5, where the bacilli are 55% and the actinomycetes 26% of the total microflora. The more active phase of mineralization of the hardly degradable organic substances in TP5 is further confirmed by the increased percentage of actinomycetes compared to the other tested areas.

In TP2 and TP3, the obtained data on the distribution of microbial groups are an indication of the predominance of the processes of transformation of more easily degradable SOM.

In the TP4, given the percentage distribution of the microbial groups, it can be concluded that the processes of transformation of complex SOM and simple SOM run in parallel, and their activity is similar.

Discussion

One of the main tasks of the present study was to analyze the influence of individual environmental factors (pH, org. C and total N) on the total microbial number and the distribution of microbial groups in the microbial communities of the studied forest soils. Previous studies have demonstrated the influence of pH, org. C and total N on soil microflora, but this environmental factors have been considered separately and not as a whole (Cho et al., 2016; Lauber et al., 2009; Khatoon et al., 2017). In the present study we discuss the complex influence of the environmental factors on soil microorganisms. The current scientific study shows a statistically significant relationship between environmental factors and changes in the total microbial number and the redistribution of microbial groups in forest microbiological communities. Here we provide evidence about the importance role of organic carbon and total nitrogen content during the development of the soil microorganisms.

Conclusions

We concluded that amount of organic C and total N were the environmental factors most strongly related to total microbial number. Soil pH was not correlated to the total microbial number, but was one of the most important factors related to the composition of the soil microorganisms – micromycetes have great abundance in profiles with acid pH. The percentage of different microbial groups shows a relationship between the amount of organic carbon and total nitrogen relative to the dominance of non-spore-forming bacteria. In this study, no relationship was found between the microbial abundance of the studied soils and altitude.

The research and the obtained data can be used in future projects as a basis for comparison and long-term monitoring of the studied sites. Further research is needed to link and analyze additional environmental conditions, such as exposure, specific vegetation, detailed mechanical composition of the soil, etc. to enrich the knowledge of the complex effects on the soil microbial community.

Acknowledgements

This research was supported by the National program "Young scientists and Postdoctoral candidates" funded by the Bulgarian Ministry of Education and Science, 2021.

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Received: 07.05.2021
Accepted: 30.08.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 57-73

Effect of Urban Park Reconstruction on Physical Soil Properties

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Abstract. Ecological restoration is an important means of managing urban natural areas with human and ecological values in mind. Urban park restoration involves significant impacts on soil cover. Soil quality is a major concern in urban park management, but little is known about the impact of park reconstruction on soil properties. The effect of urban park reconstruction on physical soil properties was investigated. The study was conducted in the recreational area of the Botanical Garden of the Dnipro National University. Data of remote sensing of the Earth's surface allowed to understand the impact of the park reconstruction on the state of the vegetation cover. The area of territories with high NDVI value, which correspond to dense tree plantations without reconstruction, strongly decreased in the reconstruction zone. The principal component analysis allowed to identify the main trends of the coordinated variability of soil features. The first principal component is obviously a reflection of the transformation of the soil properties, which occurred as a result of the reconstruction of an urban park. In the reconstructed part of the park compared to the area without reconstruction, there is an increase in soil penetration resistance, which occurs from the surface and gradually decays to a depth of 35 cm. The impact on the soil of technological processes that occurred during the reconstruction of the park, caused the compaction of the soil. The decrease in vegetation density occurred after the implementation of the reconstruction project. The consequence was that soil moisture in the reconstructed area was lower than without reconstruction.

Key words: NDVI, principal component analysis, soil compaction, soil moisture, vegetation cover.

Introduction

Urban ecosystems have a particularly critical role in providing services that directly influence human health and safety, such as air purification, noise reduction, urban cooling, and runoff mitigation (Gómez-Baggethun et al., 2013; Romzaykina et al., 2017). Urban soils and vegetation cover are heavily influenced by the human environment (Yang & Zhang, 2015). They can diverge in varying degrees from their natural analogues and vary from pseudo-natural to artificial soils and introduced plant species (Hemkemeyer et al., 2014). Urban soils play multiple roles in urban ecosystems (Setälä et al., 2014). Plant communities and soils that are similar to natural ones are typical for recreational areas and suburban zones. More disturbed plant communities and artificial soils are found in the industrial areas (Burghardt et al., 2015; Huot et al., 2017) and roadsides (Ghosh et al., 2016; Sager, 2020). The basic ecological regimes of plant life, such as soil, rainwater supply, air and light, are greatly altered in the urban environments (Li & Wong, 2007; Xiao et al., 2013; Maamar et al., 2018). Despite the high level of disturbance that characterizes most urban soils, they are able to support plant, animal and microbial organisms and mediate hydrological and biogeochemical cycles (Pavao-Zuckerman, 2008; Santorufo et al., 2012; Pouyat et al., 2020). Vegetation and soils in urban landscapes provide the key ecosystem services for the residents of a city (Pickett et al., 2008; Raciti et al., 2011) such as the biodiversity conservation, water protection, microclimate regulation, carbon sequestration, food production, and cultural and recreational needs (McKinney, 2006; Lovell & Taylor, 2013; Biliaiev et al., 2014).

The soils of urban parks create the conditions for plant growth and development (Czaja et al., 2020). Urban soils are subject to a high level of anthropogenic influence (Lehmann & Stahr, 2007; Seleznev et al., 2020). The urban environment has a unique set of specific features and processes (e.g., soil compaction, functional zoning, settlement history) that affect soil properties and their spatial variability (Vasenev et al., 2013). Temporal dynamics are the result of human-driven processes such as green space management and reconstruction (Van den Berg et al., 2014; Bae & Ryu, 2015; Kunah et al., 2019). After the moment of initial anthropogenic disturbance, the impact of urbanization decreases, which is associated with temporal dynamics of physical, biological and chemical properties of the soil (Scharenbroch et al., 2005). Re-vegetation of urban green space can improve the diversity of urban soil microbiota and bring it closer to near-natural levels by creating more wild habitat conditions (Mills et al., 2020). Urban soils have specific morphological properties (Costa et al., 2019; Prokof'eva et al., 2021). A variety of soil layers with sharp boundaries (Schoonover & Crim, 2015), abundance of anthropogenic inclusions (Sedov et al., 2017) and over compaction (Bezuglova et al., 2018) are typical for urban soils. Urban soils differ in their properties from soils in other systems. The properties of urban soils are very variable in space and time (Wiesner et al., 2016) and also vary within landscape types in urban environments. The spatial variability of soil properties in urban parks depends largely on the types of land cover and functional zoning of the area (Guo et al., 2019; Metwally et al., 2019; Romzaykina et al., 2021). The various practices of land-use and management have a significant effect on the properties of the soil (Spurgeon et al., 2013). Land-use and functional zoning are the key factors determining the spatial variability of vegetation and soils within the city area (Panday et al., 2019). The spatial heterogeneity of urban soils and vegetation within functional zones is also very high (Cadenasso et al., 2007; Mao et al., 2014). The history of land use and current land management practices are factors that determine the heterogeneity of urban soils at various scales (Fraterrigo et al., 2005; Pickett et al., 2017).

Urban green spaces can provide a thermally comfortable environment. In order for them to perform this function, parks must be designed and redesigned with climate conditions and predictions of future climate. A properly designed and reconstructed park can reduce the threat of extreme heat stress hazards (Brown et al., 2015). Urban parks reconstruction is a routine procedure (Li, 2020). Ecological restoration of urban forests is a measure to improve air quality, mitigate urban heat island effects, improve stormwater infiltration, and provide other social and environmental benefits (Johnson & Handel, 2015). Ecological restoration is becoming an important means of managing urban natural areas with human and ecological values in mind. Urban ecological restoration can contribute to a unique and positive relationship between people and nature (Gobster, 2007). The reconstruction of urban parks is associated with a significant impact on the soil cover (Shanahan et al., 2015; Sarah et al., 2015; Kumar & Hundal, 2016).

The soil quality is a major concern in urban park management, but little is known about the effects of park reconstruction on soil properties (Hou et al., 2015). The technological processes of reconstruction, such as excavation, leveling, building paths, planting trees, and adding compost, can significantly modify the spatial variability of soil properties in an urban park. The spatial variability of soil chemical properties in a city park before and after reconstruction was investigated (Romzaykina et al., 2017). However, little is known about the impact of urban park reconstruction on the physical properties of soils. Therefore, the aim of our study was to investigate the effect of urban park reconstruction on the physical properties of soils. We propose the hypothesis that the technological activities in the process of park reconstruction leads to an increase in soil penetration resistance and changes in its aggregate structure. We propose the hypothesis that the technological activity in the process of park reconstruction leads to a change in the physical properties of the soil. To test this hypothesis, it is reasonable to compare the informative indicators of soil physical condition, such as the soil penetration resistance, aggregate structure, bulk density, electrical conductivity, and the soil moisture in the area of the park that was reconstructed and without reconstruction. In order to assess the role of vegetation density change as a result of reconstruction on the physical properties of soil, we used NDVI data for the compared areas before and after reconstruction.

Materials and Methods

The study was conducted in the recreational area of the Botanical Garden of the Oles Honchar Dnipro National University (Ukraine). This artificial tree plantation was created in the 1940s on the location of a natural oak forest. A geobotanical survey of the park in 2013 revealed that plant communities within the study area were represented by 36 species (Table 1). The *Acer platanoides*, *Fraxinus excelsior*, *Gleditsia triacanthos*, *Robinia pseudoacacia* were dominated among the tree plants. The *Alliaria petiolata*, *Chelidonium majus*, *Geum urbanum*, *Viola mirabilis*, *Galium aparine* were dominated among the herbaceous plants.

In 2019, a 2.8 ha area of the park was reconstructed (Fig. 1). During the reconstruction process, walkways were rebuilt, shrubs were removed, old, damaged trees were removed, and tree crowns were trimmed. Juvenile trees were planted in the place of removed old trees. Old outbuildings, which greatly impaired the aesthetic perception of the park, were also removed. An transport and construction machinery was involved in the reconstruction. The works were carried out during the whole warm period of the year.

A study of the physical properties of the soil was conducted in 2020 after the reconstruction of the park was completed. The soil samples were taken within polygons, 2 of which were placed in the reconstruction area and 2 of which were placed in a similar section of the park where no reconstruction was performed. Each polygon consisted of 105 sample points. The points were located along 7 transects with 15 sample points in each. The distance between points in the transect as well as the distance between transects was 3 m.

Soil properties measurement

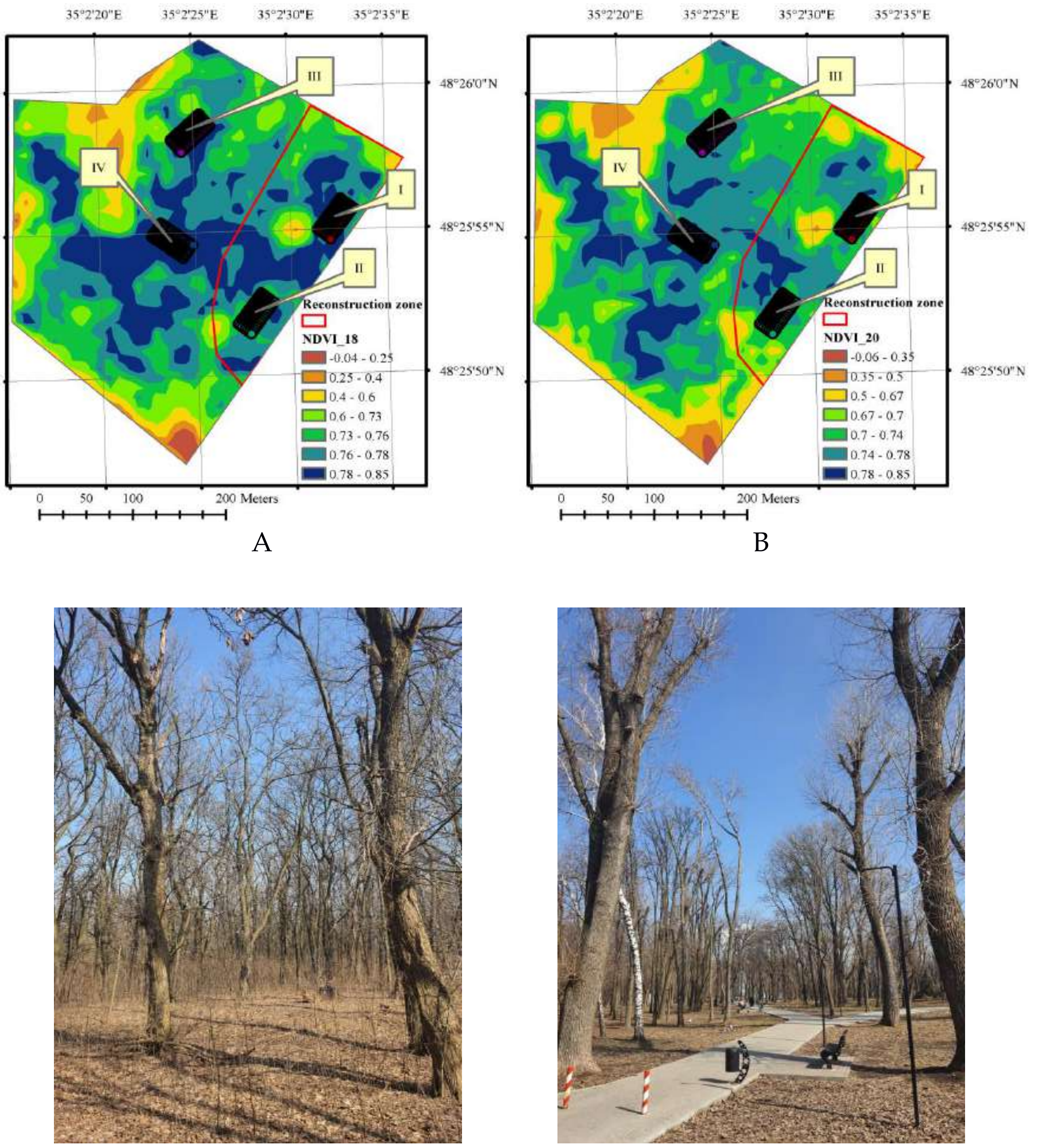
The following soil properties were measured at each test point of the polygons. The soil mechanical resistance was measured in the field using the “Eijkelkamp” manual penetrometer, to a depth of 100 cm at 5 cm intervals (Zhukov & Gadorozhnaya, 2016; Zhukov et al., 2019). The average error of the measurement results of the device is ± 8%.

The measurements were made with a cone with a cross section of 1 cm^2. At each measurement point, the soil mechanical resistance was performed in only one replication. To measure the electrical conductivity of soil *in situ* the HI 76305 sensor (Hanna Instruments, Woodsocket, R. I.), working in conjunction with the portable instrument HI 993310 were used (Yorkina et al., 2018; Kunakh et al., 2020). The soil aggregate fractions size distribution was determined in accordance with the Soil Sampling and Methods of Analysis recommendations (Kroetsch & Wang, 2008). Soil moisture was measured under the field conditions using a dielectric digital moisture meter MG–44. The core method was used for measurement of the soil bulk density (Al-Shammary et al., 2018).

Table 1. Structure of plant communities of polygons (projective coverage of plant species is represented by Braun-Blanquet density scores*) based on 2013 data. *Legend:* * – 1 – <5% cover; 2 – 5–25% cover; 3 – 25–50% cover; 4 –50–75% cover; 5 – – 75 – 100% cover.

Species and Raunkiær plant life-form	Polygon			
	Reconstructed territory		Without reconstruction	
	I	II	III	IV
Phanerophytes				
Acer campestre L.	3	0	0	2
Acer negundo L.	0	0	0	2
Acer platanoides L.	2	2	2	2
Aesculus hippocastanum L.	0	1	0	0
Ailanthus altissima (Mill.) Swingle	0	2	0	0
Betula pendula Roth	2	0	0	0
Fraxinus excelsior L.	2	3	2	2
Gleditsia triacanthos L.	2	2	2	2
Populus nigra L.	3	0	0	0
Pyrus communis L.	0	2	0	0
Quercus robur L.	2	0	0	0
Robinia pseudoacacia L.	4	2	4	2
Ulmus glabra Huds.	2	0	0	2
Nonphanerophytes				
Parthenocissus quinquefolia (L.) Planch.	0	0	0	1
Hemikryptophytes				
Alliaria petiolata (M.Bieb.) Cavara et Grande	2	2	3	2
Anthriscus sylvestris (L.) Hoffm.	0	0	2	0
Arctium minus (Hill) Bernh.	0	2	2	0
Ballota nigra L.	0	0	1	1
Carex melanostachya Bieb. ex Willd.	0	0	1	2
Chelidonium majus L.	2	3	2	2
Daucus carota L.	1	0	0	1
Fragaria viridis (Duch.) Weston	0	0	1	0
Geum urbanum L.	2	3	2	2
Plantago major L.	1	0	0	0
Poa angustifolia L.	1	0	0	0
Poa nemoralis L.	0	0	2	2
Solidago canadensis L.	1	0	0	0
Taraxacum campylodes G.E.Haglund	1	0	1	0
Viola mirabilis L.	4	2	4	1
Terophytes				
Atriplex micrantha C. A. May	1	0	0	0

Galium aparine L.	2	2	2	2
Impatiens parviflora DC.	0	0	0	2
Lactuca serriola L.	1	0	0	0
Stellaria media (L.) Vill	0	0	1	0
Geophytes				
Convolvulus arvensis L.	2	0	2	0
Humulus lupulus L.	2	0	0	0



A B

C D

Fig. 1. A spatial variation of the NDVI on July 6, 2018 (A) and July 6, 2020 (B), as well as landscape images of the area without reconstruction (C) and the area after reconstruction (D) of the park (Date of the photo 23.03.2021).

Evaluating the effects of reconstruction with Sentinel-2 images

Sentinel-2 satellite images of the study area received from Geological Survey (U.S.), & EROS Data Center on two dates: the year before reconstruction on July 6, 2018 and the year after reconstruction on July 6, 2020. Normalized difference vegetation index (NDVI) was calculated based on B8 (wavelength 842 nm) and B4 (wavelength 665 nm) channels with 10 metre spatial resolution:

$$\mathrm{NDVI} = \frac{\mathrm{B8} - \mathrm{B4}}{\mathrm{B8} + \mathrm{B4}}.$$

The value of NDVI varies from –1 to 1 (Saravanan et al., 2019). The values less than 0 indicate no vegetation cover. The values close to zero (–0.1 to 0.1) generally correspond to the barren areas of rock, sand, or anthropogenic surfaces. The values greater than 0 indicate a presence of vegetation. The closer to 1 the NDVI value is, the denser the vegetation cover is (Drisya et al., 2018). The experimental polygons were within the space that was covered by 15–17 pixels of Sentinel-2 images. The NDVI values were extracted from these images and compared using Nested design ANOVA with Reconstruction zone variables (1 – reconstruction zone; 2 – no reconstruction zone) and Polygon nested variables (1, 2, 3, 4) as predictors. The descriptive statistics, ANOVA and Principal Component Analysis were calculated using the program Statistica (Statsoft).

Results

The NDVI values were statistically significantly dependent on the reconstruction effect, year and year interaction and reconstruction effect (Table 2). Areas without reconstruction did not differ in NDVI value in 2018 and 2020, as confirmed by the test of significance for planned comparison ($F = 0.16$, $p = 0.69$). The area within which the reconstruction was conducted, there was a decrease in the NDVI index in 2020 compared to 2018 ($F = 3.81$, $p = 0.05$).

Table 2. ANOVA table between NDVI and the reconstruction effect, year and interrelation between reconstruction effect and year. *Legend:* * – Polygon – effect, which indicates the type of polygon: the polygon is in the reconstruction zone (polygon I and II) or outside the reconstruction zone (polygon III and IV).

Effect	Sum-of-squares (SS)	Degrees of freedom	Mean squares (MS)	*F*–ratio	*p*-level
Total territory comparison ($R^2_{adj} = 0.09$, $F = 290.9$, $p < 0.001$)					
Intercept	349.4	1	349.4	8105.6	<0.001
Year	0.18	1	0.18	4.26	0.04
Reconstruction	37.49	1	37.49	869.9	<0.001
Reconstruction×Year	0.13	1	0.13	2.95	0.09
Error	371.6	8620	0.043	–	–
Polygon comparison ($R^2_{adj} = 0.50$, $F = 19.2$, $p < 0.001$)					
Intercept	75.7	1	75.7	204333.9	<0.001
Polygon*	0.0414	3	0.0138	37.26	<0.001
Year	0.0041	1	0.0041	10.99	<0.001
Polygon×Year	0.0042	3	0.0014	3.79	0.01
Error	0.0441	119	0.0004	–	–

The polygon 2 was distinguished by the lowest level of NDVI, which takes a value of 0.75±0.0032 (Fig. 2). The polygons 1 and 3 did not differ in NDVI value, as confirmed by the test of significance for planned comparison (F = 0.01, p = 0.92). The polygon 4 had the highest NDVI, which took a value of 0.80±0.0033. The NDVI had a lower value in 2020 than in 2018. This difference was due to a decrease in NDVI in polygons that were in the park reconstruction area. The differences between years in polygons 3 and 4 were not statistically significant, as confirmed by the test of significance for planned comparison (F = 0.009, p = 0.92).

In turn, the differences between years in polygons 1 and 2 were statistically significant, as confirmed by the test of significance for planned comparison (F = 21.26, p < 0.001).

The data on soil properties were subjected to the principal component analysis (PCA), and the first three principal components were extracted as a result (Table 3). These components were able to explain 54.4% of the variation in the feature space. The PC 1 was able to describe 26.1% of the variation in the feature space. This component was sensitive to the conflicting directions in the trends of the soil penetration resistance at depths from the surface and up to 35 cm and at 50 cm and deeper: an increase in soil penetration resistance in the upper soil layer was accompanied by a decrease in this indicator in the lower soil layer. The PC1 indicated the presence of an opposite trend in the variability of the proportions of aggregate fractions less than 0.5 mm on one side and more than 0.5 mm on the other. The PC 2 was able to explain 15.0 % of the variability of the feature space. This component indicates consistent variability in the soil penetration resistance within the soil profile to a depth of 90 cm with two local maxima at 30–45 and 75–90 cm. The pattern explained by the PC 2 indicated an increase in aggregate fractions of 2 mm or more in size with an increase in soil penetration resistance. Also, the PC2 was sensitive to the variability in litter height. The PC 3 was able to explain 13.2 % of the variation in the feature space. This component was sensitive to the opposite variation in the soil penetration resistance from the surface to 35 cm depth on the one hand and from 40 cm depth on the other hand. The PC 3 indicated an increase in fractions larger than 2 mm with a decrease in fractions of 0.5–2 mm together with a decrease in the soil electrical conductivity, litter height, and soil density.

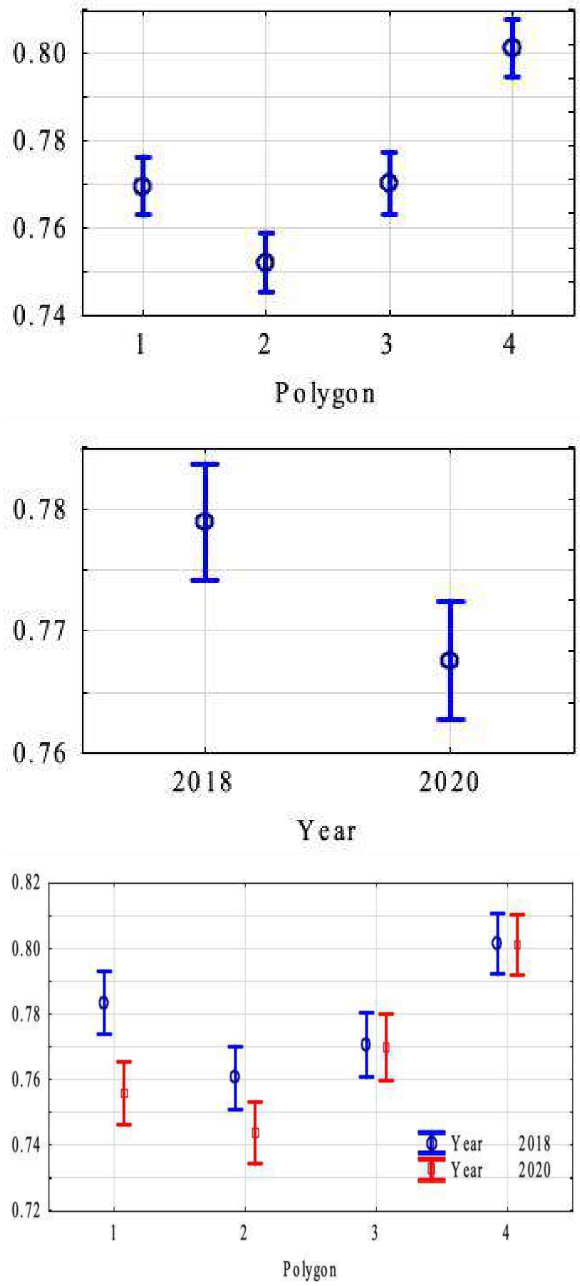


Fig. 2 Dependence of NDVI values (the ordinate axis) on the polygon, year, and interaction of year and polygon according to the results of factorial ANOVA.

The polygons had a specificity of soil properties, which largely depended on the reconstruction of the city park performed (Table 4). These predictors were able to explain 87% of the variation in PC 1. The PC 1 explained the variability of soil properties that were induced by park reconstruction (F = 23.3, p = 0.04) (Fig. 3). The PC 2 and 3 reflected a specific variation in the soil properties that had a different cause than park reconstruction (F = 0.013, p = 0.92 and F = 0.06, p = 0.83, respectively).

Discussion

The reconstruction of the park involved engineering renewal of walkways, clearing the shrub layer and undergrowth of trees, removing old diseased trees and cutting down non-viable tree branches. The technological processes were associated with the presence of a large number of workers and machinery in the park during the performance of the work. As a result, the soil was significantly affected due to compaction. The use of bulk density (BD) measurements taken at depths of up to 14 cm on a city-wide scale to compare the degree of surface soil compaction between different classes of urban green space and agricultural soils showed that in a typical British city, urban soils are in better physical condition than agricultural soils and can contribute to the provision of ecosystem services (Edmondson et al., 2011).

Our results reveal the favorable condition of the soils of the city park by the bulk density indicators in the upper layers. However, the use of the soil penetration resistance indicators shows that the soil compaction occurs to a significant depth up to 35 cm. This result leads to the conclusion that the measurement of soil penetration resistance has an advantage over the measurement of soil bulk density. The best strategy for evaluating soil compaction should be considered a combination of the soil bulk density and soil penetration resistance measurements. The vegetation canopies, especially tree crowns, can significantly regulate a solar energy input to the soil surface in the urban environments. The park reconstruction also included the removal of live branches on the outer canopy and old trees. The thinning of the tree canopy led to an increase in solar energy reaching the soil surface and a greater drying of the soil surface resulting in an increase in soil penetration resistance (Kimes & Smith, 1980; Arboit & Betman, 2017). Data of remote sensing of the Earth's surface allowed to understand the impact of the park reconstruction on the state of the vegetation cover.

The area of territories with high NDVI value, which correspond to dense old-growth tree plantations, strongly decreased in the reconstruction zone. It is also worth noting that such stands have become even more fragmented. The selected polygons are characteristic for assessing the impact of reconstruction on the soil cover. The polygons in the reconstruction zone were distinguished by a significant decrease in the NDVI index, while the polygons in the control zone did not differ significantly in this indicator. Thus, as a result of the reconstruction of the park, the changes in the structure of the vegetation cover lead to an increase in the penetration of solar energy and increase its amount, which reaches the soil surface.

The reconstruction of the park led to a variation in the soil properties, which overlapped with their patterns that existed before the reconstruction. The principal component analysis allowed to distinguish the fractions of soil property variability of the different origins. The principal component analysis revealed to identify the main trends of the coordinated variability of the soil features. The principal component 1 is obviously a reflection of the transformation of the soil properties, which occurred as a result of the reconstruction of an urban park. The principal components 2 and 3 indicate the polygon features that are independent of reconstruction. Thus, the variability induced by the park reconstruction is leading in the degree of influence on the soil properties because the principal component 1 describes the largest fraction of variation in soil features than the other components. A consequence of the effect on soil cover of park reconstruction is an increase in the soil penetration resistance, which takes place from the surface and gradually decays to a depth of 35 cm. There may be two reasons for this phenomenon. The impact on the soil of technological processes that occurred during the reconstruction of the park caused the compaction of the soil. Earlier it was shown, that the technological machines carry out significant impact on the soil, which exceeds in its size the visible boundaries of the wheel track.

This impact is manifested in the increase of the soil penetration resistance by 100–155 % in comparison with the control at the depth of 0–10 cm and by 20–30 % at the depth of 45–50 cm. It cannot be ruled out that the influence of the wheels continues deeper than the tests were conducted. It was also noted that a long period of the soil relaxation after a anthropogenic transformation can create a network of vehicle traces on the soil. In the area where the traces intersect, the negative effects increase significantly (Zhukov, 2015). Thus, the activities of construction and transport equipment during the reconstruction of the park may be the cause of an increase in soil compaction.

Also the reason for the increase in soil compaction may be a decrease in the density of the vegetation cover, which occurred after the implementation of the reconstruction project. The sparser vegetation cover is permeable to solar energy, which contributes to increased temperature and better ventilation. As a result, it is the surface soil layer that quickly dries out, which leads to an increase in the penetration resistance of the upper soil layer. This assumption is confirmed by the fact that the soil moisture in the reconstructed area was lower than that without reconstruction. The peculiarities of the variability of the aggregate structure can be explained by the technological influence on the soil. In the reconstruction zone, the proportion of aggregates less than 0.5 mm in size, which can be classified as microaggregates, increased. The reason for their emergence can be considered destruction of the larger aggregates (meso- and macroaggregates) as a result of technological activity. It should be noted that the increase in the proportion of micro-aggregates is a negative factor that deteriorates the properties of the soil as a habitat for living organisms (Zhukov et al., 2018; Zadorozhnaya et al., 2018).

Table 3. Descriptive statistics of the soil properties and the result of the principal component analysis.

Properties, mean±st. error	Polygons				Correlation coefficient (only significant at $p < 0.05$ presented)		
	1	2	3	4	PC1	PC2	PC3
Soil penetration resistance at a depth of, cm in MPa							
0–5	1.79±0.04	1.44±0.05	0.83±0.01	0.99±0.01	–0.86	0.17	0.10
5–10	2.45±0.06	1.88±0.07	1.05±0.01	1.2±0.02	–0.85	0.22	0.14
10–15	2.77±0.09	2.06±0.10	1.17±0.02	1.21±0.03	–0.81	0.30	0.19
15–20	2.73±0.09	1.97±0.09	1.33±0.04	1.19±0.03	–0.75	0.40	0.22
20–25	2.43±0.09	1.74±0.08	1.7±0.06	1.27±0.04	–0.53	0.57	0.34

25–30	2.25±0.09	1.75±0.06	2.23±0.08	1.46±0.05	–0.21	0.74	0.33
30–35	2.31±0.09	2.10±0.07	2.74±0.09	1.99±0.08	–	0.75	0.24
35–40	2.83±0.09	2.78±0.08	3.22±0.08	2.58±0.09	–	0.74	–
40–45	3.45±0.09	3.57±0.08	3.56±0.08	3.28±0.09	–	0.58	–0.37
45–50	4.04±0.08	4.13±0.08	3.76±0.07	3.79±0.07	–0.16	0.44	–0.61
50–55	4.33±0.06	4.53±0.06	4±0.07	4.23±0.06	–0.20	0.32	–0.71
55–60	4.64±0.04	4.77±0.05	4.36±0.05	4.53±0.05	–0.21	0.28	–0.77
60–65	4.87±0.04	4.91±0.05	4.62±0.05	4.75±0.04	–0.15	0.32	–0.80
65–70	4.9±0.03	4.99±0.04	4.75±0.04	4.89±0.03	–	0.26	–0.74
70–75	4.79±0.03	5.01±0.04	5.35±0.05	4.85±0.03	0.39	0.46	–0.47
75–80	4.7±0.03	4.88±0.04	5.63±0.06	4.86±0.03	0.59	0.47	–0.24
80–85	4.82±0.03	4.57±0.03	5.67±0.05	4.88±0.03	0.67	0.42	–0.14
85–90	4.99±0.02	4.15±0.03	4.53±0.04	4.98±0.02	0.16	–	–0.32
90–95	4.78±0.02	3.95±0.03	4.64±0.04	5.02±0.02	0.40	–	–0.22
95–100	4.23±0.03	3.95±0.03	4.79±0.04	5.10±0.03	0.69	––	–0.17
Aggregate fraction, in %							
>10 mm	0.06±0.003	0.05±0.002	0.13±0.006	0.07±0.003	0.59	0.36	0.33
7–10 mm	0.24±0.009	0.21±0.01	0.47±0.02	0.25±0.01	0.55	0.44	0.37
5–7 mm	0.31±0.01	0.46±0.02	0.67±0.02	0.37±0.02	0.47	0.42	0.30
3–5 mm	7.65±0.18	8.74±0.37	13.03±0.34	8.52±0.25	0.55	0.47	0.30
2–3 mm	17.84±0.29	17.94±0.41	20.48±0.45	19.94±0.42	0.43	0.26	0.17
1–2 mm	24.08±0.50	24.72±0.52	27.25±0.43	30.05±0.52	0.38	–0.27	–0.16
0.5–1 mm	16.64±0.34	18.53±0.53	19.19±0.50	20.05±0.43	0.11	–0.31	–0.28
0.25–0.5 mm	12.24±0.23	12.27±0.28	9.72±0.25	10.17±0.25	–0.53	–	–
<0.25 mm	21.00±0.76	16.54±0.58	8.82±0.35	10.23±0.47	–0.70	–	–
Other soil properties							
Electrical conductivity, dSm/m	0.45±0.006	0.41±0.008	0.29±0.007	0.35±0.007	–0.54	–	–0.16
Litter	3.47±0.06	2.26±0.06	1.83±0.05	3.54±0.05	–0.17	–0.42	–0.27
Wetness	25.87±0.31	21.57±0.39	30±0.18	27.83±0.21	0.75	–	–
Bulk density	1.01±0.01	1.09±0.008	0.8±0.006	0.89±0.006	–0.82	–	–0.13

Table 4. Nested design ANOVA results examining the effect of polygon and reconstruction (polygon nested in the reconstruction zone).

Effect*	R^2_{adj}	Model			Residual			F–ratio	p-level
		Sum-of-squares (SS)	Degrees of freedom	Mean squares (MS)	Sum-of-squares (SS)	Degrees of freedom	Mean squares (MS)		
PC1	0.87	0.75	0.75	2717.2	3	905.7	892.5	416	2.15
PC2	0.50	0.25	0.24	511.9	3	170.6	1566.6	416	3.77
PC3	0.29	0.09	0.08	157.0	3	52.3	1673.2	416	4.02

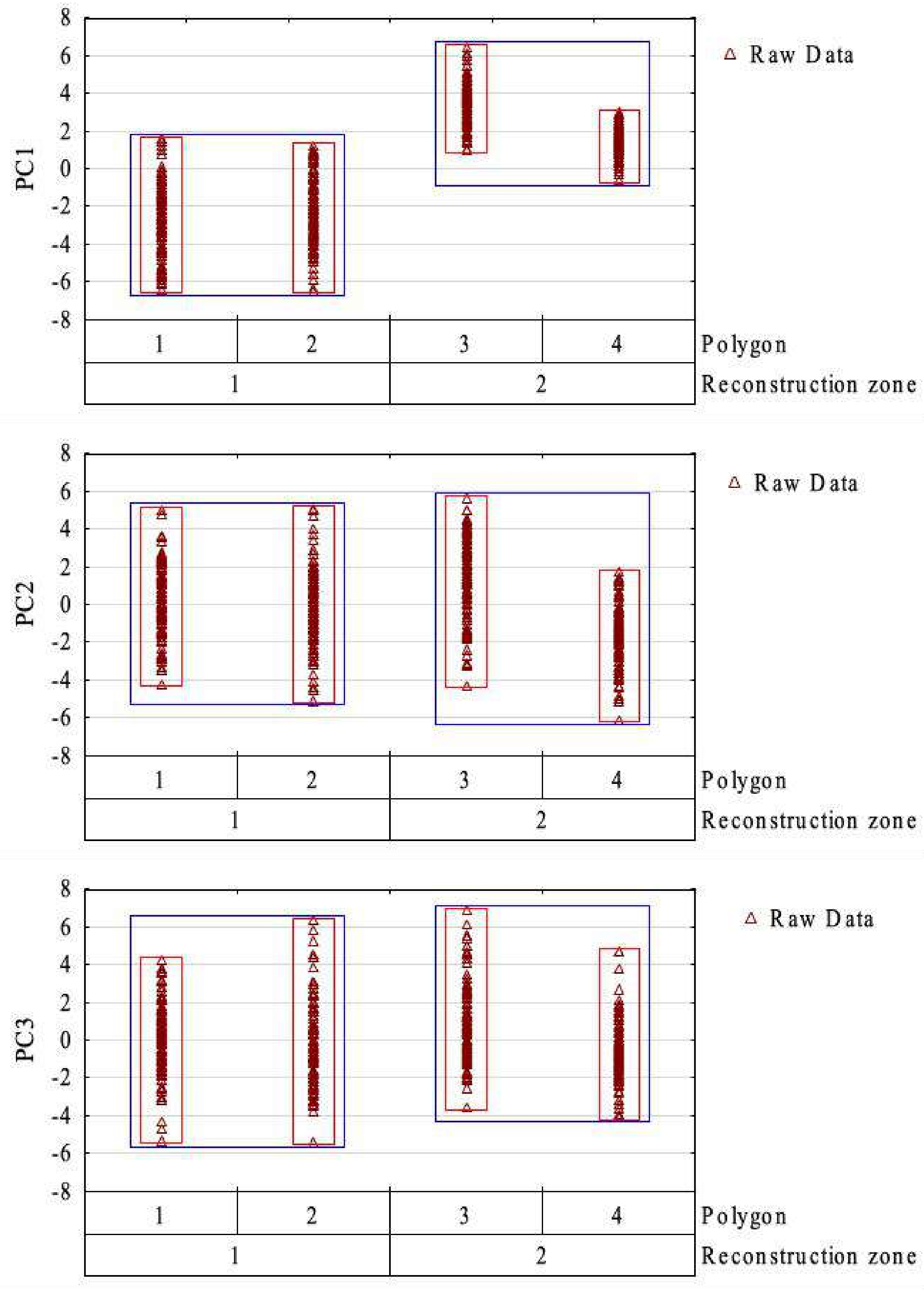


Fig. 3. Variation of the values of the principal components depending on the polygon and the effect of park reconstruction: the mean value and variance of the principal components are presented.

The risks of erosion processes also increase. Erosion can be a factor in the loss of fertility. In an urban environment, erosion processes can also accelerate the migration of toxic substances that are immobilized in the soil, which may have a secondary negative effect (Yorkina et al., 2019). The reconstruction area is located near a highway with active traffic. The electrical conductivity of the soil in this zone is higher, which may be due to the ingress of salt road de-icing agents on the soil surface.

Conclusion

The reconstruction of urban park provides many benefits for the residents of the city. The aesthetic perception of the area is improved and the comfort for recreation increases. The restoration of tree plantations should also be mentioned, which is an important component of the management of artificial forest plantations in the urban environment. However, the reconstruction of parks is associated with a number of negative effects on the soil cover. As a result of the technological processes that are carried out during the reconstruction process, the soil compactness increases to a considerable depth and the aggregate structure of the soil is disturbed. The thinning of the stand and the destruction of the shrub undergrowth greatly alter the microclimatic regime in the city park and increase the risks of excessive evaporation of water from the soil surface. These changes can have the negative consequences for the ecological services performed by the soil. Therefore, the measures to remediate the physical properties of the soil should be an obligatory element of the reconstruction of urban parks.

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Received: 16.05.2021
Accepted: 26.08.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 75-93

Genus Galanthus (Amaryllidaceae) in Bulgaria: Notes about Taxonomy, Chorology and Ecology

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Abstract. The aim of the present article is to collect and systematize the information on the distribution of *Galanthus elwesii* and *G. nivalis* in Bulgaria and to make some remarks on their taxonomy. A revision of Bulgarian herbarium specimens from Bulgarian herbariums and literature was carried out. Both species are given with their synonyms, some ecological characteristics and monitoring data of the represented populations. The distribution of both species by floristic regions is presented using revised materials and personal collections. New chorological data for the distribution of *G. elwesii* in floristic regions West Frontier Mts. (Mt. Osogovo), Mt. Belasica, Mesta Valley and Struma Valley (Northern) and *G. nivalis* from Black Sea Coast (Northern) are also presented.

Key words: *Galanthus elwesii*, *Galanthus nivalis*, chorology, ecology, distribution.

Introduction

The species of the genus *Galanthus* L. (Amaryllidaceae) are common in Europe, Asia Minor, and the Middle East. Presented like harvests of the Spring, snowdrops have economic importance as garden plants and for the content of alkaloids with pharmacological activity. Despite hundreds of cultivated varieties, *Galanthus* spp. bulbs are among the most commonly wild-collected plants in the world (Entwistle et al., 2002). This is the reason why the trade, export and import of bulbs in the world is controlled by CITES (Convention on International Trade in Endangered Species).

Genus *Galanthus* has been the subject of numerous taxonomic revisions, but each one disagrees with others in the enumeration of species, subspecies, and varieties. New taxa are regularly described - *G. trojanus* A.P.Davis & Özhatay (Davis, 2001), *G. panjutinii* Zubov & A.P. Davis (Zubov & Davis, 2012), *G. samothracicus* Kit Tan & Biei (Tan et al., 2014), *G. bursanus* Zubov, Konca & A.P.Davis (Zubov et al., 2019) and hybrid - *G.* × *valentinei* Beck nothosubsp. *subplicatus* (Zeybek) A.P.Davis (Davis et al., 2001). The most recent taxonomic revisions describe over 20 species of *Galanthus* (Davis, 1999; Ronsted et al., 2013).

In Bulgaria, the genus *Galanthus* has been the subject of many different taxonomic decisions. Over the years 5 species, 3 subspecies, 7 varieties and one forma have been described. Velenovsky

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(1891, 1898) described 3 species from Bulgaria - *G. nivalis* L., *G. gracilis* Celak. and *G. maximus* Vel. Stoyanov & Stefanov (1923, 1933, 1948) and Jordanov (1964) accept only *G. nivalis* L. as common in Bulgaria. Stoyanov et al. (1966) gave *G. elwesii* Hook. for Bulgaria for the first time. Delipavlov (1968, 1971), based on anatomical studies, defines *G. nivalis*, *G. elwesii* and *G. graecus* Orph. ex Boiss. as species of the genus *Galanthus* in Bulgaria. Anchev (1992) indicate for distributed in Bulgaria *G. nivalis* and *G. elwesii* with three subspecies. Delipavlov (2003) gave *G. gracilis* as the most widespread species in Bulgaria. (Appendix 1A). Currently, 2 species are considered to be widespread in Bulgaria - *G. nivalis* and *G. elwesii*.

The phytochemical (Berkov et al., 2008, 2011), morphometric (Sidjimova, 2009), conservation (Sidjimova, 2014), anatomical, embryological and DNA studies (Semerdjieva et al., 2019) of the genus show significant differences between *G. elwesii* and *G. nivalis* and no further subdivisions in the studied populations of the species. The revision of the chorological information and taxonomic identity of the species is the basis for further biological and phytochemical studies and the basis for appropriate measures for the conservation and protection of the species. In Bulgaria, *G. nivalis* and *G. elwesii* are protected by Biological Diversity Act (2002). According to the IUCN criteria used for the new edition of the Red Data Book of Bulgaria (Evstatieva, 2015) both species are classified as "endangered species", with the recommendation to conserve them *in situ* and *ex situ*.

No information on the ecological and population characteristics of the species has been established in the literature.

The aim of the study is to collect and systematize the available chorological and taxonomic information on the genus *Galanthus* in Bulgaria and to provide information on some ecological characteristics of *G. elwesii* and *G. nivalis*. These data will be useful in decision making for the conservation of the two protected species.

Material and Methods

The subjects of this study were *G. nivalis* L. and *G. elwesii* Hook f. s. l. (Amaryllidaceae) of Bulgarian origin. The species were identified according to the taxonomic scheme of Anchev (1992). The taxonomic feature used in the identification and revision of the species was the presence or absence of green spots at the base of the inner perianth segments. The study was conducted in Bulgaria in the period from February 2002 to March 2021.

Information on distribution and taxonomic revision of herbarium specimens was based on available literature data (56 records), specimens deposited in Bulgarian Herbariums - SOA (Herbarium in Agricultural University Plovdiv) (136 specimens), SOM (Herbarium in the Institute of Biodiversity and Ecosystem Research, BAS) (24 specimens) and SO (Herbarium in the Faculty of Biology, Sofia University "St. Kliment Ochridsky") (64 specimens) and field surveys for personal collections. All revised herbarium specimens are marked with revision notes (Appendix 1B, 1C).

Voucher specimens of 15 *G. nivalis* and 54 *G. elwesii* from personal collections were deposited in the herbarium of the Institute of Biodiversity and Ecosystem Research - Bulgarian Academy of Sciences (SOM). Localities are indicated with altitude, exposure and GPS coordinates (Table 1, 2). The distribution of species follows the floristic regions of Bulgaria (Yordanov, 1966). The results are presented graphically using Excel 2010.

Monitoring of three *G. elwesii* (Shumen, Kokaliane, Bachkovo) and three *G. nivalis* populations (Belogradchik, Pasa dere, Ropotamo) was carried out in February 2015 according to the approved monitoring methodology developed for the needs of the NSEM (National System for Environmental

Monitoring). Populations were selected based on NSEM criteria for representativeness, mainly typical habitat and accessibility of the site (Gussev & Bancheva). A "separate herbaceous plant" is used as the reporting unit. Same populations were visited in 2021.

Results

Galanthus nivalis L., Sp. Pl., ed. 1 (1753) 288; Boiss., Fl. Or., 5 (1872) 144; Velen., Fl. Bulg., (1891) 539; Velen., Fl. Bulg. Suppl., (1898) 265; Stoj. Stef., Fl. Bulg., vol. 1 (1923) 257; Hayek, Prodrom. Fl. Penins. Balc., 3 (1933) 101; Stoj. Stef., Fl. Bulg., Vol. 2 (1933) 244; Lozinska., Fl. SSSR, 4 (1935) 478; Stoj. Stef., Fl. Bulg., Vol. 3 (1948) 267; Jordanov, Fl. P.R.Bulg., 2 (1964) 318; Stoj. Stef. Kitan., Fl. Bulg., vol. 4 (1966) 237; Zahariadi, Fl. R. S Rom, 11 (1966) 407; Artjushenko, Amaryllidaceae SSSR (1970) 74; Delipavlov, Izv. Bot. I-t, 21 (1971) 165; Stepanović –Veselić, Fl. SR Srbije, 7 (1986) 597; D. A. Webb, Fl. Eur., 5 (1980) 77; P. H. Davis, Fl. Turkey, 8 (1984) 370; Jovanović, Botanica Serbica (2016); *G. nivalis L. var. montanus* (Schr.) Rouy, Delipavlov, op. c., 166. – common snowdrop.

Flowering period in Bulgaria. Winter to spring, from mid-January to mid-March, depending on altitude, immediately after snowmelt.

Habitat. Occurs in moist and shady places in deciduous forests (*Acer* spp. *Carpinus betulus*, *Fagus sylvatica*, *Quercus* spp.) on calcareous soils with east or northeast exposure. The altitude ranges from 5 m (Arcutino Reserve, SOM 162924) to 1166 m a.s.l. (Bulgarka Nature Park, SOM 177466). Populations described from Balkan range occur in beech forests, while those from Strandza occur mainly in oak, maple and hornbeam forests. During the field study, populations were also observed in open meadows, in bushes and even on sand (Arcutino Reserve). The following associated herbaceous plant species were observed during the field study: *Allium* sp., *Anemone pavonina*, *Arum maculatum*, *Corydalis bulbosa*, *C. solida*, *Crocus flavus*, *Cyclamen coum*, *Euphorbia* sp., *Lamium purpureum*, *Ornithogalum* sp., *Pulmonaria officinalis*, *Ranunculus ficaria*, *Scilla bifolia*, *Veronica* sp.

Monitoring data: The size of the studied populations is approximately the same. The total projective cover is 1-2%, which is understandable due to the small size of the plants and the fragmented populations. In two of the populations, the ratio between generative and vegetative individuals is approximately equal. In the population of Pasha Dere, the generative individuals dominate. There the density of individuals is the highest - 1.7 individuals per m^2 (Table 1).

Distribution in Bulgaria. During the revision of herbarium materials were established 37 materials from *G. nivalis* from 5 floristic regions: Black Sea Coast (Sourthern) (13), North-Eastern Bulgaria (8), Tundzha Hilly Country (2), Balkan Range (Western) (2), Mt Strandzha (12). (Appendix C, Fig.1).

Literature data gave information for distribution from Blask Sea Coast (Delipavlov, 1968, 1971; Nikolov et al., 1994; Assyov & Petrova, 2012), North–Eastern Bulgaria (Delipavlov, 1968, 1970; Assyov & Petrova, 2012), Balkan Range (Western, Central) (Delipavlov, 1968, 1971; Tzonev, 2002; Borisova & Donchev, 2003; Assyov & Petrova, 2012; Marinov et al., 2016), Forebalkan (Western) (Delipavlov, 1968, 1970; Assyov & Petrova, 2012), Danubian plain (Delipavlov 1968, 1971) and Mt Strandza (Delipavlov, 1968, 1971; Gussev et al., 1997, 2004; Assyov & Petrova, 2012).

During the investigation were collected 15 samples from 5 floristic regions, which correspond with the distribution of the species in the country (Table 2).

Galanthus elwesii Hook. f., Bot. Mag., 101 (1875) t. 6166; Stoj. Stef. Kitan., Fl. Bulg., vol. 4 (1966) 237; Artjushenko, Amaryllidaceae SSSR (1970) 77; D. A. Webb, Fl. Eur., 5 (1980) 78; Delipavlov, Izv. Bot. I-t, 21 (1971) 167; *G. elwesii* Hook.f. subsp. *minor* D.A.Webb, Bot. J.

Linn. Soc. 76(4) (1978) 312; Webb, l.c.; *G. graecus* Orph. ex Boiss., Fl. Or., 5 (1882) 145; Velen., Fl. Bulg. Suppl., (1898) 265; Hayek, Prodrom. Fl. Penins. Balc., 3 (1933) 101; Zahariadi, Fl. R. S Rom, 11 (1966) 412; Delipavlov, op. c, 163; Artjushenko, op. c., 76; *G. gracilis* Čelak., Sitz. Boehm. Ges. Wiss. (1891) i. 195 t. 9; Velen, Fl. Bulg. (1891) 539; P. H. Davis, Fl. Turkey, 8 (1984) 369; *G. nivalis* L. var. *gracilis* (Čelak.) Stoj. Stef., Fl. Bulg., vol. 1 (1923) 257; Stoj. Stef., Fl. Bulg., vol. 2 (1933) 244; Stoj. Stef., Fl. Bulg., vol. 3 (1948) 267; *G. nivalis* L. var. *graecus* (Orph.) Stoj. et Stef., Stoj. Stef. Kitan., Fl. Bulg., vol. 4(1) (1966) 237; *G. maximus* Velen., Fl. Bulg. (1891) 540; Velen., Fl. Bulg. Suppl., (1898) 266; *G. nivalis* L. var. *maximus* (Velen.) Stoj. et Stef., Fl. Bulg., vol. 1 (1923) 257; Fl. Bulg., vol. 2 (1933) 244; Fl. Bulg., vol. 3 (1948) 267; *G. graecus* Orph. ex Boiss. β *[f.] maximus* (Velen.), Hayek, op. c., 102; *G. elwesii* Hook. f. *maximus* (Velen.) Stoj et Stef., Stoj. Stef. Kitan., Fl. Bulg., vol. 4(1) (1966) 237; *G. bulgaricus* Velen., Fl. Bulg. (1891) 539; *G. nivalis* L., Jordanov, Fl. P.R.Bulg., 2 (1964) 318 pp. – Elwes's snowdrop or greater snowdrop.

Flowering period in Bulgaria. Winter to spring, from January to May, depending on location and altitude. Flowering begins immediately after snowmelt.

Habitat. Deciduous forests (*Acer* spp., *Carpinus betulus*, *C. orientalis, Fagus sylvatica*, *Quercus* spp.,) on nutrient-rich soils or floodplain soils near rivers. Also found in meadows near shrubs (*Paliurus spina-cristi*, *Rubus caesius*, *Rosa canina*). On siliceous or calcareous soils, east or northeast exposure, 60 - 1700 m a.s.l. The lowest described population is from Danubian plain, near Osam River (SOM 162988) - 61 m. a.s.l. The highest population is from Rila National Park (SOM 177471) - at 1700 m. a.s.l. The following associated herbaceous plant species were observed during the field study: *Allium* sp., *Arum maculatum*, *Asarum europaeum*, *Corydalis bulbosa*, *C. solida*, *Crocus flavus*, *Erythronium dens-canis*, *Euphorbia* sp., *Lamium purpureum*, *Ornithogalum* sp., *Potentilla micrantha*, *Pteridium aquilinum*, *Pulmonaria officinalis*, *P. rubra*, *Scilla bifolia*, *Veronica* sp.

Monitoring data: The areas of the studied populations are close to each other. The overall projective cover is 1-2%. In two of the populations (Shumen and Kokaliane) vegetative individuals dominate. In the Bachkovo population, generative individuals dominate. The density in the Bachkovo population is the highest - 1.3 individuals per m^2 (Table 3).

Table 1. Monitoring data of studied *G. nivalis* populations.

Index	**Belogradchik**	**Pasa dere**	**Ropotamo**
Area (dka)	13	15	18
Projective cover (%)	1	2	1
Vegetative individuals (%)	55	28	49
Generative individuals (%)	65	72	51
Density of individuals (m^2)	0,7	1,7	0,7

Table 2. Personal collections of *G. nivalis.*

No.	Locality
1	North-Eastern Bulgaria: Above Obrochishte village, 219 m. a.s.l. E exposition, 43.38155 N 28.07075E, 27.02.2004, coll. B. Sidjimova (SOM 162923)
2	North-Eastern Bulgaria: Carkva village, oak forest,120 m. a.s.l., E exposition, 43.416883N 27.984600E, 21.02.2002, coll. B. Sidjimova (SOM 162985)

3	Black Sea Coast (Northern): Pasha dere, “Limana”, 7 m. a.s.l., E exposition, 43.10918N 27.92358E, 26.02.2014, coll. B. Sidjimova (SOM 169988)
4	Black Sea Coast (Northern): Aladza Monastery, near Varna, 245 m. a.s.l., E exposition, 43.277478N 28.016479E, 20.02.2002, coll. B. Sidjimova (SOM 163456)
5	Black Sea Coast (Sourthern): Primorsko, near Ropotamo River, “Lavskata glava” locality, 9.7 m a.s.l., E exposition, 42.308000N 27.723533E, 19.02.2002, coll. B. Sidjimova (SOM 162920)
6	Black Sea Coast (Sourthern): near Primorsko, oak forest, 5,8 m. a.s.l., E exposition, 42.252317N 27.710150E, 19.02.2002, coll. B. Sidjimova (SOM 162921)
7	Black Sea Coast (Sourthern): Arkutino Reserve, 5 m. a.s.l., E exposition, 42.32572N 27.73056 E, 01.03.2006, coll. B. Sidjimova (SOM 162924)
8	Black Sea Coast (Sourthern): Kiten, after Diavolska river, near the road in bushes on sand, 2 m. a.s.l., E exposition, 42.25597N 27.74863E, 01.03.2006, coll. B. Sidjimova (SOM 163092)
9	Black Sea Coast (Sourthern): Camping "Coral", sea pine plantation, 7 m. a.s.l., NE exposition, 42.21507 N 27.78985 E, 25.03.2016, coll. B. Sidjimova (SOM 177463)
10	Balkan Range (Central): In Balgarka NaturePark, Balgarka Holiday Resort, 1166 m. alt, NE exposition, N42.76354 E25.49345, 05.04.2016, coll. B. Sidjimova (SOM 177466)
11	Balkan Range (Central): In Balgarka NaturePark, above Cheresha village, 1016 m. a.s.l., N exposition, N42.75097 E25.59975, 04.04.2016, coll. B. Sidjimova (SOM 177467)
12	Balkan Range (Western): Belogradchik, “Venetza”, 575 m. a.s.l., NE exposition, 43.623683N 22.676717E, 23.03.2004, coll. B. Sidjimova (SOM 162930)
13	Znepole region: Filipovci village, ”Sekirica” locality, 756 m. a.s.l., NW exposition, 42.836383N 22.696950E, 25.03.2004, coll. B. Sidjimova (SOM 162931)
14	Mt Strandza: Zabernovo village, “Tomova bahcha” locality, 167 m. a.s.l., W exposition, 42.078033N 27.582117E, 10.02.2005, coll. B. Sidjimova (SOM 162986)
15	Mt Strandza: Gramatikovo village, “Kachul” Forestry, 68 m. a.s.l., NW exposition, 42.022633N 27.650000E, 10.02.2005, coll. B. Sidjimova (SOM 162925)

Table 3. Monitoring data of studied *G. nivalis* populations.

Index	**Shumen**	**Kokaliane**	**Bachkovo**
Area, dka	13	14	16
Projective cover (%)	1	1	2
Vegetative individuals (%)	65	67	44
Generative individuals (%)	35	33	56
Density of individuals (m^2)	0,7	0,7	1,3

Distribution in Bulgaria: During the revision of herbar specimens from genus *Galanthus* in Bulgarian herbariums were established 195 materials of *G. elwesii* from 14 floristic regions: North–Eastern Bulgaria (22), Danubian plain (6), Forebalkan (1), Balkan Range (Western) (21), Sofia region (4), Znepole region (3), Vitosha region (3), Valley of River Struma (Southern) (1), Mt Pirin (2), Mt Rila (4), Mt Sredna gora (Western) (3), Rhodopes Mts. (74), Thracian plain (21), Tundza hilly region (19) (Apendix D, Fig 1). Delipavlov (1968, 1971) accept *G. graecus* like wide spread in Bulgaria and for the distribution of *G. elwesii* indicates only North–Eastern Bulgaria, Danubian plain and Forebalkan (Western). Tzonev (1997) reported *G. elwesii* from Danubian plain and Borisova & Donchev (2003) and Marinov et al. (2015) - from Balkan Range (Western, Central). According to Assyov & Petrova (2012), *G. elwesii* is widespread in Bulgaria in all floristic regions, except Mt Slavyanka, Black Sea Coast (Northern and Southern) and Mt Strandza.

During the study, 53 specimens were collected from 15 floristic regions. No data on the distribution of species in the floristic regions Black Sea Coast (Northern and Southern), Mt Slavyanka, Thracian Plain and Mt Strandza were found in the literature, herbarium collections and during the field survey. New chorological data on species distribution in the floristic regions West Frontier Mts., Mt Belasitsa, Struma Valley (Northern) and Valley of River Mesta are presented. (Table 4). Information on distribution of *Galanthus* species in Vitosha Mountain has not been confirmed for 90 years. In 2015, two populations of the species were reintroduced in places in Vitosha region, known from literature (Urumov 1930). In 2016 Gyurova & Savev published the results of their work. During my visit to the sites it was noted that the species naturalizes very successfully.

Discussion

Information on 5 species, 3 subspecies, 7 varieties and one form of genus *Galanthus* for the territory of Bulgaria was summarized from available literature and herbarium materials. This information was systematized according to the adopted taxonomic scheme (Anchev, 1992), and the synonyms of *G. nivalis* and *G. elwesii* were given in citation blocks. Thirty seven materials of *G. nivalis* and 195 of *G. elwesii* were revised with revision notes in herbarium collection in the country.

Investigations of the distribution of *G. nivalis* in Bulgaria have confirmed the findings of Delipavlov (1968, 1971) that this species has a limited distribution, especially in eastern Bulgaria. Revised herbarium specimens and personal collections show that the species occurs only in 5 floristic regions of the country. Most of the localities are from the floristic regions of Black Sea Coast (Southern) (13 revised and 5 personal) and North-Eastern Bulgaria (8 revised and 4 personal). New chorological data for two localities of *G. nivalis* from the Black Sea coast (Northern) are presented. In the Znepole region there is only one locality from a personal collection. (Fig. 1, Table 2, Appendix C).

Table 4. Personal collections of *G. elwesii*.

No	Locality
1	Danubian Plain: Vladimirovo village, by the road to „Gradeshki“ Monastery, 78 m. a.s.l., N exposition, 43.537833N 23.387833E, 22.02.2002, coll. B. Sidjimova (SOM 163459)
2	Danubian Plain: The valley of the Chernelka River, between the villages of Kartozhabene and Gortalovo, 175 m. a.s.l., SE exposition 43.334917N 23.550200E, 29.02.2004, coll. B. Sidjimova (SOM 162906)
3	Danubian Plain: Levski town, the bridge of Osam river, 61 m. a.s.l., NE exposition, 43.353500N 25.181917E, 26.02.2006, coll. B. Sidjimova (SOM 162988)
4	North-Eastern Bulgaria: On the road Popovo - Byala, before the village of Koprivets, 265 m. a.s.l., SW exposition, 43.416883N 27.984600E, 29.02.2004, coll. B. Sidjimova (SOM 162927)
5	North-Eastern Bulgaria: The village of Prolaz, before Targovishte, 323 m. a.s.l., NE exposition, 43.17177N 26.50150E, 10.02.2021, coll. B. Sidjimova (SOM 162905)
6	North-Eastern Bulgaria: The village of Moravitsa, before the town of Omurtag, 43.16415 N, 26.06418 E, 277 m. a.s.l., NE exposition , 21.02.2002, coll. B. Sidjimova (SOM 162915)
7	North-Eastern Bulgaria: Shumen plateau, the monument "1300 years Bulgaria", 454 m. a.s.l. , W exposition, 43.259783N 26.916233E,21.02.2002, coll. B. Sidjimova (SOM 162916)
8	North-Eastern Bulgaria: Shumen plateau, "Bukata" Reserve, 555 ma.s.l., NE exposition, 43.016667N 23.816667E, 28.02.2004, coll. B. Sidjimova (SOM 163462)

9	North-Eastern Bulgaria: The "Madarsky konnik" plateau, 450 m. a.s.l., NW exposition 43.266667N 27.116667E, 29.02.2004, coll. B. Sidjimova (SOM 162912)
10	North-Eastern Bulgaria: Between the villages of Sirakovo and Sarnino, 231 m. a.s.l., NE exposition, 43.650000N 28.283333E, 27.02.2004, coll. B. Sidjimova (SOM 162908)
11	North-Eastern Bulgaria: Between the villages of Kalinata and Vasilevo, "Chernata Gora" locality, 192 m. a.s.l., NE exposition, 43.62410N 28.18748E, 27.02.2004, coll. B. Sidjimova (SOM162909)
12	North-Eastern Bulgaria: The Obrochishte village, by the road to Balchik, 219 m. a.s.l., E exposition, 43.38155N 28.07075E, 20.02.2002, coll. B. Sidjimova (SOM 162922)
13	North-Eastern Bulgaria: The village of Tsarkva village , Dobrich region, 117 m. a.s.l., E exposition, 43.416883N 27.984600E, 21.02.2002, coll. B. Sidjimova (SOM 162907)
14	North-Eastern Bulgaria: Tervel Forestry, 193m. alt NE exposition, 43.68383N 27.3200E, 20.02.2002, coll. B. Sidjimova (SOM 162984)
15	Forebalkan (Western): Bozhenitsa village, Botevgrad, "Mishovite Kamani" locality, 450 m. a.s.l., NW exposition, 43.016667N 23.816667E, 24.03.2004, coll. B. Sidjimova (SOM 163093)
16	Forebalkan (Eastern): Yoglav village, Kamaka locality, 126 m. a.s.l., N exposition, 43.198055N 24.817632 E, 26.02.2003, coll. B. Sidjimova (SOM 162932)
17	Forebalkan (Eastern): Sevlievo, the forest on the way to "Momina salza" hut, 400 m. a.s.l., NE exposition, 43.196110N 25.112778E, 22.02.2002, coll. B. Sidjimova (SOM 162918)
18	Forebalkan (Eastern): Slopes along the main road Veliko Tarnovo – Targovishte, 295 m.a.s.l., NW exposition, 43.066667N 25.650246E, 26.02.2003, coll. B. Sidjimova (SOM 162907)
19	Balkan Range (Central): Balgarka Nature Park "Uzana" locality, 1016 m.a.s.l., NW exposition, 42.75097N 25.59975E, 05.04.2016, coll. B. Sidjimova (SOM 177468)
20	Balkan Range (Western): Below Baba peak, 1500 m. a.s.l., NE exposition, 42.751439N 24.006312E, 18.03.2004, coll. B. Sidjimova (SOM 162929)
21	Balkan Range (Central): above the village of Hristo Danovo, 750m. a.s.l., N exposition, 42.729432N 24.613471E, 01.04.2003, coll. B. Sidjimova (SOM 163466)
22	Balkan Range (Central): "Kozya stena" Reserve, 1500 m. a.s.l., NE exposition, 42.786854N 24.559853E, 01.04.2003, coll. B. Sidjimova (SOM 163488)
23	Balkan Range (Eastern): Nature park "Sinite kamani", on the way to "Karandila" locality, 848 m.a.s.l., W exposition, 42.747539N, 413305 E, 18.02.2002, coll. B. Sidjimova (SOM 162917)
24	Balkan Range (Eastern): Karnobat, "Markeli" locality, 212 m. a.s.l., E exposition, 42.637217N 26.897700E, 18.02.2002, coll. B. Sidjimova (SOM 162914)
25	Mt Sredna Gora (Western): Ihtiman, "Nivata" locality, over “Mativir” river, 600 m. a.s.l., N exposition, 42.418620N 23.892567E, 05.03.2004, coll. B. Sidjimova (SOM 162934)
26	Mt Sredna Gora (Western): Below Bogdan peak, 1502 m.a.s.l., NE exposition, 42.60568N 24.45145E, 21.02.2016, coll. B. Sidjimova (SOM 177463)
27	Mt Sredna Gora (Eastern): Stara Zagora, "Mechi Kladenets" locality, 348m alt, E exposition, 42.433037N 25.543833E, 10.05.2003, coll. B. Sidjimova (SOM 163370)
28	Znepole region: Trun town, “Mogilata” locality, 772 m.a.s.l., NW exposition, 42.689667N 23.857778E, 19.02.2004, coll. B. Sidjimova (SOM 162933)
29	Znepole region: Kraishte, above the village of Lyalintsi, Paramunska Mountain, below "Strazhata" peak, 954m alt, NEexposition, 42.774781N 22.751772E, coll. B. Sidjimova (SOM 177474)
30	West Frontier Mountains: Osogovo, above the village of Zhilentsi, 1349 m.a.s.l., E exposition, N42.23775 E22.61370, 17.03.2016, coll. B. Sidjimova (SOM 177469)

31	Sofia region: Kokalyane village, under the fortress "Urvich", 610m. a.s.l., NW exposition, 42.555791N 26.428444E, 20.03.2002, coll. B. Sidjimova (SOM 163471)
32	Sofia region: Makotsevo village, "Sinigerov dol" locality, 633m. a.s.l., NE exposition, 42.691044N 23.819134E, 18.03.2004, coll. B. Sidjimova (SOM163470)
33	Rila Mts: Rila National Park, above the village of Bistritsa, "Argacha" locality, 1365 m. a.s.l., NW exposition, N42.07869 E23.22630, 29.04.2015, coll. B. Sidjimova (SOM 177470)
34	Rila Mts: Rila National Park, "Chakalitsa" locality, on the road to Predela, 1700 m.a.s.l., NE exposition, N41.98442 E23.31718, 12.05.2015, coll. B. Sidjimova (SOM 177471)
35	Pirin Mts (Southern): Musomishte village, "Karacheto" locality, 550 m. a.s.l., E exposition 41.547459N 23.749398E, 19.02.2004, coll. B. Sidjimova (SOM 162910)
36	Pirin Mts (Southern): Before the village of Gospodintsi, "Trudovashkata chesma" locality, m. a.s.l., E exposition, 41.654842N 23.730121E, 19.02.2004, coll. B. Sidjimova (SOM 162910)
37	Mt Belasitsa: Near the path from "Hvoynova Polyana" hut to "Vejkata" peak, 948 m.a.s.l., NE exposition, 41.250783N 25.245366 E, 09.07.2016, coll. B. Sidjimova (SOM 173711)
38	Valley of Struma river (Northern): Below "Bliznatsite" peak, in the region of Zemen town, 589 m. a.s.l.,NW exposition, 42.452629 N 22.710993E, 21.02.2015, coll. B. Sidjimova (SOM 173472)
39	Valley of Mesta river: Hadjidimovo village, the chapel "St. Dimitar ", 450 m. a.s.l., SW exposition, 42.807886N 22.624542E, 25.03.2004, coll. B. Sidjimova (SOM 162933)
40	Rhodopes Mts (Western): On the road Belovo-Yundola, near the river Yadenitsa, 776 m. a.s.l., NE exposition, 42.144200N 23.960317E, 19.03.2003, coll. B. Sidjimova (SOM 162919)
41	Rhodopes Mts (Central): Kuklen village, near the Monastery "St. Kozma and Damyan", 450 m. a.s.l., NE exposition, 42.029321N 24.769497E, 16.03.2004, coll. B. Sidjimova (SOM 163461)
42	Rhodopes Mts (Central): Ruen village, oak forest above the chapel "St. Ilia", 600 m.a.s.l., NE exposition, 42.010823N 24.798877E, 19.03.2002, coll. B. Sidjimova (SOM 162928)
43	Rhodopes Mts (Central): Muldava village, over "St Petka Muldavska" Monastery, 443m. a.s.l., E exposition, 41.976267N 24.923018E, 16.03.2004, coll. B. Sidjimova (SOM 162905)
44	Rhodopes Mts (Central): Around Trigrad, 1350 m. a.s.l., NE exposition, 41.620924N 24.383002E, 25.03.2004, coll. B. Sidjimova (SOM 162983)
45	Rhodopes Mts (Central): Bachkovo Monastery, around the path to the "Marziganitsa" hut, 485 m. a.s.l., NE exposition, 41.938472N 24.859972E, 09.03.2002, coll. B. Sidjimova (SOM 163463)
46	Rhodopes Mts (Central): Narechenski Bani, "Soleno Izvorche" locality, beech forest, 530m. a.s.l., W exposition, 41.899729N 24.740890E, 16.03.2004, coll. B. Sidjimova (SOM 163464)
47	Rhodopes Mts (Eastern): Momchilgrad, next to the greenhouses by the river, 300m. a.s.l., E exposition, 41.511550N 25.390973E, 01.04.2003, coll. B. Sidjimova (SOM 163460)
48	Tundza Hilly Country: Topolovgrad region, around Visegrad peak, 812 m.a.s.l., NE exposition, 41.993371N 26.329675E,19.02.2002, coll. B. Sidjimova (SOM 163458)
49	Tundza Hilly Country: The hills northeast of Topolovgrad, Paliurus spina-cristi bushes, 123m. a.s.l., E exposition, 42.093056N 26.331389E, 14.03.2004, coll. B. Sidjimova (SOM 163458)
50	Tundza Hilly Country: Yambolski Bakadjik, "Inje" hut, 430m. a.s.l., E exposition,42.451083N 26.649300E, 01.03.2006, coll. B. Sidjimova (SOM 162989)
51	Tundza Hilly Country: The Jinot village, in bushes near the bridge in front of the village, 181m. a.s.l., E exposition, 42.499387N 26.650633E, 01.03.2006, coll. B. Sidjimova (SOM 163457)
52	Tundza Hilly Country: Tenevo village, Protected area "Debelata koria", 115 m.a.s.l., SE

	exposition, 42.358363N 26.541001E, 20.02.2015, coll. B. Sidjimova (SOM 177464)
53	Tundza Hilly Country: Yambol region, Hunting farm "Ormana", 129 m. a.s.l., SE exposition, N42.52818 E26.52055, 20.02.2016, coll. B. Sidjimova (SOM 177465)

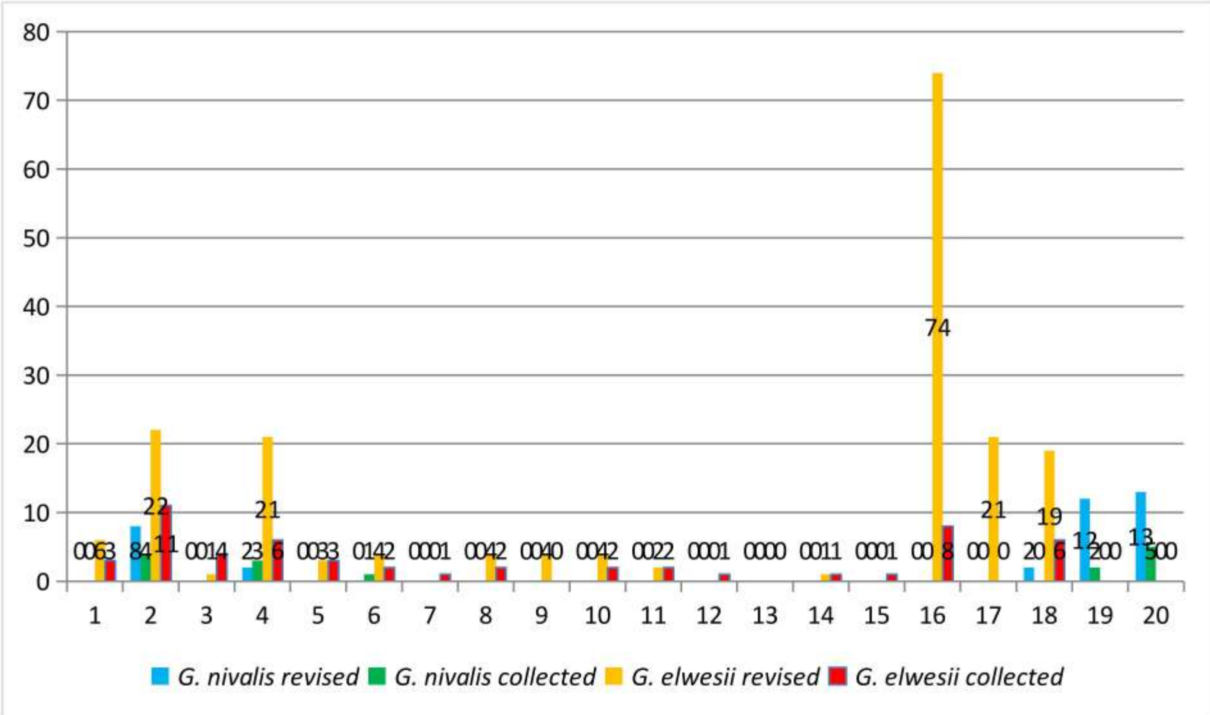

Fig 1. Distribution of *G. elwesii* and *G. nivalis* (revised chorological plates and personal collections) in the floristic regions of Bulgaria, without subregions. (1. Danubian Plain; 2. North-Eastern Bulgaria; 3. Forebalkan; 4. Balkan Range; 5. Mt Sredna Gora (Northern and Southern); 6. Znepole region; 7. West Frontier Mountains; 8. Sofia region; 9. Vitosha region; 10. Rila Mts; 11. Pirin Mts (Southern); 12. Mt Belasitsa; 13. Mt Slavyanka; 14. Valley of Struma river; 15. Valley of Mesta river; 16. Rhodopes Mts; 17. Thracian Plane; 18.Tundza Hilly Country; 19. Mt Strandza; 20. Black Sea Coast (Northern and Southern).)

G. elwesii has a wide distribution throughout the country. In the past, the species was widely distributed in the vicinity of Sofia (Lyulin Mountain, Vitosha, Lozen Mountain) (Urumov, 1929). Today, only a few sites are known in the floristic regions of Sofia. Most of the findings of the species in herbariums are from the Rhodope Mts. No any herbarium records of *G. elwesii* were found in 5 floristic regions - Western Frontier Mountains, Mt Belasitza, Mt Slavyanka, Valley of Mesta river, Mt Strandza and on the Black Sea Coast (Northern and Southern). As a result of the present study, data on the distribution of *G. elvesii* were not found only from the floristic regions of Black Sea Coast, Mt. Strandzha and Mt. Slavyanka.

The personal collections made during the survey confirm the ratio of about 5:1 between the localities of *G. elwesii* and *G. nivalis*.

Analysis of the results showed that the two species are to some extent geographically isolated. In Strandzha and on the Black Sea Coast floristic regions, only *G. nivalis* was found. In North-Eastern Bulgaria, two sympatric populations of *G. elwesii* and *G. nivalis* were established. Nearby localities of both species were identified for the Tran region (Znepole floristic region). General floristic regions for both species, but with a clear dominance of *G. elwesii*, are Balkan Range and the Tundza Hilly Country. No data were found on the distribution of *Galanthus* species in Mt Slavyanka (Fig. 1).

Both species prefer shady places with rich soils. Deciduous forests are a common habitat. Associated species are plants of the spring ephemeral complex. Observations in *G. elvesii* habitats show that the larger plants grow in sites characterized by high atmospheric and soil moisture - in oak woodlands on nutrient-rich soils or on floodplain soils near rivers. Smaller plants are found in dry, open habitats, under shrubs, or in *Carpinus orientalis* forests. These results confirm the conclusions of Sidjimova (2009) that smaller plants are the result of phenotypic variability in the adaptation of the species to drier habitats.

Populations of *G. nivalis* and *G. elwesii* with approximately equal size and mosaic structure were monitored. The age structure shows a slight predominance of vegetative individuals in *G. elwesii* and generative individuals in *G. nivalis*. The project cover is about 1% and the density is about 1/m^2. From the monitoring data, it can be concluded that all monitored populations are in a favorable condition at the time of monitoring.

Conclusions

The systematized taxonomic information on the genus *Galanthus* in Bulgaria and the revision of 37 *G. nivalis* and 195 *G. elwesii* herbarium specimens according to the taxonomic scheme of Anchev (1992) provide a basis for further studies of the species. The collected new chorological data on the distribution of *G. elwesii* in West Frontier Mts. (Osogovo), Mt Belasica, Valley of Struma river (Northern) and the Valley of Mesta river, new chorological data of *G. nivalis* from Black Sea Coast (Northern) and the confirmation of old data, unconfirmed for decades, as well as the described ecological characteristics of the species are of great importance for taking appropriate protective measures for bouth protected species. Initial monitoring data for the species indicate that their populations are in stable condition. The measures taken to protect both species (Biodiversity Act, 2002; Evstatieva, 2015) in Bulgaria are adequate and ensure good development of the species.

Acknowledgements: The author acknowledge financial support of Bulgarian Ministry of Education and Science (Project D01-230).

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Received: 20.05.2021
Accepted: 10.08.2021

APPENDIX

A *Galanthus* taxa, regarded as distributed in Bulgaria in different literature sources. In brackets are given synonyms of the taxon, according to this source.

Literature sources	Taxon, synonyms in brackets
Velenovsky (1891)	*Galanthus nivalis* L.
Velenovsky (1898)	*Galanthus gracilis* Celak. (*G. bulgaricus* Vel.) *Galanthus maximus* Vel.
Stoyanov & Stephanov (1923)	*Galanthus nivalis* L. var. *gracilis* Čelak. var. *maximus* Vel. (*G. maximus* Vel.)
Stoyanov & Stephanov (1933)	*Galanthus nivalis* L. var. *gracilis* Čelak. (*G. gracilis* Celak; *G. graecus* Orph.) var. *maximus* Vel. (*G. maximus* Vel.)
Hayek (1933)	*Galanthus nivalis* L. *Galanthus graecus* Orph.ex Boiss (*G. bulgaricus* Vel.; *G. gracilis* Celak)
Stoyanov & Stephanov (1948)	*Galanthus nivalis* L. var. *gracilis* Čelak. (*G. gracilis* Celak; *G. graecus* Orph.) var. *maximus* Vel (*G. maximus* Vel.)
Jordanov (1964)	*Galanthus nivalis* L. s.l
Stoyanov & all. (1966)	*Galanthus nivalis* L. var. *nivalis* var. *graecus* (Orph.) Stoj. et Stef. (*G. gracilis* Celak; *G. graecus* Orph., *G. bulgaricus* Vel.) *Galanthus elwesii* Hook. f. *maximus* Vel. (*G. maximus* Vel.)
Artjusenko (1970)	*Galanthus nivalis* L. *Galanthus. elwesii* Hook. var. *maximus* (Vel.) (*G. maximus* Vel.; *G. graecus* Orph. var. *maximus* (Vel.)Hayek.) *Galanthus graecus* Orph. ex Boiss (*G. nivalis* ssp. *graecus* (Orph. ex Boiss.) Gottlieb-Tannenhain; *G. bulgaricus* Vel.; *G. gracilis* Celak.)
Delipavlov (1971)	*Galanthus nivalis* L. var. *montanus* (Schur.)Rouy. var. *nivalis* L. *Galanthus graecus* Orph.ex Boiss (*G. bulgaricus* Vel.; *G. gracilis* Celak; *Galanthus nivalis* L. var. *gracilis* (Čelak.) Stoj. et Stef.) var. *maximus* (Vel.) Beck. (*G. maximius* Vel.; *G. nivalis* var. *maximus* Vel.; *G. elwesii* f. *maximus* (Vel.) Stoj.,Stef.,Kitan.; *G. elwesii* var.*maximus* (Vel.)Beck.) *Galanthus elwesii* Hook.
Webb (1978)	*Galanthus nivalis* L. *Galanthus elwesii* Hook. fil. (*G. maximus* Vel.; *G. elwesii* var. *maximus* (Vel.) G.Beck.; *G. graecus* var. *maximus* (Vel.)Hayek; *G. nivalis* var. *maximus* (Vel.)Stoj. et Stef.; *G. graecus* Orph. ex Boiss.; *G. nivalis* ssp. *graecus* (Orph. ex Boiss.) Gottl.-Tann.) - ssp. *elwesii* ssp. *minor* D.A.Webb.

Anchev (1992) In:	*Galanthus nivalis* L.
Kozuharov (ed)	*Galanthus elwesii* Hook. fil.
	ssp. *elwesii*
	ssp. *minor* Webb. (*G. graecus* auct. bulg.)
	ssp. *maximus* (Vel.) Kož. et Andr.
Anchev (1999) In:	*Galanthus nivalis* L.
Petrova & all.	*Galanthus elwesii* Hook. fil.
Davis (2000)	*Galanthus nivalis* L.
	Galanthus elwesii Hook. fil. (*G. graecus* Orph. ex. Boiss, *G. maximus* Vel.; *G. bulgaricus* Vel.)
	Galanthus gracilis Čelak. (*G. elwesii* Hook. f. subsp. *minor* Webb; *G. graecus* auct. non Orph. ex Boiss., pro parte: Stern)
Delipavlov (2003)	*Galanthus nivalis* L.
	Galanthus elwesii Hook. fil
	Galanthus gracilis Čelak. (*G. graecus* Orph.)

B *Galanthus* taxa found in the chorological literature and herbariums in Bulgaria

1. Galanthus elwesii Hook. f.: Delipavlov (1968), Delipavlov (1971), Tzonev (2002), Delipavlov & Chesmedziev (ed) (2003); SO 97162 (Tzonev); SOA 04145 (Delipavlov); SO 58673 (Koeva); SOM 121447, SO 12984, 12983, 12985 (Simeonovsky); SOM 159628, 159631, 159829, 159634, 159630 159633, 159632 (Sopotlieva).
2. *Galanthus elwesii Hook. f. subsp. elwesii*: Anchev (1992).
3. *Galanthus elwesii Hook. f. subsp. maximus* (Velen.)Beck.: Kozuharov (1992), SO 99150 (Tzonev); SOA 32414 (Delipavlov); SOM 13606 (Davidoff, J.M.Tzar Boris III); SOM 13603 (Davidoff, Radev), SOM 13613 (Davidoff).
4. *Galanthus elwesii Hook. f. subsp. minima*: Anchev (1992), Tzonev(1997).
5. *Galanthus elwesii Hook. f. subsp. balcanicus* Dav.: SOM 13604, 13605 (Davidoff); SOM 13608 (J.M.Tzar Boris III).
6. *Galanthus elwesii Hook. f. var. maximus* (Velen.)*:* SOM 13612 (Davidoff, Nejchev); SOM 13609 (Toshev, Davidoff).
7. *Galanthus elwesii* Hook. f. *var. orbelicus* Dav.: SOM 13610, 13611 (Davidoff).
8. *Galanthus elwesii* Hook. f. f. *maximus* (Velen.)Stoj. & Stef.: SO 29898 (Ganchev, Vichodcevsky).
9. *Galanthus gracilis* Čelak.: Čelakovsky (1891), Toshev (1903), Urumov (1897, 1901, 1906, 1909, 1913, 1917, 1923, 1929, 1935a, 1935b, 1937); SO 12994, 12993, 12991, 12992 (Stribrny).
10. *Galanthus graecus* Orph. ex Boiss.: Delipavlov (1968); SOA 45539, 45540 (Dimitrov, Delipavlov); SOA 33823, 33818, 32462, 04154, 04155, 32409, 32408, 32445, 04142, 04143, 04150, 32441, 32442, 32396, 32397, 33827, 32399, 32436, 32437,32443, 32459, 32458, 32451, 32453, 32440, 32425, 32424, 32454, 32455, 32438, 32431, 04159, 04163, 45536, 45541, 45542, 27444, 32410, 32444 (Delipavlov); SOA 04161-04165, 40510, 04149, 45543, 32405, 32406, 04179, 36613, 04175, 36608, 04153 (Delipavlov, Popova); SOA 45537, 45538 (Delipavlov, Chaushev); SOA 36602, 42998, 42999, 45536, 50739, 04147, 34766, 44804, 40510, 50734 (Popova); SOA 27446 (Popova, Tzvetanov); SOA 34738 (Stribrny); SOA 04176, 32398, 44863, 04175, 04176, 30439, 40542, 31172, 31173, 31174 (Ceshmedziev).
11. Galanthus graecus Orph. ex Boiss. var. maximus: SOA 04171, 04156, 32407, 04146, 32441, 04157, 04166, 04177, 04161, 04162, 04164, 04165 (Delipavlov); SOA 27448 (Popova).

12. ***Galanthus nivalis*** **L.:** Yavashev (1890), Velenovsky (1891, 1898), Toshev (1895, 1903), Kovachev (1890, 1892), Urumov (1897, 1898, 1901, 1904, 1905, 1906, 1908a, 1908b,1908c, 1909, 1913, 1917, 1919, 1925, 1926, 1928, 1929, 1930, 1935a, 1935b); Stoyanov (1966); Delipavlov & Angeliev (1970); Delipavlov (1968, 1971), Kozhuharov (1992); Nikolov et al. (1994); Evstatieva & Vitkova (1999); Evstatieva & Hardalova (2000); Gussev et al. (1997, 2004); Delipavlov & Cheshmedzhiev (2003); Assyov & Petrova (2012); SOA 32413, 27441, 04181, 04182, 04183, 32402, 32403, 04183, 324000, 324001, 32434, 32429, 32430, 32435, 32412, 32424, 04182, 32404, 32415, 32416, 32417, 32418, 32419, 32432, 32433, 32446, 32420, 32421, 32422, 32446, 32447, 32448, 32427, 32428 (Delipavlov); SOA 27447, 27449 – (Delipavlov, Slovakova); SO 83719 (Drianovsky); SO 98451 (Georgiev); SO 13025,13026, 13022, 13027, 13028 (Georgiev); SO 13018 (Grigorov, Jordanov); SO 30097 (Ivancheva); SO 13033, 13019, 13019 (Jordanov); SO 26805, 92655 (Jordanov, Ac. Янев); SOA; SO 13032 (Kitanov); SO 35722 (Mustafov); SO 13030, 13021 (Nejchev); SOM 139481 (Rohod); SO 13037 (Rohod, Vichodcevsky); SOA 36609, 36562, 29564 (Popova); SO 13016, 13026 (Toshev); SO 13035 (Tzvetkov, N. Vihodcevsky); SO 100669, 00703 (Tzonev); SOM 13036, 103195, 13034, 46662- N. Vihodcevsky; SO 29550 (Vasileva); SO 18304, 29555, 29563, 29562, 29559, 29558, 20772, 29557, 20777, 20778, 20775, 20774, 20776, 29551, 29553, 29554, 29556, 20771, 20779, 20780, 20773, 29552, 29561, 29560, 29550 (Vasileva); SO 13029 (Valev), SO 86636 (Yanev).
13. ***Galanthus nivalis*** **L. var.** ***gracillis*** **Celak.:** SO 13024 (Jordanov)
14. ***Galanthus maximus*** **(Velen.) Stoj. & Stef.:** Kovachev (1900); Velenovsky (1898, 1891), Nejchev (1903, 1908), Urumov (1898,1901, 1905, 1906, 1908, 1909, 1913,1925, 1928, 1929,1930, 1935); SOM 13602, 13607 (Davidoff); SOA 32423, 33819, 33820, 04169, 04170, 04172, 04173, 04172 (Delipavlov); SO 12982 (Georgiev); SO 12999 (Nejchev); SO 13001, 13000, 83718, 12996; 12997,12998, 12995, 34738 (Stribrny); SOM 103197, 103716, 13002 (Vihodcevsky).

C. Revised herbarium plates from *Galanthus nivalis,* arranged by floristic regions
Black Sea Coast (Sourthern) (13): SOA 04181, 04182, 04183 (4.02.1964, 23.02.1966, Delipavlov); SOA 32413, 27441 (3.02.1963, Delipavlov); SOA 32402, 32403, 32404, 32415, 32416, 32417 (3.02.1963, Delipavlov); SOA 32418, 32419 (04.02.1964, 04.02.1964, Delipavlov)
North-Eastern Bulgaria (8): SOA 324000, 324001 (28.03.1965, Delipavlov); SOA 32434 (27.02.1968, Delipavlov); SOA 32429, 32430 (Delipavlov); SOA 32435, 32412 (27.03.1964, Delipavlov); SOA 32424 (27.02.1968, Delipavlov)
Tundza Hilly Country (2) SOA 27447, 27449 (Delipavlov, Slovakova)
Balkan Range (Western) (2) SOA 36609, 36562 (04.03.1979, Popova)
Mt Strandza (12): SO 98451 (20.02.1977, Georgiev); SOA 32432, 32433 (15.03.1964, Delipavlov); SOA 32446, 32420, 32421, 32422, 32446, 32447, 32448, 32427, 32428 (05.02.1964, Delipavlov)

D. Revised herbarium plates from *Galanthus elwesii* arranged by floristic regions
1. North–Eastern Bulgaria (22): SO 13025, 13026 (05.03.1894, Georgiev); SO 35722 (20.03.1973, Mustafov); SOA 33823, 33818 (01.03.1969, Delipavlov); SOA 32462 (27.03.1964, Delipavlov); SOA 27446 (25.02.1972, Popova, Cvetanov); SOA 32441 (25.03.1964, Delipavlov); SOA 32444 (29.03.1964, Delipavlov); SOA 04154, 04155 (23.03.1964, Delipavlov); SOA 32409, 32408 (26.03.1965, Delipavlov); SOA 04142, 04143, 04150 (24.03.1964, Delipavlov); SOA 32442 (29.03.1964, Delipavlov); SOA 27444 (10.03.1968, Delipavlov); SOA 04169, 04170 (15.03.1967, Delipavlov); SOM 13607 (03.03.1895, Б. Davidoff); SOA 32410 (05.03.1965, Delipavlov); SOA 04172 (25.03.1964, Delipavlov)

2.Danubian plain (6):SO 97162 (07.04.1993, Tzonev); SO 99150 (15.02.1977, Tzonev); SO 100669 (03.2000, Tzonev); SOA 32414 (12.02.1966, Delipavlov); SOA 04145 (24.03.1964, Delipavlov); SOA 04160 (18.03.1966)
3.Forebalkan (1):SOM 13612 (02.1896, Б. Davidoff; Nejchev).
4. Balkan range (21): SO 29898 (28.01.1960, Ganchev, Vichodcevsky); SO 58673 (31.03.1974, Koeva); SO 13002 (30.03.1960, Vichodcevsky); SO 12999 (03.1900, Nejchev); SO 13030 (1889, Nejchev); SO 13021 (22.01.1897, Nejchev); SO 13023, 13026 (13.02.1897, Toshev); SO 13022 (22.04.1891, Georgiev); SOA 27448 (28.02.1972, Popova); SOA 40542 (01.05.1984, Ceshmedziev); SOA (02.1904); SOA 31172, 31173, 31174 (12.03.1977, Ceshmedziev); SOA 34766 (16.04.1978, Popova); SOA 04171 (25.03.1964, Delipavlov); SOM 13606 (13.04.1921, Davidoff; J.M.Tzar Boris III).; SOM 103197 (30.03.1960, Vichodcevsky); SOM 103716 (30.03.1960, Vichodcevsky); SOM 13609 (02.04.1893, Toshev; Davidoff); SOM 13602 (23.04. 1915, Б. Davidoff).
5. Sofia region (4): SO 13032 (10.04.1949, Kitanov)**;** SO 13034 (26.02.1960, Vichodcevsky); SOA (03.1920); SOM 13603 (4.03.1923, Davidoff, Radev); SOM 103195 (26.02.1960, Vichodcevsky).
6. Znepole region (4):SO 13027, 13028 (25.03.1892, Georgiev)**;** SO 30097 (10.04.1971, Ivancheva); SOA (03.1914)
7. Vitosha region (4): SO 12982 (02.04.1889, Georgiev); SO 13029 (10.04.1961, Valev); SO 83719 (02.1930, Drianovsky); SOA (05.1923)
8. Valley of Struma riverStruma Valley (1): SOA 32445 (14.02.1966, Delipavlov)
9. Pirin Mts (2): SOA 45537; 45538 (03.03.1970, Delipavlov, Chaushev)
10. Rila Mts (4): SOM 13605 (25.03.1911, B Davodoff); SOM 13604 (25.02.1912, Б. Davidoff); SOM 13610; 3611 (25.02.1912, Б. Davidoff)
11. Sredna gora Mt (Western) (3): SO 12983, 12985 (07.04.1968, Simeonovsky); SOM 121447 (7.04.1968, Simeonovsky);
12. Rhodopes Mts (74): SO 12994 (23.03.1894, Stribrny); SO 12993 (23.03.1895, Stribrny); SO 12991, 12992 (03.1895, Stribrny)SO 13001, 13000 (13.03.1898, Stribrny); SO 83718 (Stribrny); SO 12995 (03. 1895, Stribrny); SO 12996, 12997 (13.03.1893, Stribrny); SO 12998 (03.1893, Stribrny); SO 18304 (04.1970, Vasileva); SO 29555; 29563 (08.02.1972, Vasileva); SO 29562 (06.02.1972, Vasileva); SO 29559 (15.02.1972, Vasileva); SO 29558 (13.02.1972, Vasileva); SO 20772 (14.02.1972, Vasileva); SO 29557 (11.02.1972, Vasileva); SO 20777; 20778 (04.02.1972, Vasileva); SO 20775 (09.02.1971, Vasileva); SO 20774 (05.02.1971, Vasileva); SO 20776 (07.02.1971, Vasileva); SO 29551 (18.03.1972, Vasileva); SO 29553 (19.02.1972, Vasileva); SO 29554 (19.02.1972, Vasileva)SO 29556 (13.02.1972, Vasileva); SO 20771; 20779 (14.02.1972, Vasileva); SO 20780 (14.02.1971, Vasileva); SO 20773 (24.02.1971, Vasileva); SO 29552 (20.03.1972, Vasileva); SO 29561 (06.02.1972, Vasileva); SO 29560 (17.03.1972, Vasileva); SO 29550 (28.03.1972, Vasileva); SOA 04147 (12.03.1963, Popova); SOA 04161-04165 (16.03.1964, Delipavlov, Popova); SOA 40510, (20.02.1966, Delipavlov § Popova); SOA 36613 (06.02.1976, Delipavlov § Popova); SOA 36608, 04153 (14.02.1979, Delipavlov, Popova); SOA 32398 (13.03.1965, Ceshmedziev); SOA 04149, 04166 (23.03.1964, Delipavlov); SOA 44863 (26.03.1985, Ceshmedziev); SOA 45543 (26.02.1967, Delipavlov); SOA 50734 (31.01.1971, Popova); SOA 33819, 33820 (16.03.1969, Delipavlov); SOA 32405, 32406 (30.03.1964, Delipavlov); SOA 44804 (23.03.1986, Popova); SOA 04156, 04157 (20.03.1964, Delipavlov); SOA 32406 (30.03.1964, Delipavlov); SOA 04176 (09.02.1958); SOA 34738 (03.1905, Stribrny); SOA 04175 (01.03.1953); SOA 40510 (06.02.1976, Popova); SOA 04177 (01.03.1959, Delipavlov); SOA 04179 (07.04.1963, Delipavlov);

SOM 159628 (08.03.2002, Sopotlieva); SOM 159631 (08.03.2002, Sopotlieva); SOM 159829 (08.03.2002, Sopotlieva); SOM 159633 (05.03.2002, Sopotlieva); SOM 159632 (06.03.2002, Sopotlieva); SOM 159634; 159635 (04.03.2002, Sopotlieva); SOM 159630 (07.03.02, Sopotlieva);
13. Thracian plain (21): SO 29564 (25.02.1972); SO 13037 (11.02.1968, Rohod, Vichodcevsky); SOA 33827 (10.03.1968, Delipavlov); SOA 32396, 32397 (07.03.1965, Delipavlov); SOA 04163, 32399 (19.02.1966, Delipavlov); SOA 32443 (06.03.1964, Delipavlov); SOA 32459, 32458, 32451, 32453, 32454, 32455 (27.03.1970, Delipavlov); SOA 32438 (15.03.1965, Delipavlov); SOA 32431 (12.02.1966, Delipavlov); SOA 45539, 45540 (28.02.1970, Delipavlov, Dimitrov); SOA 50739 (02.03.1971, Popova); SOA 04159 (23.03.1960, Delipavlov); SOA (04.1929); SOM 139481 (03.02.1979, Rohod).
14. Tundza hilly country (19): SO 13033 (09.03.1972, Jordanov); SO 13018 (04.1927, Grigorov & Jordanov); SOA 32436, 32437 (25.03.1970, Delipavlov); SOA 04173, 04172 (22.03.1975, Delipavlov); SOA 32407, 04146 (12.03.1964, Delipavlov); SOA 45536 (22.03.1970, Popova); SOA 30439 (06.03.1974, Ceshmedziev); SOA 45541, 45542 (09.03.1970, Delipavlov); SOA 42998, 42999 (02.04.1985, Popova); SOA 32440 (07.03.1970, Delipavlov); SOA 32425 (29.03.1964, Delipavlov); SOA 32424 (30.03.1965, Delipavlov); SOA 36602 (16.03.1079, Popova); SOM 13613 (02.1926, Davidoff).

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 95-105

Weed Infestation and Control on a Miscanthus giganteus Plantation in the Marginal Lands of Ukraine

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Abstract. The research was carried out on the waterlogged marginal lands in the zone of unstable moisture of the Central Forest-Steppe of Ukraine. The generalization of the dynamics of changes in the number of weeds in the miscanthus plantations of the first year of growing allows to divide the growing season into three specific periods. The dynamics of weed infestation in *Miscanthus x giganteus* J.M. Greef et Deuter ex Hodk. et Renvoize plantations of the first and subsequent years of growing, monthly accumulation of weed green mass starting from 10th June was studied. The number of weeds in the plantations of miscanthus increased most intensively in the period from the middle of May to early June. In subsequent periods, with the achievement of full projective coverage of the field by plants, the intensity of new weed seedlings' appearance decreased. The most abundant weed species were *Echinochloa crus-galli (L.)P. Beauv.*, *Setaria glauca (L.) P.Beauv.*, *Thlaspi arvense* L., *Sinapis arvensis* L., *Sonchus arvensis* L., and *Chenopodium album* L. All considered herbicides showed the highest level of toxic action to the seedlings of annual cereal weed species. Seedlings of perennials *Sonchus arvensis* L., *Cirsium arvense (L.) Scop.* have not been affected by soil herbicides. The following herbicides: Master Power OD at an application rate of 1.5 l/ha (efficiency 95.0%) or Milagro 040 SC at an application rate of 1.25 l/ha (efficiency 88.0%.) are recommended during the season of plant vegetation.

Key words: miscanthus, weeds infestation, herbicides, marginal lands.

Introduction

Currently, numerous species of *Miscanthus* were actively studied to investigate the influence of genetic and environmental factors (Hodgson et al, 2010; Kharytonov et al., 2019).

Meantime in absolute measures, the largest values were noted for *Miscanthus x giganteus* biomass indexes (Christian et al., 2008). A biological feature of miscanthus plants (*Miscanthus × giganteus* J.M.Greef et Deuter ex Hodk. et Renvoize) is a long period from planting to emergence (from 25 to 30 days) and slow growth and development in the first half of the growing season. Accordingly, the slow initial growth of miscanthus significantly reduces its ability to compete with weeds. In addition, the low planting density leaves a large number of unfilled spaces where weeds can grow. The soil is loosening in the process of planting miscanthus, thereby promoting the germination of weed seeds. In the early stages, young miscanthus seedlings can be easily suppressed by weeds (McCalmont et al., 2017; Winkler et al., 2020). The greatest competition with weeds is observed during the first year of growing and partly in the second year (Borkowska & Molas, 2010; Koncekova et al., 2014; Winkler et al., 2020). Cold tolerance and over-winter survival of first-year stands is also a concern in temperate areas with cold winters and little snow cover (Anderson et al., 2011a). It was indicated also a major risk to viability when soil temperatures drop below -3°C at the 5-cm soil level, with lethal rates of up to 50% at first-year cold (Clifton-Brown & Lewandowski, 2000). Field experiments suggest that although glyphosate and tillage can reduce miscanthus biomass, complete control of a mature stand likely will require more than one growing season (Anderson et al., 2011b). It was shown also the active substances and their maximum application rates, which did not imply a significant phytotoxic effect on miscanthus plants (Anderson et al., 2015). At the same time, other studies have shown that the application of herbicides of both pre- and post-emergence action causes a decrease in miscanthus yield in the first and second years compared to the control treatment, where the crop was grown clear of weeds (Everman et al., 2011; Maksimovich et al., 2016).

There are several tasks of the research as following: a) to clarify the features of weed infestation in *Miscanthus x giganteus* Greef & Deuter plantations in the first and subsequent years of growing; b) to investigate the peculiarities of weed species composition and the dynamics of their germination and to assess the level of the negative impact of weeds on crops; c) to determine the biological efficiency of the herbicides of soil action and herbicides applied during the vegetation of miscanthus plants.

Materials and Methods

The research was carried out in the years 2015–2019 on the experimental field of the State Enterprise Experimental Farm Salyvinky (Ksaverivka-2, Vasylkiv district, Kyiv region), which is located in the zone of unstable moisture in the Central Forest-Steppe of Ukraine with the moderate continental climate. The soil of the experimental field is podzolic chernozem. The experimental field is located in waterlogged lowland and corresponds to unfavourable marginal lands. *Miscanthus × giganteus* variety "Osinnii Zoretsvit" (IBCSB NAAS of Ukraine) was used. Rhizomes were planted in the soil in rows at row spacing of 70 cm and plant density of 15 thousand plants per hectare. The planting design was 70 cm x 90 cm. The main tillage was carried out in August-September with disc cultivators to a depth of 10–12 cm in two tracks in a cross manner with an angle of attack of the discs of 30–35°. Deep furrow ploughing carried out 10−15 days later, after stubbing to a depth of 28−30 cm. To level the field surface, control weed seedlings, and create favourable conditions for the accumulation of soil moisture in winter, continuous cultivation of the field surface to a depth of 5−7 cm carried out on the 10^{th}−15^{th} day after ploughing. Pre-planting soil treatment carried out before planting to a depth of 12–15 cm. This measure provides good conditions for planting rhizomes to a given depth. Rhizomes were planted by hand. The next day after planting the field was rolled. The level of survival of young plants was high and over the years of research varied from 86 to 90%. The number of weed seedlings was counted in the area of 0.25 m^2 in four replications on each site by types of weeds with the subsequent recalculation per 1 m^2. It was supposed to conduct calculations on the following dates: the dynamics of the number of weed seedlings (plants/m^2) and accumulation of weed green mass on the 10th May; 10th June; 10th July; 10th August, and 10th September. The dynamics of the weed mass accumulation in the plantations was determined by the method of total cutting of aboveground parts of weeds within the borders of the counting frame (0.25 m^2) on four plots (total area 1 m^2) in each replication of a treatment. That is, in all replications of all treatments, the aboveground weed mass was calculated on 16 sites. The cut weed biomass was sorted by species and weighed. The gross mass was converted into average mass per 1 m^2. The experiments involved a weed control system in the first year with the use of soil herbicides. Herbicide solution spread on the soil surface and formed a protective herbicide screen after planting miscanthus rhizomes. Active ingredient partially penetrated the upper layer of the soil (0–3 cm) and was available in the soil moisture. It diffused into the seedlings of annual weeds and led to their die off with soil moisture. The solution was applied with a special wheeled gas slit-type sprayer with a bar. The working pressure was 2.2 atmospheres, herbicide solution consumption was 240 l/ha. The level of efficiency of the protective action of soil herbicides assessed by comparing the number of weed seedlings per unit area of treatment with control. Evaluation of the herbicide efficiency was done based on a comparison of the number of weed seedlings in the accounting area before spraying and 20 days after spraying. The difference in the number of seedlings expressed as a percentage of the control. We used the following soil herbicides after planting rhizomes: 1) Frontier Optima SC (dimethenamid - P, 720 g/l) at an application rate of 1.4 l/ha; 2) Dual Gold EC (S-metolachlor, 960 g/l) at an application rate of 1.6 l/ha; 3) Merlin 750, WG (isoxaflutol, 750 g/kg) at an application rate of 0.15 kg/ha. Six consecutive manual weeding carried out in the control treatment. The area of a single plot was 36 m^2. The accounting area was 25 m^2. The experiment was conducted in a randomized block design with four replications. The experiments involved weed control in miscanthus plantations in the first year using herbicides, which were applied by spraying weed seedlings in the tillering stage. We used the following herbicides: 1) Master Power OD (foramsulfuron, 31.5 g/l + iodosulfuron-methyl sodium, 1 g/l + thiencarbazone-methyl, 10 g/l + cyprosulfamide, 15 g/l) at an application rate of 1.5 l/ha; 2) Milagro 040 SC (nicosulfuron, 40 g/l) at an application rate of 1.25 l/ha. The herbicide solution sprayed with a special wheeled gas slit-type sprayer with a bar. The working pressure was 2.2 atmospheres, working solution consumption was 210 l/ha. The yield of the aboveground biomass of miscanthus evaluated by the method of continuous cutting of terrestrial parts of crop plants in the accounting areas (25 m^2) in all replications of treatments. The obtained biomass was weighed, converted to a standard humidity, and expressed per 1 hectare. The obtained experimental data were processed by statistical methods, such as the method of variance, correlation, regression analysis using personal computer and software Excel, and Statistica 12.0.

Results

Rhizomes of miscanthus in the years of the experiment were planted in the middle or late April. Total cultivation of the field before planting provided destruction of wintering weed sprouts and a part of early spring weeds. Favourable weather conditions in a combination with large stocks of weed seeds in the soil ensured the rapid emergence of new weed sprouts soon after planting rhizomes. In the areas of plantations where weed control measures were not applied, new weed sprouts were observed as early as on the 7th-10th day after pre-planting tillage (Fig.1).

Fig. 1. General view of miscanthus plantations of the first year of growing without weed control measures.

At the time of the first calculation of weeds in the miscanthus plantations (10th May) we recorded (on average over the years of the experiment) sprouts of *Thlaspi arvense* L . in the amount of 4.3 plants/m^2, *Sinapis arvensis* L. 3.2 plants/m^2, *Fumaria officinalis* L. 2.9 plants/m^2, Galium 2.3 plants/m^2, *Polygonum convolvulus* L. 2.3 plants/m^2, *Melandrium album* (Mill.) Garke 1.3 plants/m^2, *Cirsium arvense* L. irsium arvvense Scop. 1.2 plants/m^2, and other species. The total number of weed sprouts in this period averaged 38.8 plants/m^2 (Table 1).

At the same time, the green mass of weeds was insignificant and amounted to 18 g/m^2. The emergence of new weed sprouts within one species normally was extended in time and lasted from two weeks to the end of the growing season (Fig.2).

Relatively evenly emerged the sprouts of annual cereal weeds such as *Echinochloa crus-galli*, *Setaria glauca*, and *Polygonum* species. The most extended in time was the emergence of sprouts of *Chenopodium album* L., *Amaranthus retroflexus* L., and some other species. During the next month of joint vegetation of miscanthus plants with weeds, the number of weeds in the plantations increased significantly. At the time of subsequent calculation of weeds (10th July) average number in the plantations was 119.5 plants/m^2. Compared to the previous period, it was increased by 3 times. The total green mass of all weeds in the plantations of miscanthus was 950 g/m^2. It increased significantly in comparison with the indicators of the weed accumulation at the time of last calculation. The largest biomass accumulation was demonstrated by plants of *Chenopodium album* L., 172 g/m^2 (18.1%), *Sinapis arvensis* L., 166 g/m^2 (17.5%), *Thlaspi arvense* L., 60 g/m^2 (6.3%), *Echinochloa crus-galli* L., 57 g/m^2 (5.9%), *Cirsium arvense* L., 50 g/m^2 (5.3%) and other species. The largest indicators of the total accumulation of the aboveground mass of weed plants in the miscanthus plantations in the years of research were recorded on the 10th August 2906 g/m^2.

Table 1. Dynamics of weed accumulation in miscanthus plantations (plants/m^2).

Weed species	Date of calculations				
	10 May	10 June	10 July	10 August	10 September
Chenopodium album L.	1.6	4.6	4.8	4.9	4.9
Amaranthus retroflexus L.	2.2	4.1	4.5	4.7	4.7
Echinochloa crus-galli (L.) *P.Beauv.*	3.4	20.9	31.3	31.5	31.5
Setaria glauca (L.) *Pal Beauv.*	1.9	15.8	16.7	16.9	16.9
Polygonum convolvulus L.	2.3	5.9	5.9	5.9	5.9
Polygonum persicaria L.	2.7	5.7	5.7	5.7	5.7
Thlaspi arvense L.	4.3	6.1	6.1	6.1	6.5
Viola arvensis Murr.	0.9	1.2	1.2	1.2	1.2
Fumaria officinalis L.	2.9	4.2	4.2	4.2	4.4
Galium aparine L.	2.3	1.0	1.0	1.0	1.3

Sinapis arvensis L.	3.2	6.1	6.1	6.1	6.4
Solanum nigrum L.	1.1	2.7	2.9	3.0	3.0
Polygonum aviculare L.	2.1	3.2	3.3	3.3	3.3
Melandrium album (Mill.) Garcke.	1.3	1.9	2.0	2.0	2.0
Sonchus arvensis L.	1.1	5.7	5.9	6.1	6.1
Cirsium arvense L.	1.9	3.4	3.7	3.7	3.7
Other species	3.6	13.3	14.2	14.5	14.8
Total	**38.8**	**105.8**	**119.5**	**120.8**	**122.3**
$LSD_{0.05}$	0.12	0.20	0.22	0.23	0.26

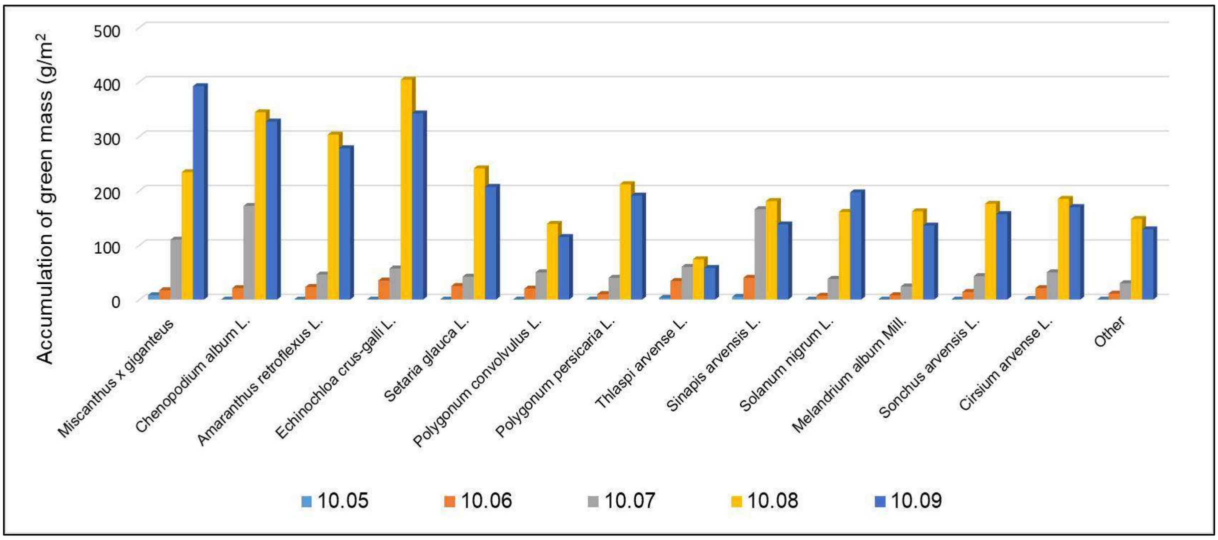

Fig. 2. Dynamics of weed green mass accumulation in miscanthus plantations (g/m^2). *Note:* The total weed green mass includes the following weed species: *Viola arvensis* Murr., *Fumaria officinalis* L., *Galium aparine* L., and *Polygonum aviculare* L. These weeds formed a small amount of green mass (‹50 g/m^2). The total mass of weed green mass on 10 May – 18 g/m^2 ($LSD_{0.05}$ 0.1); 10 June – 330 g/m^2 ($LSD_{0.05}$1.2); 10 June – 950 g/m^2 ($LSD_{0.05}$ 7.8); 10 August – 2906 g/m^2 ($LSD_{0.05}$ 12.5); 10 August – 2585 g/m^2 ($LSD_{0.05}$ 13.1).

The intensity of emergence of new seedlings of wild species decreased significantly in the following periods of joint vegetation of miscanthus and weeds. It can be explained by the gradual and increasingly dense optically projective coverage of the soil surface with leaves of plants that began vegetation earlier.

Calculations conducted on the 10th of August recorded the average values of the accumulation of weed biomass at the level of 2585 g/m^2, which was less than in the previous accounting period by 11.1%. The most widespread in the plantations of miscanthus were *Echinochloa crus-galli* L., 31.3 plants/m^2, *Setaria glauca* L., 16.7 plants/m^2, *Thlaspi arvense* L., 6.1 plants/m^2, *Sinapis arvensis* L., 6.1 plants/m^2, *Sonchus arvensis* L., 5.9 plants/m^2, *Chenopodium album* L., 4.8 plants/m^2 and other species. Based on the experimental data, we found the relationship between the duration of joint vegetation and the accumulation of the green mass of weeds and miscanthus. There is a very strong correlation between the accumulation of the green mass of weeds and the duration of their joint vegetation with miscanthus: -r = 0.9388. The obtained regression equation has a polynomial type of curve: $y = 0.116x^2 + 2.711x$. It is natural to change the accumulation of the green mass of miscanthus depending on the duration of the joint vegetation with weeds.

The regression equation, in this case, has a strong inverse correlation: -r = -0.8628. The value of the decrease in yield is influenced by the following indicators: weed species composition, the intensity of competition between crops and weeds for life factors, the amount of biomass accumulation, as well as the duration of the period of joint vegetation. 37.2 plants/m^2 of weeds was formed to 10th May in the plantations of miscanthus of the second year. The green mass of weeds at this time was quite low, 17.6 g/m^2. It should be noted that in the second year of growing, miscanthus plants grew and developed more intensively, which indirectly affected the number of weed seedlings. There was a decrease in the total number of weeds to 19.2 plants/m^2 for the next accounting period (10th July) in the miscanthus plantations of the second year but the green mass of weeds increased to 85.3 plants/m^2. Such a significant reduction in their numbers was due to the dominance of miscanthus plants and, as a consequence, their complete shading of the field surface. It was due to both a decrease in the number of weed plants and the extinction of some of their species. In particular, such species as *Viola arvensis* Murr, *Gallium aparine* L., *Sinapis arvensis* L., *Echinochloa crus-galli* L., and *Setaria glauca* L. completely disappeared. As of the 10th September, the total number of weeds in miscanthus plantations decreased to 9.8 plants/m^2, green mass of weeds to 28.2 plants/m^2, and in addition to the above species completely absent were such weed species as *Fumaria officinalis* L., *Solanum nigrum* L., and *Melandrium album* Mill (Fig.3). In the third year, it was possible to fully determine the efficiency of weed competition for nutrients and light. It was found that the total number of weeds in the fields of miscanthus in the third year of growing, at the time of the first calculations (10th May) was 26.7 plants/m^2, which was by 28.2 plants/m^2 less compared to the same period in the miscanthus plantations of the second year.

As of 10th July, the total number of weeds in miscanthus plantations of the third year decreased to 9.6 plants/m^2, which was 50% less than in the second year. At the date of the last calculation (10th September), the number of weeds in the plantations decreased to 2.4 plants/m^2, which was less than in the previous year by 75.5%.

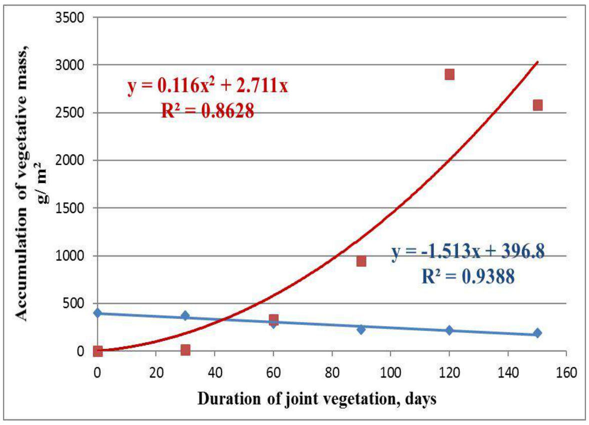

Fig. 3. The relationship between the duration of joint vegetation and the accumulation of green mass of weeds and *Miscanthus x giganteus.*

Many of the species were not present on the field, they could not accumulate vegetative mass, and those that remained did not pose any serious threat to miscanthus plants in the third year of growing. In the process of selecting possible herbicides registered in Ukraine attention was paid to the following requirements: herbicides should not show significant negative effects on crop plants. They are effective against seedlings of annual weeds of both monocotyledonous and dicotyledonous class to provide maximum duration of the protective action. After planting rhizomes we applied soil herbicide Frontier Optima 72% SC. The soil herbicide controlled well dicotyledonous annual weed species such as *Chenopodium album* L. (75%), *Amaranthus retroflexus* L. (77%), *Thlaspi arvense* L. (75%), and *Polygonum persicaria* L. (65%), and annual cereal weed species *Echinochloa crus-galli* L. (83%) and *Setaria glauca* L. (81%). The overall decrease in the number of weed seedlings over the years of research was 72.6% (Fig.4).

The overall decrease in the number of sprouts of dicotyledonous annual weed species was as following: *Gallium* - 47%, *Polygonum* *convolvulus* L. - 51%, *Polygonum persicaria* L.- 54%, *Fumaria officinalis* L. - 57%, *Solanum nigrum* L. - 60%, *Chenopodium album* L. - 64% in the areas of crop plantations where soil herbicide Dual Gold 96% EC was used after planting the rhizomes of miscanthus. Seedlings of perennial weeds of *Sonchus arvensis* L. and *Cirsium arvense* L. were resistant to the herbicide Dual Gold 96% EC. The active ingredient of the herbicide S-metolachlor compared to the previous herbicide was more toxic primarily to seedlings of annual weeds. The decrease in the number of seedlings of annual weeds compared to the indicators in the control treatment averaged 85%. Dicotyledonous weeds were more resistant to the active substance of the herbicide Dual Gold 96% EC. The overall decrease in the number of weed seedlings averaged 66.0%. Effective control of annual weed species emergence by herbicide Dual Gold 96% EC in the plantations of miscanthus lasted about 40 days. New weed seedlings appeared almost simultaneously with similar processes in the areas of plantations, where the soil herbicide Frontier Optima 72% SC was applied. Protection of miscanthus plantations from weeds in the first year of growing with the use of the herbicide Merlin 75% proved to be quite effective in all years of research. The herbicide reliably controlled the seedlings of the most common annual cereal weeds. The decrease in the number of seedlings of *Echinochloa crus-galli* L. was 87%, *Setaria glauca* L. 84% compared to control. The reduction in the number of seedlings of *Chenopodium album* L. was 77%, *Amaranthus retroflexus* L. 80%, *Solanum nigrum* L. 72%, *Thlaspi arvense* L. 68% and *Polygonum convolvulus* L. 67%. The overall reduction in the number of weeds during the years of research was 74.9%, i.e. was higher than the efficiency of all previously tested herbicide formulations.

Using of the postemergence herbicides Master Power OD and Milagro 040 SC was also effective (Fig.5).

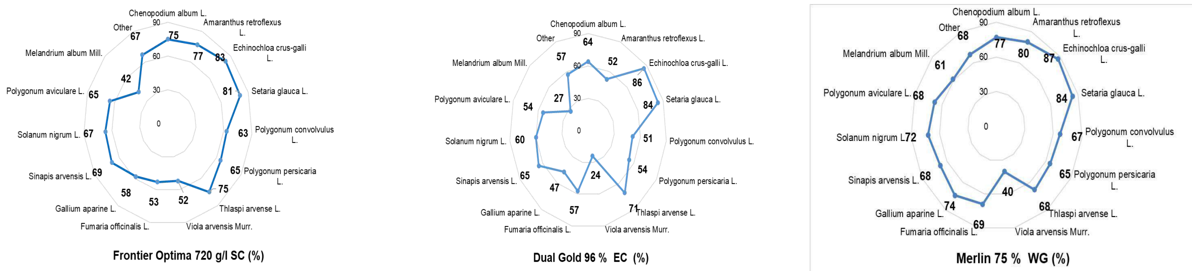

Fig. 4. The efficiency of soil herbicides.

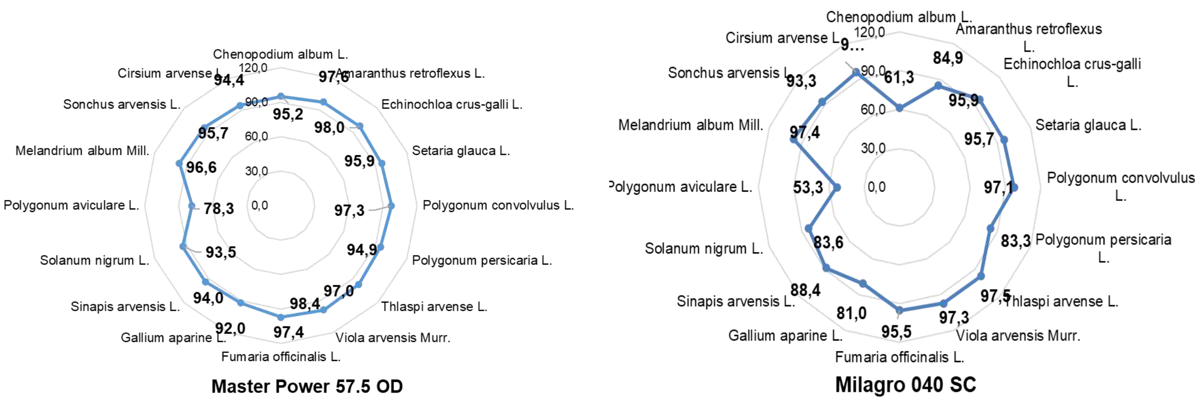

Fig. 5. The efficiency of the post-emergence herbicide Master Power OD (left) and Milagro 040 SC (right).

Application of herbicide Master Power OD (foramsulfuron, 31.5 g/l + iodine sulfuron-methyl sodium, 1 g/l + thiecarbazonmethyl, 10 g/l + cyprosulfamide, 15 g/l) at a normal application rate of 1.5 l/ha in the tillering stage provided a high level of weed control. The number of weed seedlings before spraying averaged 95.1 plants/m ^{2}. After spraying, the die-off rate of weed seedlings reached 95.0%. Most species of weeds that were present in the plantations were quite sensitive to the action of the herbicide. Seedlings of *Chenopodium album* L. died off by 95.2%, *Amaranthus retroflexus* L. by 97.6%, *Echinochloa crus-galli* L. by 98.0%, *Setaria glauca* L. by 95.9%, *Polygonum convolvulus* L. by 97.3%, *Thlaspi arvense* L. by 97.0%, *Viola arvensis* Murr. By 98.4%, *Fumaria officinalis* L. by 97.4%, *Melandrium album* (Mill.) by 96.6%, and *Sonchus arvensis* L. 95.7%.

The average number of weed seedlings in plantations after spraying with herbicide Milagro 040 SC was 93.2 plants/m ^{2}. Calculations after application recorded weeds in the amount of 11.2 plants/m^2. The efficiency of the protective action averaged 88.0%. The highest level of sensitivity to the herbicide Milagro 040 SC demonstrated sprouts of *Thlaspi arvense* L. 97.5%, *Melandrium album* (*Mill.*) 97.4%, *Viola arvensis* Murr. 97.3%, *Polygonum convolvulus* L. 97.1%, *Cirsium arvense* L. 96.2%, and other species.

The areas that were treated with the Master Power herbicide during the tillering stage provided a dry biomass yield of 1.71 t/ha, while with Milagro 040 SC the yield was 1.70 t/ha. The maximum yield of dry biomass of miscanthus (1.75 t/ha) was demonstrated in the control treatment without weeds (6 consecutive manual weedings were carried out).

Discussion

There are some difficulties in growing miscanthus in the period after planting rhizomes. In the initial stages of organogenesis, young crops develop a free ecological niche, which is a new plantation. 27 weed species belonging to different classes were noticed by polish scientists (Sekutowski & Rola, 2009). Three dominating weeds were noticed: *Elymus repens* (50-75% of soil coverage), *Artemisia vulgaris* (5-10% of soil coverage), and *Anthemis arvensis* (5-8% of soil coverage). Species diversity of weeds included 34 species, including the Monocotyledons, *Liliopsida,* 5 species, and Dicotyledons, *Magnoliopsida,* 29 species. Soil herbicides are effective in controlling annual weed species in miscanthus plantations. Among herbicides used in the experiments, the highest and most stable results of weed control were shown by Merlin 75% WG 74.9%, while the efficiency of the protective action of herbicides Dual Gold 96% EC was 66.0%, and Frontier Optimum 720 g/l SC 71.3%. The general convenience of using soil herbicides is that they are able to limit the emergence of seedlings of many annual weeds for a long time (in our experiments it was 35−40 days). However, the availability of free ecological niches last persists much longer in the plantations of miscanthus of the first year. After all, young plants due to several objective factors are not able to quickly occupy free space in the plantations. Meantime, the disadvantage of soil herbicides is that they are not able to fully control perennial weeds that form significant reserves of underground parts: roots, rhizomes, tubers, bulb and therefore easily overcome the toxic effects of herbicide formulations (Smith et al., 2015). Accordingly, perennial weeds must be destroyed, or such measures should be provided that do not use soil herbicide formulations against weeds in young miscanthus plantations. A positive feature of all soil herbicides used in our research is that they did not show a significant negative impact on crop plants, which confirmed by other researchers (Anderson et al., 2010; Song et al., 2016). Weed infestation in miscanthus plantations is extended in time and therefore there is always a problem with the right timing of herbicide spraying. Spraying plants in the cotyledons – two-leaf stage provides the highest level of biological activity of all post-emergence herbicides. However, it is not always advisable to focus only on the time of the first weed sprouts appearance. Most weed seedlings come to the surface of the soil after spraying with herbicides. Such seedlings avoid chemical exposure and successfully grow in plantations until autumn. Postponing the time of spraying to a later date, when most weeds are already sprouted, is also not rational. Several postponing herbicides (fluazifop, pyrithiobac, and sulfometuron) may be viable options to control this species if it becomes invasive were to evaluate invasive potential of giant miscanthus (Li et al., 2013). Seedlings of weeds that first come to the soil surface before the time of spraying plantings have time to form 6-8 leaves and acquire significant resistance to the action of herbicide. As a rule, such weeds survive after the action of herbicides. The highest level of weed control efficiency of the post-emergence herbicides tested in miscanthus plantations was shown by Master Power OD and Milagro 040 SC. The reduction in the number of weed seedlings as a result of their action was 95.0% and 88.0%, respectively.

Conclusion

The generalization of the dynamics of changes in the number of weeds in the miscanthus plantations of the first year of growing allows dividing the growing season into three specific periods. The first period – the beginning of active vegetation of all plants on the plantations and their intensive growth and development. Plants occupy available free ecological niches in plantations and develop a protective covering that prevents sunlight coming to the field surface. The second period – the fiercest competition in plantations for the factors of life, especially for the energy of light between crops and weeds of different species. Under such conditions, the emergence of new weed plants almost completely stops. The third period – plants of weeds and crops gradually begin to reduce the area of their leaves, complete the formation of seeds, and reduce the optical density of the projective cover of the soil surface in the plantations.

The number of weeds in the miscanthus plantations grew most intensively in the period from middle May to early June. In subsequent periods, with the achievement of full projective coverage of the field by plants, the intensity of the new weed sprouts emergence decreased. The maximum number of weeds in the plantations was 122.3 plants/m^2. The accumulation of the green mass of weeds in miscanthus plantations was very intense, especially in the period from middle June to middle August. The largest weed green mass accumulation in the years of research was recorded in the middle of August and averaged 2906 g/m^2. It was found that in the second year of growing, miscanthus plants developed more actively. It was indirectly noted in the number of weed seedlings. Thus, as of 10th June, the total number of weed seedlings remained at the previous level, 33.5 plants/m^2, whereas, in the first year of growing their number was 105.5 plants/m^2.

The presence of moisture in the upper layer of the soil in combination with the light, available seed stock in soil, and mineral nutrition ensures the emergence of new weed seedlings, mainly of wintering species. It is a clear the study of weed population dynamics requires the development of new strategies. After all, the plant community unites species completely different in their biology. Post-emergence herbicides allow protecting miscanthus plantations creatively, taking into account the characteristics of the weed species composition, growth stage, the specifics of the herbicide action. Spraying weeds seedlings with herbicides in the cotyledon – the two-leaf stage is the most effective. The most effective weed control measure for the plantations of *Miscanthus x giganteus* before the emergence of the seedlings of the crop will be to carry out spraying the soil with herbicide Merlin 750 WG at an application rate of 0.15 kg/ha. During the growing season, taking into account the specifics of weeds (tillering stage), spraying herbicides Master Power OD 1.5 l/ha or Milagro 040 SC 1.25 l/ha is recommended.

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Received: 15.06.2021
Accepted: 16.08.2021

ECOLOGIA BALKANICA
2021, Vol. 13, Issue 2 December 2021 pp. 107-114

Trends in the Change of the Ecological Condition of the River Mesta After 10 Years Period of Research

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Abstract. Long-term trends of the ecological status assessed by biological quality element macrozoobenthos, of the transboundary Mesta River (South Bulgaria) during the period 1978-2020 were analyzed. Macrozoobenthos samples were taken and physico-chemical parameters were measured twice at 7 sites situated on the main river and its tributaries during the low water period (August-September 2019 and 2020). Selected sites represented various ecological situations that are characterized by different types of human impact. Data on the current status of the river were compared with the last studied periods 2009-2010. The investigation was an extension of long-term research of the Mesta River that associated the river with the European Long Term Ecological Research Network (LTER). During the last decades, the self-purification processes and recovery of the bottom invertebrate communities and processes of stabilization of the aquatic ecosystem have been observed, after stopping the heavy organic pollution from industries in the Razlog town. This resulted in improvement of the water quality and the Mesta River ecological status. The current study demonstrated local sources of load on the aquatic ecosystem (agriculture, lack of urban wastewater treatment plants), which has a negative effect on the ecological situation in the river. Measures aimed at stimulating environmentally friendly agricultural practices and the construction of treatment plants are needed to preserve the integrity of the aquatic ecosystem and to maintain the regulated good ecological status.

Key words: Mesta River, macrozoobenthos, water quality, ecological status assessment.

Introduction

Mesta River was a subject of periodical hydrobiological (Kovachev, 1977; 1991; Psilovikos et al., 2005; Uzunov et al., 2011; Varadinova et al., 2013 a), ecological (Kovachev, Uzunov, 1986; Varadinova & Uzunov 2002; Varadinova et al., 2013c) and hydrological (Mimides et al., 2007; Diadovski et al., 2007, Hristova, 2012) studies during the last 40 years.

A special emphasis in the previous research was the selection of the Mesta River as a model object for the study of the functional trophic groups of the macrozoobenthos communities (Varadinova et al., 2008; 2013b; Kerakova et al., 2017; Varadinova & Kerakova, 2018).

Due to the long range of systematic biological, physico-chemical, and hydrological

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data storage the river is a representative lotic site in the national network for long-term research, as well as in the European ecological network LTER (Varadinova et al., 2013a; Sani et al., 2012). Mesta River was an excellent model object for investigation processes of the heavy organic matter pollution before 1990, from industries in the Razlog Valley, discharging untreated waters in its right tributary - the Iztok River and domestic wastewater from the town of Gotse Delchev. The powerful self-purification potential, as well as the lack of other significant sources of organic load, led downstream to self-purification processes and after the site MKU (Table 1), improvement of the ecological situation (beta-mesosaproby) was registered (Kovachev, 1977; Kovachev & Uzunov, 1986). After stopping the operation of the industries in 1990, a transformation of the biotic communities and recovery of the lotic ecosystems have been found by Kovachev (1993). In the new saprobic conditions a gradual shortening of the recovering area of the river (Varadinova & Uzunov, 2002) and improvement of the ecological situation was registered (Varadinova et al., 2013c). The results of long-term researches were summarized in the monographic study for the Mesta River (Varadinova et al., 2013a).

The present paper was aimed at assessing the current ecological status of the Mesta River through macrozoobenthos, as well as analyzing the trends in the situation of the aquatic ecosystem 10 years after the last benthological study.

Material and Methods

Study area

The studies were carried out in a low water period (August-September) in 2019 and 2020. A total of seven 7 sampling sites (five situated on the main river and two on its' tributaries - the Cherna Mesta River and the Bunderitsa River (Fig. 1) were investigated.

Current results were compared with published data from the last studied period 2009-2010 (Varadinova et al., 2013a; Varadinova et al., 2013c). Selected seven sites (Table 1) were common for the two studied periods.

Sampling methods

The macrozoobenthos was taken following the multi-habitat sampling approach (Cheshmedjiev et al., 2011) following the standards BDS EN ISO 5667-1:2007 and BDS EN ISO 5667- 3:2018. *In situ*, the basic physico-chemical parameters (pH, electrical conductivity (μS/cm), dissolved oxygen concentration (mg/l), and saturation) were measured with a portable Windaus Labortechnik Package.

The collected material was fixed with 70% ethanol *in situ*. In the laboratory, the macroinvertebrates were sorted by taxonomic groups. The species composition and abundance were analyzed. After primary processing and identification of macrozoobenthos, feeding groups' affiliation was assigned according to Cheshmedjiev & Varadinova (2013).

The assessment of the ecological status through Total Number of Taxa (TNT) and Biotic Index (BI) based on the regulations in the Ordinance № H-4/2012 was made.

In addition, the trophic index RETI based on functional feeding groups according (Chesmedjiev & Varadinova, 2013) was calculated.

The map of the surveyed lotic water bodies was prepared with the help of the software product Quantum GIS Version 2.18 Las Palmas.

Results and Discussion

According to the Bulgarian typology, studied sites situated on the main stream (MYA, MGK, MIZ, MKU, MHD) were defined as water bodies R5 type "Semi mountain rivers" in ecoregion 7 – Eastern Balkans. The smaller tributaries belong respectively– Bunderitsa River to R1 "Alpine rivers" and Cherna Mesta River to R3 type "Mountain rivers".

The presence, abundance, and structure of the macrozoobenthos communities in lotic ecosystems are strongly influenced by various abiotic factors such as temperature, dissolved oxygen, pH, substrate, and conductivity of the bottom water (Beuchel et al., 2006). The dynamics of the values of the aquatic parameters at the studied sites during 2019 and 2020 (Fig. 2, 3) characterized water ecosystems in good-high status (Table 2). Slight deterioration of oxygen parameters at the site BUN (Fig. 2, 3 - 2019-8.5mg/l; 2020-6.84 mg/l) in 2020 was due to the registered local extensive stream of tourists and higher water temperature at the moment of sampling (2019-10.5°C; 2020-14.7°C). Comparative analysis between the years 2009-2010 and 2019-2020 showed that the studied sites retain the standards for high and good status (Varadinova et al., 2013c).

The dynamics of the benthological indices during the 2019 and 2020 (Fig. 4, 5) demonstrated that the values of TNT, BI, and RETI were characterized with a trend of decreasing downstream. This corresponded to the deterioration of the ecological situation in the lower river sections.

Table 1. Main features and coding of the studied sampling sites. *Legend:* R. – river, u/s – upstream the site, d/s – downstream the site, R – referent conditions; SA – slightly affected; A – affected.

Code	Sampling point	Degree of human impact	N	E	Altitude (m a.s.l.)
BUN	Bunderitsa R. u/s Vihren hut	R	41.756062	23.416446	1983
CHM	Cherna Mesta R., u/s Cherna Mesta Village	R	42.058139	23.727472	1011
MYA	Mesta R. u/s Yakoruda	SA	42.03115	23.6914	935
MGK	Mesta R., General Kovatchev (u/s Iztok River)	A	41.88705	23.57172	750
MIZ	Mesta R. d/s Iztok R.	A	41.8825	23.57335	756
MKU	Mesta R., at Kupena	A	41.70987	23.70255	599
MHD	Mesta R., at Hadzhidimovo	A	41.525033	23.87175	460

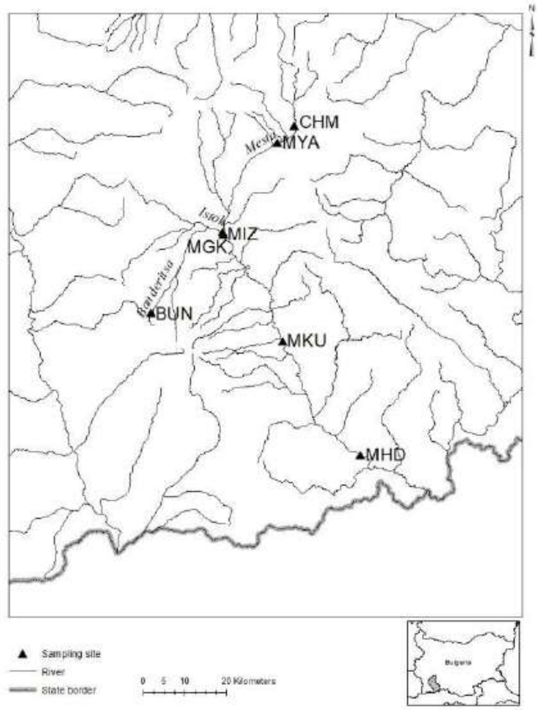

Fig. 1. Scheme of Mesta River valley with the sampling sites.

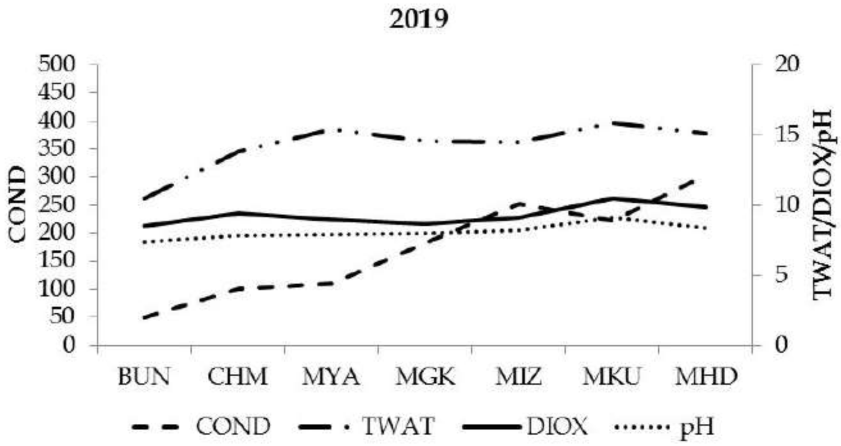

Fig. 2. Longitudinal dynamics of the values of physico-chemical parameters (dissolved oxygen (DIOX), pH, conductivity (COND) and water temperature (TWAT) in the studied sites during 2019.

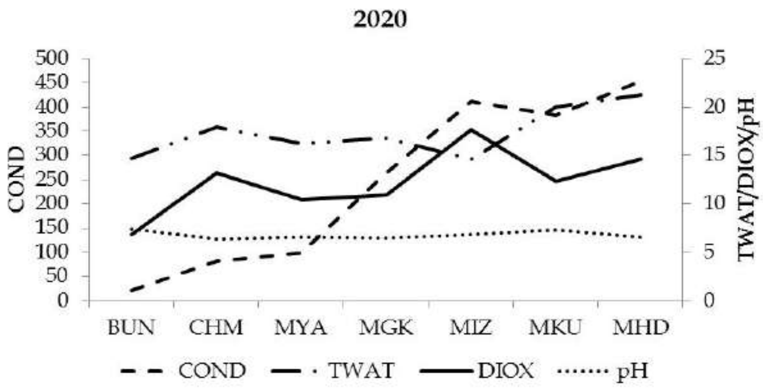

Fig. 3. Longitudinal dynamics of the values of physico-chemical parameters (dissolved oxygen (DIOX), pH, conductivity (COND) and water temperature (TWAT) in the studied sites during 2020.

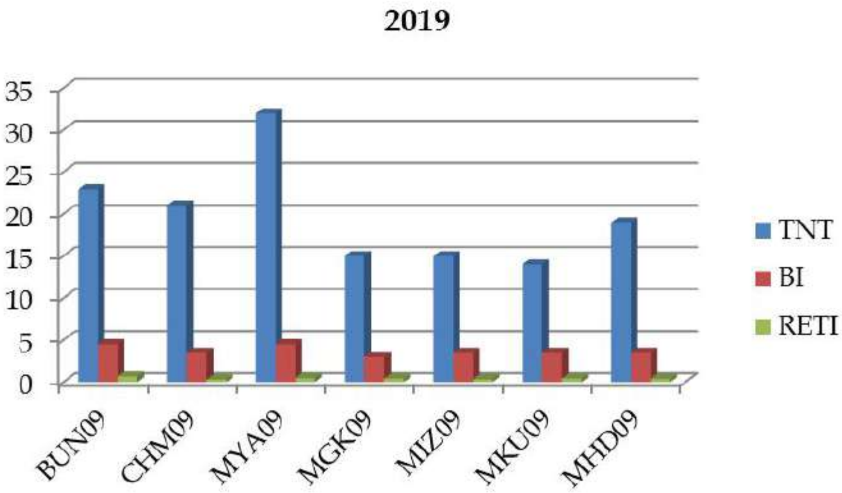

Fig. 4. Ecological state assessment, based on TNT, BI and RETI at studied sites during 2019.

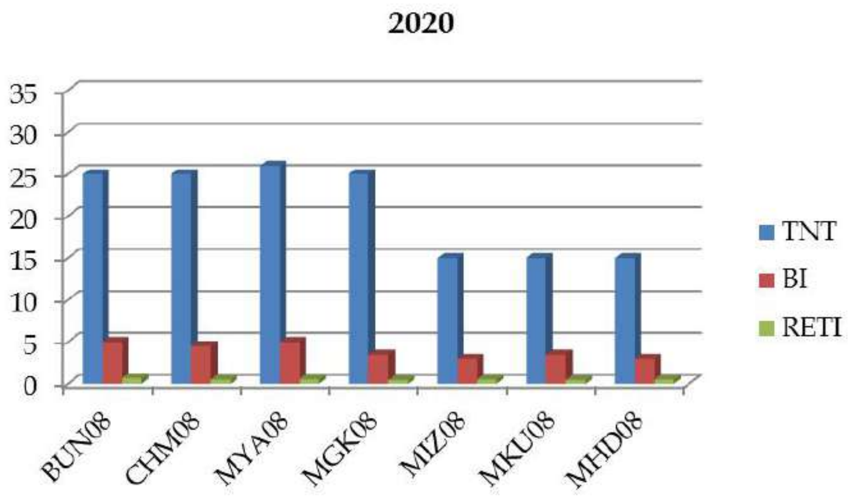

Fig. 5. Ecological state assessment, based on TNT, BI and RETI at studied sites during 2020.

The ecological status evaluation determined by BI values showed high-moderate status of the studied sites (tributaries and main river) (Table 2). Officially, BUN and CHM were defined in the active River Basin Management Plan (West Aegean RBMP, 2016-2021) as sites with referent conditions. During the current study and in the previous studied period (Varadinova et al., 2013c), biological indices (TNT and BI) calculated for BUN and CHM retained the requirements for high and good ecological status.

In the section of the Mesta River, after the town of Yakoruda (MGK), to the last studied site MHD, the aquatic ecosystem was influenced in varying degrees from negative anthropogenic influences (West Aegean RBMP, 2016-2021), which redounded in a decrease of BI values (Fig. 2) and a variation in the assessment between good and moderate status (Table 2). Fluctuations in the indices values represent the changes in the benthic communities, caused by the anthropogenic impact and different kinds of land use that affect the macroinvertebrates in the streams (Cooper et al., 2013).

A similar situation was observed at site MHD, situated nearby settlements Hadzhidimovo and Blatska. MHD showed deterioration of the ecological assessment – moderate-good (2019-2020, compared to good-high status, defined 10 years ago (2009-2010)). The sampling site was affected by the hydromorphological changes in the river bank by the local sandpit. In addition, the self-purification capacity of the lotic ecosystem was not able to cope with the organic pollution from the fertilizers and detergents used in agriculture and wastewaters inflows from the settlements. The planned wastewater treatment plant in the Hadzhidimovo municipality will significantly alleviate the load on the river, which will contribute to improving the ecological condition in the transboundary part of the Mesta River (River Basin Management plans, 2016-2021).

The values of the RETI trophic index showed a significantly lower assessment, compared to the TNT and BI regulated in the legislation (Table 2). The reason for this could be found in the higher sensitiveness of the trophic structure towards different hydromorphological changes, registered downstream as well unfavourable local impact caused by agricultural activities close to the riverbed, which reflected on the sensitive functional feeding groups' (shredders and scrapers) and caused trophic structure transformation (Fu et al., 2016; Moreyra et al., 2015).

Table 2. Evaluation of the ecological status of the studied sampling sites in 2019/2020.

	Site Code/ Year	BUN	CHM	MYA	MGK	MIZ	MKU	MHD
O_2 mg/l	2019	high	high	high	high	high	high	high
	2020	good	high	high	high	high	high	high
pH	2019	good	good	good	good	good	good	good
	2020	good	good	good	good	good	good	good
COND µS/cm	2019	high	high	high	high	high	high	high
	2020	high	high	high	high	high	high	high
EQR/ BI	2019	high	good	high	moderate	good	good	good
	2020	high	high	high	good	moderate	good	moderate
EQR/ RETI	2019	good	poor	moderate	moderate	poor	moderate	moderate
	2020	good	good	good	moderate	moderate	moderate	moderate
TNT	2019	high	high	high	good	good	good	high
	2020	high	high	high	high	good	good	good

Conclusion

The upper river sections and mountain tributaries of the Mesta River fully met the requirements for referent conditions. Aquatic invertebrate communities were characterized by high taxonomic richness and aquatic ecosystems were defined as unaffected and balanced. However, downstream the aquatic ecosystem integrity was highly vulnerable, because of the accumulated local effects of the human impact. These processes had a negative effect on the assessment, which in certain periods deteriorated and fell below the recommended in Europe and national water legislation good ecological status.

This requires measures aimed at implementing environmentally-friendly agricultural practices near the riverbed and the construction of treatment plants in the adjacent settlements.

Acknowledgements

This study is funded by the project "Upgrading distributed scientific infrastructure "Bulgarian Network for Long-Term Ecosystem Research (LTER-BG)" implemented within the National Roadmap for Research Infrastructure (2020-2027) (DO1-405/18.12.2020)

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Received: 02.07.2021
Accepted: 01.09.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 June 2021 pp. 115-124

The Effect of Some Heavy Metals (Cd, Cu, Pb, Zn) and Substrates on Chelidonium majus L. Seed Germination and Seedling Growth

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Abstract. The increasing heavy metal presence in the environment affects plant growth and physiology, seed production and crop quality. Moreover, it could indirectly influence people's health and the quality of the environment. *Chelidonium majus* L. is an object of study in order to investigate its sensitivity to the toxic effects of heavy metals because of its growth in populated areas and common occurrence. The results of this study revealed that the heavy metal influence on seed germination and seedling growth depended on the type and concentration of the heavy metals and the type of the substrate they supplemented. The studied heavy metals (Cd, Cu, Pb, Zn) did not inhibit the germination of the seeds on filter paper but retarded root and hypocotyl growth on this substrate, except Pb^{2+}, which did not. On agar substrate the metals inhibited both the germination (excl. Pb^{2+}) and seedling growth regardless of concentrations applied.

Key words: Greater celandine, heavy metal toxicity, tolerance index, filter paper, water agar.

Introduction

Heavy metals are among the main pollutants in the environment. They affect plant development, seed germination, slow down photosynthesis and decrease yield, seed production and crop quality. Apart from their direct influences, soil quality could indirectly influence people's health and the quality of the environment (Oliver, 1997; Stasinos et al., 2014). Anthropogenic activities such as mining, smelting, burning of fossil fuels and agricultural practices involving the excessive use of pesticides, fungicides, fertilizers and sewage sludge have led to an increase in heavy metal content in soil in different areas (Ahmad et al., 2012; Chen et al., 2005). The increasing environmental pollution requires an environmentally-friendly technology such as phytoremediation. It is a low-cost sustainable approach which is based on the capacity of some plant species to purify contaminated soils and water (Laghlimi et al., 2015). Screening for plant species with promising characteristics for phyto-remediation is the first but basic and important step in broadening the knowledge of phytoremediation and its future perspectives. *Chelidonium majus* L. is a perennial medicinal species which is widely distributed across Europe (Euro+Med, 2006-2020). In Bulgaria the species is distributed across rocky and damp shadowed habitats, hedgerows and as a ruderal species beside woodlands. It is widely distributed across the country, except along the Black sea coast, Struma valley, Tundzha hilly plain up to 1,500 m above sea level (Jordanov, 1970).

The species' fast growth, relatively high biomass production and common occurrence in populated areas make the species an appropriate object of study in order to investigate its sensitivity to the toxic effects of heavy metals as a potential species for phytoremediation. A few reports suggested the species' ability to accumulate organic and inorganic soil pollutants such as phenanthrene, arsenic, lead, mercury and cadmium (Badea, 2015; Zhang et al., 2011, 2014). The germination and seedling growth are frequently used to assess the tolerance of a plant species to heavy metals because these charactaristics often are very sensitive to the environmental conditions (Peralta et al., 2001; Seregin & Ivanov, 2001).

The aim of the study was to evaluate seed germination ability and seedling development on two types of substrates supplemented with different concentrations of Cd, Cu, Pb, Zn in order to define the species tolerance towards them. The heavy metal sensitivity is species-specific, therefore, the measures of germination, plant growth and tolerance index were used as indicators to assess the metal toxicity to *Ch. majus*. It is known that different substrates and conditions could impact the germination and root growth. Filter paper which is most commonly used in germination experiments is considered to interfere with metal ions (Di Salvatore et al., 2008). Therefore, it was necessary to compare it to another substrate (which is water agar) to analyze the influence of the substrate type and this type of *in vitro* conditions on seed germination in this species and to assess the substrate influence on metal toxicity. The studied Cd and Pb are among the commonly occurring heavy metals. They do not participate in physiological processes in plants and are very toxic metals which are harmful even in minor quantities. On the other hand, Cu and Zn are essential microelements necessary for plant growth and development, but they are toxic in excessive concentrations.

The studies on *Ch. majus* were mainly connected with its medicinal properties and effects in use based on the alkaloids which the species biosynthesize (Maji & Banerji, 2015). Up to now studies on heavy metal toxicity to *Ch. majus* germination and seedling growth have not been reported.

Material and Methods

Seed origin. Seeds were gathered from native plants of *Ch. majus* grown in the village of Mramor, near Sofia.

Seed sterilization. Seeds were pre-soaked in distilled water for 24 hours. They were first surface sterilized with 70% ethanol for 2 minutes, then were soaked in 0.1% $HgCl_2$ for 2 minutes and rinsed three times in sterile distilled water. As a last step in surface sterilization seeds were sterilized in commercial bleach (chlorine < 2.5%) half diluted with sterile water for 10 minutes and afterwards the seeds were rinsed again three times with sterile distilled water.

Seed germination and seedling growth conditions. Compounds which were used to create metallic stress were $Pb(NO_3)_2$, $ZnSO_4 \cdot 7H_2O$, $CdCl_2 \cdot 2½H_2O$ and $CuSO_4 \cdot 5H_2O$. They were applied in three concentrations of Pb^{2+}, Zn^{2+} and Cu^{2+} ions: 100; 150; 250 mg/l, and Cd^{2+} ions were added in concentrations of 1; 5 and 10 mg/l. The chosen concentrations were determined taking into account the Bulgarian standards for permissible content of harmful elements in soils (Ordinance № 3, 2008). The sterilized seeds were put onto two types of substrate – filter paper and water agar. Sterilized glass petri dishes (90 mm in diameter), were covered with two layers of sterilized filter paper (Whatman № 1), moistened with 4 ml of water solutions of heavy metals in the different concentrations or only with distilled water for the control. For the agar medium, water solutions of the heavy metals were solidified with 7 g/l agar (Duchefa, NL) and autoclaved at 121ºC for 20 minutes. In the water agar experiment $CuSO_4 \cdot 5H_2O$ was not added because of the severe decrease in pH and the incapability of agar to harden. The petri dishes used were 90 mm in diameter. The control variants contained distilled water agar only.

All petri dishes were wrapped with Parafilm. They were kept in dark at 8±2°C for 7 days. After that, they were placed at 23±2°C under dark conditions for an additional 2 weeks and then were cultivated at 23±2°C with photoperiod of 16h light/8h dark.

Each treatment had 3 or 2 replicates (for filter paper and water agar dishes, respectively) with 20 seeds in each. The seed coat protrusion was considered a sign of germination.

Statistical analyses. The statistical analyses were made using SigmaPlot v. 14.0 and statistical significance was evaluated with Student's t-test ($p=0.05$).

Studied heavy metal toxicity parameters. Several parameters were measured in order to determine the species' ability to germinate and develop in heavy metal presence: germination percentage, relative germination rate, mean root length, mean hypocotyl length, root/shoot ratio, tolerance index, phytotoxicity percentage (Rasafi et al., 2016).

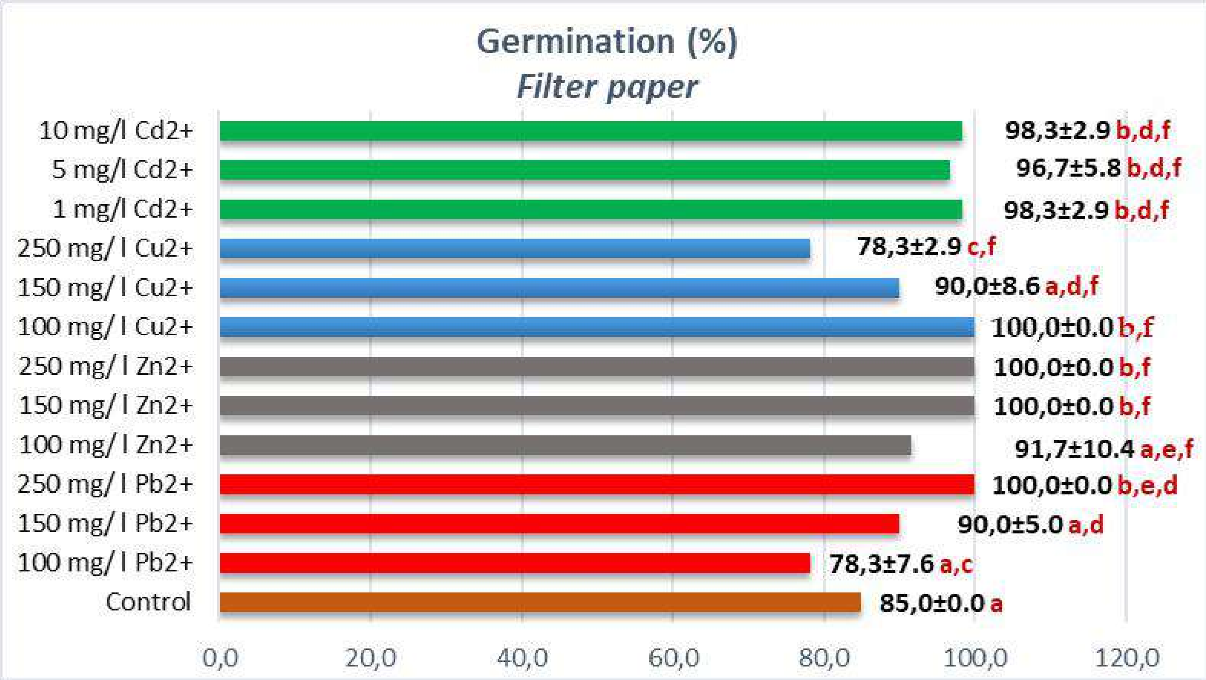
Results

Germination percentage. The germination percentage of the seeds sown on filter paper was high (over 75%) in all concentrations applied and for all heavy metals used (Fig. 1-A). On agar substrate the germination was low – less than 50%. In contrast, only for Pb^{2+} in all its applied concentrations the germination was 75% and more, which was even higher than the control (Fig. 1-B).

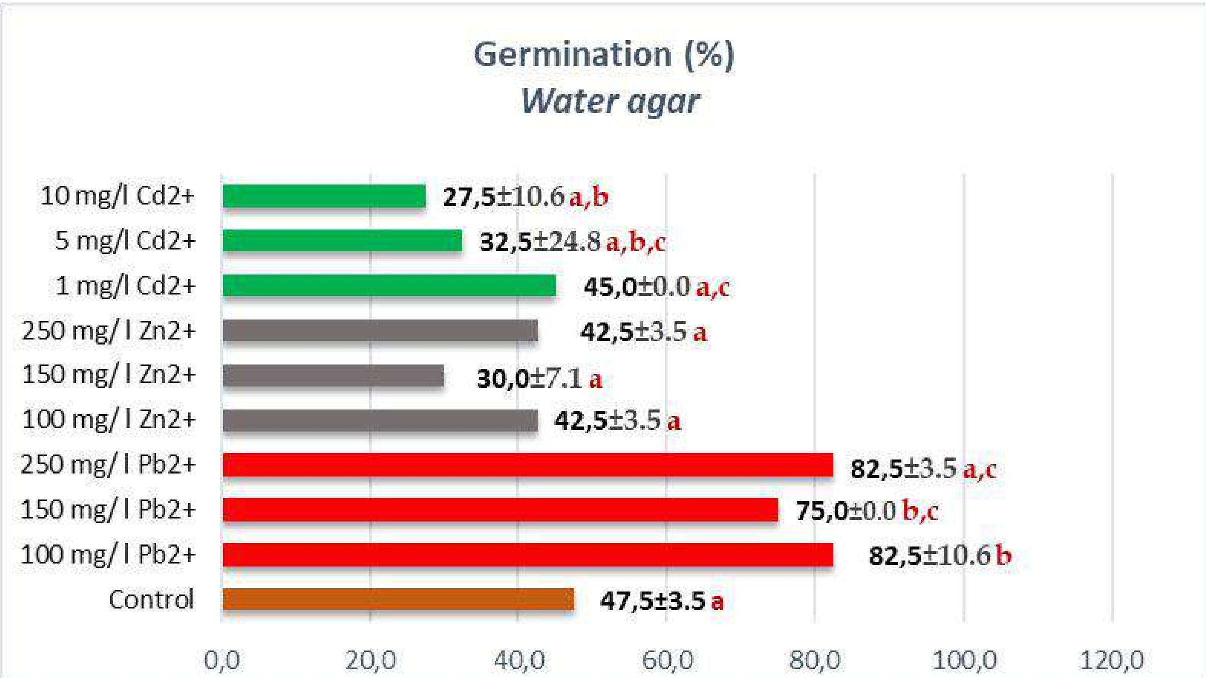
Effect of the applied heavy metals on relative germination rate. There was a strong inhibiting influence of the heavy metals in water agar. Germination did not decrease with the increase in heavy metal concentrations on filter paper. However, there was a rise in the germination percentages for all heavy metals used and compared with the control (Table. 1). On filter paper only for Cu^{2+} was observed progressive inhibition of the germination with the increase in Cu^{2+} concentration. In water agar this tendency was observed for the Cd^{2+} variants.

Mean root length under heavy metal stress. The roots of seedlings which were growing on filter paper moistened with Pb^{2+} solution were significantly longer than all variants on this type of substrate (Fig. 2-A). There was a little decrease in length at the highest Pb^{2+} concentration even though it remained higher than that of the control. Seedlings grown on filter paper dampened with the lowest Cd^{2+} concentration had roots with comparatively high length, but it sharply decreased at the higher concentrations used. In terms of the variant with Zn^{2+}, there was a significant reduction in root length, which was proportional to the increase in Zn concentration. Root growth was completely halted in the variant where Cu^{2+} was used. The inhibiting influence of heavy metals was considerably more expressed in the substrate of water agar (Fig. 2-B). When it was supplemented with Pb^{2+} and Zn^{2+}, the roots were black and underdeveloped, except for Cd^{2+}, where the inhibiting effect was weaker.

Mean hypocotyl length under heavy metal stress. For all heavy metals, the higher applied concentrations were, the shorter the hypocotyl length was. It was higher at lower concentrations of lead and cadmium than it was in the control seedlings. Zn^{2+} and Cu^{2+} inhibited hypocotyl growth, where the most significant inhibition occurred for Cu^{2+} treated seedlings and was observed even at the lowest concentrations of the element applied (Fig. 3-A). The growth preventing effect of heavy metals on hypocotyls was again significantly stronger in water agar. The highest hypocotyl length of seedlings developed in water agar supplemented with heavy metals was observed in 1 mg/l Cd^{2+} treatment. In concentrations of 5 and 10 mg/l Cd^{2+} the inhibiting effect was more prominent, close to that observed in the variant treated with lead and Zn^{2+}. Hypocotyls did not develop at the highest Zn^{2+} concentration (Fig. 3-B).

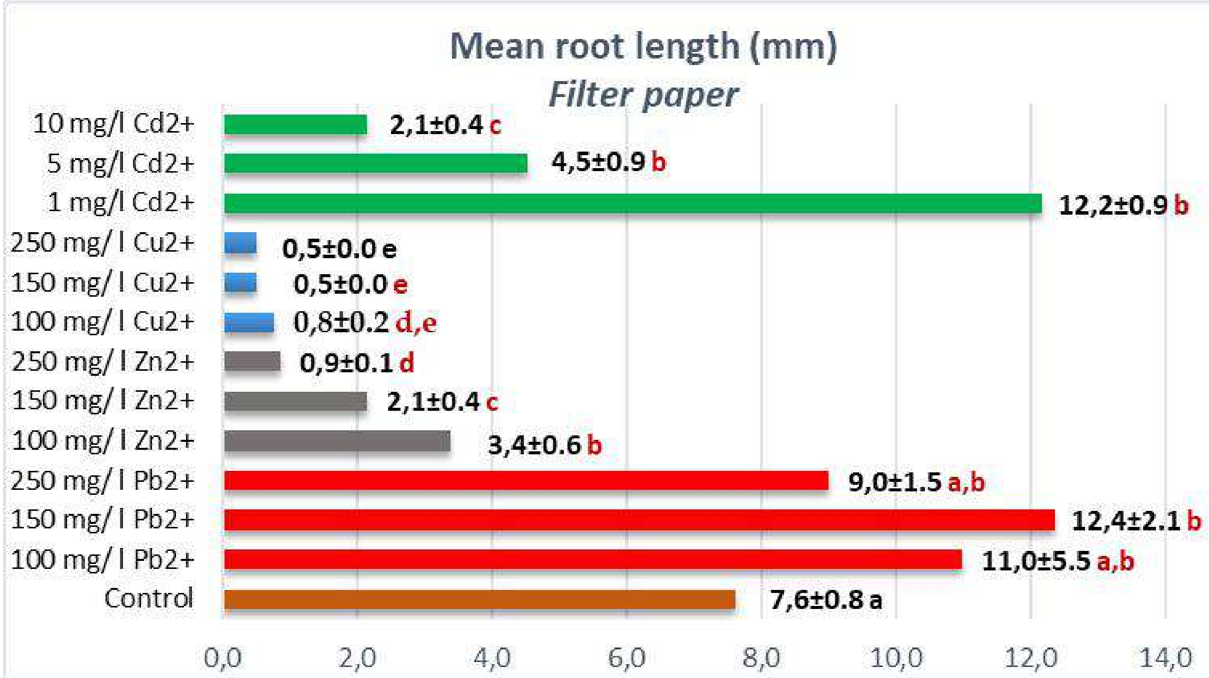


A)

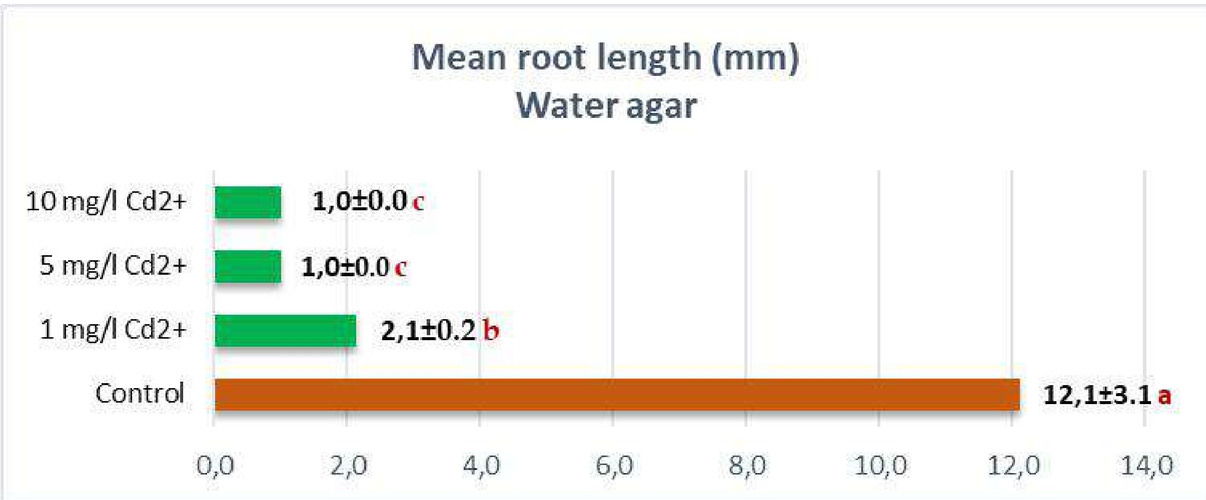


B)

Fig. 1. Germination percentage (%) ± Standart deviation on filter paper **(A)** and water agar **(B)**. Values with different letters are significantly different.



A)



B)

Fig. 2. Mean root length (mm) of seedlings grown on filter paper (A) and water agar (B) supplemented with different concentrations of heavy metals. Values with different letters are significantly different.

Root/shoot ratio. This indicator showed that the Pb^{2+} effect on seedling development did not vary widely at the different concentrations on filter paper, but it was absolutely detrimental for the root development on agar substrate (Table. 1). Of all applied metals on filter paper, Cu^{2+} most strongly inhibited seedling growth. The ratio tended to decrease for Zn^{2+} and Cd^{2+} with the increase in their concentrations on filter paper. The toxic influence was huge on water agar regardless of the concentration applied.

Tolerance index. The tolerance index decreased with the concentration increase of all heavy metals although their influence on germination was small. On filter paper the lowest tolerance of the plants was for Cu^{2+} and for Cd^{2+} there was a sharp drop at the highest concentration of 10 mg/l Cd^{2+} (Table. 1). Zn^{2+} and Pb^{2+} ions completely halted seedling development on the water agar substrate and there was low tolerance for Cd^{2+} on the same substrate.

Phytotoxicity. Cu^{2+} had the strongest phytotoxic effect on *Ch. majus*, followed by Zn^{2+}, Cd^{2+} and Pb^{2+} in filter paper. Phytotoxicity increased when the heavy metals were added to the agar medium. Cd^{2+} was less phytotoxic in filter paper, but as it can be seen, Cd^{2+} was less phytotoxic than Pb^{2+} and Zn^{2+} in water agar instead of in filter paper (Table. 1).

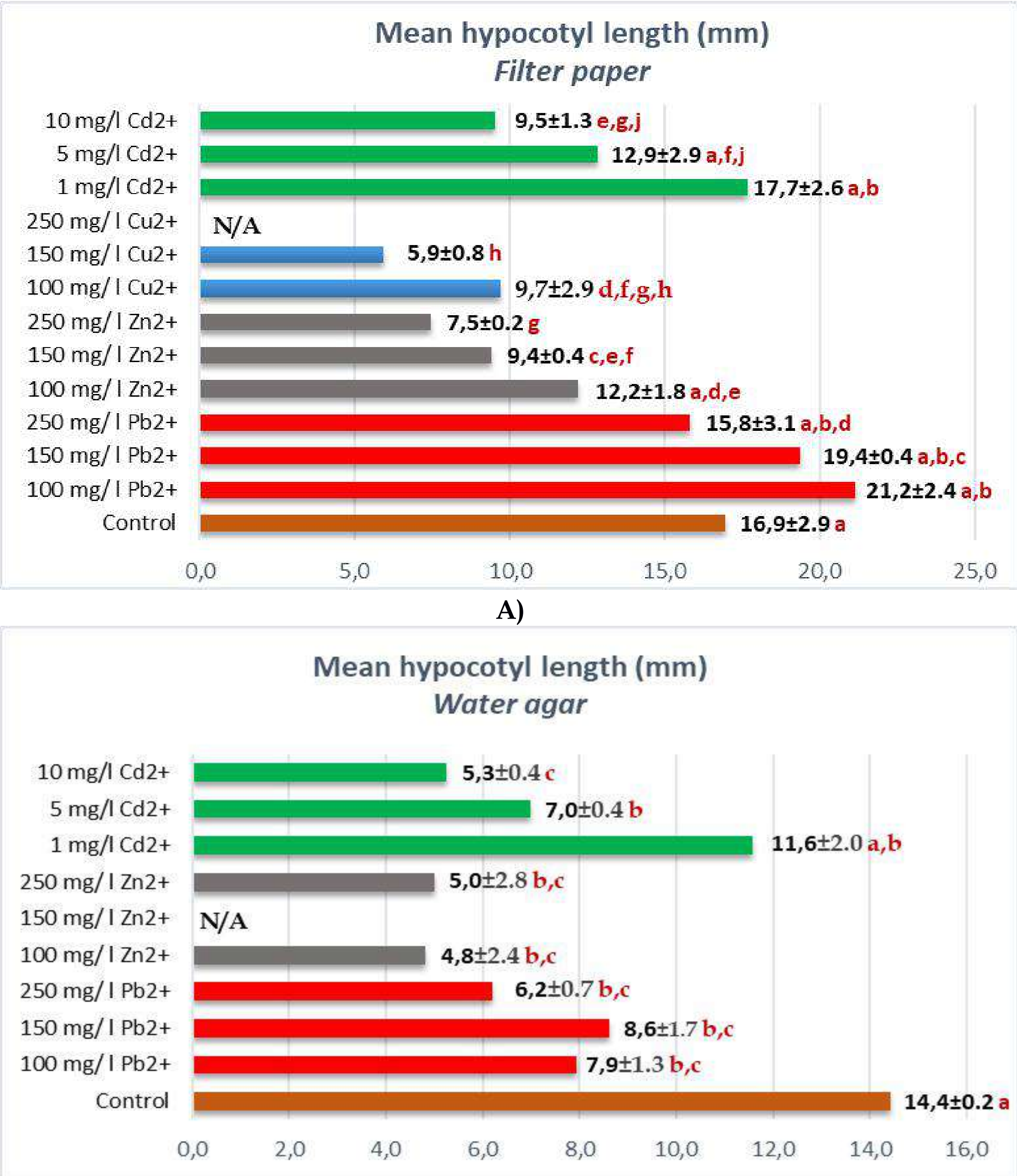


Fig. 3. Mean hypocotyl length (mm) of seedlings grown on filter paper (A) and water agar (B) supplemented with heavy metals. Values with different letters are significantly different.

Table 1. Values of some of the studied parameters on filter paper and water agar supplemented with heavy metals. *Legend:* Values with different letters are significantly different.

Concentration (mg/l)	Relative germination rate		Tolerance index		Phytotoxicity percentage (%)		Root/shoot ratio	
	Filter paper	Water agar	Filter paper	Water agar	Filter paper	Water agar	Filter paper	Water agar
Control								
-	7.6±0.8	12.1±3.1	16.9±2.9	14.4	0.0	0.0	0.5	0.8
Pb^{2+}								
100	10.9±5.5	N/A	21.2±2.4	7.9	-0.4	N/A	0.5	N/A
150	12.4±2.3	N/A	19.4±0.4	8.6	-0.6	N/A	0.6	N/A
250	9.0±1.5	N/A	15.8±3.1	6.2	-0.2	N/A	0.5	N/A
Zn^{2+}								
100	3.4±0.6	N/A	12.2±1.8	4.8	0.6	N/A	0.3	N/A
150	2.2±0.4	N/A	9.4±0.4	5.0	0.7	N/A	0.2	N/A
250	0.9±0.1	N/A	7.5±0.2	N/A	0.9	N/A	0.1	N/A
Cu^{2+}								
100	0.8±0.2	N/A	9.7±2.9	N/A	0.9	N/A	0.1	N/A
150	0.5±0.0	N/A	5.9±0.8	N/A	0.9	N/A	0.1	N/A
250	0.5±0.0	N/A	N/A	N/A	0.9	N/A	N/A	N/A
Cd^{2+}								
1	12.2±0.9	2.1±0.2	17.7±2.6	11.6	-0.6	0.8	0.7	0.2
5	4.5±0.9	1.0±0.0	12.9±2.9	6.9	0.4	0.9	0.4	0.1
10	2.2±0.4	1.0±0.0	9.5±1.3	5.3	0.7	0.9	0.2	0.2

Discussion

The results obtained showed that the heavy metal influence on seed germination and seedling growth depended on the type and concentration of the heavy metals and the type of the substrate they supplemented.

Overall, heavy metals did not inhibit the germination of the seeds which were germinated on filter paper, but retarded root and hypocotyl growth on this substrate. Such a tendency has been observed in *Arabidopsis thaliana*, *Glycine max*, *Triticum aestivum* (Araujo & Monteiro, 2005; Li et al., 2005; Yang et al., 2010). It was considered to be related to the seed coat. Many chemicals could not be absorbed by the seeds due to the seed coat. Moreover, their reserves make the germination possible even in unfavourable conditions (Kapustka, 1997). Besides this, the seed coat protrusion does not necessarily mean growth through cell division, but it could be due to cell elongation (Chon et al., 2004; Haber & Luippold, 1960).

It is known that root growth is more sensitive to heavy metal influence than germination (Araujo & Monteiro, 2005). The inhibiting effect of heavy metals on seedling root growth after germination is observed in a wide variety of studied species. The diminished root and hypocotyl growth, which affected the values of the other calculated parameters, revealed the extent of the toxic effect of a given element on the species. The retarted growth of hypocotyls and roots could be due to the impact of heavy metals on cell division and metabolite activity (Hargemeyer & Breckle, 1996; Naseer et al., 2001). The strong reduction in root growth might be because of their greater susceptibility to heavy metals being the first contact point which is exposed to their toxic impact (Shah et al., 2010; Yang et al., 2010).

Roots absorb and accumulate metals more often and that is why metals impact roots more strongly than the aboveground parts of the plant (Öncel et al., 2000). The different presence of heavy metals in plant organs resulted in the different degree of root and hypocotyl growth inhibition, because the heavy metals remain in the cell walls and close to the place of intake. Metals are found to be more concentrated in roots than in stems (Greger, 2004).

In this study it was shown that the substrate type is a factor which influences the toxicity of the heavy metals and that is why it is important to be taken into account. All studied parameters of seeds germinated and developed on agar substrate were worse than those on filter paper, which revealed the higher toxic impact of the heavy metals on agar substrate. The lower heavy metal toxicity on filter paper substrate might be due to the interaction of metal ions with the filter paper, which led to the decrease in the heavy metal concentration, lower bioavailability and subsequently weaker impact on the seeds and seedling growth on filter paper than on water agar (Di Salvatore et al., 2008). Perhaps Pb^{2+} binded to the filter paper more easily than the other metals, which could be the reason for better seedling development.

The *in vitro* conditions themselves on agar substrate might influence germination negatively because the percentage of the control, that was just water agar, was low, too. We could explain this observation with the conditions (such as temperature and humidity in cultivation vessels), which don't seem to be favourable for *Ch majus*' seed germination.

These adverse conditions in combination with the negative impact of the heavy metals resulted in poor seedling development. This was observed even in the agar supplemented with Pb^{2+}, which initially had stimulating impact on germination, but subsequently the Pb^{2+} presence in the substrate inhibited seedling growth.

Research of seed germination and seedling growth of *Ch. majus* is the first step of a study on its heavy metal tolerance. In this respect, it is necessary to take into account the higher degree of severity of the metal impact in laboratory conditions because of their direct influence on the seeds and seedling development. Generally, heavy metals bind in different degrees in soil conditions which decreases their bioavailability. As a continuation of this study seed germination and seedling growth are going to be explored in soils supplemented with heavy metals. Moreover, the impact of metals on *in vitro* cultivated plants is going to be investigated.

Conclusion

The results showed that the heavy metal type, concentration and substrate interact to form the conditions which the species seeds have to overcome in order to germinate and develop. Taking into account all studied parameters, the heavy metals applied in filter paper could be put in the following toxicity scale according to degree of toxicity and impact on seedling development: Cu>Zn>Cd>Pb. The heavy metals supplemented in agar could be sequenced in the scale as follows: Zn>Pb>Cd.

Acknowledgment. This research was supported by the Bulgarian National Science Fund, Bulgarian Ministry of Education and Science (Project КП-06-М26/4 from 01.12.2018).

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Received: 18.07.2021
Accepted: 27.08.2021

ECOLOGIA BALKANICA
2021, Vol. 13, Issue 2 December 2021 pp. 125-134

Comparative Genome Analysis of Phormidesmis priestleyi ULC007 and Some Representatives of Genus Phormidium (Cyanobacteria)

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Abstract. Cyanobacteria live in a wide range of habitats encompassing freshwater, marine, and terrestrial ecosystems. They are one of the most morphologically diverse groups. Techniques based on polyphasic taxonomy were introduced to cyanobacterial systematics in an attempt to overcome the shortcomings brought by the traditional emphasis on morphological features. Recent advances in genomics are greatly accelerating our understanding of cyanobacterial taxonomy. Genomes available in NCBI's GenBank were selected to comprehensively clarify the genetic similarities and differences of representatives of genus *Phormidium* and *Phormidesmis priestleyi* ULC007 (former *Phormidium priestleyi*). We performed a comparative genomic analysis, which includes analysis of genome characteristics and annotation of the subsystem, classification of functionally annotated common genes and automatic annotation of secondary metabolic gene clusters. Results showed that about 83% of the genes in the studied cyanobacterial genomes were identified as genes with unknown functions, and those with annotated functions were mainly involved in: (I) cofactors, vitamins, prosthetic groups, and pigments; (II) cell wall and capsule; (III) RNA metabolism; (IV) protein metabolism; (V) DNA metabolism, (VI) fatty acids, lipids, and isoprenoids; (VII) respiration; (VIII) stress response; (IX) amino acids and derivatives; and (X) carbohydrates We found that *Phormidesmis priestleyi* ULC007 possess seven genes involved in the chemotaxis. The *in silico* identification, annotation and analysis of the secondary metabolite biosynthesis gene clusters of the most promising targets within the studied cyanobacterial genomes showed presence of specific secondary metabolite genes, which need further detailed analyses.

Key words: Cyanobacteria, genome, genomics, *Phormidium*, *Phormidesmis*, secondary metabolites.

Introduction

The phylum Cyanobacteria includes oxygenic phototrophic bacteria, that are considered as the ancestors of the plant chloroplasts. They have thrived on our planet for at least 2.33–2.4 billion years ago (Bekker et al., 2004; Schirrmeister et al., 2015). Cyanobacteria are able to inhabit most of Earth's environments (oceans, lakes, soils, deserts and hot springs) because they evolved mechanisms to detect and rapidly adapt to environmental changes by using a complex of signaling molecules (including secondary metabolites) to regulate the

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physiology or metabolism (Agostoni & Montgomery, 2014).

Morphologically, cyanobacteria are one of the most diverse groups of organisms distributed worldwide. Techniques based on polyphasic taxonomy were introduced to cyanobacterial systematics in an attempt to overcome the shortcomings caused by the traditional emphasis on morphological features (Hoffmann et al., 2005; Komárek et al., 2014; Vandamme et al., 1996). The sequence analysis of the 16S rRNA gene is commonly used for the revision of cyanobacterial genera, but it is unsuitable for a clear and undoubtful assessment of the subgeneric categories (Johansen & Casamatta, 2005).

Genomics is currently the most promising framework for correcting mistakes caused by the traditional taxonomics and clearing out the complicated evolutionary relationships of several polyphyletic taxa persisting in the cyanobacterial classification. Sequencing of genomes from cyanobacteria allows not only increasing the knowledge of the molecular genetics of this phylum, but also to understand the evolution and diversity related to the morphology, photosynthesis, secondary metabolism, and endosymbiosis (Dagan et al., 2013; Shih et al., 2012). Advances in the genome sequence technology have caused a deluge of genome sequences for cyanobacteria. Their genomes are different in size (from 1.44 to 12.07 Mb), ploidy (from two to more than 20 copies of the circular genome per cell) or GC content (30–60%), probably due to processes of gaining and losses of genes transferred by plasmids, insertion sequences (Cassier-Chauvat et al., 1997) and/or cyanophages (Hess, 2011; Shih et al., 2012). The number of cyanobacterial genomes is approximately 0.6% of all prokaryotic genomes available at this moment (Banack et al., 2012; Schirrmeister et al., 2015). More than 20 years after the publication of the first cyanobacterial genome (*Synechocystis sp.* PCC 6803), just a few over 400 cyanobacterial genomes are available in public databases, a number that pales in comparison to more than 30,000 complete genomes available for strains classified in 50 bacterial and 11 archaeal phyla (Land et al., 2015).

In 2009, another new genus (*Phormidesmis, Leptolyngbyaceae*) was separated from the genus *Phormidium* (*Oscillatoriaceae*) based on morphological, ultrastructural, and molecular analyses. *Leptolyngbyaceae* and *Oscillatoriaceae* belong to the most difficult cyanobacterial taxa for proper identification (Komárek et al., 2014). Recently, Raabová et al. have conducted revision of the genus *Phormidesmis* analyzing 26 *Phormidesmis* strains by using morphological, ultrastructural and phylogenetical features. Based on the results, they classified two new species (*Phormidesmis arctica* and *Phormidesmis communis*) and transferred *Leptolyngbya nigrescens* to *Phormidesmis nigrescens* (Raabová et al., 2019). Despite the revision, the ambiguities within genus *Phormidesmis* remained unresolved. These organisms are limited ecologically, and some species were found only in a specific ecological niche (Raabová et al., 2019). *Phormidesmis priestleyi* ULC007 is a freshwater cyanobacterium isolated from Antarctica. The strain *Phormidesmis priestleyi* ULC007 was originally isolated from Lake Bruehwiler, a shallow freshwater lake of 1 ha (Hodgson et al., 2004). *Phormidesmis molle*, which is a type species of the genus *Phormidesmis*, contains strains from tropical and subtropical swamps in Central America and was described from similar localities across tropical and subtropical regions (Turicchia et al., 2009).

Different cyanobacterial strains produce a variety of secondary metabolites, some of which are toxic (Dittmann et al., 2015; Pearson et al., 2016). On the other hand, many marine, terrestrial and freshwater cyanobacteria produce a wealth of natural products with interesting biological activities (Jones et al., 2009; Jones et al., 2010; Welker & Von Döhren, 2006). The genetic origins of specialized metabolites and the mechanisms, which drive their evolution are poorly understood. About 5-6% of the cyanobacterial genomes are used for production of secondary metabolites (Calteau et al., 2014). The known cyanobacterial natural products includes more than 1100 secondary metabolites with complex and interesting chemical structures. They can be assigned as peptides, polyketides, isoprenoids, alkaloids, lipids, and terpenes (Jones et al., 2009; Welker & Von Döhren, 2006).

The aim of this study was to identify similarities and differences between *Phormidesmis priestleyi* ULC007 (former *Phormidium priestleyi*) and other representatives of genus *Phormidium* by using a comparative genomics analysis, which includes genome feature analysis and subsystem annotation, classification of functionally annotated common genes and automatic annotation of secondary metabolite gene clusters.

Material and Methods

Available genomes of representatives of the genus *Phormidium* (*Phormidium ambiguum* IAM M-71, *Phormidium pseudopriestleyi* FRX01, *Phormidium tenue* FACHB-1052, and *Phormidium willei* BDU 130791) along with *Phormidesmis priestleyi* ULC007 were retrieved from the GenBank and the Joint Genome Institute's Integrated Microbial Genomes database (JGI-IMG). The RAST-Server (Rapid Annotation using Subsystems Technology) and the SEED viewer were used for the automatic annotation of genes and functional classification in subsystems (Aziz et al., 2008; Overbeek et al., 2014). For genome-wide identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in cyanobacterial genomes was used the antiSMASH Server (Blin et al., 2019).

Results and Discussion

Nowadays, many cyanobacterial genomes have been sequenced and annotated. This information can be used to identify biological pathways presented in all cyanobacteria as the proteins involved in such processes are encoded by a so called core-genome. The complete genome sequences for four *Phormidium* species (*Ph. ambiguum* IAM M-71, *Ph. pseudopriestleyi* FRX01, *Ph. tenue* FACHB-1052, and *Ph. willei* BDU 130791) were compared with the complete genome sequence of a representative of the genus *Phormidesmis* (*Phormidesmis priestleyi* ULC007). General genome features (genomic statistics and subsystem statistics-SEED) for all complete genomes used in this study are shown in Table 1.

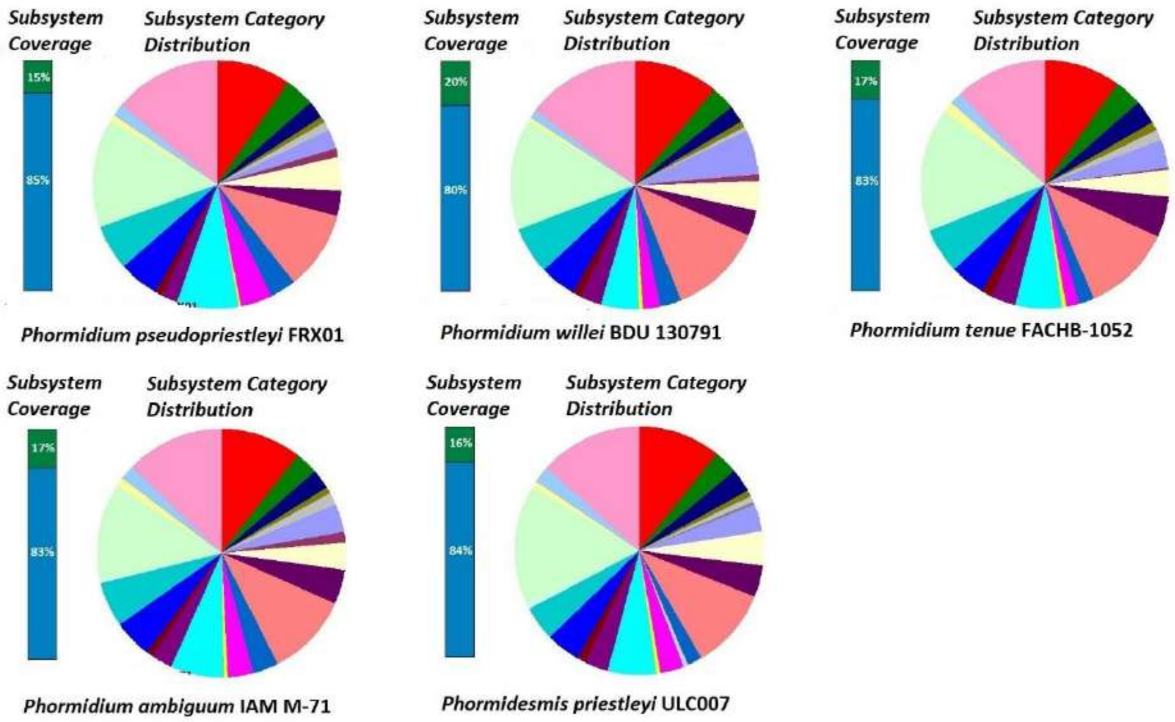
Genomes ranged in size from 4.60 Mb (*Phormidium willei* BDU 130791) to 7.41 Mb (*Phormidium ambiguum* IAM M-71). The genome of *Phormidesmis priestleyi* ULC007 (5.71 Mb with an overall GC content of 48.6 %) is similar in size to genomes of the representatives of *Phormidium*. *Phormidium tenue* FACHB-1052, and *Phormidium willei* BDU 130791 are characterized by a higher GC content (56.0% and 53.4%, respectively). *Phormidium ambiguum* IAM M-71 has the lowest GC content (39.5%). Results showed that there are large differences in the number of contigs and the N50 value between the different cyanobacterial strains. Contigs ranged from 39 (*Phormidium tenue* FACHB-1052) to 678 (*Phormidium pseudopriestleyi* FRX01). *Phormidesmis priestleyi* ULC007 has 167 contigs. *Phormidium pseudopriestleyi* FRX01, and *Phormidium willei* BDU 130791 are characterized by a lower N50 value (10908 and 34265, respectively) (Table 1). The number of subsystems (243-290), coding sequences in subsystems (15-20%) and coding sequences not in subsystems (80-85%) are similar between all genomes (Table 1). Selection pressures may cause changes in genetic factors such as genome size, G-C percentage, gene number, and evolutionary rates. While cyanobacteria may develop individual strategies for interacting with the environment, several of their systems are globally conserved (Simm et al., 2015).

Table 1. Comparison of the genomic features and subsystem annotation of the studied strains.

Statistics	ULC007	BDU 130791	FACHB-1052	FRX01	IAM M-71
Genomic statistics					
N of contigs	167	171	36	678	90
Total size (Mb)	5714281	4600567	5821333	5965908	7410502
Proteins	5196	3929	5051	4719	6262
Genes	5455	4077	5200	5101	6474
Pseudogenes	211	103	100	317	141
GC content (%)	48.6	53.4	56.0	47.4	39.5
N50	106941	34265	248526	10908	196225
Number of RNAs	42	42	43	59	65
Subsystem statistics – SEED					
N of subsystems	278	243	278	264	290
N of coding sequences	6075	4424	5687	6794	6842
Coding sequences in subsystems/ %	950 16%	853 20%	955 17%	968 15%	1158 17%
Coding sequences not in subsystems/ %	5125 84%	3571 80%	4732 83%	5826 85%	5684 83%

The gene composition and putatively identified functional differences between the cyanobacterial species were assessed by using Rapid Annotation and Subsystems Technology (RAST-Server) (Fig. 1.). The RAST server automatically produces two classes of asserted gene functions: subsystem-based assertions are based on recognition of functional variants of subsystems, while nonsubsystem-based assertions are filled in using more common approaches based on integration of evidence from a number of tools. The results of the RAST Server and SEED viewer subsystem annotation showed that analyzed cyanobacterial genomes are considerably similar (Fig. 1). An average of 83% of genes in the studied genomes were identified as genes with unknown functions, and those with annotated functions were mainly involved in: (I) cofactors, vitamins, prosthetic groups, and pigments; (II) cell wall and capsule; (III) RNA metabolism; (IV) protein metabolism; (V) DNA metabolism, (VI) fatty acids, lipids, and isoprenoids; (VII) respiration; (VIII) stress response; (IX) amino acids and derivatives; and (X) carbohydrates. The most prominent difference was observed for motility and the chemotaxis. Not all of the genes involved in the motility and chemotaxis were presented in all studied cyanobacterial strains. Most of them were detected only in *Phormidesmis priestleyi* ULC007 (Fig. 1). In the cyanobacterial chemotaxis, these genes encode proteins defined as: positive regulator of CheA protein activity (CheW), chemotaxis protein CheV (EC 2.7.3.-), chemotaxis response regulator protein-glutamate methylesterase CheB (EC 3.1.1.61), multdomain signal transduction protein including CheB-like methylesterase, maltose/maltodextrin ABC transporter, substrate binding periplasmic protein MalE, methyl-accepting chemotaxis protein I (serine chemoreceptor protein), chemotaxis protein methyltransferase CheR (EC 2.1.1.80).

Cyanobacteria are subject of scientific investigation in regard to their production of toxic and non-toxic secondary metabolites. The presence of secondary metabolite biosynthesis gene clusters (BGCs) was tested using the antiSMASH platform (Blin et al., 2019). The genome of *Phormidesmis priestleyi* ULC007 contains 8 regions encoding BGCs (terpene; type II polyketide synthase (T2PKS); NRPS-like fragment; non-ribosomal peptide synthetase (NRPS) and type I polyketide synthase (T1PKS)).



Subsystem Feature Counts	ULC007	BDU 130791	FACHB -1052	FRX01	IAM M-71
Cofactors, Vitamins, Prosthetic Groups, Pigments	139	125	132	123	164
Cell Wall and Capsule	38	33	44	52	46
Virulence, Disease and Defense	37	25	40	25	39
Potassium metabolism	12	9	14	13	12
Photosynthesis	0	0	0	0	0
Miscellaneous	9	6	18	12	26
Phages, Prophages, Transposable elements, Plasmids	4	2	1	3	1
Membrane Transport	44	64	47	32	56
Iron acquisition and metabolism	2	7	4	15	21
RNA Metabolism	57	43	46	56	55
Nucleosides and Nucleotides	51	37	68	40	70
Protein Metabolism	132	136	152	132	167
Cell Division and Cell Cycle	26	31	22	42	48
Motility and Chemotaxis	7	0	0	0	0
Regulation and Cell signaling	38	24	24	50	51
Secondary Metabolism	5	4	5	5	10
DNA Metabolism	83	56	80	103	105
Fatty Acids, Lipids, and Isoprenoids	35	30	44	26	45
Nitrogen Metabolism	14	14	18	12	12
Dormancy and Sporulation	1	1	1	2	1
Respiration	59	52	55	66	75
Stress Response	54	65	77	75	90
Metabolism of Aromatic Compounds	7	3	4	3	5
Amino Acids and Derivatives	199	161	204	178	197
Sulfur Metabolism	9	6	15	9	16
Phosphorus Metabolism	27	13	24	22	28
Carbohydrates	164	153	149	165	194

Fig. 1. Classification of functionally annotated common genes between cyanobacterial strains ULC007, BDU 130791, FACHB-1052, FRX01, and IAM M-71. Between eighty percent and eighty-five percent of genes in these "core" genomes are hypothetical and are not represented in the figure.

Results showed a large number of regions encoding BGCs in the genome of *Phormidium ambiguum* IAM M-71 (11 regions encoding BGCs - lanthipeptide-class-V; non-ribosomal peptide synthetase (NRPS); siderophore; terpene; phenazine; lassopeptide; linear azol(in)e-containing peptides (LAP) and lanthipeptide class-II) (Table 2). The number of regions encoding BGCs in the other representatives of genus *Phormidium* is between 4 and 8. The different types of secondary metabolite clusters are included here (arylpolyene; terpene; siderophore; RiPP-like; phenazine; thioamitides, thiopeptide; resorcinol; NRPS; T1PKS and NRPS-like). The terpene is specific for all studied cyanobacterial species; non-ribosomal peptide synthetase (NRPS) is specific for all strains without *Phormidium pseudopriestleyi* FRX01; *Phormidesmis priestleyi* ULC007 and *Phormidium willei* BDU 130791 encode NRPS-like and T1PKS; *Phormidium pseudopriestleyi* FRX01 and *Phormidium tenue* FACHB-1052 encode arylpolyene; siderophore is specific for *Phormidium pseudopriestleyi* FRX01 and *Phormidium ambiguum* IAM M-71*;* some secondary metabolites are species-specific - T2PKS (*Phormidesmis priestleyi* ULC007), RiPP-like (*Phormidium pseudopriestleyi* FRX01), thioamitides, thiopeptide and resorcinol (*Phormidium tenue* FACHB-1052), lanthipeptide-class-V, lassopeptide, LAP and lanthipeptide-class-II (*Phormidium ambiguum* IAM M-71*)* (Table 2). Genomic content can be changed by neutral processes or due to adaptation of the organism to different environmental conditions (Barrick et al., 2009; Koonin, 2009; Tenaillon et al., 2016). Taking in account that 83% of the identified genes have an unknown function, it is very difficult at this stage to conclude from the encoded secondary metabolites that there is a significant difference between the two genera *Phormidesmis* and *Phormidium*. In addition to the morphology and molecular phylogeny, the isolation and identification of specific gene clusters or metabolites (or groups of metabolites) would give a more complete and realistic picture of the justification for separating the representatives of these two genera into different taxonomic categories.

Table 2. Secondary metabolites produced by studied cyanobacterial species.

Region	Type	From	To	Most similar known cluster	Similarity
Phormidesmis priestleyi **ULC007**					
Region 11.1	Terpene	49,266	70,072	-	-
Region 22.1	Terpene	25,098	45,913	-	-
Region 45.1	T2PKS, NRPS-like	63,729	160,746	Cichopeptin, NRP	15%
Region 46.1	NRPS, terpene	37,585	75,615	-	-
Region 51.1	NRPS, T1PKS, NRPS-like	1	59,806	Puwainaphycin F, Minutissamide A, B, C, D NRP	76%
Region 78.1	Terpene	1	15,726	-	-
Region 79.1	NRPS	1	29,783	-	-
Region 132.1	NRPS	24,888	73,841	Nostopeptolide A2, Polyketide + NRP:Cyclic depsipeptide	-
Phormidium pseudopriestleyi **FRX01**					
Region 26.1	Arylpolyene	1	7,941	-	-

Region 138.1	Terpene	10,796	31,616	-	-
Region 373.1	Siderophore	1	11,575	-	-
Region 552.1	RiPP-like	1	3,072	-	-
***Phormidium tenue* FACHB-1052**					
Region 2.1	Phenazine	130,964	151,422	-	-
Region 4.1	Arylpolyene	43,773	84,786	-	-
Region 7.1	Thioamitides, thiopeptide	6,918	32,005	-	-
Region 19.1	Resorcinol	33,576	58,279	-	-
Region 20.1	NRPS	22,721	52,507	Cyanopeptin, NRP	75%
Region 23.1	Terpene	7,855	39,848	Hopene, Terpene	15%
Region 34.1	Terpene	42,905	63,831	-	-
Region 36.1	Terpene	16,019	37,248	-	-
***Phormidium willei* BDU 130791**					
Region 28.1	Terpene	16,008	36,898	-	-
Region 36.1	T1PKS, NRPS	1	36,780	Nostopeptolide A2, Polyketide + NRP: Cyclic depsipeptide	25%
Region 47.1	NRPS-like	1	33,117	-	-
Region 77.1	Terpene	5,548	22,637	-	-
Region 82.1	NRPS-like	1	20,129	-	-
Region 95.1	Terpene	1	17,535	-	-
Phormidium ambiguum IAM M-71					
Region 1.1	Lanthipeptide-class-V	132,930	175,212	-	-
Region 2.1	NRPS	14,197	63,330	Nostocyclopeptide A2, NRP	28%
Region 2.2	Siderophore	109,211	126,128	-	-
Region 14.1	Terpene	25,551	46,480	Trichamide, RiPP: Cyanobactin	18%
Region 31.1	Phenazine	1	16,230	Kedarcidin, Polyketide:Iterative type I + Polyketide:Enediyne type I	1%
Region 36.1	Lassopeptide	15,654	37,959	-	-
Region 39.1	LAP	6,892	30,538	-	-
Region 57.1	Terpene	1	32,508	Malleobactin A, B, C, D, NRP: NRP siderophore	7%
Region 64.1	Lanthipeptide-class-II	1	13,657	-	-
Region 67.1	Lanthipeptide-class-II	226,317	249,727	-	-
Region 89.1	Terpene	201,922	224,279	Geosmin, Terpene	100%

Conclusions

A comparative genomics analysis was conducted (including a genome feature analysis and subsystem annotation, the classification of functionally annotated common genes and the automatic annotation of secondary metabolite gene clusters) to identify similarities and differences between *Phormidesmis priestleyi* ULC007 (previously classified as *Phormidium priestleyi*) and other representatives of genus *Phormidium*.

Using a comparative genomic approach, we found that *Phormidesmis priestleyi* ULC007 possess more genes involved in the motility and chemotaxis compared to the *Phormidium* species. The comparative genome analysis of studied cyanobacterial species allowed us to establish that some of the cyanobacterial species have specific secondary metabolite biosynthesis gene clusters, but this is not enough to support the recent taxonomic separation of these species. Many of the secondary metabolites are common to most cyanobacterial strains.

Acknowledgements

This research was financially supported by the National program "Young scientists and postdoctoral candidates" funded by the Bulgarian Ministry of Education and Science.

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Received: 10.08.2021
Accepted: 11.11.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 135-143

Research of Cambisols in Western Balkan Mountains

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Abstract. The research deals with Cambisols soil classification and parameters in the area of Western Balkan Mountains. The studied soils are spread in the Lower altitude area of oak forests (0 to 600 m a.s.l.) and in the Middle mountain area of beech and coniferous forests (600 to 2000 m a.s.l.). The gradient of the slopes varied from flat to steep. The landscape is strongly rugged and covered with forest. The studied Cambisols are formed on granites, granite gneisses, shales, syenites and rhyolites. These soils are characterized by the following features: they have shallow surface horizon A and a deeper B*w cambic* horizon. The horizon sequence of soil profiles is O- A-Bw-CR. Soil pH $_{H2O}$ has mean values of 5.3 in A horizon and decreases in B*w cambic* horizon. The low pH values are result of the leaching of the basic cations in the profile, organic acids washed form soil surface and acidic rocks. Profiles 2, 6 and 7 have base saturation more than 50 %, which defines them as Eutric Cambisols. These soils were identified in the lower parts of the Western Balkan Mountains formed on more basic soil forming rocks. The other soil profiles were classified as *Dystric* Cambisols having base saturation under 50 %. They were developed on more acidic silicate rocks, higher altitude and colder and humid climate of Western Balkan Mountains.

Key words: soil properties, soil classification, Cambisols.

Introduction

Western Balkan Mountain is a part of the Balkan Mountains range ("Stara Planina" in Bulgarian). Geologically, the Balkan Mountains block morphostructure is a complex mosaic of geological structures of different types and ages, composed of weakly metamorphosed, magmatic rocks of Paleozoic age and sedimentary rocks of Mesozoic and Neozoic age. In its heterogeneous structure, the constituent structures are set at different geological times. The modern relief has a relatively massive character, formed on the complex fold structure of the Berkovitsa anticlinorium and the Svoge anticline (Haydutov et al., 1995). Soil-forming rocks are very diverse, Pliocene and Quaternary sediments, poor carbonate and non-carbonate materials, red-brown clays and hard rocks as granites (Zagorchev & Dinkova, 1991). This is a prerequisite for the presence of soils with different chemical composition, properties and base saturation.

Most of Cambisols in Bulgaria are distributed between 800 and 1800 m a.s.l. This altitude range covers most of the Middle mountain area of beech and coniferous forests (600 to 2000 m a.s.l.) also called the middle forest belt. The relief is strongly rugged, and the vegetation is mostly forest, composed mainly of beech

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(*Fagus sylvatica* L.), but in the lower part also of oak (*Quercus spp.*), hornbeam (*Carpinus betulus* L.) and birch (*Betula pendula* R.) (Bondev, 2002). The soil-forming rocks on which Cambisols soils are spread are mainly granites, granite gneisses, shales, syenites, rhyolites, etc. (Koinov et al. 1998). Studies by Malinova & Petrova (2019) indicate that unsaturated brown forest soils (*Dystric* Cambisols) predominate in the middle forest belt in the Western Balkan Mountains.

In Bulgaria Cambisols occupy significant areas from 0 - 2000 m a.s.l. mainly in forest areas (Ninov, 2002). In Northern Bulgaria, these soils occupy the foothills and slopes of the Balkan Mountains. For the most part, these are areas with sloping or moderately hilly lowland relief, with characteristic for the oak-hornbeam belt forest, shrubby and grassy vegetation. Cambisols are the main soil type in the mountainous regions, and they are located almost entirely under forest vegetation. They are spread on steep terrains in the higher parts of the mountain areas. Therefore, it is difficult to study their genesis, properties and classification (Bogdanov et al., 2014; Karatoteva et al., 2017; Bogdanov, 2018; Malinova, 2019).

According to Malinova (2016) the soils of Central Balkan Mountains are insufficiently studied. There is a lack of information and data about their accurate classification. It is known that the *Eutric* Cambisols occupy 32.1% of the total Cambisols area in this part of the Balkan Mountains but it is not clearly specified that the remaining area (56.6%) is occupied by *Dystric* Cambisols.

Generally, the mountainous soils in Bulgaria are not well studied. That is why the aim of the research is to establish the main morphological, chemical and physical processes and properties of Cambisols, which are the main soil type in Western Balkan Mountains.

Materials and Methods

Profiles with numbers 1, 2, 3, 4, 5, 6 and 7 were studied and described with the following features: location, altitude, slope, parent rocks, vegetation and land use. Morphologically the soil profiles were described according to the guidelines for soil description (Jahn et al., 2006).

The soil characteristics were analyzed according to the following methods: organic carbon (org. C, %) by the modified Turin´s method (Kononova, 1963; Filcheva & Tsadilas, 2002); total nitrogen (total N, %) content, with a modified version of the classic Kjeldahl´s method with Kjeltec Auto 1030 Analyzer; soil acidity (pH_{H2O}; pH_{CaCl2}) – measured potentiometrically with WTW 720 pH meter (ISO 10390); soil texture (sand - 2 mm - 63 µm, %; silt - 63 µm - 2 µm, %; clay < 2 µm, %), using the sedimentation method (ISO 11277:2009); C/N ratio – calculation method; exchangeable acidity (extraction of 0.1 mol/l solution of $BaCl_2$) and exch. Ca, exch. Mg, and exch. K (ISO 11260&14254); Microsoft Office 10 for graphs, statistics and tables.

Results and Discussion

The main soil properties of Cambisols are presented in Table 1.

Cambisols can be found in all regions of Bulgaria, but usually these soils are spared in hilly and mountainous sloppy areas under forest. They have surface soil horizon, which is lighter in color, especially in leached soils where it is usually brownish or yellowish (Fig. 1).

The studied soils were characterized by the following features: they have shallow surface horizon A and a deeper B*w cambic* horizon. Surface A horizon varies from 1 cm to 22 cm depth, but mean values is 7.8 cm. The horizon sequence of the soil profile is O-A-B*w*-CR.

The most important horizon of these soils is subsurface *cambic* horizon (B*w*). According to WRB (IUSS Working Group, 2015), the pedogenetic alteration of a *cambic* horizon can also be established by contrast with one of the overlying mineral horizons that are generally richer in organic matter and therefore have a darker and less intense colour. These features can be seen in Fig. 1, with darker surface A horizon and alternation in subsurface B*w* horizon.

Table 1. Main soil properties of Cambisols.

Horizon/ Layer	Depth cm	Sand >0.063 mm	Silt 0.063 -0.002 mm	Clay <0.002 mm	Org. C %	Total N %	C/N	pH_{H2O}
				Profile 1. *Dystric* Cambisols Loamic				
O	2-0	-	-	-	26.66	1.487	10.40	5.2
A	0-6	48.85	25.21	25.93	4.46	0.270	9.58	4.6
Bw1	6-30	49.14	25.57	25.29	4.00	0.243	9.55	4.3
Bw2	30-45	48.13	25.40	26.47	2.39	0.166	8.35	4.4
				Profile 2. *Eutric* Cambisols Loamic				
O	1-0	-	-	-	31.53	1.3	14.08	5.4
A	0-5	58.98	22.95	18.07	7.52	0.38	11.60	6.0
Bw	5-20	56.25	23.92	19.83	2.04	0.10	11.38	4.7
C	20-47	-	-	-	0.81	0.01	52.20	4.9
				Profile 3. *Dystric* Cambisols Loamic				
O	1-0	-	-	-	33.46	2.02	9.60	5.1
A	0-4	51.23	28.83	19.94	6.35	0.34	10.90	4.8
Bw1	4-30	40.62	39.33	20.05	2.39	0.12	11.55	4.5
BC1	30-41	41.52	49.58	8.90	0.76	0.04	11.91	4.4
BC2	41-↓	64.12	29.46	6.43	0.65	0.03	15.08	4.6
				Profile 4. *Dystric* Cambisols Loamic				
O	1-0	-	-	-	32.84	1.89	10.09	5.0
A	0-1	52.41	28.13	19.46	3.95	0.21	10.96	4.9
ABw	1-10	47.93	30.62	21.45	1.54	0.11	7.98	4.2
Bw	10-34	47.21	31.43	21.37	1.74	0.09	11.73	4.2
BC	34-55↓	52.06	28.3	19.64	1.13	0.06	10.40	4.3
				Profile 5. *Dystric* Cambisols Loamic				
O	2-1	-	-	-	33.77	1.83	10.72	5.2
OH	1-0	-	-	-	15.58	1.39	6.49	5.3
A	0-4	75.09	16.52	8.40	2.52	0.19	7.61	4.4
Bw1	4-23	61.46	27.02	11.51	1.62	0.14	6.66	4.5
Bw2	23-32	63.68	29.06	7.26	0.62	0.09	3.95	4.6
BC	32-70↓	67.85	23.43	8.72	0.25	0.04	3.63	5.1
				Profile 6. *Eutric* Cambisols Loamic				
O	1-0	-	-	-	35.21	1.46	13.99	5.8
A	0-10	40.86	36.57	22.56	2.37	0.20	6.80	5.1
Bw1	10-18	38.25	38.16	23.6	1.41	0.13	6.10	4.6
Bw2	18-30	39.98	35.57	24.45	0.88	0.12	4.29	4.8
BC	30-110↓	51.9	24.8	23.3	0.18	0.08	1.39	5.7
				Profile 7. *Eutric* Cambisols Loamic				
A	0-4	50.02	27.6	22.38	3.4	0.32	6.26	6.7
Bw1	4-17	53.49	26.41	20.11	2.61	0.28	5.50	5.6
Bw2	17-31	51.67	25.93	22.4	1.5	0.17	5.15	5.7
BC	31-53	48.47	25.36	26.18	0.64	0.10	3.83	5.6
C	53-↓	-	-	-	0.19	0.08	1.38	5.3

Fig. 1. Profile 2 (left) and Profile 3 (right) – typical shallow Cambisols in Western Balkan Mountains.

The other diagnostic criteria for the definition of cambic horizon are, 15 cm deep and more, soil texture of sandy loam or finer. Similar properties can be seen in all soil profiles (Table 1). The soil texture was assessed as sandy loam, loam and sandy clay loam (Fig. 2). That defines all soil profiles as Loamic according to the second level qualifiers in WRB (IUSS Working Group, 2015).

Soil texture varied in a wide range mainly depending on the soil-forming materials. Cambisols are characterized by low clay content and significant number of skeletal elements the amount of which increases in depth.

Studied soils are characterized by high organic carbon content, about 4.5% in mineral A horizons, the reason for relatively high content is that these soils are developed under the influence of forest vegetation and they never have been cultivated. In the Bw horizons the humus decreases to the average value of 2.44% (Table 1 and Fig.3). The highest values of soil organic matter are in surface organic (O) layers. These soil layers are dominated by organic materials consisting of undecomposed or partially decomposed litter formed by the litterfall of the trees.

The C/N ratio in more of the soil horizons is under 15, which means that the type of humus is *mull* with mean value of 9.7. According to Vanmechelen et al. (1997) in South Europe there is advanced degree of transformation of organic matter and formation of stable humic substances. *Mull* humus is characterized by a fast turnover rate and an intimate mixture with mineral soil materials, and it is the most frequently observed humus type in Bulgaria. The *mull* type often consists fresh, undecomposed leaves, while older organic materials have already been incorporated in the mineral soil. In the metamorphic (B*w*) horizons the ratio decreases.

The amount of total nitrogen in Cambisols formed under the influence of forest vegetation is very dynamic indicator depending on the amount of moisture in the soil in different seasons and conditions of the processes of ammonification and nitrification. The content of total nitrogen in the soil follows the same trend as carbon with values from 0.01 up to 0.38 %.

The soil pH_{H2O} in *A* horizon varied between 6.7 (profile 7) and 4.2 (profile 4) - In subsurface B*w* horizon the pH is low and reaches the lowest obtained values. The soil reaction in the studied Cambisols was assessed as neutral to strongly acid. The low values indicated that there is ongoing destructive process of exchangeable Al over the soil mineral compositions. Its quantity is not high due to the low value of the cation exchange capacity (CEC). The low pH values are result of the leaching of the basic cations in the profiles.

The acidity of the studied profiles increases in *cambic* horizons, due to the vertical migration of acidic products from the soil formation process, including free organic acids. This defines it as more acidic than the surface A horizon. The soil acidity of soils is a result of silicate soil-forming rocks and organic residues. The exchangeable acidity varied (exch. Al) form 0.2 – 5 cmol(+).kg^{-1}. Highest values of exch. Al were assessed in B*w cambic* horizon (Table 2). These soil profiles (1, 2, 3, 4 and 5) were defined as *Dystric* Cambisols. Therefore, basic cations such as exch. Ca, exch. Mg, and exch. K have higher values in the *Eutric* Cambisols (profiles 2, 6 and 7). Results showed that calcium cations have highest values in comparison to the magnesium and potassium cations.

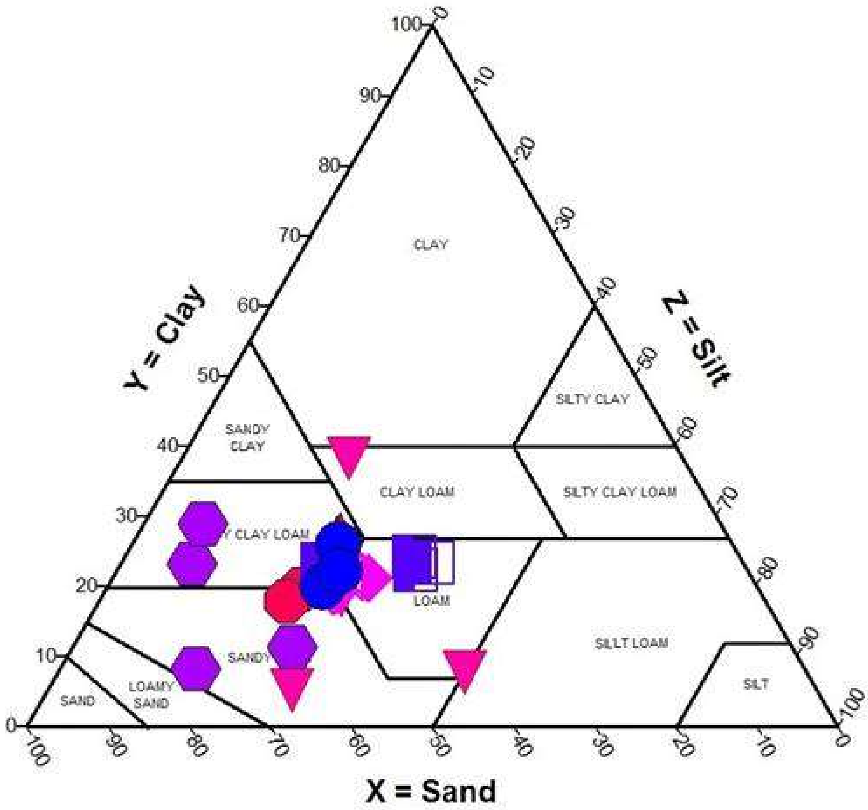

Fig. 2. Soil texture triangle with textural classes. (Profile 1 pink diamonds, Profile 2 red triangle, Profile 3 in pink triangles, Profile 4 red hexagon, Profile 5 in purple hexagon, , Profile 6 in purple square, Profile 7 in blue circle).

Table 2. Physicochemical values of Cambisols.

Horizon/ Layer	Depth cm	pH $_{CaCl2}$	Exch. Al	Exch. Ca	Exch. K	Exch. Mg	CEC	BS %
			cmol(+).kg^{-1}					
			Profile 1. *Dystric* Cambisols Loamic					
O	2-0	4.9	1.00	28.85	1.16	4.71	39.18	97
A	0-6	3.9	2.73	1.74	0.12	0.28	4.98	44
Bw1	6-30	3.8	3.45	0.93	0.02	0.14	4.63	24
Bw2	30-45	3.9	2.82	0.79	0.00	0.11	3.90	24
			Profile 2. *Eutric* Cambisols Loamic					
O	1-0	4.6	0.73	40.77	2.41	4.71	53.38	98
A	0-5	5.4	0.64	17.59	0.00	3.53	22.44	97
Bw	5-20	4.1	5.00	7.69	0.03	2.61	15.75	67
C	19-47	4.6	0.55	13.03	0.03	3.33	17.36	97
			Profile 3. *Dystric* Cambisols Loamic					
O	1-0	4.8	1.00	36.99	2.32	8.34	53.71	98
A	0-4	4.2	1.27	3.75	0.25	1.12	6.54	80
Bw1	4-30	4.1	2.27	0.50	0.04	0.34	3.20	28
BC1	30-41	4.0	1.91	0.94	0.00	0.67	3.59	46
BC2	41-80	4.0	1.09	0.53	0.01	0.33	1.98	45
			Profile 4. *Dystric* Cambisols Loamic					
O	1-0	4.8	1.36	35.77	1.57	10.39	54.43	97
A	0-1	4.3	1.73	1.85	0.40	0.98	5.06	65
ABw	1-10	3.8	3.09	0.60	0.05	0.37	4.18	25
Bw	10-34	3.8	2.82	0.21	0.01	0.12	3.19	11
BC	34-55	3.7	3.27	0.34	0.00	0.19	3.86	14
			Profile 5. *Dystric* Cambisol Loamic					
O	2-1	4.6	1.09	40.45	1.13	6.90	55.27	98
OH	1-0	4.6	0.55	29.59	0.41	3.05	36.01	98
A	0-4	3.7	2.36	1.12	0.10	0.21	3.84	38
Bw1	4-23	3.9	2.00	0.25	0.01	0.07	2.36	14
Bw2	23-32	3.9	1.36	0.40	0.02	0.08	1.87	27
BC	32-70	4.2	0.55	0.94	0.00	0.24	1.74	68
			Profile 6. Eutric Cambisols Loamic					
O	1-0	5.2	0.64	35.13	1.61	10.12	52.49	99
A	0-10	4.5	0.64	7.82	0.26	1.73	10.68	94
Bw1	10-18	4.0	1.45	6.11	0.13	1.65	9.54	84
Bw2	18-30	4.2	0.73	7.35	0.10	2.01	10.41	93
BC	30-110	5.3	0.27	10.71	0.11	4.76	16.51	98
			Profile 7. *Eutric* Cambisols Loamic					
A	0-4	6.3	0.18	16.42	0.35	2.01	19.43	99
Bw1	4-17	5.0	0.45	10.30	0.07	1.05	12.10	96
Bw2	17-31	5.2	0.27	10.01	0.02	0.68	11.19	98
BC	31-53	4.7	0.45	11.45	0.03	0.86	13.08	96
C	53-80	4.3	0.64	12.42	0.03	1.08	14.60	95

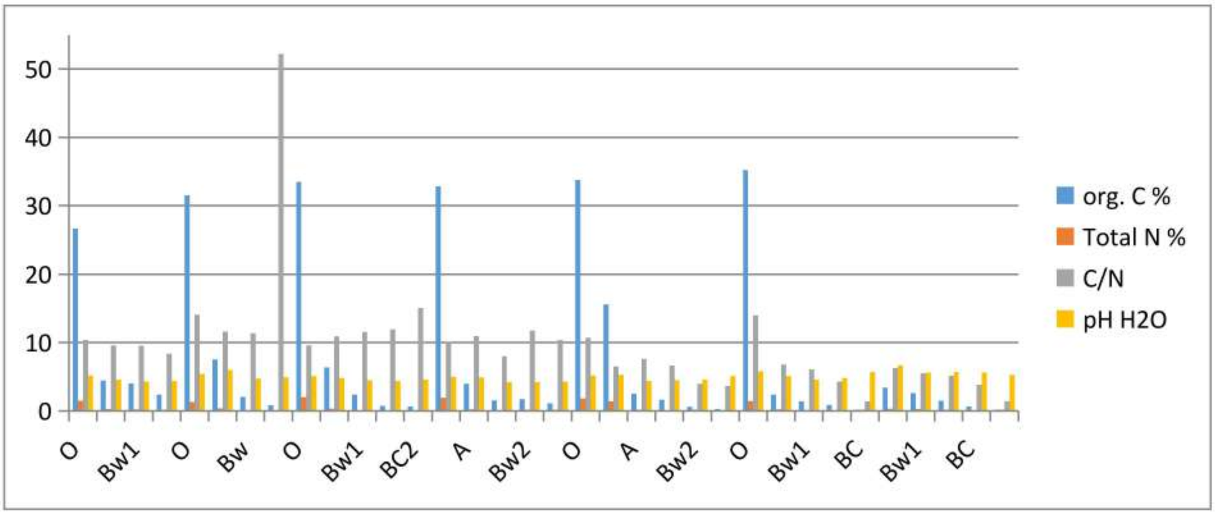

Fig. 3. Main chemical properties – org. C %, Total N %, C/N, pH_{H2O}.

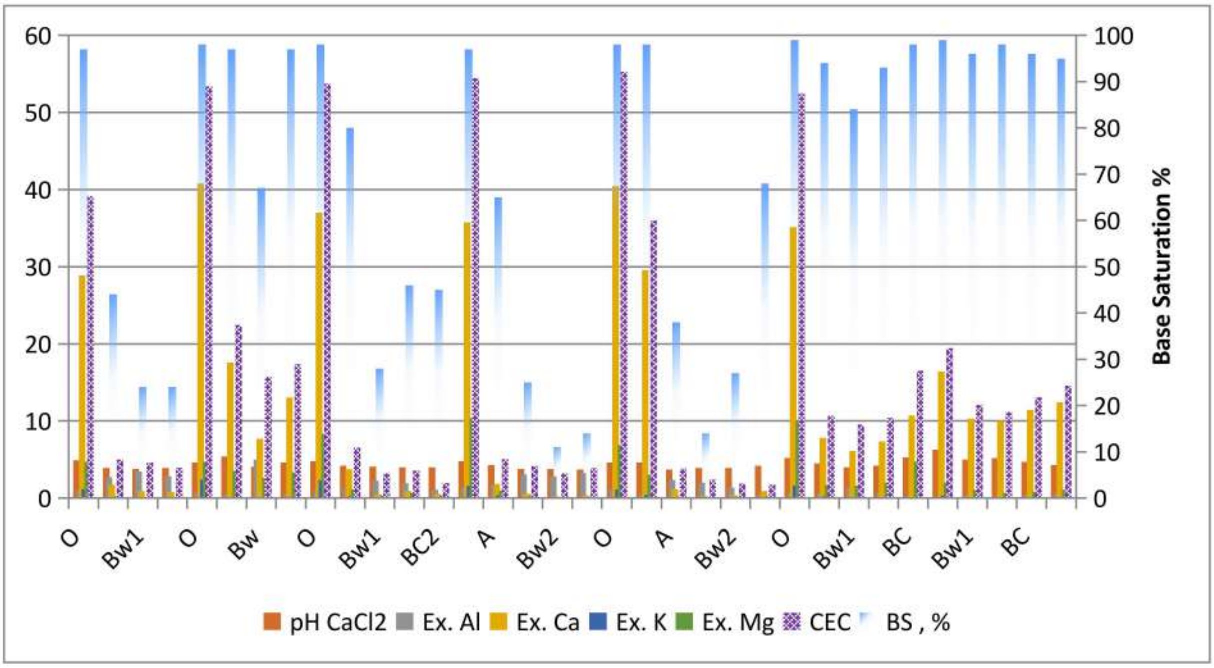

Fig. 4. Base Saturation and physicochemical properties of Cambisols.

According to Ganev (1989) the acidic instability of clay minerals is determined by the soil colloids which are in the state of incomplete neutralization with basic cations. In *Dystric* Cambisols the destructive processes (high soil acidity and exch. Al) have negative effect on clay colloids formation. Quantity of basic cations is also low in the C horizons and in litterfall (O horizon). Therefore, the acidification is a naturally occurring process.

The results indicated that CEC decreased with the progress of the soil acidification. The CEC is usually low with mean value about 8 cmol(+).kg^{-1} (from 2.3 up to 27.6 cmol(+).kg^{-1} in the mineral horizons). In organic (O) layers, it was very high and varied from 19 up to 54 cmol(+).kg^{-1}.

The base saturation (BS) is one of the main chemical properties used for soil classification. The sum of basic exchange cations (exch. Ca, exch. Mg, exch. K) define the degree of base saturation. It is typical for all organic layers to have high base saturation and the studied ones are not an exception (table 2 and fig.4). Profiles 2, 6 and 7 have base saturation in *cambic* diagnostic horizon (B*w*) over 50 %, which defines them as *Eutric* Cambisols according WRB (IUSS Working Group, 2015). *Eutric* Cambisols in the studied area were spread in the lower parts of the Western Balkan Mountains. They were formed on more basic soil forming rocks with favorable soil properties and moderate fertility.

The other soil profiles were classified as *Dystric* Cambisols, because they had base saturation under 50 % in *cambic* horizon. These soils are developed on more acidic silicate rocks with higher altitude in colder and humid parts of the Western Balkan Mountains. *Dystric* Cambisols have low fertility and in most of the cases they are threaten by degradation and erosion.

Conclusions

Cambisols are the main soil type in Western Balkan Mountains developed on silicate rocks mainly granites, granite gneisses, shales, syenites, rhyolites. The horizon sequence of soil profile is mainly O-A-Bw-CR, with shallow surface organic layers and mineral horizons and deeper *cambic* horizons.

Soil texture differs depending on the soil-forming materials. It varied between sandy loam, loam and sandy clay loam. Cambisols have low clay content and higher number of skeletal fragments.

Soils are characterized by relatively high organic carbon content of 4.5% in the surface A horizon, but in B*w* horizon it decreases nearly twice. The mean value of calculated C/N ratio is 9.7. Therefore, the type of humus is *mull* in almost all of the studied profiles.

The studied Cambisols have neutral to strongly acidic reaction (pH $_{H2O}$). There is ongoing destructive process due to the impact of exchangeable aluminum on the secondary minerals of the soil. Soil acidity increased in *cambic* horizons. The obtained lower pH values were due to the released acidic products in soil from weathering rocks, free organic acids leached from the soil surface and low quantities of basic soil cations.

Profiles 2, 6 and 7 were classified as *Eutric* Cambisols (Loamic) because they have base saturation over 50 % in *cambic* horizon and loamic soil textural qualifier. Profiles 1, 3, 4, 5 were identified as *Dystric* Cambisols (Loamic) because their base saturation is under 50 % in *cambic* diagnostic horizon.

Acknowledgments

The current research was performed in the framework of the Project B-1072/16.03.2020 “Research of the soils in the lower and middle mountain belt from the northern slope of the Western Balkan Mountains” funded by Science Research Sector of University of Forestry.

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Received: 20.05.2021
Accepted: 13.10.2021

ECOLOGIA BALKANICA
2021, Vol. 13, Issue 2 December 2021 pp. 145-154

Survey on Medicinal Plants Used in the Folk Medicine of Current Bulgarian Society as a Basic Information for Plant Protection

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Abstract. Traditional herbal medicine has gained increasing interest. Modern ethnobotany is focused on documentation of traditional knowledge as a base data for plant diversity protection. The present survey aimed to estimate the status of uses of medicinal plants in current Bulgarian society. The study is focused on most commonly used wild plants in studied regions as a basic information for development of plant protection strategies. Ethnobotanical information was collected during semi-structured interviews. Respondents have been selected based on their knowledge – people known to be particularly interested in traditional health practices. Respondents declare empiric knowledge as the major source of information. So, results revealed medicinal plants explored for decades in studied regions. A total number of 88 plant species, belonging to 43 families were recorded. The most dominant plant families were Asteraceae (28%), Lamiaceae (26%) and Rosaceae (19%). Majority of cited species are wild (60.2%), including protected species *Galanthus nivalis* L., *Primula veris* L., *Asplenium scolopendrium* L. and *Sideritis scardica* Griseb. (Balkan endemic). Herbs represent the majority (72.7%) of the reported plant species. The most used parts of the plants were leaves (40.9%). To our knowledge this is the first survey in Bulgaria only with selected respondents. The number of plants reported reflects well-preserved traditional knowledge in the investigated region. Quantitative analysis revealed the significance of species for local people. Majority of cited species are wild. From the point of view of sustainable ecology information to local people about the importance of preservation of natural habitats of wild species should be provided.

Key words: medicinal plants, questionnaire survey with key informants, plant protection.

Introduction

Plants have been used from ancient times for the improvement of overall well-being, for diverse physical and mental disorders (Hussain et al., 2019). In contemporary society a lot of people have returned to traditional medicine in spite of the access to conventional medicine (Karunamoorthi et al., 2013). Local people usually use both wild and cultivated plants. Nowadays ethnobotanical surveys represent a valuable tool for the environmental protection. Increased demand for natural remedies exerts a negative impact on the native habitats of many species. Documentation of indigenious knoweldge evaluated the utilization of wild species and provides basic data for development of appropriate conservation strategies (Axiotis et al., 2018; Tugume et al., 2016).

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Contemporary surveys in Bulgaria revealed increasing utilization of natural remedies and consequently – the necessity of development of sustainable strategies.

Despite of the small national territory geographic specificity of Bulgaria has resulted in the development of a great plant diversity (Assyov et al., 2012). Ethnobotanical surveys in Bulgaria aimed to preserve and maintain traditional knowledge passed down through generations (Kozuharova et al., 2013). Different approaches have been used in order to collect data in modern ethnobotany. Some studies involved a large number of random informants, others – selected informants, or both of them (Leporatti & Impieri, 2007; Rajaei & Mohamadi, 2012; Tsioutsiou et al., 2019). Since variability of traditional knowledge between different countries and cultures in contemporary surveys researchers have applied different quantitative ethnobotanical indices (Hussain et al., 2019).

The aim of this study is twofold: to estimate the status of the uses of medicinal plants in current Bulgarian society and to document the most commonly used wild plants in studied regions as a basic information for development of plant protection strategies.

Materials and Methods

This research was conducted by collecting ethnobotanical information during semi-structured interviews with local knowledgeable persons. Field study was carried out over a period of approximately 2 years in central and northeastern Bulgaria. Ethnobotany Club bachelor student members (Faculty of Natural Sciences, University of Shumen, Bulgaria) carried out the survey. The students were trained to conduct an ethnobotanical survey. Questionnaires were prepared in order to document the most frequently used herbs and their most popular application. Respondents (key informants) have been previously selected based on their knowledge – few people known to be particularly interested in traditional health practices. The demographic features of the people who accepted to participate in the interview were determined. Voluntary participation (informed consent obtained) was documented in questionnaires. Local plant names, growth form, source of plants (wild, cultivated, purchased) and utilized parts of the plants were recorded. As a first step of questionnairy, respondents were asked to show a sample of reported plant – raw or dried. The mentioned plants were identified by Zheni Stoynova, expert plant taxonomist. Botanical species names were presented according to WFO (2021): World Flora Online. The family list is structured according to Angiosperm Phylogeny Group IV (Stevens, 2017).

Quantitative ethnobotanical indices were estimated as described in other studies (Rehman et al., 2017; Tardío & Pardo-de-Santayana, 2008; Umair et al., 2017). The ethnobotanical indices are founded on the data obtained in the study: one of the respondents mentions the use of the reported species in the special use-category. Relative Frequency of Citation (RFC) is obtained by dividing the number of respondents who mention the use of the species, by the number of all respondents. It represents the fraction of informants that used a given plant and the local significance of plant species. Use Value (UV) demonstrates the significance of a species by considering number of use reports. This index represents the sum of number of use-reports of participants who mentioned each use of the specie, divided by the number of all respondents.

Results

One approach to collect ethnobotanical information is purposefully selection of informants (Gao et a.l, 2019). In present survey a total of 15 interviews were conducted with knowledgeable respondents from urban and rural areas in Bulgaria (Fig. 1).

Fig. 1. Regions in Bulgaria where the interviews were conducted.

Data about the source of ethnobotanical knowledge of informants is summarized in Table 1. The age of the informants ranged from 40 to 88 years old, more than half of the informants aged between 60 and 80. The majority of the respondents are well educated. Only two of them, women living in rural area at age of 88 and 73, had not finished high school. Female informants predominated in our study. The respondents declared to have an experience in traditional practices for more than ten years. A matter of interest is the source of ethnobotanical knowledge (The sum of percentage is more than 100 since there are more than one answer). Majority of respondents (80.00%) declared as a source of knowledge their relatives. Friends were pointed from 33.33% of respondents. A large proportion of respondents (66.67%) acquires information from specialized books. Only 20.00% have used internet as a source of information.

Table 1. Source of ethnobotanical knowledge of respondents. *Legend:* W – woman, M – man.

Gender	Age (years)	Place of living	Education	Experience as a herbalist (years)	Source of ethnobotanical knowledge			
					Relatives	Friends	Books	Internet
W	40	Small town	university	>15	Relatives			x
W	40	City	high school	>20	Relatives	x		
W	43	City	high school	> 0	Parents		x	x
W	45	Village	high school	>20	Relatives		x	
W	56	Small town	high school	>10	Mother			
W	61	Small town	high school	>15	Relatives		x	
W	63	Small town	high school	>14		x	x	
W	63	City	university	>40			x	
W	70	Village	high school	>50	Relatives	x	x	
W	71	Small town	high school	>30	Grandmother herbalist		x	
W	73	Village	basic school	>30		x	x	
W	88	Village	primary school	>70	Mother& grandmother			
M	49	City	university	>10	Parents		x	x
M	60	Village	high school	>15	Father			
M	68	Village	high school	>20	Relatives	x	x	

Reported species are listed in Table 2, where plant families are cited in alphabetical order. Data on scientific name, local name, status (wild, cultivated or purchased) and parts used are included in the table. In this study were recorded a total number of 88 plant species, belonging to 43 families. The most dominant plant families were Asteraceae (28%), Lamiaceae (26%) and Rosaceae (19%). The second mentioned were Amaryllidaceae (9.3%) and Apiaceae (9.3%).

Majority of cited species (53 species) are wild, including protected species *Galanthus nivalis* L., *Primula veris* L., *Asplenium scolopendrium* L. and *Sideritis scardica* Griseb. (Balkan endemic). Furthermore, 32 plant species are cultivated in the gardens/houses. Among cultivated plants 17 species are non native – 14 of them are grown in gardens as food or for decorative purposes; remaining 3 exotic plants are reported to be purchased.

Herbs represent the majority (72.7%) of the reported plant species. The second group are shrubs (10.2%) and trees (11.4%). Two species are subshrubs and only one representative – shrub/small tree, climbing shrub and climbing herb. The most used parts of the plants (above ~ 20%) were leaves (40.9%), followed by aerial parts (27.27%), fruits (21.6%). The second group (above ~10%) included flowers/flower parts (19.3%), root and rhizomes (12.5%) and seed/ nuts/ kernel (9.1%). Rarely used are tubers (2.3%), barks (2.3%), stems (1.1%) and bulbs (1.1%).

In ethnobotanical surveys RFC and UV indices are used to select potential plant species for further scientific research **(Table 2).** The highest RFC was calculated for *Hypericum perforatum* (0.47), followed by *Sambucus nigra*, *Achillea millefolium*, *Thymus* sp. div., *Urtica dioica* (0.40) and *Matricaria chamomilla* (0.33). In the present study UV values of the reported species varied from 0.07 to 2.53. *Hypericum perforatum* (2.53), *Urtica dioica* (2.07), *Matricaria chamomilla* (1.80), *Achillea millefolium* (1.73), *Thymus* sp. div. (1.53) and *Anethum graveolens* (1.13) were the most utilized medicinal plant species with highest used value.

Table 2. List of plants used by respondents. *Legend:* Status: W – Wild, C – Cultivated, P – Purchased; Habit: H – herb, T – tree, S – shrub, sS – subshrub; S/T – shrub or small tree, Clh – climbing herb, Cls – climbing shrub; Part used: R – Root/rhizomes, St – Stem, L – Leaf, Fl – Flower, Fr - Fruit, Se – Seed, N – Nuts, AP – Aerial Parts, Tu – Tuber, B – Bulb, Bd – Buds, Ba – Bark, Stigma – Sgm, K – Kernel.

Family/Scientific name	Local name	Status	Habit	Part used	RFC	UV
Adoxaceae						
Sambucus ebulus L.	Trevist buz (Buzak)	W	H	Fr, L	0.07	0.47
Sambucus nigra L.	Cheren buz	W	S/T	Fr, Fl, R	0.40	0.87
Amaryllidaceae						
Allium sativum L.	Chesun	C	H	L, Bu	0.13	0.40
Allium ursinum L.	Div chesun (Levurda)	W	H	L	0.07	0.33
Galanthus nivalis L.	Kokiche	W	H	Fl	0.07	0.13
Allium siculum Ucria.	Samardala	C	H	L	0.07	0.40
Anacardiace						
Cotinus coggygria Scop.	Smradlika	W	S	L	0.2	0.87
Apiaceae						
Anethum graveolens L.	Kopur	C	H	Fr, AP	0.2	1.13
Apium graveolens L.	Tselina	C	H	R, L	0.07	0.6
Levisticum officinale W.D.J.Koch	Devisil	C	H	R, L	0.07	0.33
Pimpinella anisum L.	Anason	C	H	Fr	0.07	0.13
Asparagaceae						
Convallaria majalis L.	Momina sulza	C	H	Fl	0.07	0.13
Araceae						
Arum maculatum L.	Zmiyarnik	W	H	Tu	0.07	0.07
Araliaceae						
Hedera helix L.	Brushlyan	W	Cls	L	0.07	0.2
Asphodelaceae						
Aloe vera L.	Aloe	C	H	L	0.07	0.8

Aspleniaceae						
Asplenium scolopendrium L.	Volski ezik	W	H	L	0.07	0.2
Asteraceae						
Achillea millefolium L.	Byal ravnets	W	H	Fl, AP	0.40	1.73
Arctium lappa L.	Repey	W	H	R, L	0.13	0.40
Artemisia absinthium L.	Byal pelin	W	H	AP	0.07	0.07
Calendula officinalis L.	Neven	C	H	L, Fl	0.2	0.73
Carduus acanthoides L.	Magareshki trun	W	H	Fl	0.07	0.2
Cyanus segetum Hill	Sinya metlichina	W	H	Fl	0.07	0.2
Cichorium intybus L.	Sinya zhluchka	W	H	AP, R	0.13	0.40
Matricaria chamomilla L.	Layka	W	H	Fl	0.33	1.80
Tussilago farfara L.	Podbel	W	H	L	0.07	0.2
Silybum marianum (L.) Gaertn.	Byal trun	W	H	Fr	0.07	0.13
Solidago virgaurea L.	Zlatna pruchitsa	W	H	AP	0.07	0.07
Taraxacum campylodes G.E.Haglund	Glukharche	W	H	AP, R	0.2	0.93
Betulaceae						
Betula pendula Roth	Byala breza	C	T	L, Bd	0.07	0.27
Corylus avellana L.	Leska	W	S	L, Fr, Ba	0.13	0.47
Boraginaceae						
Symphytum officinale L.	Zarasliche	W	H	AP, R	0.07	0.07
Brassicaceae						
Armoracia rusticana P.Gaertn., B.Mey. & Scherb.	Khryan	C	H	R	0.13	0.60
Brassica nigra (L.) W.D.J.Koch	Cheren sinap	W	H	Se	0.07	0.27
Cannabaceae						
Humulus lupulus L.	Khmel	W	Clh	Fl	0.07	0.20
Caryophyllaceae						
Herniaria glabra L.	Izsiplivche	W	H	AP	0.13	0.20
Crassulaceae						
Sedum maximum (L.) Suter	Debela mara	W	H	L	0.07	0.07
Cucurbitaceae						
Cucurbita pepo L.	Tikva	C	H	Fr, Se	0.07	0.53
Ecballium elaterium (L.) A.Rich.	Luda krastavitsa	W	H	Fr	0.07	0.13
Ericaceae						
Vaccinium vitis-idaea L.	Chervena borovinka	W	sS	Fr, L	0.07	0.40
Equisetaceae						
Equisetum arvense L.	Polski khvosht	W	H	AP	0.07	0.33
Fabaceae						
Galega officinalis L.	Zhablek	W	H	AP	0.07	0.13
Phaseolus vulgaris L.	Fasul	C	H	Fr -husk	0.07	0.20
Gentianaceae						
Centaurium erythraea Rafn	Cherven kantarion	W	H	AP	0.07	0.20
Geraniaceae						
Geranium macrorrhizum L.	Zdravets	C	H	L	0.07	0.27
Pelargonium zonale (L.) L'Hér. ex Aiton	Mushkato	C	H	L	0.07	0.07
Ginkgoaceae						
Ginkgo biloba L.	Ginko	C	T	L	0.07	0.20
Hypericaceae						
Hypericum perforatum L.	Zhult kantarion	W	H	AP	0.47	2.53
Iridaceae						

Crocus sativus L.	Shafran	C	H	Sgm	0.07	0.07
Juglandaceae						
Juglans regia L.	Orekh	C	T	L, N, green husk	0.13	0.80
Lamiaceae						
Lavandula latifolia Medik.	Lavandula	C	H	L, Fl	0.13	0.93
Marrubium vulgare L.	Pchelnik	W	H	AP	0.07	0.07
Melissa officinalis L.	Matochina	W	H	AP	0.27	0.53
Mentha spicata L.	Dzhodzhen	W	H	AP	0.13	0.53
Mentha sp.	Menta	W	H	AP	0.13	0.60
Ocimum basilicum L.	Bosilek	C	H	L	0.13	0.53
Origanum vulgare L.	Rigan	W	H	AP	0.27	0.87
Rosmarinus officinalis L.	Rozmarin	C	S	L	0.07	0.27
Salvia officinalis L.	Gradinski chay	C	sS	L	0.07	0.27
Sideritis scardica Griseb.	Mursalski chay	W	H	AP	0.13	0.67
Thymus sp.div.	Mashterka	W	H	AP	0.40	1.53
Lauraceae						
Laurus nobilis L.	Dafinov list	P	T	L	0.07	0.13
Malvaceae						
Malva sylvestris L.	Kamilyak, slez	W	H	L, Fl	0.07	0.33
Tilia sp.	Lipa	W	T	Fl	0.2	0.47
Moraceae						
Ficus carica L.	Smokinya	C	T	L, Fr	0.13	0.93
Papaveraceae						
Chelidonium majus L.	Zmiysko mlyako	W	H	AP	0.13	0.33
Pinaceae						
Pinus sylvestris L.	Byal bor	W	T	L, young shoots	0.13	0.33
Plantaginaceae						
Plantago major L.	Zhivovlek	W	H	L	0.13	0.47
Poaceae						
Avena sativa L.	Oves	C	H	Se, St	0.13	0.60
Zea mays L.	Tsarevitsa	C	H	Se, Sgm	0.13	0.60
Polygonaceae						
Persicaria hydropiper (L.) Delarbre	Vodno piperiche	W	H	AP	0.07	0.13
Primulaceae						
Primula veris L.	Iglika	C	H	Fl, R	0.07	0.20
Ranunculaceae						
Nigella sativa L.	Posevna chelebitka	C	H	Se	0.07	0.07
Rosaceae						
Agrimonia eupatoria L.	Kamshik	W	H	AP	0.07	0.07
Aronia melanocarpa (Michx.) Elliott	Aroniya	C	S	Fr	0.07	0.20
Crataegus monogyna Jacq.	Glog	W	S	L, Fr, Fl	0.40	0.87
Fragaria vesca L.	Gorska yagoda	W	H	L, Fr	0.07	0.27
Prunus persica (L.) Batsch	Praskova	C	T	Fr, K	0.07	0.47
Rosa canina L.	Shipka	W	S	Fr	0.27	0.80
Rosa damascena Herrm.	Roza	C	S	Fl	0.07	0.27
Rubus idaeus L.	Malina	C	S	L, R, Fr	0.13	0.60
Rubiaceae						
Galium verum L.	Enyovche	W	H	AP	0.07	0.07
Salicaceae						
Salix alba L.	Byala vurba	W	T	Ba	0.07	0.33
Sapindaceae						
Aesculus hippocastanum L.	Konski kesten	W	T	Se	0.07	0.13

Solanaceae						
Lycium barbarum L.	Godzhi beri	C	S	Fr	0.07	0.40
Solanum tuberosum L.	Kartof	C	H	Tu	0.07	0.07
Urticaceae						
Urtica dioica L.	Kopriva	W	H	AP L R	0.40	2.07
Zingiberaceae						
Curcuma longa L.	Kurkuma	P	H	R	0.07	0.40
Zingiber officinale Roscoe	Dzhindzhifil	P	H	R	0.13	0.47

Discussion

Analysis of the source of ethnobotanical knowledge of respondents demonstrate persistence of oral empiric folk knowledge in Bulgaria nowadays. Specialized books usage is connected with well documented during years knowledge and valuable books written in the past (Kozuharova et al., 2013). It should be noted, that people who pointed internet as a source of information declared also relatives. So, ethnobotanical data revealed medicinal plants explored for decades in these regions.

Recently, results of numerous ethnobotanical studies in different parts of the world have been published. Such researches are necessary to protect ethnobotanical data and to estimate dynamic of local knowledge in modern society (Menale et al., 2016; Pardo-de-Santayana et al., 2015). Depending on geographic, sociological and cultural distinctives every local study contributes to global knowledge. Our data could be compared with other data from the same region. We found some differences about prevalent plant families, but Lamiaceae, Asteraceae and Rosaceae are amongst the most represented families in other studies in Greece (Tsioutsiou et al., 2019), Turkey (Akaydin et al., 2013) and previous surveys in Bulgaria (Kozuharova et al., 2013; Kültür & Sami; 2009; Leporatti & Ivancheva, 2003).

Different plant parts are used to treat different diseases because of specific chemical constituents (Nguyen et al. 2019). Reported preferred use of leaves could be considered as a sustainable harvesting approach, since the whole plant is not damaged. Similar results have been presented in other studies (Axiotis et al., 2018; Chaachouay et al., 2019; Rehman et al., 2017). Leaves are easily collected and being metabolitically most active parts of plant contain numerous valuable chemical compounds (Khan et al., 2018).

Results of present study revealed predominant use of wild plants. Cited exotic plants revealed dynamics of traditional knowledge. It is known that during centuries foreign medicine plants have been incorporated into local practices (Quave et al., 2012). Wild plant species are cited in other surveys as a major source for remedies, including endemic and endangered species (Tsioutsiou et al., 2019). In Bulgaria, *Galanthus nivalis* L. and *Primula veris* are included in Biological Diversity Act (2002), Annex 3 and Annex 4. *Asplenium scolopendrium* L. and *Sideritis scardica* Griseb are under a regime for special protection and use determined by Ordinance № RD – 162/25.02.2021 (State Gazzette, № 20/2021) issued by the Minister of Environment and Water. Their gathering for trade use is prohibited throughout the country. The same order sets eligible quantities for collection of *Primula veris*. Habitat destruction is a major problem of exploration of wild medicinal plants (Axiotis et al. 2018). It should be noted that one and the same medicinal plant grown in particular regions could possess different therapeutical properties. Epigenetic factors may explain phenotypic variations. Contemporary studies have established that plant chemical constituents depend on environmental conditions (Hao & Xiao, 2018). So, education of local people, sustainable utilization of plants and preservation of natural habitats are of global importance.

In recent decades ethnobotanical studies have been provided in different regions of Bulgaria, but quantitative methods have only very recently received attention. Relative frequency of citation (RFC) and use value (UV) revealed prevalent use of well-known species and their proven pharmacological properties. RFC signifies the local importance of each species (Umair et al., 2017). Reported species with highest RFC are widely distributed in the studied regions. Obviously, these plants are commonly used by respondents. In some cases, the most common species reported are in agreement with those cited from Bulgaria neighbouring countries – *Matricaria chamomilla* (Axiotis et al., 2018; Tsioutsiou et al., 2019), *Urtica dioica, Hypericum perforatum* (Akaydin et al., 2013). Only *Hypericum perforatum* and *Matricaria chamomilla* were mentioned in previous survey in Bulgaria (Kozuharova et al., 2013). UV evaluates use reports of the species known locally (Axiotis et al., 2018). The high UV indicates the extensive use of listed species for the treatment of various ailments (Hussain et al., 2019).

Conclusion

To our knowledge this is the first survey in Bulgaria only with selected respondents. The number of plants species reported reflects well-preserved traditional knowledge in the studied region. Relatives were pointed as a basic source of information indicating that this valuable heritage passed down through the generations. So, traditionally used in the studied region species were listed in the paper. Quantitative analysis revealed the significance of species for local people. Majority of cited species are wild. From the point of view of sustainable ecology information to local people about the importance of preservation of natural habitats of wild species should be provided.

Acknowledgments

This work has been supported by the Bulgarian Ministry of Education and Science, grant no. RD-08-67/25.01.2021.

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Received: 09.06.2021
Accepted: 17.11.2021

ECOLOGIA BALKANICA
2021, Vol. 13, Issue 2 December 2021 pp. 155-159

A Comparative Study on Callus Induction and Indirect Morphogenesis in Two Papaveraceae Species

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Abstract. The aim of the study was callus induction of two species of Papaveraceae family (*Chelidonium majus* L. and *Glaucium flavum* Crantz) using various types of explants on *in vitro* media supplemented with different combinations of plant growth regulators (PGRs). Their indirect morphogenetic capacity has been subsequently investigated, too. Entire *in vitro* seedlings or organs excised from them were used as explants for callus induction. High percentage of seedlings and root explants from *G. flavum* formed calli in almost all tested combinations of PGRs. In *C. majus*, the roots were the most appropriate among all tested explant types, followed by seedlings and hypocotyls, regardless of the PGRs used for stimulation of callogenesis. For both studied species, only in the combination with N-(2-Chloro-4-pyridyl)-N'-phenylurea (4-CPPU) no callogenesis was observed. The combination of α-Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) stimulated morphogenetic processes in the seedlings – shoot organogenesis in *C. majus* and somatic embryogenesis in *G. flavum*.

Key words: Greater celandine, *Chelidonium majus* L., Yellow horned poppy, *Glaucium flavum* Crantz, *in vitro* cultivation.

Introduction

Chelidonium majus L. and *Glaucium flavum* Crantz are both medicinal species belonging to Papaveraceae family and biosynthesize large amounts of isoquinoline alkaloids with promising biological activities. *G. flavum* is a vulnerable species with a decreasing area of occurrence, whose gathering is forbidden from its native populations in Bulgaria according to the Medicinal Plants Act (2000). The main alkaloids in this species are glaucine and protopine. *G. flavum* is mainly famous for its antitussive activity (Aleshinskaya, 1976; Stoykov, 1964). Additionally, its newly found antiproliferative and anticancer activities have recently been investigated (Bournine et al., 2013). Its medicinal properties and narrow distribution demand its *ex situ* conservation. *C. majus*, on the other hand, is a valuable medicinal species with common occurrence. It contains many alkaloids, among them chelidonine, chelerythrine, sanguinarine, protopine, and berberine. It has a wide variety of biological activities: antiulcer, anticancer, hepatoprotective, anti-arthritic, anti-inflammatory and analgesic, antibacterial,

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antifungal, antiviral, etc. (Maji & Banerji, 2015). The species is an ingredient in some remedies and becoming more and more popular object of numerous studies aimed at revealing its cytotoxic and antitumor activity. It is cultivated as a crop in some countries in Europe (Zielinska et al., 2018). The rising interest in the species makes it a topical object of different ways of *in vitro* cultivation that might be useful in future breeding of *C. majus'* genotypes which could possess desired characteristics. Overall, it is important that different *in vitro* methods be selected which could facilitate the multiplication of both species.

Indirect morphogenesis is among the most successful *in vitro* methods applied to the species of Papaveraceae family to which *G. flavum* and *C. majus* belong. In the indirect morphogenesis the cultures pass through a stage of callus formation, which facilitates the use of methods such as indirect somatic embryogenesis and morphogenesis, suspension and protoplast cultivation. The successful application of suitable *in vitro* methods for rapid propagation would be beneficial for the multiplication of cultivars with higher alkaloid content than that of plants grown in their natural habitats. Auxins and cytokinins are the most commonly used plant growth regulators (PGRs) and usually combined in the media for *in vitro* tissue cultures. It is well known that auxins play crucial role in callus formation and cytokinins support their effect in the callus induction (Zakaria et al., 2011). The number of PGR combinations applied for callogenesis in Papaveraceae species is small. The combinations α-Naphthaleneacetic acid (NAA) + Kinetin (Kin); NAA + 6-Benzylaminopurine (BAP); 2,4-Dichlorophenoxyacetic acid (2,4-D) + BAP; and 2,4-D + Kin are the most commonly used in studies. *G. flavum* is a recalcitrant for *in vitro* cultivation species, which explains the limited number of research papers on this aspect (Doycheva et al., 2017; Mohamed et al., 2014). However, *C. majus* is more popular as an object of study through different *in vitro* methods – callogenesis, somatic embryogenesis, direct and indirect organogenesis (Otgonpurev et al., 2013; Vântu, 2011; Vinterhalter & Vinterhalter, 2002; Zielinska et al., 2018). The object of the study was callus induction of two species of Papaveraceae family (*C. majus* and *G. flavum*) and comparison of their propagation response using various types of explants on *in vitro* media supplemented with different combinations of plant growth regulators.

Materials and Methods

Seeds were gathered from native plants of *C. majus* grown in the village of Mramor, near Sofia (N 42.78855, E 23.27943), and from native plants of *G. flavum* grown on the Black sea coast located near the city of Pomorie, Bulgaria (N 42.58634, E 27.63191). Sterilized seeds were germinated on a B5 agar solidified medium (Gamborg et al., 1968). Entire *in vitro* seedlings or organs excised from them (hypocotyls and cotyledons from *C. majus* and roots from both species) were used as explants for callus induction. Explants were cultivated in Petri dishes with MS-based medium (Murashige & Skoog, 1962) supplemented with different combinations and concentrations of the following PGRs: NAA, 2,4-D, Kin, BAP, Thidiazuron (TDZ), N-(2-Chloro-4-pyridyl)-N'-phenylurea (4-CPPU), p-Chlorophenoxy-acetic acid (4-CPA), and 4-Amino-3,5,6-trichloropicolinic acid (Picloram). Seven media differing in their PGRs combinations and/or concentrations were tested: 1.0 mg/l 2,4 D + 0.5 mg/l TDZ + 0.5 mg/l BAP (medium MS1); 1.0 mg/l 2,4 D + 0.5 mg/l TDZ + 0.2 mg/l BAP (MS2); 1.0 mg/l 4-CPPU + 0.5 mg/l TDZ + 0.2 mg/l BAP (MS3); 1.0 mg/l 4-CPA + 0.5 mg/l TDZ + 0.2 mg/l BAP (MS4); 1.2 mg/l NAA + 0.7 mg/l BAP (MS5); 1.0 mg/l NAA + 0.5 mg/l BAP (MS6); 1.0 mg/l Picloram + 0.5 mg/l TDZ + 0.2 mg/l BAP (MS7). The media were solidified with 7 g/l Plant agar and autoclaved at 121°C for 20 minutes.

Cultivation was performed at 23,2°C under dark conditions. Each treatment had 3 replications with 20 explants in each. The frequency of callus formation is presented as a percentage of the number of primary explants, and its amount is determined approximately. The statistical significance was evaluated using Student's t-test. ($p \leq 0.05$).

Results and Discussion

High percentage of seedlings and root explants from *G. flavum* formed calli in all tested combinations of PGRs (Table 1). Only in the combination with 4-CPPU no callogenesis was observed. During the sub-cultivation of the formed calli on the same media on which they were induced, somatic embryogenesis (SE) was observed when the combination NAA + BAP was applied to *G. flavum* on media MS5 and MS6, supplemented with different concentrations of these PGRs. SE was observed in 51.59%±22.81 of the seedlings cultivated on MS6 (1.0 mg/l NAA+0.5 BAP) while 31.02%±17.58 of those cultivated on MS5 (1.2 NAA + 0.7 BAP) formed somatic embryos. Although SE occurred in higher frequency on the medium supplemented with lower concentrations of NAA and BAP, the average number of somatic embryos formed per explant did not differ considerably on media MS6 and MS5: 23±6.18 (1.0 NAA + 0.5 BAP) and 22±0.51 (1.2 NAA + 0.7 BAP). SE was not observed on root explants on any of the applied PGR combinations. Successful callogenesis on MS6 was reported in previous studies on *G. flavum* (Doycheva et al., 2017; Mohamed et al., 2014). However, until now this combination of PGRs has not induced SE in this species. The little increase in NAA and BAP concentrations did not increase the amount of calli induced or the number of somatic embryos formed in *G. flavum*.

Table 1. Callus formation and quantity of seedlings and root explants of *G. flavum*. *Legend:* Mean ± standard deviation; mean values followed by different letter within the same column are statistically different. N/A – not available. (-) no callus; (+) poor callus quantity; (++) moderate callus quantity; (+++) large callus quantity.

Medium and PGRs (mg/l)	Seedlings		Roots	
	Callus formation (%)	Callus quantity	Callus formation (%)	Callus quantity
MS + 1.0 2,4 D + 0.5 TDZ + 0.5 BAP	100.0a±0.0	(++)	100.0a±0.0	(++)
MS + 1.0 2,4 D + 0.5 TDZ + 0.2 BAP	100.0a±0.0	(+)	90.0a±0.0	(++)
MS + 1.0 4-CPPU + 0.5 TDZ + 0.2 BAP	0.0b±0.0	(-)	0.0b±0.0	(-)
MS + 1.0 4-CPA + 0.5 TDZ + 0.2 BAP	100.0a±0.0	(++)	100.0a±0.0	(+)
MS + 1.2 NAA + 0.7 BAP	100.0a±0.0	(++)	100.0a±0.0	(+)
MS + 1.0 NAA + 0.5 BAP	100.0a±0.0	(++)	100.0a±0.0	(+++)
MS + 1.0 Picloram + 0.5 TDZ + 0.2 BAP	100.0a±0.0	(+)	N/A	N/A

Of all the PGRs used for stimulation of callogenesis in *C. majus*, roots were the most appropriate for callogenesis among all tested explant types (Table 2). That was observed by other authors in *Papaver somniferum* L., too (Laurain-Mattar et al., 1999; Pathak et al., 2012). In the case with *C. majus* roots were followed by seedlings and hypocotyls. Cotyledons formed calli at the smallest degree. Callogenesis in this species was the most common in the explants cultivated on media MS5 and MS2. The PGR combination 4-CPPU + TDZ + BAP did not induce callogenesis in any of the used explant variants. The increase in the concentration of NAA and BAP (media MS6 vs. MS5) resulted in an increase in the callogenesis on hypocotyls and especially on cotyledons from *C. majus*.

The combination of NAA and BAP stimulated morphogenetic processes in the seedlings of *C. majus* and *G. flavum*, but shoot organogenesis and not somatic embryogenesis was stimulated in the former. But in contrast to *G. flavum*, only 10% of *C. majus* seedlings formed shoots. On average, 58 shoots per explant were induced. The shoots were transferred on B5 medium, where rhizogenesis was observed.

The combination of PGRs which included 4-CPPU inhibited callogenesis in both species. This could be explained with the presence of this cytokinin itself or with the disruption of the auxin:cytokinin ratio, in which normally auxins predominated over cytokinins. The supplementation of auxins ten times more than cytokinins is the most frequently applied ratio in PGRs combinations for callogenesis. Such proportion was used in all other combinations used except for MS1 where auxins and cytokinins were in equal concentrations.

Conclusions

The current research in *C. majus* and *G. flavum* revealed dependence of *in vitro* callogenesis on the type of the explant used. Thus, root explants were the most appropriate for callus formation in both species. Moreover, callogenesis could be affected by the type of PGRs applied, their combinations and concentrations. Therefore, the PGR combination containing 4-CPPU didn't induce callogenesis in both species.

The morphogenetic response of callus also depended on the plant species. The two studied species took different morphogenetic directions – somatic embryogenesis in *G. flavum* and shoot organogenesis in *C. majus*.

Acknowledgements

The scientific research for *Chelidonium majus* was supported by the Bulgarian National Science Fund, Bulgarian Ministry of Education and Science (Project КП-06-M26/4 from 01.12.2018). The research for *Glaucium flavum* was financially supported by the Bulgarian Ministry of Education and Science under the National Research Programme "Young scientists and postdoctoral students" approved by DCM № 577 / 17.08.2018.

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Received: 16.08.2021
Accepted: 18.10.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 161-171

Phytochemicals and Antimicrobial Potential of Dry Ethanol Extracts from Ailanthus altissima – An Invasive Plant Species for the Bulgarian Flora

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Abstract. Dry ethanol extracts of flowers, leaves, and stem bark of *Ailanthus altissima* were subject to the determination of the chemical components by GC/MS analysis. Fresh plant material was used for obtaining the plant extracts (*in vacuo*). Forty-seven compounds were identified from different plant parts. The oxygenated aliphatics were the best represented group in the extracts (flower – 55.44%, leaf – 51.57%, bark – 33.19%), followed by oxygenated monoterpenes (bark – 25.42%; leaf – 15.87%), and diterpenes (flower 14.22%). The major constituent in the flower and leaf extracts was (3Z)-hexenyl hexanoate (28.59%, 12.61%, respectively), while in the bark extract - α-terpinyl acetate (15.55%). The distribution by functional groups, in relation to the total oxygen-containing components, pointed out that the esters were predominated in the three extracts analyzed, followed by alcohols and acids. The EtOH extracts of *A. altissima* were studied for *in vitro* antimicrobial activity against gram-positive and gram-negative bacterial strains. The study results showed that the leaf and bark extracts of *A. altissima* inhibited the growth of six of the tested pathogenic strains. The largest zones of inhibition were measured of ethanol leaf extracts against *Bacillus subtilis* ATCC 6633 (24.00 ± 0.11 mm) and *Klebsiella* (clinical isolate – 23.00 ± 1.24 mm). To the best of our knowledge, this is the first study that investigates the phytochemical composition and the antimicrobial activity of the extracts obtained from *Ailanthus altissima* from Bulgaria. Furthermore, our results revealed that the leaf and bark ethanol extracts could be used as natural antimicrobial agents.

Key words: *Ailanthus altissima,* aerial parts, ethanol dry extract, GC/MS, antimicrobial activity, Simaroubaceae.

Introduction

Ailanthus altissima (Mill.) Swingle (Simaroubaceae) is a tree from the Far East, introduced in Europe in the 18th century for decorative purposes and now it is found on all continents except Antarctica (Kowarik &

Säumel, 2007; Sladonja et al., 2015). Today it is considered one of the worst invasive plant species, both in Europe and in various regions of the world. For Bulgaria, the species is in the "top 10" among the most problematic alien plant species (Petrova et al., 2012).

On the other hand, it has been found that the tree contains several valuable phytochemicals such as alkaloids, flavonoids, quassinoids, phenylpropanoids, triterpenoids, volatile oils, sterols and others, which are responsible for the many proven biological activities - antimicrobial, antifungal, antiviral, antitumor, herbicidal, anti-inflammatory, and others (De Martino & De Feo, 2008; Kundu & Laskar, 2010; Albouchi et al., 2013; Kožuharova et al., 2014; Sladonja et al., 2015; Li et al., 2021). The subject of research is different plant parts of *A. altissima* (mostly bark, leaves, fruits), with various extraction methods and identification of the chemical components. All this is the reason for the differences in the quantitative and qualitative composition of the studied plant products - extracts and essential oils (Andonova et al., 2021; Hadadi et al., 2020; Chouhan et al., 2017).

There are many studies on the biological activity of *A. altissima* extracts, but data for component determination by gas chromatography-mass spectrometry analysis are scarce. Caboni et al. (2012) identified a total of 14 components of wood, leaf, bark, and root methanol extracts, and they found that unsaturated aldehydes are responsible for the nematicide activity. Fewer components (twenty-seven) in leaf ethanol extracts of *A. altissima* determined Lungu et al. (2016) when studying their antioxidant and phytotoxic activity. Forty-one chemical components in fruit methanol extracts and thirty-five components from fresh leaves of *A. altissima* were identified by Panasenko et al. (2020). They point out the presence of compounds with antioxidant and anti-inflammatory activities, such as α-tocopherol in fruits.

Increasing microbial resistance to drugs due to excessive use of antibiotics, and on the other hand, the negative perception of synthetic preservatives increase the interest in the search for new natural alternatives (Swamy et al., 2016; Chouhan et al., 2017). Many studies have focused on the antibacterial properties of plant extracts and their possible use as reliable inhibitors of bacterial and fungal growth (Zazharskyi et al., 2020). Aissani et al. (2018) prove the antimicrobial activity of *A. altissima* wood aqueous extract against *Pseudomonas aeruginosa* and conclude that this extract can be used as a natural antimicrobial agent. Other authors report that the methanol extracts of *A. altissima* leaves and their hydro-distilled residues are efficient against Gram-positive bacteria, but not active against Gram-negative bacterial strains and the yeast *Candida albicans* (Albouchi et al., 2013). The composition of ethanol fruit extracts (*in vacuo*) of *A. altissima* and their antimicrobial activity was investigated by Zhao et al. (2005) and they found that individual ingredients (stigmasterols) showed moderate activity against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhimurium*. The N-butanol fraction of methanol fruit extract is well active against *P. aeruginosa* and *S. typhi* and moderately active against the fungus *Microsporum canis* (Khan et al., 2016). Significant inhibitory activity against *E. coli* and *S. aureus* is demonstrated in the green synthesis of zinc oxide and copper oxide nanoparticles with water extracts of *A. altissima* fruit and leaves (Awwad & Amer, 2020; Awwad et al., 2020).

In Bulgaria, the first chemical investigations on *A. altissima* species belong of our team. They are related to the carotenoid content of stem bark (Zhelev et al., 2016) and on the essential oil composition of its various plant parts (Andonova et al., 2021). The results of our previous research necessitated our interest in investigating other possible sources of phytochemicals of this plant species. Therefore, the aim of the present study was to evaluate the chemical composition of dry ethanol extracts obtained from the bark, leaves, and flowers of *A. altissima*, and their antimicrobial activity against human pathogenic strains.

Materials and Methods

Plant material and obtaining the plant extracts

Flowers, leaves, and stem bark of *A. altissima* were collected from the city of Plovdiv, Bulgaria, in June-July 2020, and were identified by Prof. Ivanka Dimitrova-Dyulgerova at the Department of Botany, University of Plovdiv "Paisii Hilendarski" (Fig.1). Fresh plant parts (100 g flowers, leaves, and bark) were grounded and soaked in an Erlenmeyer flask with 1l (96%) ethanol (1:10 w/v), to complete exhaustion of the herb for 10 days with intermittent stirring. The extracts were filtered using Whatman filter paper no.1, and the filtrates were evaporated under reduced pressure and dried using a rotary evaporator (Buchi, Rotavapor R-300) at 50°C. All plant samples were stored in the dark at 4°C before analysis. For sample preparations for microbiological analysis, 2 g of each extract was dissolved in distilled water in a ratio of 1:2.

(a) (b) (c)

Fig. 1. Analyzed plant parts of *Ailanthus altissima*.
(a) - flowers, (b) - leaves, and (c) - stem bark (the photos taken by the authors).

Gas Chromatography/Mass Spectrometry (GC/MS) and GC-FID analyses

Extract of 10.0 mg, dissolved in 1.0 ml absolute alcohol, was injected into a gas chromatograph Agilent GC 7890A and in a mass spectral detector Agilent MD 5975C, column HP-5MS with parameters: length – 30 m, diameter - 0.32 mm, and film-coating thickness - 0.25 µm. The temperature program was as follows: initial temperature – 100°C, 2 min retention, increase to 180°C with 15°C/min, 1min retention, increase to 300°C with 5°C/min, 10min retention; injector and detector temperatures – 250°C. Helium was used as a carrier gas, at flow speed - 1.0 ml/min; mass-detector scan range - m/z=50-550; injected sample volume - 1 µL in flow split ratio 20:1. The compounds were identified by comparing retention times and relative Kovats (RI) indices with those of standard substances and mass spectral data from The Golm Metabolome Database - GMD (Hummel et al., 2010) and NIST'08 - National Institute of Standards and Technology, USA.

Antibacterial activity assay

Test microorganisms

Strains of pathogenic bacteria that are reported as causing infections, toxic infections, and toxicosis were used. The strains were supplied by the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC). The following Gram-positive bacteria were used: *Listeria* *monocytogenes* NCTC 11994*, Staphylococcus aureus* ATCC 25093, *Bacillus subtilis* ATCC 6633, *Bacillus cereus*; and the Gram-negative bacteria - *Escherichia coli* ATCC 8739, *Salmonella enterica subsp. Enterica serovar abony* NCTC 6017, *Pseudomonas aeruginosa* ATCC 6027*, Proteus vulgaris* ATCC 6380*, and Klebsiella* (clinical isolate).

Determination of the titer of microbial suspensions

From two consecutive tenfold dilutions of the suspensions in saline, surface sowing was done on the relevant selective media for the test cultures - 1 mL of an appropriate dilution of each suspension was evenly distributed in four plates with previously poured and dried media. The titer of the suspension was determined by the formula:

$N = \Sigma C / V \times (n1 + 0{,}1 \times n2) \times d$, cfu / cm^3 (1)

where:

V – sowing material volume; ΣC – sum of the listed colonies;

n1 – number of plates from the first dilution in which colonies are listed;

n2 – number of plates from the second dilution in which colonies are listed;

d – dilution factor corresponding to the first dilution in which colonies are listed.

The antibacterial activity was determined by a modification of the agar diffusion method, through measuring the zones of pathogen growth inhibition around metal rings, in which a certain amount of the tested material was imported. Selective media for the test cultures were inoculated with pathogen suspensions prepared from a 24-hour culture on oblique PCA. From a suitable tenfold dilution of the suspension, the molten and cooled to 45 - 50°C selective media were inoculated. The current concentration of the cells in the culture medium was equated to the concentration of the dilution suspension because 1 mL of suspension was inoculated into 99 mL of medium. After solidification of the media, sterilized metal rings of diameter Ø = 6 mm were placed on their surfaces, in which 0,10 and 0,15 µL of filtrate from the extract were imported, respectively. The test cultures were incubated at 37°C. The diameter (cm) of the growth inhibition zones of the test cultures was measured at the 24th and 48th hours, and a comparative assessment of their antibacterial activity was made. The final DMSO content was 5% (v/v), and this solution was used as a negative control. Chlorhexidine (100 µL) was used as a positive control.

Results and Discussion

Chemical composition of ethanol extracts from aerial parts of Ailanthus altissima

The extracts' yields are as follows: bark - 0.608 g, leaf – 0.630 g, flower – 0.573 g. They are viscous liquids of dark-brown and dark-green color, with a specific odor. A total of 47 components were identified in the three extracts tested (Table 1) and their chemical composition is as follows:

In flower extract, 31 components were identified (representing 99.27% of the total content). Twenty-one of them were in a concentration above 1%, and the other ten – were less than 1%. The major compounds (over 3%) were: (3Z)-hexenyl hexanoate (28.59%), oleic acid (11.04%), dihydro-eudesmol (6.66%), (3Z)-hexenyl butanoate (5.46%), phytol (5.23%), phenyl ethyl 2-methylbutanoate (4.42%), (6E,10E)-pseudo phytol (3.25%) and linoleic acid (3.09%).

There is a lack of literature data on the composition of plant's flower extracts. A parallel could be drawn with our previous study on volatiles in *A. altissima* flowers (Andonova et al., 2021), in which 44 components were identified, 8 of them were major compounds (over 3%): *β*-caryophyllene (16.98%), germacrene D (16.24%), n-tricosane (8.33%), methyl hexadecanoate (7.87%), linoleic acid (6.84%), *α*-humulene (5.30%), n-pentacosane (3.70%), and linolenic acid (3.38%). As can be seen, only linoleic acid is among the main constituents of the essential oil and the ethanol extract.

In leaf extract, 31 components were identified, which represents 98.45% of the total composition. Twenty-two of them were in a concentration above 1%, and the other nine – were less than 1%. The main components in the extract (over 3%) were: (3Z)-hexenyl hexanoate (12.61 %), isopropyl hexadecanoate (10.77%), lavandulyl acetate (9.65%), (3Z)-hexenyl 2-methyl butanoate (8.05%), β-caryophyllene (5.88%), dihydro-eudesmol (5.57%), phenyl ethyl butanoate(5.20%), (2E,4E)-nonadienol (5.07%), linoleic acid (4.61%), and palmitic acid (4.39%). Lungu et al. (2016) identify 27 components in 70% ethanol extracts of dry grounded leaves of *A. altissima*, growing spontaneously in Romania (obtained by the reflux and ultrasound methods), among which are: saturated fatty acids, sterols, vitamin E, neophytadiene, phytol, and others. In methanol leaf extract, Caboni et al. (2012) identify four plant metabolites: acetic acid, 5-hydroxymethylfurfural, nonanal and hexanoic acid. Panasenko et al. (2020) found a greater variety of phytochemicals in *A. altissima* methanol leaf extracts from Ukraine (35 biologically active compounds), as eleven of them – were over 3%, and the main ones among them were phytol – 21.15%, hexadecanoic acid – 8.53%, α-tokospiro A – 8.14%, 2-C-methyl-myo-inositol – 7.78%. In comparison with our results, the difference in the main components is obvious (only palmitic acid is present in both types of extracts). Our previous GC/MS analysis (Andonova et al., 2021) on the essential oil of *A. altissima* leaves identify 41 components with fatty acids predominating (oleic acid is the best-represented compound – 22.94%).

In stem bark extract, 42 components were identified, which represents 98.76% of the total composition. Twenty-two of them were found to be in a concentration above 1%, and the other twenty – less than 1%. The main components in the extract (over 3%) were: α-terpinyl acetate (15.55%), oleic acid (9.99%), tetracosane (9.04%), linoleic acid (8.85%), α-curcumene (5.75%), palmitic acid (3.71%), nerol (3.42%), β-eudesmol (3.36%), and phenyl ethyl butanoate (3.02%). Caboni et al. (2012) determined in methanol bark extract by GC/MS-analysis only three metabolites: acetic acid, nonanal and hexanoic acid. Andonova et al. (2021) found 41 chemicals in stem bark essential oil, among which five are the major compounds - oleic acid (30.21%), β-caryophyllene (6.7%), γ-cadinene (5.94%), palmitaldehyde (5.87%) and palmitic acid (4.31%).

The distribution of the components by chemical groups (Fig. 2) showed that aliphatic oxygen derivatives predominated in the three extracts, followed by oxygenated monoterpenes (in bark and leaves), diterpenes (in flowers), sesquiterpene hydrocarbons (in bark), and the other groups were less represented and their distribution can be seen in Fig. 2.

Oxygenated aliphatics also predominated in the essential oils of leaves and bark, while sesquiterpene hydrocarbons predominated in the essential oil of flowers (Andonova et al., 2021).

The distribution of the components by functional groups in the ethanol extracts showed the highest content of esters (33.83% in bark – 61.33% in leaves), followed by alcohols (14.88% in leaves – 24.89% in flowers), acids, and hydrocarbons (Fig. 3). Aldehydes and ketones were poorly represented, lactones were present only in bark extracts, and phenols were not identified in the extracts.

The differences between the quantities of the identified components in the extracts and the literature data are due to the plant's growing conditions, the technological parameters of the extraction, and its analysis.

Antibacterial activity of Ailanthus altissima ethanol extracts

The zones of growth inhibition of the test cultures are presented in Table. 2 and Fig. 4. The study results showed that *A. altissima* leaves had the most substantial inhibitory effect against *Bacillus subtilis* ATCC 6633 – 24.00 ± 0.11 mm and *Klebsiella* (clinical isolate) – 23.00 ± 1.24 mm.

Table 1. Chemical composition of the ethanol extracts of *Ailanthus altissima* aerial parts. *Legend:* RT - Retention Time; RI - Kovats Retention Index, calculated by authors; TIC - Total Ion Current; nd - not detected.

Peak	RT	RI	Compound	*Ailanthus altissima*, % of TIC		
				flower	**leaf**	**stem bark**
1	4.49	1174	Furfuryl butanoate	nd	1.28	nd
2	4.68	1190	(3Z)-Hexenyl butanoate	5.46	0.34	0.68
3	5.20	1218	(2E,4E)-Nonadienol	nd	5.07	0.39
4	5.30	1233	Nerol	nd	0.55	3.42
5	5.41	1231	(3Z)-Hexenyl 2-methyl butanoate	0.99	8.05	0.56
6	5.60	1288	Lavandulyl acetate	0.78	9.65	1.29
7	6.11	1316	δ-Terpinyl acetate	nd	1.04	0.82
8	6.23	1345	α-Terpinyl acetate	1.17	2.49	15.55
9	6.55	1366	α-Methyl benzyl butyrate	2.02	0.73	0.96
10	6.64	1375	(3Z)-Hexenyl hexanoate	28.59	12.61	1.42
11	6.82	1386	Isobutyl phenylacetate	1.10	2.35	0.73
12	7.01	1401	n-Tetradecane	0.48	2.10	1.85
13	7.08	1416	β-Caryophyllene	1.23	5.88	1.91
14	7.16	1439	Phenyl ethyl butanoate	1.79	5.20	3.02
15	7.26	1453	Geranyl acetone	0.74	1.28	1.37
16	7.41	1465	Linalool isovalerate	nd	nd	1.84
17	7.55	1476	α-Curcumene	0.55	2.27	5.75
18	7.64	1481	γ-Curcumene	0.48	0.89	0.42
19	7.72	1486	Phenyl ethyl 2-methylbutanoate	4,42	1.50	nd
20	7.76	1488	β-Selinene	nd	nd	2.86
21	7.97	1497	α-Selinene	nd	nd	1.77
22	8.15	1511	β-Curcumene	1.07	0.21	0.49
23	8.26	1556	Lauric acid	nd	nd	1.05
24	8.41	1562	Geranyl butanoate	nd	nd	0.56
25	8.50	1568	Octyl hexanoate	2.73	1.64	0.42
26	8.56	1576	Decyl butyrate	0.20	0.58	0.34
27	8.71	1611	Tetradecanal	1.54	1.43	0.77
28	9.07	1640	Phenyl ethyl hexanoate	2.59	0.67	0.91
29	9.17	1649	β-Eudesmol	1.92	1.32	3.36
30	9.26	1654	α-Eudesmol	2.01	2.50	1.43
31	9.37	1662	dihydro-Eudesmol	6.66	5.57	2.70
32	9.61	1671	epi-β-Bisabolol	nd	nd	1.11
33	9.67	1675	β-Bisabolol	nd	nd	0.69
34	9.86	1690	(Z)-α-trans-Bergamotol	nd	nd	0.86
35	10.21	1699	(2Z,6Z)-Farnesol	nd	nd	0.74
36	10.50	1706	δ-Dodecalactone	nd	nd	0.82
37	10.82	1718	Methyl eudesmate	0.33	0.87	0.28
38	11.93	1826	(E)-Nerolidyl isobutyrate	0.17	0.61	0.25
39	14.49	1953	Phytol	5.23	nd	nd
40	14.61	1971	Palmitic acid	1.39	4.39	3.71
41	14.73	1992	Ethyl palmitate	nd	nd	2.80
42	16.67	2018	(6E,10Z)-Pseudo phytol	5.64	nd	nd

43	16.85	2026	Isopropyl hexadecanoate	nd	10.77	0.98
44	16.94	2055	(6E,10E)-Pseudo phytol	3.25	nd	nd
45	18.05	2133	Linoleic acid	3.09	4.61	8.85
46	18.28	2142	Oleic acid	11.04	nd	9.99
47	21.58	2400	Tetracosane	0.61	nd	9.04
			Total identified,%	**99.27**	**98.45**	**98.76**

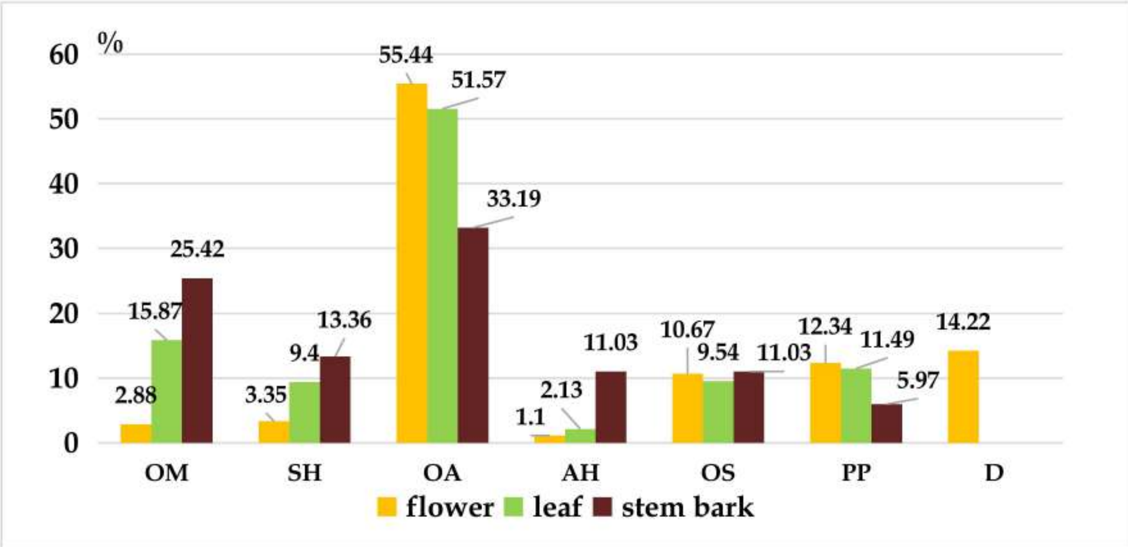

Fig. 2. Composition by chemical groups of ethanol extracts, obtained from fresh aerial parts of *Ailanthus altissima* (%). *Legend:* OM – Oxygenated monoterpenes; SH - Sesquiterpene hydrocarbons; OA - Oxygenated aliphatics; AH - Aliphatic hydrocarbons; OS - oxygenated sesquiterpenes; PP - Phenyl propanoids; D – Diterpenes.

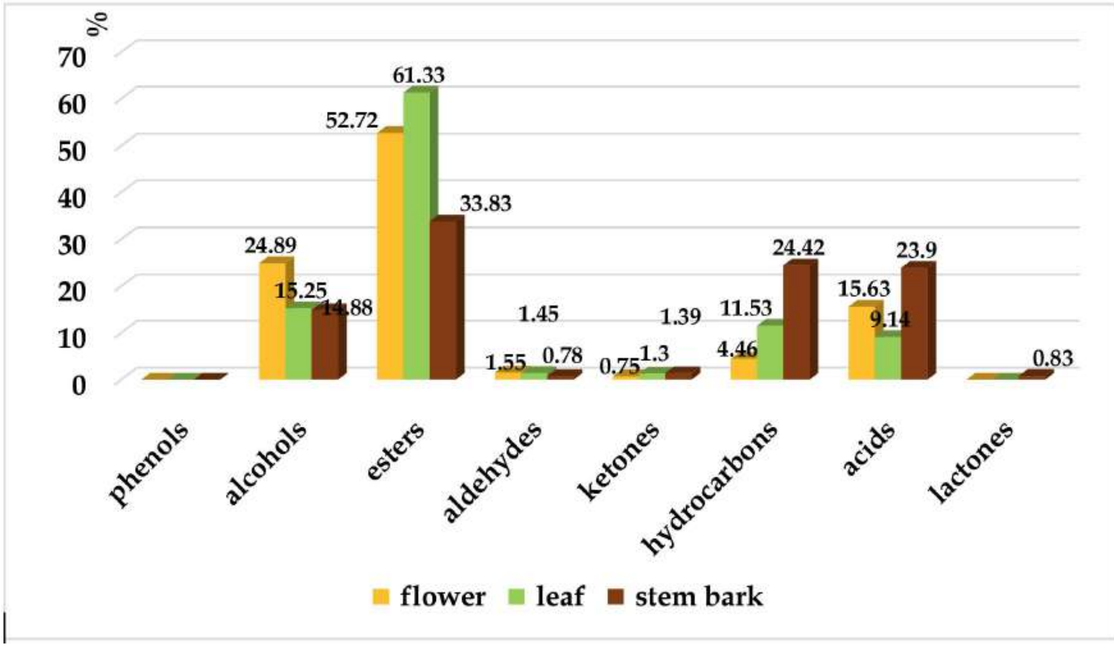

Fig. 3. Composition by functional groups of ethanol extracts, obtained from fresh aerial parts of *Ailanthus altissima* (%).

The most susceptible to the effect of the *A. altissima* leaves was *B. subtilis* which was still lower than that of the positive control (39.00 ± 0.12). The inhibitory activity of the plant's leaf extract against *Klebsiella* was determined as closest, even higher than that of the positive control sample (21.00 ± 0.81). It is known that Gram (+) bacteria are generally more sensitive to plant extracts than Gram (-). Gram (-) bacteria have an outer membrane and a unique periplasmic space that is not found in Gram (+). Gram (-) bacteria's resistance to antibacterial substances is due to their outer membrane, rich in hydrophilic molecules, serves as a barrier to the penetration of many antibiotic molecules. Resistance also binds to enzymes in the periplasmic space that can destroy molecules that have entered from the outside. Some plant extracts do not fully follow this trend. The antimicrobial activity of plant extracts was widely studied by several authors. The methanol leaf extract and its different polar subfractions inhibited significantly the growth of gram-positive bacteria (*Listeria monocytogenes, Staphylococcus aureus, Bacillus subtilis*) and gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli)* (Rahman et al., 2009). The methanol extracts of *A. altissima* leaves and their hydrodistilled residues were effective against Gram-positive bacteria (*S. aureus, Enterococcus feacium, Streptococcus agalactiae* and *Bacillus subtilis*). Neither the Gram-negative bacteria (*E. coli, Enterococcus feacalis* and *Salmonella typhimurium*) nor the yeast (*Candida albicans*) were inhibited by these extracts (Albouchi et al., 2013). The *A. altissima* acetone leaf extract was active against *E. coli*. Both acetone and methanol: dichloromethane extracts had higher activity against *C. albicans* than a standard drug amphotericin B (Poljuha et al., 2017). Aissani et al. (2018) studied the antimicrobial activity of aqueous and methanol extracts of wood and bark of *A. altissima* against a set of Gram-positive and Gram-negative bacteria using disc diffusion and broth microdilution methods and reported antimicrobial activity of the wood extract against *P. aeruginosa* with a MIC of 10.46 mg/mL. The volatile oil and phenolic constituents of *A. altissima* leaves were efficient against Gram-positive bacteria, but not active against Gram-negative bacterial strains and the yeast *C. albicans* (Aissani et al., 2018). The results obtained in this study might be attributable to the occurrence of some specific components such as linoleic acid, β-caryophyllene, germacrene D, and methyl hexadecanoate. The antimicrobial potential of ethanol leaf extracts was found by Zazharskyi et al. (2020), but the zone of inhibition of *E. coli* and *Listeria monocytogenes* is weaker compared to our results.

Table 2. Inhibition zones of the pathogenic bacteria growth, mm. *Legend:* VF- Value of the filtrate; FE - Flower extract; LE - Leaf extract; SBE - Stem bark extract; CH – Chlorhexidine; *- There was no inhibitory activity.

Test microorganism	VF	Inhibition zone/mm			
		FE	LE	SBE	CH
Gram-negative bacteria					
Escherichia coli ATCC 8739	150 µL	-*	9.00 ± 0.47	6.00 ± 0.81	28.00 ± 0.02
	100 µL	-	-	-	
Salmonella enterica NCTC 6017	150 µL	-	-	-	24.00 ± 0.09
	100 µL	-	-	-	
Klebsiella (clinical isolate)	150 µL	-	23.00 ± 1.24	-	21.00 ± 0.81
	100 µL	-	-	-	

Pseudomonas aeruginosa ATCC 6027	150 µL	-	8.00 ± 0.09	5.00±1.01	18.00 ± 0.24
	100 µL	-	5.00 ± 0.24	-	
Proteus vulgaris ATCC 6380	150 µL	-	8.00 ± 1.24	8.00±1.47	-
	100 µL	-	7.00 ± 0.12	7.00±0.24	
Gram-positive bacteria					
Staphylococcus aureus ATCC 25093	150 µL	-	-	-	25.00 ± 0.18
	100 µL	-	-	-	
Bacillus subtilis ATCC 6633	150 µL	-	24.00 ± 0.11	14.00 ± 1.24	39.00 ± 0.12
	100 µL	-	10.00 ± 0.19	10.00 ± 0.18	
Bacillus cereus	150 µL	-	-	-	-
	100 µL	-	-	-	
Listeria monocytogenes NCTC 11994	150 µL	-	-	11.00 ± 0.09	21.00 ± 0.81
	100 µL	-	-	5.00 ± 0.51	

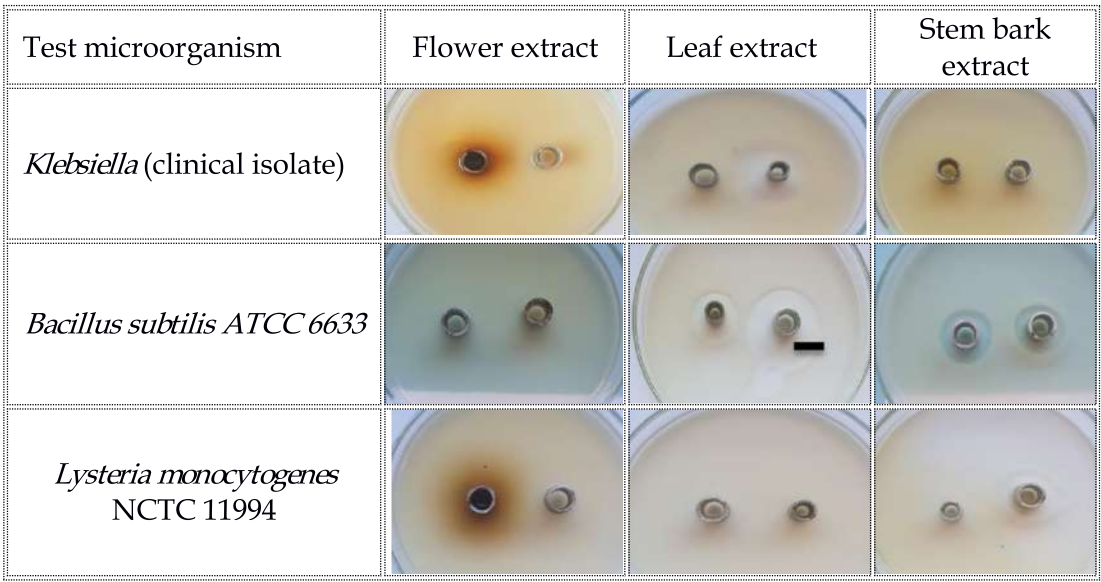

Fig. 4. Photos of the growth inhibition zones (over 10 mm) (Scale bar indicated 20 mm).

Our study confirms the antimicrobial activity of leaf and stem bark extracts. The flower extracts were not effective at all. As it is known, the activity of the main components of essential oils is in the following sequence: *phenols > alcohols > aldehydes > ketones > esters > hydrocarbons* (Gabrielli et al., 1988). In the extracts studied, the content of esters predominates, which would explain the lower antimicrobial activity against some of the strains studied.

Conclusions

Bringing clarity to the composition of the extracts of *A. altissima* is an important step for discovering new compounds into the composition of various natural products. The results obtained can add valuable information to the knowledge available about the species. In Bulgaria, the composition and antimicrobial activity of dry ethanol extracts of various aerial parts of the invasive alien tree *A. altissima* were studied for the first time. Forty-seven compounds were identified from different plant parts. The oxygenated aliphatics were the best-represented group in the extracts, followed by oxygenated monoterpenes, diterpenes, while other groups were poorly represented. Our findings show that the leaf and bark ethanol extracts of *A. altissima* exhibited antibacterial activity (especially of ethanol leaf extract against *Bacillus subtilis* ATCC 6633 and *Klebsiella*, clinical isolate). The extracts could be used as a natural medicinal resource, which requires further research.

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Received: 04.09.2021
Accepted: 02.11.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 173-197

Effect of Stand Density and Diversity on the Tree Ratio of Height to Diameter Relationship in the Park Stands of Southern Ukraine

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Abstract. The article investigated the influence of factors on the dependence of tree height on tree diameter in a park stand. The role of tree damage, density, and stand diversity as predictors in the dependence of tree height on its diameter was revealed. The hypothesis of the scale dependence of the influence of stand density on plant growth was tested. The number of plants that are within a radius of 3, 5, 7, 10 meters was determined for each of the recorded plant specimens. The diversity according Shannon of the plant community was estimated based on the information on the species composition of plants within a radius of 10 meters from the focal plant. The age of plants in the community was positively correlated with the diversity of vegetation in the surroundings of a particular plant. About 74.1% of the trees were found to have the signs of pathological damage. The best model to explain tree damage was a model that included as predictors plant species, its age, the diversity of the surrounding stand, and its density estimated from a 7-m radius sampling site. The GLM approach allowed to reveal that 83% of tree height variation can be explained by the information on tree and shrub species, plant condition (healthy plant or damaged one), its diameter and stand density. The stand density and the square of this index were found to be statistically significant predictors if the density was calculated for a sample area with a radius of 7 meters.

Key words: recreation, allometry, park management, ecosystem services, optimal density.

Introduction

Urban trees deliver ecosystem services including environmental regulation, resource provisioning, increasing biodiversity, and aesthetic enhancement (Song et al., 2020; Willis & Petrokofsky, 2017). The transformation of forest cover and the replacement of natural vegetation by buildings, roads, exotic vegetation, and other urban infrastructures is one of the greatest dangers to global biodiversity (Pereira et al., 2012). The trees in parks support biodiversity, store carbon, and improve microclimatic conditions (Heo et al., 2019; Kunakh et al., 2021). The trees in park plantations provide carbon sequestration by storing carbon as biomass (Nowak & Crane, 2002). As more and more land is set aside for urban development, identifying the effective wildlife management tools for urban forests becomes crucial for providing urban forest habitat to sustain bird and other wildlife populations (Lerman et al., 2014). The strategies for conserving and maintaining ecosystem services include efforts to reduce fragmentation, such as the creation of ecological networks. The parklands provide a landscape matrix that reduces fragmentation and contributes to biodiversity conservation (Watts et al., 2010). The formation of sustainable forest plantations in the urban environment provides normal living conditions for urban residents.

During the inventory process, landscape managers gather detailed information about trees that is needed for research and management (Berland & Lange, 2017; McPherson, 2014). The height to diameter ratio (HDR) is a tree-level slenderness index and is used to assess the stability of trees and stands (Vospernik et al., 2010). The variation in tree height-diameter ratios is important for a wide range of forest ecological problems. The variability in tree height-diameter ratios explains plant acclimation to the environment and tree competition for resources (Canham et al., 2006). Information about HDR is necessary to better understand forest ecological processes in a forest stand because this indicator depends on tree species, stand age, stand structure and density, soil type and moisture content, litter thickness, slope, elevation and exposure, stand development stage, climatic and natural disturbances (light, wind, snow, icing), forest care and species origin (Burton, 1993; Henry & Aarssen, 1999; Kamimura & Shiraishi, 2007).

Theoretical considerations and empirical studies of the height-diameter relationship show that it can be represented by an allometric function of diameter to the power of 2/3 (Greenhill, 1881; Norberg, 1988; O'Brien et al., 1995). The diameter of a tree's trunk can easily be measured (Song et al., 2020). The important tree parameters such as biomass and total leaf area can only be measured by destructive sampling. The allometric relationships allow to find the relationship between these parameters and can be used to study how tree biomass and structure change during growth (Mcpherson & Peper, 2012), and how the tree performance and benefits change over time (McPherson et al., 2016). An elastic stability and bending require trees to take a shape in which length increases in proportion to diameter in the power of 2/3 (McMahon, 1973). This relationship is appropriate for describing the properties of a column with equal bending and buckling resistance, which is a valid model for the tree trunks exposed to wind (Schniewind, 1962; Jaouen et al., 2007) and snow (King & Loucks, 1978; Fournier et al., 2013) in addition to gravity (Alméras & Fournier, 2009; Dargahi et al., 2019). Such a column maintains elastic similarity along the trunk (Rich, 1987). An elastic similarity results in $b=2/3$ and allows the tree to maintain a constant safety factor on both fracture and bending under the weight of the tree and wind force (McMahon, 1973; Dahle et al., 2017). There are also biological mechanisms beyond purely structural reasons for the existence of allometric dependence. Trees evolved not so much to align strength along the trunk (Fisler et al., 2020) as to even out damage to ensure survival (Vogt et al., 2015). Below the crown, this biological requirement is the same as the mechanical requirement, because in this range a break would destruct the tree to death. Inside the crown, the situation is different. The trees can survive the loss of a significant portion of the crown. Therefore, storing mass in the upper part of the trunk and crown is not biologically reasonable. Indeed, trees often lose their tops, most often in the upper third of the crown.

HDR is an index that is used to assess the sustainability of trees and stands (Sharma et al., 2019). Silvicultural systems that lead to high HD trees increase the risk of wind damage (Schelhaas, 2008; Wonn & O'Hara, 2001). High HDR values indicate that either the trees grew in a crowded stand with mutual support of neighboring trees, or they grew in an extremely open stand with no significant competition (Valinger &

Fridman, 2011; Vospernik et al., 2010). The variability in height to diameter ratio within and between habitat types may be either an adaptive or passive response to environmental gradients such as light, elevation, slope, aspect, or proximity to the coast (Schmidt et al., 2011). The ratio of tree height to trunk diameter also depends on the density of the stand: in a dense stand, trees with the same diameter are higher than in a less dense stand (Zeide & Vanderschaaf, 2002). The ratio of tree height and diameter is more variable in multi-species and multi-layered forests than in single-age, single-layer, and monodominant stands. The relationship between individual tree height and diameter in multispecies forests depends on the spatial distribution of trees in the stand, which is complicated by disturbances and changing stand dynamics (Schmidt et al., 2011). The relationship between height and diameter depends on height, aspect, slope, climate, and competition (Huang et al., 2000; Temesgen & Gadow, 2004). A stand at the forest edge, a stand in a large gap, or an unstable fragment of a stand are characterized by HDR features (Lohmander & Helles, 1987; Mitchell, 2013). HDR varies with tree spacing, even for the same species in the same stand, and extreme HDR can reach both extremely free stands and crowded stands (Nykänen et al., 1997). HDR also varies considerably in trees with an upper or lower canopy layer in the stand (Schmidt et al., 2010). HDR also depends on the root system of the tree, the width and length of the crown (Nykänen et al., 1997). The greatest influence on HDR is the distance between trees, competition, and stand density (Mäkinen et al., 2002; Slodicak & Novak, 2006).

The use of HDR information is essential for effective silviculture and forest management. By understanding the extent to which trees and stands are more susceptible to snow, icing, and wind damage, forest managers can better develop silvicultural prescriptions based on a range of HDRs (Nykänen et al., 1997; Valinger & Fridman, 1997; Wonn & O'Hara, 2001). HDR can be used to assess the efficiency and effectiveness of thinning, as thinning significantly affects HDR, both mid-stems and even the top layer of trees in a stand (Opio et al., 2000). The ratio of height to diameter is considered as an alternative to conventional procedures for assessing competition between cultivated trees and other vegetation (Opio et al., 2003). In addition, HDR can be used as an important predictor to describe the effects of competition in various forest models (MacDonald et al., 1990; Morris & MacDonald, 1991; Temesgen et al., 2005; Yang & Huang, 2018). HDR is used to assess tree viability and health. A tree with a higher HDR may have lower overall viability (Opio et al., 2000). HDR derived from any empirical HDR model can be viewed as a reference value that can be compared to HDR derived from tree height and diameter increase models, as well as height-diameter dependence models. Since HDR correlates significantly with tree crown ratio, trees with unfavorable properties of both HDR and crown ratio can be removed (Opio et al., 2003).

Thus, current knowledge suggests that HDR in natural forests or artificial plantations depends on tree species, stand age, stand structure, stand density, and a variety of other ecological parameters. The natural forests are examples of the most diverse ecological communities, while the forest plantations tend to be monodominant ecosystems. Urban parks have an intermediate position in terms of diversity between the natural forests and artificial plantations. However, there is very little information about what HDR in an urban park depends on. The objective of the study was to identify the factors that affect the dependence of tree height in a park stand on its diameter. We formulated the following hypotheses. Hypothesis 1. The probability of damage to trees in urban park plantations is decreased in more diverse stands. Hypothesis 2. The ratio of tree height to tree diameter depends negatively on damage, density, and positively on stand diversity. Hypothesis 3. The effect of urban park stand density is scale-dependent.

Materials and Methods

The Park-Monument of the landscape art of local importance "Healing Springs" was created by the decision of the Zaporizhzhya regional council from August 17, 1999 № 7 with the purpose of protection of the artificially created forest-park zone with natural healing springs. The park area of 3 hectares is located in the northern part of the village Terpeniya of Melitopol district of Zaporizhzhya region (Fig. 1). The area of the Park has a geometrically irregular configuration and extends from north-west to south-east for 392 m, and from north to south for 177 m. The average multi-year air temperature varied up to 2005 within 9.5°C, from 2005 till present time it was recorded at the level of 11.5°C. The average temperature in January was from –3.3°C to –3.5°C, in July 23.8°C with a tendency to increase. Since 2005, the minimum temperature was recorded at –26.3 (23.01.2006), and the maximum was 41°C (07.08.2010) (Solonenko et al., 2020). The temperature regime is unstable, especially in the spring and autumn periods, which negatively affects the artificial tree and shrub plantations (Koshelev et al., 2020a; Mirzoeva & Zhukov, 2021). Soil presented by Luvic chernozem developed in loess under native vegetation on ravine slopes (Soil profile classification according to IUSS Working Group WRB, 2015). In addition to precipitation, they receive surface runoff from the surrounding areas; groundwater may be encountered within 23 m (Yakovenko & Zhukov, 2021).

The transition of the average daily air temperature through 0°C occurs in spring on March 12, and in winter on December 5. Number of days with average daily temperature above 0°C is more than 270 days (Koshelev et al., 2020b).

The transition of the average daily air temperature over 10 ° C occurs in spring on April 20, and in autumn on October 17, and the number of days with a temperature above 10°C is 180 days. The sum of active air temperatures above 10°C exceeds 3200°C. The duration of frost-free period in the air is about 200 days. According to the scheme of agroclimatic zoning, the study area is located within the very warm and very dry agroclimatic area. The precipitation pattern is continental in character, with maximums in spring and summer and minimums in winter. The annual precipitation is unstable and fluctuates within 320–360 mm. The lowest precipitation is observed in March–April (25–30 mm). Then a gradual increase in precipitation begins, which lasts until June. Precipitation falls in the form of rain and snow, in the summer period there are often showers (Shcherbyna et al., 2021).

The assessment of the state of damage to trees and shrubs in the park was conducted in accordance with municipal standards that are specified in the "Instruction on technical inventory of green areas of SCN 03.08.007–2007 in cities and towns of urban type in Ukraine (2007) (Instruction, 2002). According to the Instruction damaged trees were determined by the following attributes: trees are very weakened, trunks curved, crowns poorly developed, dry and drying branches, growth of one-year shoots is insignificant, mechanically damaged trunks, there are hollows. During the inventory, the following information was recorded to the accounting information: the number of the accounting area (quarter), plant coordinates, type of plantings, species name of trees or shrubs, age, height, trunk diameter at 1.3 m height, number of trees in the quarter and their state. Species name of trees and shrubs were given according to the database The Plant List (TPL). During the tree survey, the length of the trunk circumference was measured, and then the trunk diameter was calculated using the formula $C = 2\pi R$. Tree heights were measured with a Nikon "Forestry 550" laser rangefinder. The age of the trees was determined by the analysis of biometric indicators (measurements of the stem diameter and height) (Erokhina et al., 1987). The ecological groups of trees and shrubs were represented according to V. Tarasov (Tarasov, 2012). The information obtained is summarized in a geographic database in ArcMap 10.8. ESRI Inc.

The number of plants that are within a radius of 3, 5, 7, 10 meters was determined for each of the recorded plant specimens (focal plant). Smaller distances are limited by the resolution of the tree coordinate record. Preliminary calculations showed that distances greater than 10 meters give statistically poorer results than estimates based on 10 meters, so we chose this distance as the upper limit. Based on the data obtained, the density of the stand was calculated:

$$H = \sum_{i=1}^{S} (-1 \times \frac{n_i}{N} \times log_2 \frac{n_i}{N}),$$

where H is Shannon diversity index, S is the species number, i is sequence number of the species in the community, n_i is the number of specimens of the i-species, N is the number of all plants within 10 meters of the focal plant.

To explain the probability of plant damage, Generalized Additive Models (GAMs) were tested with plant species identity, plant age, diversity of the surrounding plant community within 10 meters, and stand density (within 3, 5, 7, and 10 meters of the focal plant). Akaike weights were used to select the best model (Wagenmakers & Farrell, 2004).

The dependence of the height was described by an allometric function on the diameter in the power b equal to 2/3 (Greenhill, 1881; Zeide & Vanderschaaf, 2002):

$$H = a D^b,$$

where H – tree height, D – tree diameter, a and b – allometric coefficients.

The species identity of the plant, plant condition (healthy and damaged), and stand density (within 3, 5, 7, and 10 meters of the focal plant) were considered as additional predictors of the height-diameter relationship. For statistical analyses, the information on 13 plant species that were encountered more than 10 times was used. A total of 14 species were encountered less than 10 times, so information about them was used only for the ecological analysis. The dependence was fitted using the General Linear Model (GLM). The descriptive statistics, GLM, and GAMs were calculated using the software program Statistica (Statsoft).

Results

The plant community in the park was represented by 27 species of trees and shrubs (Table 1).

The most frequent species was *Ulmus caprinifolia*. This species accounted for 35.0% of the total number of tree specimens within the park (Table 2, 3). *Acer negundo* and *Crataegus pentagyna* were also important in the plant community, accounting for 20.9 and 11.1%, respectively. Phanerophytes accounted for 66.7% of the community species, and nanophanerophytes accounted for 33.3%, respectively. Mesotrophs (74.1%) dominated among the ecological groups that were distinguished on the basis of plant preference for soil fertility conditions. Accordingly, megatrophs were 25.9%. In respect to preference of moisture conditions, mesoxerophytes prevailed in the community (42.2% of the total number of species) and the proportion of xeromesophytes was very high in the community (33.3%).

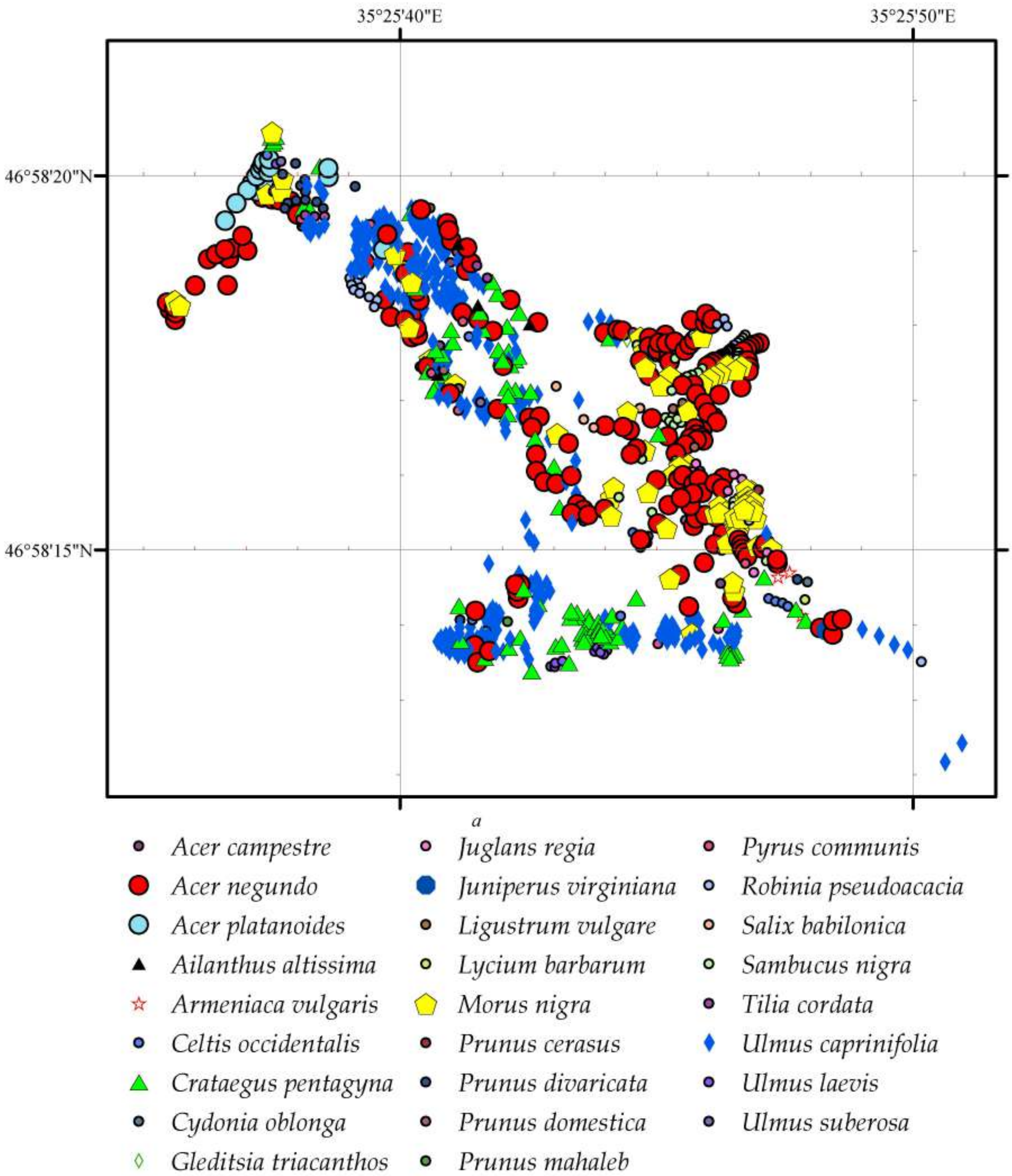

Fig. 1. Position of the park "Healing Springs" and location of tree species within the park.

Table 1. Age, species diversity and ecological groups of trees and shrubs in the park. *Legend:* *: Ph – phanerophytes; nPh – nanophanerophytes; **: MsTr – mesotrophic (plants prefer moderately fertile soils), MgTr – megatrophic (plants prefer highly fertile soils); ***: X – xerophytes (plant adapted to life in a dry habitat), MsX – mesoxerophytes (xerophytes are more demanding to the presence of available moisture), XMs – xeromesophytes (mesophytes, which are able to inhabit more dry conditions), Ms – mesophytes (a plant adapted to life in a moderately humid habitat or, in other words, not adapted to particularly

dry or particularly humid habitats); ****: He – heliophytes (plants of open places, which are located under direct sunlight), ScHe – scyoheliophytes (plants of light forests and shrubs, or tall herbaceous communities; the lower layers are in the shade), HeSc – helioscyophytes (plants of light coniferous and sparsely closed deciduous forests), Sc – scyophytes (plants of typical deciduous forests).

Species	Raunkiær plant life-form*	Ecological groups in relation to			Origin	Age, years		
		soil fertility**	soil humidity***	light****		Mean±st. error	Minimum	Maximum
Acer negundo L.	Ph	MsTr	XMs	HeSc	Adventive	44.2±2.17	3	100
Acer campestre L.	Ph	MsTr	XMs	Sc	Autochthonous	24.6±13.88	7	80
Morus nigra L.	Ph	MsTr	XMs	He	Autochthonous, Cultural	28.3±3.19	5	95
Acer platanoides L.	Ph	MgTr	Ms	Sc	Autochthonous	8.1±1.04	6	23
Celtis occidentalis L.	Ph	MsTr	XMs	Sc	Adventive	24.1±5.78	2	90
Crataegus pentagyna Waldst. & Kit. ex Willd.	nPh	MsTr	MsX	ScHe	Autochthonous	41.1±1.32	18	80
Tilia cordata Mill.	Ph	MgTr	XMs	Sc	Autochthonous	40.5±15.65	3	85
Prunus cerasifera Ehrh.	nPh	MsTr	MsX	He	Adventive, Cultural	19.7±1.55	10	30
Prunus domestica A.Sav.	Ph	MsTr	MsX	He	Adventive, Cultural	36.8±4.75	11	85
Pyrus communis Gouan	Ph	MgTr	MsX	Sc	Autochthonous	21.6±0.87	5	90
Ulmus minor Mill.	Ph	MsTr	X	ScHe	Autochthonous	20.9±2.18	13	32
Ulmus laevis Pall.	Ph	MsTr	Ms	Sc	Autochthonous	18.3±1.67	15	20
Ulmus suberosa Moench	Ph	MsTr	MsX	Sc	Autochthonous	11.2±2.60	5	20
Ailanthus altissima (Mill.) Swingle	Ph	MsTr	XMs	ScHe	Adventive	19.8±1.60	10	30
Rosa sp.	nPh	MsTr	XMs	He	Autochthonous	–	–	–
Robinia pseudoacacia L.	Ph	MgTr	MsX	ScHe	Adventive	21.0±4.25	5	70
Styphnolobium japonicum (L.) Schott	Ph	MsTr	XMs	ScHe	Adventive	60.0±20.00	40	80
Sambucus nigra L.	nPh	MgTr	Ms	Sc	Autochthonous	38.4±1.66	20	50
Gleditsia triacanthos L.	nPh	MsTr	MsX	He	Adventive	10.0	10	10
Ligustrum vulgare L.	nPh	MsTr	MsX	Sc	Adventive	20.0	20	20
Salix babylonica L.	nPh	MsTr	Ms	HeSc	Adventive	38.8±8.51	25	60
Juglans regia L.	Ph	MgTr	MsX	He	Adventive	34.6±8.94	12	90
Prunus cerasus L.	Ph	MgTr	XMs	Sc	Cultural	40.0	40	40
Prunus armeniaca L.	Ph	MsTr	MsX	He	Adventive, Cultural	23.3±8.82	10	40
Lycium barbarum L.	nPh	MsTr	MsX	He	Adventive, Cultural	2.0	2	2
Cydonia oblonga Mill.	nPh	MsTr	MsX	He	Adventive, Cultural	25.0	25	25
Juniperus virginiana L.	Ph	MsTr	MsX	ScHe	Adventive	10.0	10	10

Xerophytes were rare (3.7%). The proportion of mesophytes in the community was not high (14.8%). The plant community was dominated by species of marginal ecological groups in the light regime gradient, such as heliophytes (33.3%) and sciophytes (37.0%). The species of transitional ecological groups were less present in the community. The heliophytes accounted for 7.4% of the total number of species in the community, and the sciogeliophytes accounted for 22.2%. Adventive species accounted for 59.3% of the total number of species, and autochthonous species accounted for 40.7%. Cultivated species accounted for 25.9%.

Plant community diversity ranged from 0.05 to 2.85 bits/species (mean 1.44±0.019 bits/species) (Fig. 2). Species differed in the diversity of their surroundings (F = 7.47, p < 0.001). The lowest diversity of surroundings was found for *Acer campestre* (0.66±0.08 bit/species), *Robinia pseudoacacia* (1.24±0.11 bit/species), *Ulmus caprinifolia* (1.27±0.03 bit/species), *Crataegus pentagyna* (1.36±0.07 bit/species). The highest diversity of surroundings was found for *Ailanthus altissima* (1.80±0.20 bit/species), *Prunus domestica* (1.80±0.10 bit/species), *Ligustrum vulgare* (1.81±0.12 bit/species), *Prunus divaricata* (2.09±0.07 bit/species). There was a negative correlation between diversity and vegetation density (r = –0.21, p < 0.001). Thus, more dense vegetation cover was more uniform. A positive correlation was found between plant diversity and stem width of *Ulmus caprinifolia* (r = 0.13, p < 0.001), *Ulmus laevis* (r = 0.74, p < 0.001), *Sambucus nigra* (r = 0.39, p < 0.001). A negative correlation was found between diversity of surroundings and trunk width of *Ligustrum vulgare* (r = –0.57, p < 0.001). No correlation of diversity was found with plant height. The plant community diversity in the surroundings of healthy plants was higher than in the surroundings of damaged plants (F = 35.9, p < 0.001). The diversity in the surroundings of healthy plants averaged 1.66±0.032 bits/species, and in the surroundings of damaged plants averaged 1.37±0.023 bits/species.

The Gaussian mixture model allowed to estimate the distribution parameters of the three distributions that compose the final distribution of tree ages in the park plantation: age 14.0±4.50 years (48.3% of plants in the community), age 38.2±13.64 years (44.1% of plants in the community), and age 85.5±6.84 years (7.5% of plants in the community) (Fig. 3). The age of plants in the community as a whole was positively correlated with the diversity of vegetation in the surroundings of a particular plant (r = 0.11, p < 0.001). A correlation of individual plant species age and plant community diversity was found for *Ulmus caprinifolia* (r = 0.12, p = 0.038), *Ulmus laevis* (r = 0.70, p = 0.025), *Juglans regia* (r = 0.83, p = 0.020).

Table 2. Descriptive statistics of morphometric traits of tree and bush species in park plantations.

Species	Total (N, mean ± st. error)			Healthy tree (N, mean ± st. error)			Tree with signs of pathology (N, mean ± st. error)		
	N	H, meters	D, cm	N	H, meters	D, cm	N	H, meters	D, cm
A. negundo	170	8.48 ± 0.29	25.86 ± 1.21	100	8.50 ± 0.39	30.36 ± 1.65	70	8.44 ± 0.43	19.43 ± 1.45
A. campestre	5	5.10 ± 1.27	14.33 ± 5.83	2	4.00 ± 1.00	6.37 ± 1.27	3	5.83 ± 2.09	19.64 ± 8.81
M. nigra	71	6.75 ± 0.51	18.02 ± 1.83	19	9.42 ± 1.28	33.57 ± 4.64	52	5.77 ± 0.46	12.33 ± 1.06
A. platanoides	17	5.50 ± 0.53	8.39 ± 0.70	8	4.75 ± 0.53	8.28 ± 0.99	9	6.17 ± 0.86	8.49 ± 1.03
C. occidentalis	15	5.82 ± 0.96	14.67 ± 3.62	3	7.17 ± 3.42	28.87 ± 14.24	12	5.48 ± 0.94	11.12 ± 2.43
C. pentagyna	90	5.06 ± 0.25	12.86 ± 0.59	22	5.39 ± 0.71	16.06 ± 1.65	68	4.96 ± 0.23	11.82 ± 0.52
T. cordata	6	7.90 ± 2.70	25.47 ± 9.48	3	10.43 ± 3.92	35.77 ± 13.99	3	5.37 ± 3.82	15.16 ± 12.16
P. cerasifera	16	4.66 ± 0.18	9.89 ± 0.97	4	5.00 ± 0.21	14.89 ± 0.68	12	4.54 ± 0.23	8.22 ± 0.82
P. domestica	12	5.13 ± 0.74	11.73 ± 1.74	5	4.20 ± 1.46	13.25 ± 4.02	7	5.79 ± 0.72	10.65 ± 1.18
P. communis	16	7.64 ± 0.85	19.61 ± 2.79	5	8.40 ± 1.75	27.26 ± 6.25	11	7.29 ± 0.99	16.13 ± 2.45

U. minor	285	6.71 ± 0.21	12.74 ± 0.47	25	5.76 ± 0.94	17.62 ± 2.91	260	6.80 ± 0.21	12.27 ± 0.43
U. laevis	11	9.77 ± 0.77	16.68 ± 1.49	5	9.50 ± 1.79	19.11 ± 3.02	6	10.00 ± 0.35	14.65 ± 0.45
U. suberosa	3	3.67 ± 0.67	9.77 ± 1.66	–	–	–	3	3.67 ± 0.67	9.77 ± 1.66
A. altissima	5	5.40 ± 0.87	9.55 ± 1.65	2	7.00 ± 1.00	13.06 ± 1.27	3	4.33 ± 0.88	7.22 ± 1.29
Rosa sp.	1	2.00	1.00	–	–	–	1	2.00	1.00
R. pseudoacacia	19	5.65 ± 0.67	10.66 ± 1.87	5	6.80 ± 1.74	19.04 ± 5.19	14	5.24 ± 0.68	7.67 ± 0.99
S. japonicum	2	11.00 ± 1.00	30.57 ± 8.92	–	–	–	2	11.00 ± 1.00	30.57 ± 8.92
S. nigra	36	2.81 ± 0.10	11.78 ± 0.72	13	2.58 ± 0.22	15.73 ± 1.03	23	2.95 ± 0.09	9.55 ± 0.58
G. triacanthos	1	3.50	6.37	–	–	–	1	3.50	6.37
L. vulgare	14	2.71 ± 0.10	2.93 ± 0.05	6	2.33 ± 0.11	2.83 ± 0.11	8	3.00 ± 0.08	3.00 ± 0.07
S. babylonica	4	15.50 ± 2.99	50.62 ± 14.62	2	20.00 ± 2.00	71.94 ± 6.69	2	11.00 ± 3.00	29.30 ± 18.15
J. regia	8	5.70 ± 1.20	15.84 ± 3.92	3	6.33 ± 2.85	21.44 ± 10.04	5	5.32 ± 1.22	12.48 ± 2.29
P. cerasus	1	9.00	26.11	–	–	–	1	9.00	26.11
P. armeniaca	3	5.00 ± 2.00	16.24 ± 5.79	1	3.00 ±	15.92 ±	2	6.00 ± 3.00	16.40 ± 10.03
L. barbarum	1	1.20	1.00	–	–	–	1	1.20	1.00
C. oblonga	1	3.00	9.55	–	–	–	1	3.00	9.55
J. virginiana	1	3.00	7.96	–	–	–	1	3.00	7.96
Total	814	6.58 ± 0.13	16.07 ± 0.44	233	7.20 ± 0.29	24.31 ± 1.11	581	6.36 ± 0.14	12.76 ± 0.34

As the radius of the sample site increases, the density raster generalizes, but the value of stand density decreases (Fig. 4). A regression relationship was found between the radius of the sample site and the average density of the stand:

$$Y = 7.2 \times X^{-0.73} \ (R^2 = 0.99),$$

where Y is the mean density of the stand, ex./100 m^2, X is the radius of the sampling site, m.

A regression relationship between the radius of the sample site and the maximum density of the stand was found:

$$Y = 58.7 \times X^{-1.0067} \ (R^2 = 0.99),$$

where Y is the maximum of the stand density, ex./100 m^2, X is the radius of the sampling site, m.

About 74.1% of the trees were found to have the signs of pathological damage. The plant species that were rarely found in the plantation had signs of damage in 100% of cases. For the rest plants, there was a tendency for the degree of damage to increase with increasing presence in the community. A positive correlation ($r = 0.69$, $p < 0.001$) was found between the level of damage of a particular species and its abundance in the community. *A. negundo* was an exception to this trend. This species was second in the community in terms of dominance, but its damage rate was relatively low (41.2%). The best model to explain tree damage was a model that included as predictors plant species, its age, the diversity of the surrounding stand, and its density estimated from a 7-m radius sampling site (Table 4). As the age and density of the stand increases, the probability of pathological plant damage increases. The diversity of the stand around a given plant reduces the probability of plant damage.

Table 3. Descriptive statistics of height to diameter ratio (HDR) and allometric coefficient of tree and bush species in park plantations. *Note:* a^* – allometric coefficient derived from the equitation $H = a D^b$ where H – tree height, D – tree diameter, b=2/3.

Species	Total (N, mean ± st. error)			Healthy tree (N, mean ± st. error)			Tree with signs of pathology (N, mean ± st. error)		
	N	HDR	a^*	N	HDR	a	N	HDR	a
A. negundo	170	2.97±0.08	0.93±0.03	100	3.52±0.09	1.04±0.04	70	2.19±0.09	0.77±0.04
A. campestre	5	2.55±0.42	0.62±0.16	2	1.61±0.08	0.38±0.05	3	3.18±0.30	0.78±0.23
M. nigra	71	2.51±0.09	0.71±0.05	19	3.56±0.11	1.10±0.11	52	2.13±0.04	0.57±0.03
A. platanoides	17	1.56±0.05	0.45±0.02	8	1.74±0.06	0.45±0.03	9	1.40±0.04	0.46±0.04
C. occidentalis	15	2.39±0.27	0.62±0.10	3	4.00±0.35	0.99±0.33	12	1.99±0.19	0.52±0.08
C. pentagyna	90	2.62±0.06	0.60±0.02	22	3.16±0.11	0.69±0.05	68	2.45±0.06	0.57±0.02
T. cordata	6	2.83±0.29	0.87±0.25	3	3.36±0.10	1.15±0.34	3	2.30±0.37	0.58±0.35
P. cerasifera	16	2.10±0.17	0.50±0.03	4	2.98±0.14	0.67±0.02	12	1.81±0.14	0.45±0.03
P. communis	16	2.53±0.17	0.78±0.07	5	3.27±0.20	0.98±0.15	11	2.20±0.14	0.69±0.07
U. minor	285	1.99±0.04	0.58±0.01	25	3.25±0.12	0.71±0.07	260	1.87±0.03	0.57±0.01
U. laevis	11	1.78±0.14	0.72±0.04	5	2.16±0.20	0.78±0.09	6	1.46±0.05	0.66±0.01
U. suberosa	3	2.69±0.26	0.50±0.06	–	–	–	3	2.69±0.26	0.50±0.06
A. altissima	5	1.76±0.06	0.49±0.06	2	1.88±0.09	0.62±0.04	3	1.68±0.05	0.41±0.05
P. domestica	12	2.46±0.24	0.56±0.05	5	3.26±0.26	0.60±0.12	7	1.89±0.13	0.53±0.04
Rosa sp.	1	0.50	0.11	–	–	–	1	0.50	0.11
R. pseudoacacia	19	1.82±0.14	0.51±0.06	5	2.73±0.13	0.77±0.14	14	1.49±0.08	0.42±0.04
S. japonicum	2	2.73±0.56	1.08±0.21	–	–	–	2	2.73±0.56	1.08±0.21
S. nigra	36	4.37±0.31	0.57±0.02	13	6.36±0.40	0.69±0.03	23	3.24±0.18	0.50±0.02
G. triacanthos	1	1.82	0.38	–	–	–	1	1.82	0.38
L. vulgare	14	1.09±0.03	0.23±0.00	6	1.22±0.01	0.22±0.01	8	1.00±0.04	0.23±0.03
S. babylonica	4	3.00±0.54	1.46±0.33	2	3.60±0.03	1.92±0.12	2	2.39±1.00	1.01±0.45
J. regia	8	2.77±0.18	0.68±0.10	3	3.33±0.08	0.82±0.26	5	2.43±0.11	0.59±0.07
P. cerasus	1	2.90	0.98	–	–	–	1	2.90	0.98
P. armeniaca	3	3.46±0.96	0.69±0.17	1	5.31	0.70	2	2.53±0.41	0.68±0.30
L. barbarum	1	0.83	0.11	–	–	–	1	0.83	0.11
C. oblonga	1	3.18±	0.50	–	–	–	1	3.18	0.50
J. virginiana	1	2.65	0.44	–	–	–	1	2.65	0.44
Total	814	2.44±0.04	0.67±0.01	233	3.41±0.08	0.88±0.03	581	2.05±0.03	0.58±0.01

The GLM approach allowed to reveal that 83% of tree height variation can be explained by the information on tree and shrub species, plant condition (healthy plant or damaged one), its diameter and stand density (Table 5, 6). The width of the tree trunk was the most informative predictor of height. Other predictors should be considered as the factors that deviate the biologically determined dependence from the ideal form. The pathologic states of trees and shrubs resulted in an average 10.3% reduction in the tree height at a given diameter (F = 3656.3, p = 0.001) (Table 5). The effect of damage on the height of trees and shrubs depended on the species. For *Ailanthus altissima* and *Acer campestre* species, the individuals with signs of pathology were more elongated compared to healthy ones. For other species, healthy individuals were more elongated. This trend was greatest for *Sambucus nigra*, *Celtis occidentalis*, and *Acer negundo*. The calculation of the degree of height reduction for the lowest weighted estimates of the mean indicated a value of 29.1%. The estimation of the role of stand density to explain the variation in tree height depended on the radius of the sample site size. The stand density and the square of this index were found to be statistically significant predictors if the density was calculated for a sample area with a radius of 7 meters. The density quadrant was a significant predictor if the density was calculated for sampling areas with a radius of 5 meters. The stand densities were not statistically significant predictors if the measurements were made for 3-meter and 10-meter radius sampling areas. A quadratic form of the dependence indicates the presence of the maximum of the function. In natural units, the regression coefficients take values of 0.1164 for the linear term and –0.00623 for the quadratic term. The calculations showed that the first derivative of the quadratic function is equal to zero at a density of 9.3 individuals in a sample area with a radius of 7 meters, or 3.0 plants per 100 meters^2. Accordingly, the plant height reaches the maximum at these values. When density deviates upward or downward, the plant height at a given diameter decreases. The predictor that indicated the interaction of species and stand density factor was not statistically significant. Therefore, the above optimal value of park stand density, at which the greatest plant growth can be expected, should be recognized as applicable for all the species studied.

Discussion

The studied park plant community has a high level of taxonomic diversity. It includes both autochthonous and adventive species. The adventive species are widely used in the design of park plantings (Blinkova, 2017; Burda & Koniakin, 2019). These species are often marked by an aesthetic attraction and a high ecological stability (Andrea et al., 2020). However, the acclimation of non-native species in botanical gardens and parks has its own negative consequences. Many adventive species, which are now weeds, were acclimatized to botanical gardens as exotic ornamental plants (Mayer et al., 2017). Despite the diversity of geographical sources of the flora of the park plantation, the plant species are highly ecologically similar. This similarity can be explained by the action of the ecological filter, which creates conditions for the normal existence only for species that are adapted to the conditions presented in the park environment (Duflot et al., 2014). The main factor determining the successful introduction of alien species into resident plant communities is ecological filtration, which is expressed in a similar distribution of ecological traits (Zhukov et al., 2017; Divíšek et al., 2018). Such similarity is also the basis for considering plant species as indicators of environmental properties.

Herbaceous plants play a special role in phytoindication of environmental conditions (Zhukov et al., 2018, 2019;Zhukova et al., 2020). Trees have a special role in the typology of forest vegetation (Belgard, 1950; Nazarenko, 2016). The forest type is considered as a combination of trophic and moisture conditions of the ecotope. Trees are considered to be more sensitive to the trophic conditions, while herbaceous plants are more sensitive to the moisture conditions (Zhukov & Shatalin, 2016; Yorkina et al., 2020). These assumptions apply to the natural forests in the steppe zone, but our results confirm this conclusion for artificial park plantations as well (Zhukov et al., 2021). The community is dominated by plants that are very sensitive to the soil fertility. The Calcic chernozem, on which the studied park is formed, is known to have a high level of fertility (Yakovenko, 2017). The positive effect of artificial tree plantations on the fertility of this soil type was also recorded (Gorban et al., 2020). At the same time, ecological groups in relation to the moisture conditions are represented by a wide range from xerophiles to mesophiles. This suggests that the trophic conditions of the ecotope that are the limiting factor that acts as an ecological filter for the selection of species capable of existing in the community. In this connection, a hypothesis can be formulated that species whose ecological optimum deviates from the typical ecotope ecological regimes will be confirmed to be at greater risk of damage.

Naturalization is the process of transforming urban forests into a state compositionally, structurally, and functionally similar to the natural forests that are in the proximity of the city (Maltsev et al., 2017; Kunakh et al., 2020). The positive correlation between the age of plants and the diversity of their environment can be explained by the processes of community development and naturalization. The different-aged plants were found to be represented in the community. The probability of occurrence of younger plants of another species in the surrounding increases with the age of the focal plant (Moreira et al., 2017). Neighboring plants can decrease or increase the likelihood of damage to each other through associative resistance or susceptibility, respectively (Kim, 2017). This suggests an alternative interpretation, which is that in a more diverse community, the probability of surviving to a greater age is higher. This assumption is supported by the fact that the number of healthy plants is higher in more diverse communities.

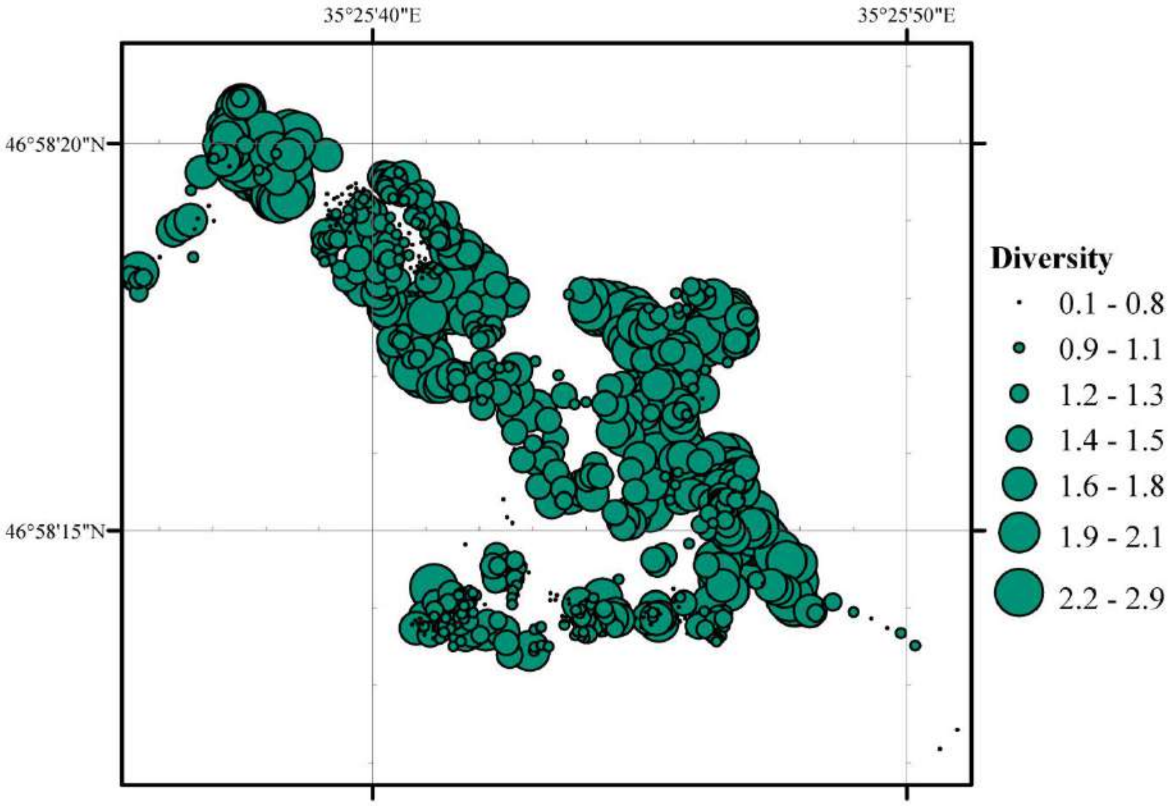

Fig. 2. Shannon diversity of the stand within 10 meters of the focal tree.

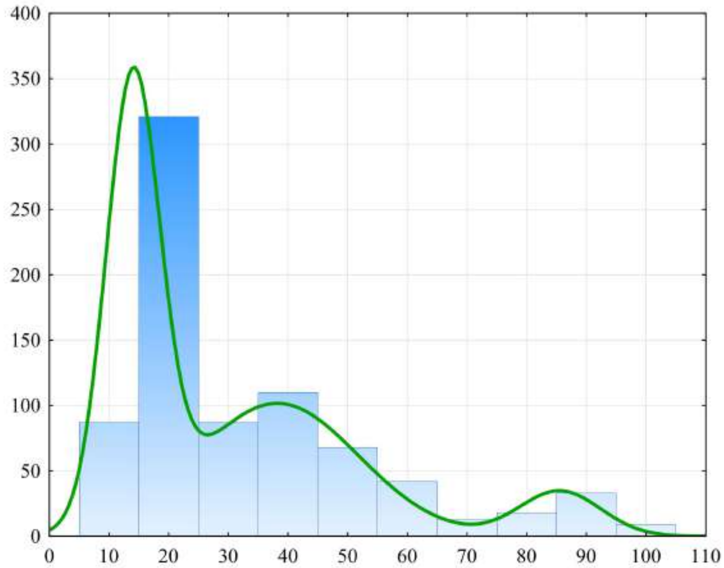

Fig. 3. Age distribution of tree and shrub plants in the park plantation. The abscissa axis is the age of trees, years; the ordinate axis is the number of trees. The green line denotes the hypothetical mixture of the three distributions. Gaussian mixture model revealed parameters of the three distribution: age 14.0±4.50 years (48.3% of plants in the community), age 38.2±13.64 years (44.1% of plants in the community), and age 85.5±6.84 years (7.5% of plants in the community).

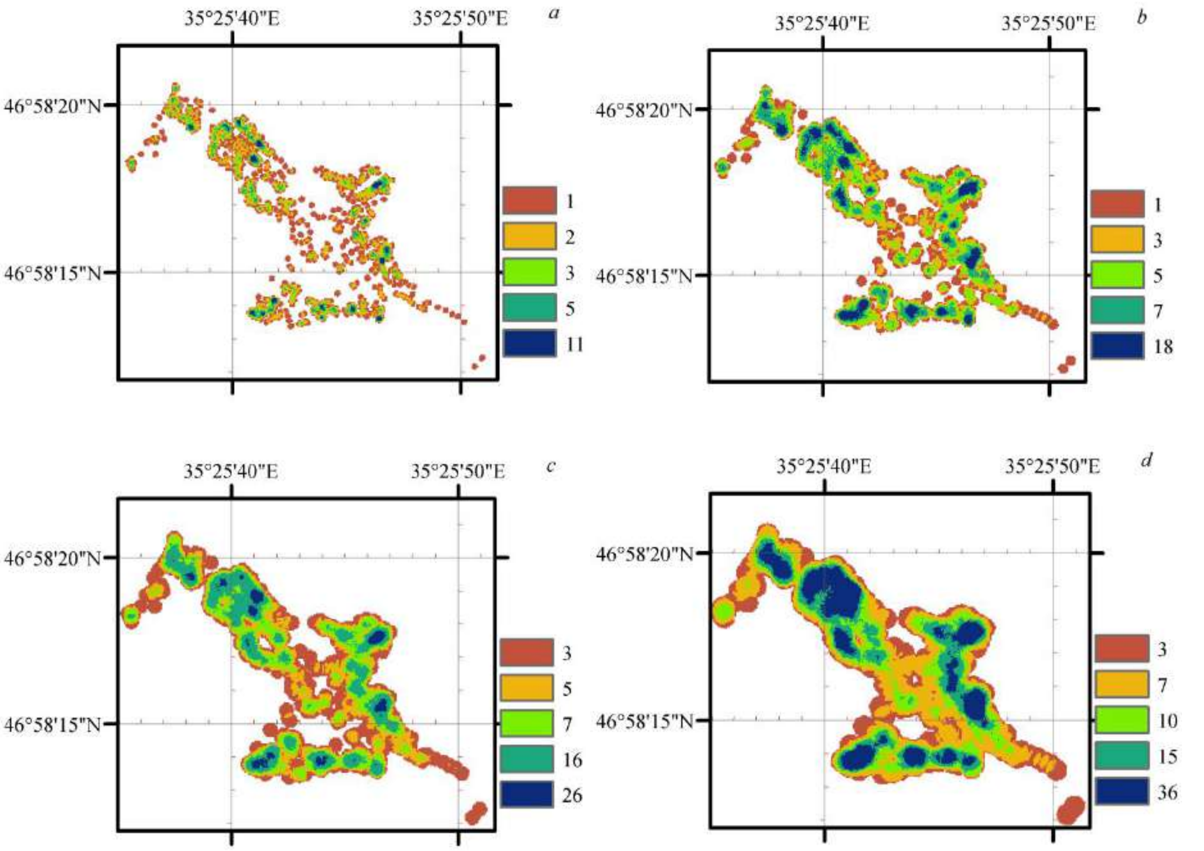

Fig. 4. Tree stand densities in the park plantation at the different radius of the neighboring space: *a* – radius of 3 meters, *b* – radius of 5 meters, *c* – radius of 7 meters, *d* – 10 meters.

Table 4. Model comparison results for mixed-effects models evaluating the effect of plant species, age, and stand density on pathological damage to trees in a park stand. *Legend:* * – k is the number of estimated parameters for the model; $\log(L_i)$ is natural logarithm of maximum likelihood for model i; AIC_i is the Akaike information criterion value for model i; Δ_i is AIC differences, relative to the smallest AIC value for given models; $w_i(AIC_c)$ is the rounded Akaike weights.

i	Model	k_i*	$\log(L_i)$	AIC_i	Δ_i	$\exp(-1/2*\Delta_i)$	$w_i(AIC_c)$
1	Species	1	–401.1	834.2	140.1	0.000	0.000
2	Species+Age	2	–351.1	736.1	42.0	0.000	0.000
3	Species+Age+Diversity	3	–343.9	723.8	29.7	0.000	0.000
4	Species+Age+Diversity+$Density_3$	4	–334.3	706.6	12.5	0.002	0.001
5	Species+Age+Diversity+$Density_3$+D_3^2	5	–333.7	707.4	13.3	0.001	0.001
6	Species+Age+Diversity+$Density_5$	4	–331.5	701.0	6.9	0.032	0.015
7	Species+Age+Diversity+$Density_5$+D_5^2	5	–330.9	701.8	7.7	0.021	0.010
8	Species+Age+Diversity+$Density_7$	4	–328.1	694.1	0.0	1.000	0.484
9	Species+Age+Diversity+$Density_7$+D_7^2	5	–327.4	694.9	0.8	0.670	0.324
10	Species+Age+Diversity+$Density_{10}$	4	–330.1	698.2	4.1	0.129	0.062
11	Species+Age+Diversity+$Density_{10}$+D_{10}^2	5	–328.6	697.2	3.1	0.212	0.103

Table 5. GLM of the effects of tree species, condition and diameter, and density of the tree stand on tree height (N = 788). *Legend*: * – tree condition: healthy tree or dangerous tree.

Predictor	Estimation of stand density with different test site radii							
	3 m radius, R_{adj}^2 = 0.83, F = 200.1, P < 0.001		5 m radius, R_{adj}^2 = 0.83, F = 111.9, P < 0.001		7 m radius, R_{adj}^2 = 0.83, F = 113.3, P < 0.001		10 m radius, R_{adj}^2 = 0.83, F = 110.9, P < 0.001	
	F-ratio	P-level	F-ratio	P-level	F-ratio	P-level	F-ratio	P-level
Intercept	7.2	0.01	11.6	0.001	15.6	0.001	6.5	0.01
Species (S)	6.4	0.001	5.1	0.001	6.4	0.001	5.7	0.001
State (St)*	250.0	0.001	252.2	0.001	247.7	0.001	244.2	0.001
S x St	94.7	0.001	95.5	0.001	100.3	0.001	99.5	0.001
$Diameter^{2/3}$	2707.3	0.001	2793.3	0.001	2785.7	0.001	2793.9	0.001
Stand density (D)	0.5	0.50	1.4	0.23	3.8	0.05	0.1	0.81
D^2	0.1	0.73	5.2	0.02	6.2	0.01	0.9	0.34
S x D	0.7	0.75	0.6	0.88	0.9	0.55	0.9	0.54

Our results are in agreement with findings indicating that more diverse communities have higher and more stable ecosystem functioning over time than less diverse communities (Allan et al., 2011; Bussotti et al., 2018; Turner - Skoff & Cavender, 2019; Budakova et al., 2021). The forest health depends on the ecosystem processes and the sustainability of the ecosystem (Raffa et al., 2009; Domnich et al., 2021). There is a fundamental difference between tree health and forest health, especially when forest health is viewed through the lens of ecosystem management.

Tree defoliation and crown dieback, tree mortality and pathogenic damage are the main aspects considered when assessing tree health. The health of an individual tree depends on the diversity of neighboring trees (Bussotti et al., 2018). Healthy forests include not only healthy trees, but also include diseased, injured, and non-viable individual trees (Manion, 2003; Sniezko & Koch, 2017). The diversity reflects the qualitative aspect of the plant surroundings, and the vegetation density reflects the quantitative aspect. Both of these indicators are scale-dependent (Zymaroieva et al., 2021).

Increasing the radius of the sampling area increases the diversity of the plant community in the plant environment and this indicator tends for all plant species to the diversity of the community as a whole. Thus, as the sampling area radius increases, the specificity of the estimate of plant community diversity at each point in the space, decreases. As the size of the sampling radius increases, the estimate of vegetation density decreases as more and more plant-free space is included in the area calculation. In an infinitely large plant community, density with increasing sampling radius will tend toward the average density of the entire community. For an island plant community, the density will tend toward zero. Obviously, of interest is the scale dependence in the radius range, which corresponds to the spatial coverage of possible interactions between individual plants. The high values of HDR indicate the trees are growing in a crowded stand with the reciprocal support of adjacent trees (Valinger & Fridman, 1997). Absence of competition in an extremely open stand can be indicated by the high HDR values (Wonn & O'Hara, 2001; Valinger & Fridman, 2011). A smaller value of HDR indicates greater crown length, greater crown projection area, better developed root system, lower position of the center of gravity, and higher mechanical stability of trees. HDR is a characteristic of tree and stand stability and their sensitivity to natural disasters (Nykänen et al., 1997; Jiao-jun et al., 2003; Castedo-Dorado et al., 2009).

Table 6. Regression coefficients derived from GLM-procedure of the effects of tree species, condition and diameter, and density of the tree stand on tree height (N= 788).

Effect	Level of effect	T–value	P–level	β-regression coefficient		
				mean ± st. error	–95%	+95%
Species	*Acer negundo*	–1.46	0.14	–0.065 ± 0.045	–0.15	0.02
	A. campestre	0.09	0.93	0.004 ± 0.041	–0.08	0.08
	A. platanoides	2.16	0.03	0.103 ± 0.048	0.01	0.20
	Morus nigra	–1.49	0.14	–0.063 ± 0.043	–0.15	0.02
	Celtis occidentalis	–2.13	0.03	–0.083 ± 0.039	–0.16	–0.01
	Crataegus pentagyna	–3.29	0.001	–0.129 ± 0.039	–0.21	–0.05
	Prunus divaricata	–0.71	0.48	–0.032 ± 0.045	–0.12	0.06
	Pyrus communis	–0.21	0.83	–0.008 ± 0.036	–0.08	0.06
	Ulmus caprinifolia	2.80	0.01	0.137 ± 0.049	0.04	0.23
	U. laevis	4.35	0.001	0.161 ± 0.037	0.09	0.23
	Ailanthus altissima	1.16	0.25	0.055 ± 0.047	–0.04	0.15
	Prunus domestica	0.18	0.86	0.009 ± 0.047	–0.08	0.10
	Robinia pseudoacacia	0.62	0.53	0.028 ± 0.044	–0.06	0.12
	Sambucus nigra	–4.71	0.001	–0.199 ± 0.042	–0.28	–0.12
	Ligustrum vulgare	0.98	0.33	0.096 ± 0.098	–0.10	0.29

State	Tree with signs of pathology	–15.74	0.001	–0.276 ± 0.018	–0.31	–0.24
Diameter$^{2/3}$	–	52.78	0.001	0.969 ± 0.018	0.93	1.01
Stand density (D)	–	1.95	0.05	0.134 ± 0.069	0.00	0.27
D^2	–	–2.49	0.01	–0.154 ± 0.062	–0.28	–0.03

A decrease in stand density increases the risk of windfalls (Wallentin & Nilsson, 2014). Trees with a large HDR are susceptible to uprooting by wind and breakage by wind (Urata et al., 2012). Tree species and stand height are the most important risk factors for storm damage. Stand density, soil and site condition, and topographic variables are important in explaining susceptibility to storm damage (Albrecht et al., 2012). Tree height-to-diameter ratio or time since last thinning were not significant predictors of vulnerability of forest stands to storms (Schütz et al., 2006). Mixture and aspect combined with gradient are reliable predictors of stand sensitivity to extreme weather events (hurricane). An admixture of 10% or more broadleaved trees or wind-resistant conifers significantly reduced the vulnerability of spruce stands (more than threefold). On wind-exposed slopes, damage was more than twice the average. Tree height-to-diameter ratio or time since last thinning were not significant predictors (Schmidt et al., 2010). The trees with a large HDR may not be adapted to the conditions of increased mechanical disturbance (Moore, 2000; Bošeľa et al., 2014). The mechanical properties of the trunk wood can be evaluated with HDR, e.g., trees with small HDR may have higher bending movements than trees with large HDR of similar height (Peltola, 2006). The maximum bending resistance moment correlates most significantly and positively with the diameter at breast height and tree height (Peltola et al., 2000).

Tree mortality is an important ecological process that alters the structure and function of the forest and influences forest management decisions. Tree mortality is considered as a general process that includes all the forms of tree mortality, from tree parts to large-scale disturbances. Multiple processes can lead to mortality, each with its own set of controls (Harmon & Bell, 2020). The mortality of trees is the result of density-dependent and density-independent processes (Larson et al., 2015; Gendreau-Berthiaume et al., 2016). The density-dependent mortality is caused when trees lose the competition for growing space (Bottero et al., 2017). A density-dependent mortality is a process in which the resources available for plant life are limited and mortality must occur for stand development (Hamilton et al., 1995). The mechanism of density-dependent mortality is believed to be primarily related to competition for light (Weiskittel et al., 2009), as opposed to competition for nutrients (Gafta & Crişan, 2010). However, mortality associated with causes other than competition was reported to be density-dependent as well, especially in mixed species stands (McCarthy-Neumann & Kobe, 2008; Piao et al., 2014). The probability of plant damage is species-specific, proportional to age and density, and inversely proportional to density. The nonlinear dependence of damage risks on density is not excluded, which indicates the possible existence of the extreme density of vegetation, at which the probability of damage will be the highest. The role of vegetation density as a damage risk factor is greatest when sampling with a radius of 7 meters. As the radius size increases or decreases, the role of density in the variation of plant damage probability decreases. The damage and mortality of plants may be independent of density. A density-independent mortality is a species-specific death of a stand due to processes that are unrelated to natural mortality during succession or stand maturation (Lintz et al., 2016). The density-independent mortality of trees in stable forests is caused by disturbance and physical damage, which makes trees more susceptible to other disturbance agents, resulting in tree mortality (Franklin et al., 1987). The management of risks to the safety of people and structures is a high priority in urban forest landscapes (Suchocka & Kimic, 2019; Wolf et al., 2020).

A methodological simplification in our work was the estimation of age based on allometric dependence. The direct determination of age is especially important in natural forests, where trees of different ages are represented and the tree size strongly depends on intra-ecosystem interactions. In park stands in the steppe zone, the artificial stands are not of great age, and the age of a stand is determined by the time of stand formation. Therefore, allometric dependences only for artificial plantations can give an acceptable approximation of tree age. It should be taken into account that the age category in our study has a largely allometric component. The direct measurements of tree age are a prospect for further research.

The plant damage affects the allometric dependence of plant height and trunk width. The leading predictor of plant height is trunk width (Greenhill, 1881; Norberg, 1988; O'Brien et al., 1995), but other predictors also influence the overall pattern. The damaged plants have a relatively lower height for a given trunk width. GLM revealed that the HDR of undamaged trees is 47.6% higher than that of damaged trees if plant species and trunk diameter are taken as covariates ($F = 235.5$, $p < 0.001$). Obviously, the damage of different nature negatively affects the rate of increase in plant height. This pattern seems to apply to all community species. The rate of plant growth is density-dependent. The greatest value as a predictor is density, which is estimated using a sampling area with a radius of 7 meters and this dependence is non-linear. Non-linear form of dependence leads to existence of optimal value of plant community density at which the plant growth will be the highest. Obviously, this density contributes to the formation of a healthy park plantation, which has the greatest functional and aesthetic value.

Conclusion

The probability of tree damage in a park plantation increases with plant age and stand density, but decreases as the diversity of the plant community increases. The allometric dependence of height on tree diameter depends on plant damage and stand density. The damaged plants have a lower height for a given trunk diameter. The dependence on density is scale-dependent. The effect of stand density on the allometric dependence is greatest at the density accounting radius, which is 7 meters. The optimal stand density in park stands in the southern steppe zone of Ukraine, which forms the best conditions for the growth of trees, is 3.0 plants per 100 meters^2.

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Received: 21.09.2021
Accepted: 02.11.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 199-209

Impact of Biological Fertilizing on the Composition and Productivity of Degraded Mesophytic Meadow in Mountain Conditions

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Abstract. A field experiment was conducted at the Research Institute of Mountain Stockbreeding and Agriculture (Bulgaria) to establish the impact of annual organic fertilizing on the bioproductive characteristics of a degraded grassland with a predominant share of grasses in mountain conditions. A significant effect on the biological productivity of the at all levels of fertilizing was found. The excess in the values of the indicator compared to the control was from 49.1% to 98.8%. Desired changes in the individual groups and species of the botanical composition of the pastures were observed. *Lotus corniculatus, Vicia sativa, Trifolium hybridum* (from legumes) and *Festuca arundinaceae* Scherb., *Festuca rubra, Anthoxanthum odoratum, Agrostis capillaris* (grasses) showed the highest resistance and adaptability to mountain conditions. The elimination of *Trifolium repens, Trifolium aureum* Pollich., *Vicia villosa* Roth. (second year), *Vicia cracca, Medicago lupulina* (third year) and *Trifolium pratense* (fourth year) from the composition of the grass mass affected the fodder and nutritional value of the grasslands. The grasslands treated with 1000 and 4000 kg/da dose of manure have the highest concentration of crude protein, respectively 14.39 and 14.05%. The values of the indicator exceeded the control by 20.0% and 17.2%, respectively. With a slight difference in protein content were grasslands treated with 2000 kg/da (12.03%) and 3000 kg/da (12.52%). The excess compared to the control variants was 0.3% и 4.4%, respectively. The highest fiber (44.37%) is the biomass with an imported dose of 3000 kg/da. The fertilizing variants registered with 2.3% to 11.4% higher content of crude ash compared to the control.

Key words: productivity, organic fertilizing, botanical and chemical composition of degraded grassland.

Introduction

The quality of natural grasslands is a prerequisite for optimal consumption and efficiency in the use of fodder. The resistance, adaptation and diversity of plant species in the composition of biocenoses affect the nutritional value of grass mass and the effect on the productive and reproductive indicators of animals (Slavkova & Shindarska, 2017). Changes in climatic characteristics, specific management practices and widespread extensive animal husbandry in mountain areas lead to some changes in the structure of floristic composition and the stability of ecosystems (Allan et al., 2015; Blüthgen et al., 2016; Habel et al., 2013).

Surface fertilizing of meadows and pastures is a major agronomic measure that increases yields, improves the botanical composition of mesophytic grasslands in the process of degradation (Popescu & Churkova, 2015; Samuil et al., 2013). Feeding the plants with organic fertilizers creates conditions for the development of valuable and high quality species (Kurhak et al., 2020; Vargová et al., 2020), which positively affects the composition of hay and pasture in mesotrophic and other types of meadows (Kirkham et al., 2014). Nonorganic and organic fertilizing has been shown to increase the percentage share of grasses and dry matter yield (Iliev, 2018; Iliev et al., 2017; Nemera et al., 2017) and has a less pronounced effect on plant height in degraded grasslands (Ahmed & Ibrahim, 2013).

In the mountaine and foot-hill regions of Bulgaria, the ratio of individual plant groups in the composition of natural meadows and pastures, as well as the yield obtained from them, are indicators of the effectiveness of applied agrotechnical events (Blanke et al., 2012; Leps, 2014; Naydenova et al., 2013; Tomashkin et al., 2013).

The objective of the study was to determine the impact of the annual organic (cattle manure) surface fertilizing on the bioproductive indicators of degraded meadow grassland (with predominant share of *Festuca rubra* L.) in the region of the Central Balkan Mountain.

Material and Methods

The experiment was conducted at the Research Institute of Mountain Stockbreeding and Agriculture, Troyan (Bulgaria) in the period 2016-2019, on degraded grassland, in the conditions of the Central Balkan Mountain. The experiment was based on the block method, in 4 replications with a plot size of 5 m^2.

Variants in the study were:

- Control (untreated)
- Fertilizing with cattle manure at a dose of 1000 kg/da;
- Fertilizing with cattle manure at a dose of 2000 kg/da;
- Fertilizing with cattle manure at a dose of 3000 kg/da;
- Fertilizing with cattle manure at a dose of 4000 kg/da.

The cattle manure treatment was applied annually and once, every year, manually, by spraying, before the onset of active vegetation in the grass cover.

The experimental areas were harvested in the phenophase of tasseling-ear formation (for grass species) until the beginning of the phenophase of flowering.

Studied indicators:

Yield of fresh and dry mass (kg/da) - the yield of fresh and dry mass was determined by mowing the area of each harvest plot (in replications) followed by drying the plant samples (0.5 kg) in a laboratory dryer at 105ºC and recalculating for an area of 1 da, based on dry mass content.

Botanical analysis of grassland (%) was determined by weight by analysis of grass samples taken immediately before mowing. The percentage share of each species per year from the group of grasses and legumes, of motley grasses (total) in both modes of use and their total ratio in the main botanical groups (grasses, legumes and motley grasses) was established.

Chemical analysis was conducted on: Crude protein (CP, %) according to *Kjeldahl* (following BDS/ISO-5983); Crude fiber (CFr, %); Crude fat (CF, %) (following BDS/ISO-6492) through rxtraction in an extractor *Soxhlet*; Ash (%) (following BDS/ISO-5984) through decomposition of the organic matter by gradual combustion of the sample in a muffle furnace at 550°C; Dry matter (DM, %) was empirically calculated from the % of moisture.

The results were analyzed by the method of analyzing the variance of a single-factorial trial (ANOVA) using the SPSS 4.5 software. The significance of differences in mean values of the treatments was tested by the LSD test.

Results and Discussion

Productivity of degraded grassland after manure fertilizing

In the first experimental year, fertilizing with manure had the greatest impact on the productivity of grassland at the imported rate of 2000 kg/da (Table 1). The amount of dry matter (303.5 kg/da) of the variant significantly exceeds the untreated control by 127.7%. The degraded grassland reacted positively to the imported higher norms of manure (3000 and 4000 kg/da). Dry matter yield of the variants increased by 70.4% and 89.1%, respectively, compared to the control. An increase in grassland productivity was also reported at the lowest rate (1000 kg/da) of fertilizer application, but without statistical significance. The amount of dry matter (207.7 kg/da) exceeded the variant without fertilizing by 55.8%.

Table 1. Dry matter yield (kg/da) of foot-hill mesophytic meadow, after organic fertilizing (over the years and average for 2016-2019).

Variants	2016		2017		2018		2019		2016-2019	
	kg/da	% compared to C	kg/da	% compared to C	kg/da	% compared to C	kg/da	% compared to C	kg/da	% compared to C
Control	133.3	100.0	104.3	100.0	358.7	100.0	197.5	100.0	198.5	100.0
1000	207.7	155.8	167.6	160.8	447.7	124.8	306.1	154.9	282.3	149.1
2000	303.5	227.7	259.2	248.6	569.3	158.7	316.8	160.4	362.2	198.8
3000	227.1	170.4	313.0	300.1	551.4	153.7	318.3	161.1	352.4	196.3
4000	252.0	189.1	276.4	265.1	503.3	140.3	356.2	180.3	347.0	193.7
GD 5 %	***86.2***	***64.8***	***105.4***	***100.8***	***110.7***	***30.9***	***85.7***	***43.3***	***56.4***	***26.7***
GD 1 %	***121.1***	***91.0***	***147.9***	***141.5***	***155.4***	***43.4***	***120.3***	***60.8***	***79.1***	***37.4***
GD 0.1 %	***170.9***	***128.5***	***208.8***	***199.8***	***219.5***	***61.2***	***169.9***	***85.8***	***111.7***	***52.8***

In the second year (2017) the dynamics of dry matter productivity follows the trend of the first experimental year. The degraded grassland reacted to the highest degree when fertilizing at a rate of 3000 kg/da. Dry matter yields of the variant (313.0 kg/da) were proven to exceed by 200.0% ($p<0.001$) the untreated control. A significant positive reaction of the degraded grassland to the applied fertilizing rates of 2000 and 4000 kg/da was observed. Dry matter yields of the variants are proven to exceed the fertilized variant by 148.6% and 165.1%, respectively. The excess of dry matter yield (by 60.8%) in the variant with the lowest test dose (1000 kg/da) remained statistically unproven.

Agroecological conditions and fertilizing dose affect the productivity of natural grasslands (Iliev et al., 2020). In the third experimental year (2018), the effect of the accumulated amount of manure in the degraded grassland, as well as the favorable climatic conditions during the year allowed the formation of the highest amount of dry biomass for the entire experimental period. Yields range from 358.7 to 569.3 kg/da. The variants with doses: 2000 kg/da (by 58.7%), 3000 kg/da (by 53.7%) and 4000 kg/da (by 40.3%) are again proven to exceed the control. The effect of fertilizing at norm of 1000 kg/da was less. The registered yield of the variant was 447.74 kg/da and marked a statistically unproven increase (by 24.81%) compared to the untreated control.

Under the impact of organic fertilizing, in the fourth year (2019) of the experiment, the increase in yields had proven positive differences in all treated variants. The excess of the indicator compared to the control varied from 54.9% (at a dose of 1000 kg/da) to 80.3% (at a dose of 4000 kg/da). When monitoring the productivity results, the fertilizing rates of 2000 and 3000 kg/da registered similar values (316.8 and 318.3 kg/da), where the dry matter yields were increased by 60.4% and 61.1%, respectively, compared to the control variant.

On average for the four-year experimental period (2016-2019), the annual manure fertilizing had the maximum impact on dry matter productivity in degraded mountain grassland. A reliable effect on the biological productivity of grassland has been found in all fertilizing variants. The excess in the productivity of the treated variants was statistically proven compared to the control and varied from 49.1% (1000 kg/da) to 98.8% (2000 kg/da).

Botanical and quality composition of degraded grassland after fertilizing with manure

The changes in the botanical composition of the grassland (by groups and years) are shown in Table 2. Fertilizing with cattle manure increases most significantly the share of legumes in the degraded grassland.

Table 2. Botanical composition (%) of foot-hill mesophytic meadow (by years and groups), after applied organic fertilizing.

Variants	2016			2017			2018			2019		
	grasses	legumes	motley grasses	grasses	legumes	motley grasses	grasses	legumes	motley grasses	grasses	legumes	motley grasses
Control	19.5	32.7	47.8	11.1	56.7	32.2	18.5	42.4	39.1	23.8	39.0	37.2
1000	9.2	53.0	37.8	1.4	79.2	19.4	9.5	70.2	20.3	44.8	29.2	26.0
2000	30.7	34.7	34.6	11.7	61.1	27.2	28.9	46.7	24.4	29.4	50.0	20.6
3000	33.3	50.8	15.9	6.7	62.0	31.2	22.1	46.1	31.8	46.0	38.0	16.0
4000	29.5	36.9	33.6	3.4	64.2	32.4	18.3	48.7	33.0	25.9	52.5	21.6

The variants with the lowest fertilizing dose (1000 kg/da) had the highest presence of the legume component. In the first, second and third experimental years, legumes accounted for 53.0%, 79.2% and 70.2% of the grassland, respectively. Similar results (with a predominance of the legume component in the grassland during the first three years of experiment) were observed in the variants with annual application of 3000 kg/da of manure. The values of the indicator were 50.8% (in 2016), 62.0% (in 2017) and 46.1% (in 2018). In the fourth experimental year, the share of legumes in the biomass of both variants was lower compared to the group of grasses by 53.4% (in the variants with manure at a dose of 1000 kg/da) and by 21.1%% (in the variants with manure at a dose of 3000 kg/da). Throughout the test period, the percentage share of legumes compared to grasses prevailed in the grassland of the variants with fertilizing rates at 2000 kg/da (34.7%, 61.1%, 46.7%, 50.0%) and 4000 kg/da (36.9%, 64.2%, 48.7%, 52.5%). Dominant representatives of legumes were mainly *Trifolium hybridum* and *Vicia sativa*, and of grasses - *Festuca rubra* and *Festuca arundinaceae* Scherb. The group of motley grasses had the highest share in the grassland of unfertilized control - from 32.2 to 47.8%.

The organic fertilizing of the degraded grassland affected the changes in the percentage share of the species in the different grass groups.

In the first experimental year grass species, such as *Festuca arundinaceae* Scherb. and *Anthoxanthum odoratum* participated in the grassland of all variants (Fig. 1). Their share varied respectively from 4.1% (variants with manure at a dose of 1000 kg/da) to 21.9% (variants with manure at a dose of 3000 kg/da) and from 1.6% (variants with manure at a dose of 2000 kg/da) to 7.7% (variants with manure at a dose of 3000 kg/da).

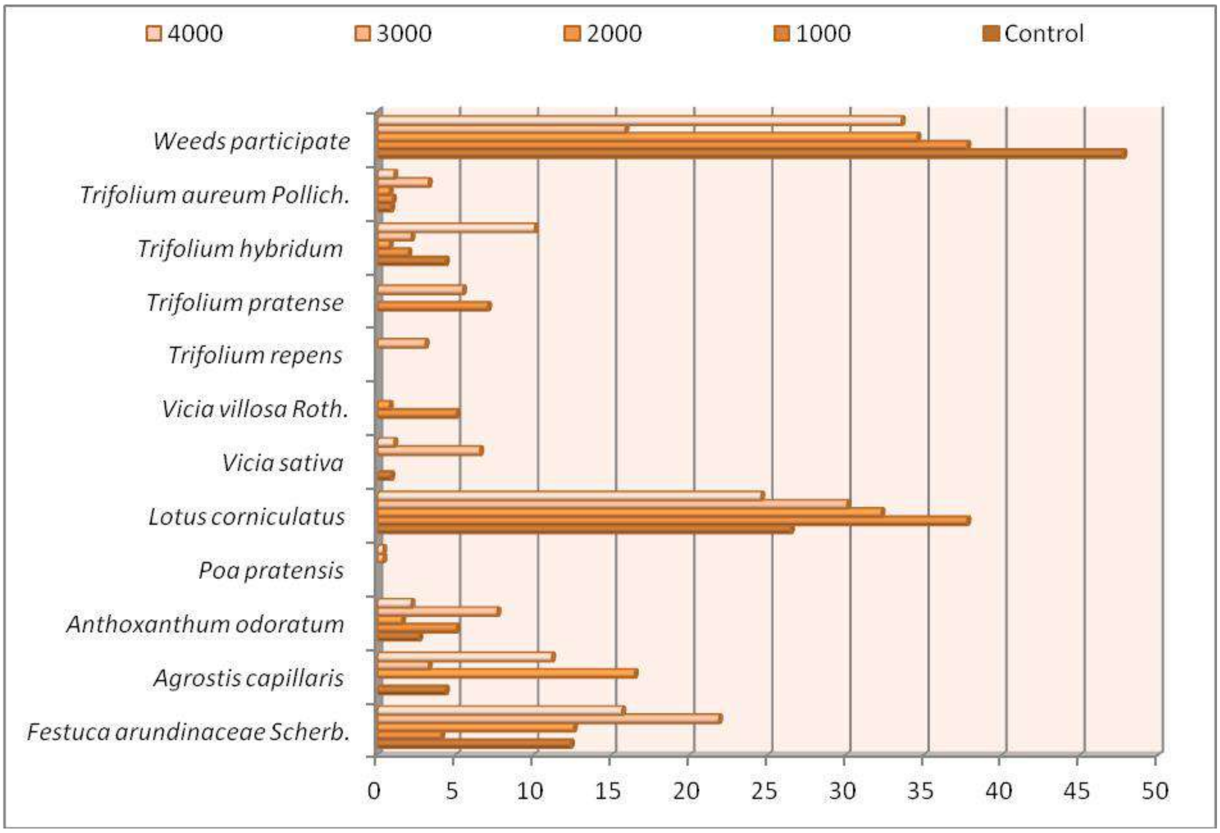

Fig. 1. Botanical composition by species (%) of foot-hill mesophytic meadow, after organic fertilizing (first experimental year - 2016).

The presence of other species was established:

- *Agrostis capillaris* - from 3.3% (variants fertilized with manure - dose - 3000 kg/da) to 16.5% (variants fertilized with manure - dose - 2000 kg/da) and
- *Poa pratensis* - 0.4% (variants fertilized with manure - dose - 3000 and 4000 kg/da).

Legumes: Lotus corniculatus - 24.6% (fertilized with manure at a dose of 4000 kg/da) to 37.8% (fertilized with manure at a dose of 1000 kg/da), *Trifolium hybridum* - 0.8% (fertilized with manure at a dose of 2000 kg/da) up to 10.1% (fertilized with manure at a dose of 4000 kg/da) and *Trifolium aureum* Pollich. - 0.8% (fertilized with manure at a dose of 2000 kg/da) to 3.3% (fertilized with manure at a dose of 3000 kg/da) participated in the grasslands of all tested variants.

The presence of other species was established:

- *Trifolium pratense* - from 5.5% (fertilized with a dose of 3000 kg/da manure) to 7.1% (fertilized with a dose of 1000 kg/da manure)
- *Vicia sativa* - from 0.9% (Control) to 6.6% (fertilized with a dose of 3000 kg/da manure)
- *Vicia villosa* Roth. - 0.8% (fertilized with a dose of 2000 kg/da manure) to 5.1% (fertilized with a dose of 1000 kg/da manure) and
- *Trifolium repens* - 3.1% (fertilized only with a dose of 3000 kg/da of manure).

In the second experimental year we observed a significant increase in useful legumes and lower participation of grasses. In the case of legumes, which had fallen out of the grassland, the species are: *Trifolium* *repens*, *Trifolium aureum* Pollich. and *Vicia villosa* Roth. (Fig. 2). *Vicia cracca* (1.1-7.4%) and *Medicago lupulina* (1.2-1.5%) registered additional share in the botanical composition of the grassland. *Lotus corniculatus* had the highest share in the variant with fertilizer application - 1000 kg/da (37.0%), *Trifolium hybridum* in fertilizer application - 4000 kg/da (42.8%). The grasslands in these variants had the lowest share of grasses. The percentage share of *Vicia sativa* in the botanical composition of the grassland was from 2.2% (variants fertilized with manure at a dose of 1000 kg/da) to 22.6% (variants fertilized with manure at a dose of 3000 kg/da).

In the group of grasses additional presence of *Cynosurus cristatus* (0.5-0.8% - in the variants fertilized with a dose of 4000 and 3000 kg/da) and *Festuca rubra* (1.1% - in the variants fertilized with a dose of 4000 kg/da was found; 2.3% in the variants fertilized with a dose of 3000 kg/da and 3.7% in the unfertilized control).

In the third experimental year (2018), the applied organic fertilizing continued to have a positive effect, mainly the share of legumes in the grassland compared to that of forage grasses (Fig. 3).

Trifolium hybridum and Vicia sativa participated in the biomass of all studied variants. In grasslands that were fertilized, the percentage share of these species varied from 29.9% (at a dose of 2000 kg/da) to 50.0% (at a dose of 1000 kg/da) and from 1.7% (at a dose of 1000 kg/da) to 3.6% (at a dose of 3000 kg/da). *Lotus corniculatus* registered a presence of 4.6% (dose - 4000 kg/da) to 17.4% (dose - 1000 kg/da) only in the fertilized variants. The share of *Trifolium repens* and *Trifolium pratense* in the grassland was 0.4% (Control) to 1.1% (dose - 1000 kg/da) and from 0.9% (dose - 3000 kg/da) to 2.8% (dose - 2000 kg/da). During the year species, such as - *Vicia cracca* and *Medicago lupulina* were not present in the botanical composition of the grassland.

Subsequent fertilizing at a norm of 2000 kg/da, enriched the species diversity of grasses in the grassland, where traces of *Bromus arvensis* (0.9%) were found. *Festuca arundinaceae* Scherb., *Festuca rubra*, *Agrostis capillaris* and *Anthoxanthum odoratum*, registered the highest presence respectively in the variants fertilized annually with 2000 kg/da (11.2%), 3000 kg/da (8.2%), 4000 kg/da (7.3%).) and 2000 kg/da (8.4%). In the year, *Poa pratensis* dropped out of the grassland.

The dynamics in the change of vegetation (after the subsequent fertilizing in the fourth year - 2019) includes an increase in the degree of grass component in the composition of the grassland (Fig. 4).

Festuca rubra and *Agrostis capillaris* were dominant. Their share in the fertilized variants varied from 8.6% (for fertilizing with a dose of 1000 kg/da) to 20.5% (for fertilizing with a dose of 2000 kg/da) and from 5.5% (for fertilizing with a dose of 2000 kg/da) to 30.0%. (when fertilizing with a dose of 3000 kg/da). *Festuca arundinaceae* Scherb. predominated in the grassland of the untreated control (10.2%). *Bromus arvensis* (5.7%) and *Anthoxanthum odoratum* (2.0%) participated in the composition of the grassland only in the variants fertilized with a dose of 1000 kg/da and 3000 kg/da, respectively.

Trifolium hybridum had the largest share of legumes in the grassland - from 5.7% (at a dose of 1000 kg/da) to 38.0% (at a dose of 3000 kg/da). *Medicago lupulina* (8.6%), *Vicia sativa* (5.5%) and *Lotus corniculatus* (21.6%) registered the highest share in the variants fertilized with 1000 kg/da, 2000 kg/da and 4000 kg/da, respectively.

In the last experimental year (2019), *Trifolium pratense* and *Cynosurus cristatus* did not participate in the botanical composition of the treated grasslands, and the group of weeds showed a decreasing trend compared to the year when the experiment was set (2016). The percentage share of weeds in the variants with imported manure was reduced (except for the variant with imported dose of 3000 kg/da) by 45.4% (at a dose of 1000 kg/da), 55.6% (at a dose of 4000 kg/da) and 68.0% (at a dose of 2000 kg/da).

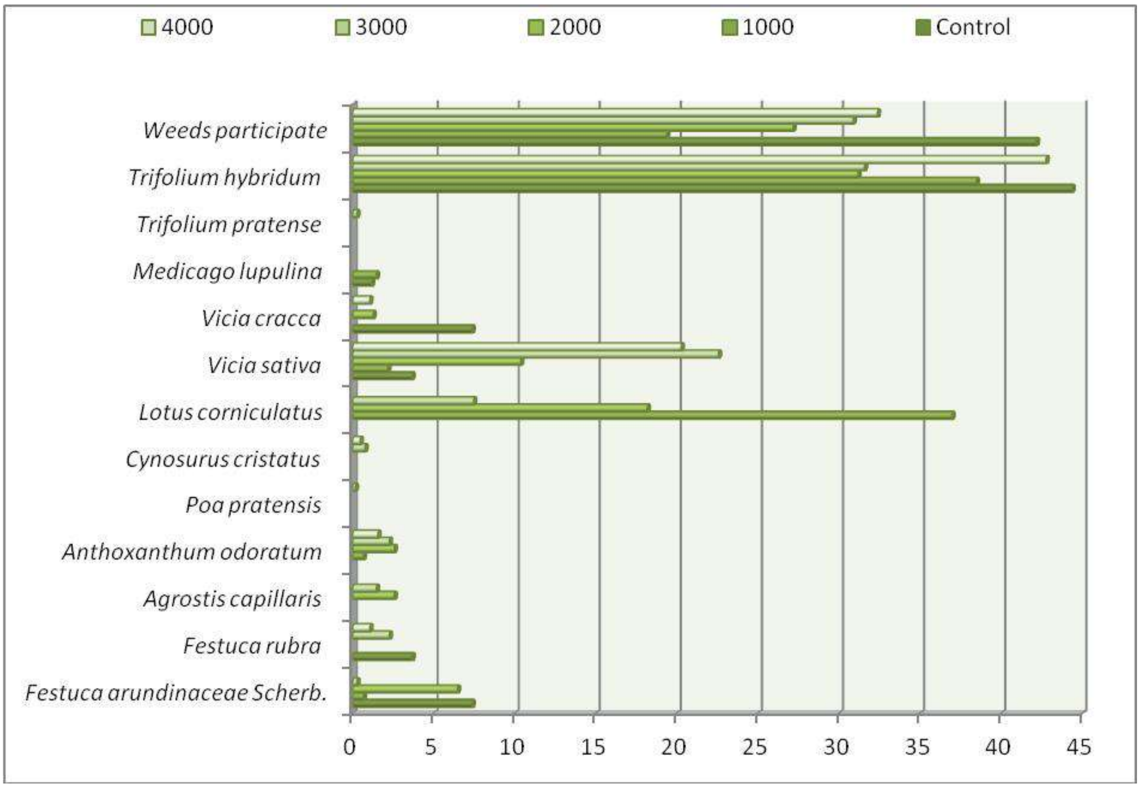

Fig. 2. Botanical composition by species (%) of foot-hill mesophytic meadow, after organic fertilizing (second experimental year - 2017).

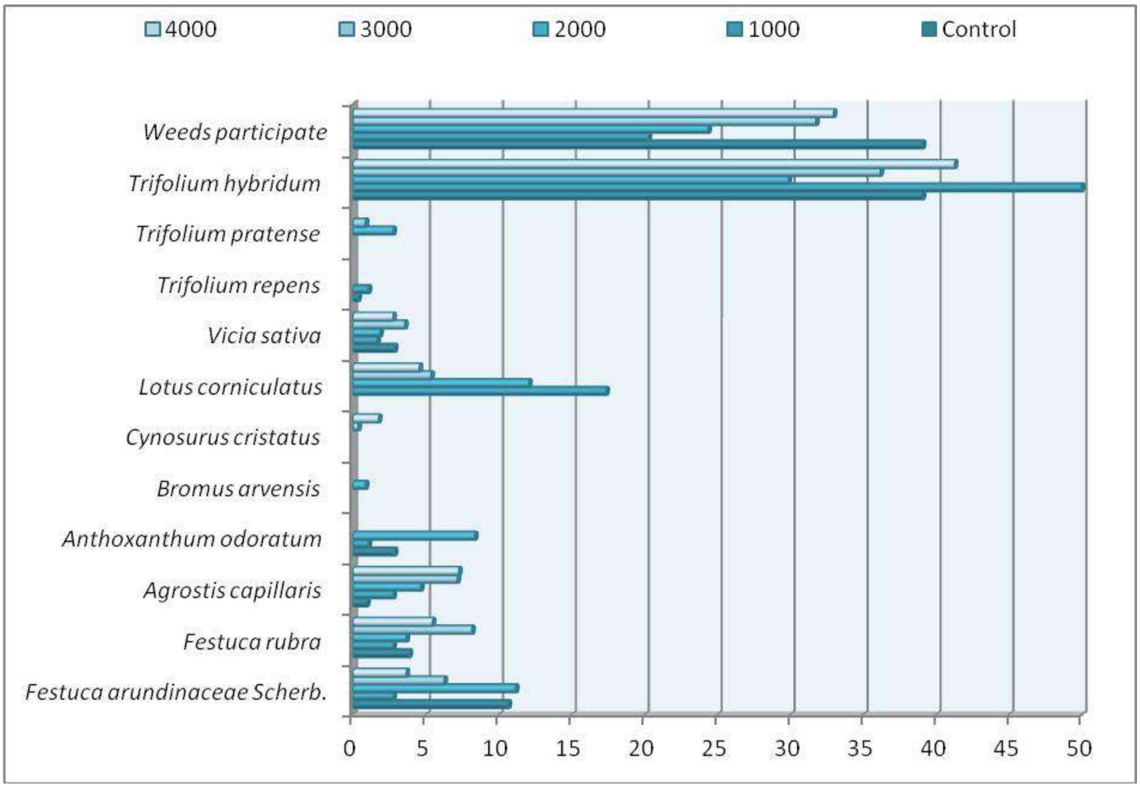

Fig. 3. Botanical composition by species (%) of foot-hill mesophytic meadow, after applied organic fertilizing (third experimental year - 2018).

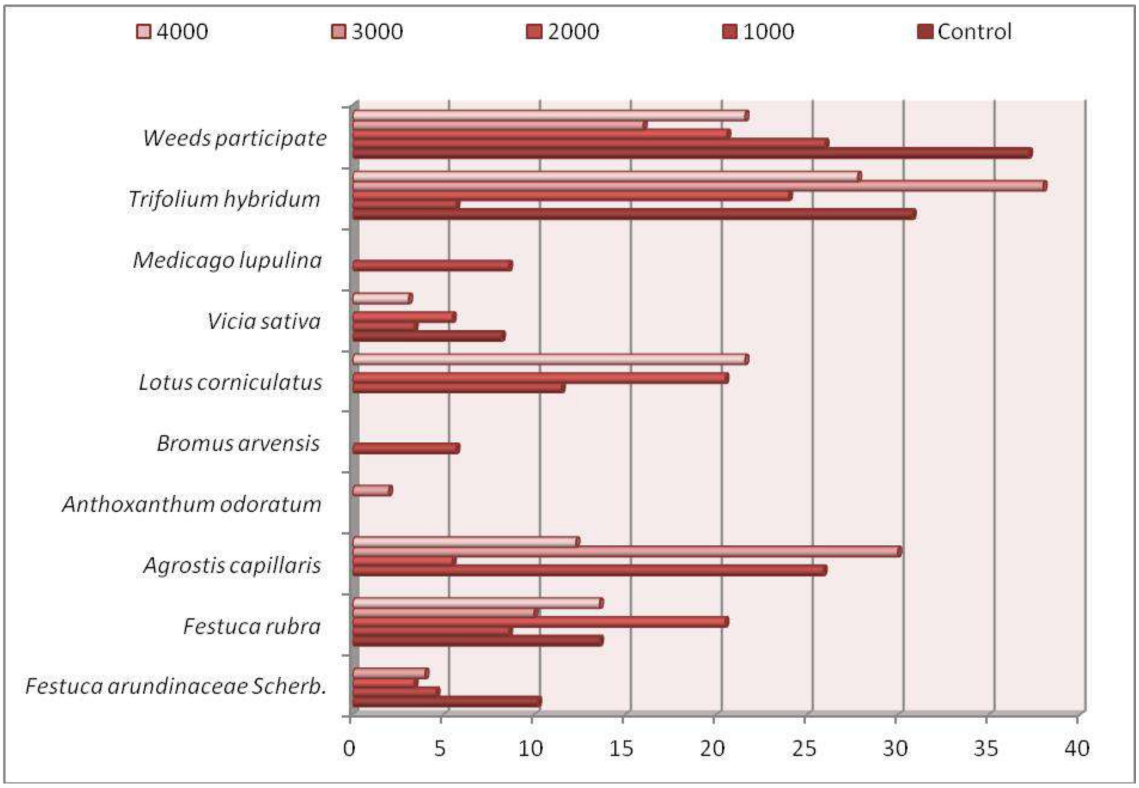

Fig. 4. Botanical composition by species (%) of foot-hill mesophytic meadow, after organic fertilizing (fourth experimental year - 2019).

Legumes were an unstable and changing component in the composition of natural grasslands. During the test period (after the application of cattle manure) the highest stability and adaptability were shown by species, such as: *Lotus corniculatus*, *Vicia sativa* and *Trifolium hybridum*. As for grass species, *Festuca arundinaceae* Scherb., *Festuca rubra*, *Anthoxanthum odoratum* and *Agrostis capillaris* showed the highest resistance to the mountain conditions of the Central Balkan Mountain. *Festuca arundinaceae* Scherb., *Festuca rubra*, *Anthoxanthum odoratum* and *Agrostis capillaris*.

Botanical composition is a factor that affects the feed and nutritional value of grass biomass (Churkova & Churkova, 2020; 2021; Naydenova et al., 2015; Naydenova & Vasileva, 2016). The dominance of legumes from the first to the third experimental year is clearly expressed in all variants of fertilizing, which implies the formation of biomass with a higher crude protein content. During the fourth (last) experimental year, organic fertilizing increased the percentage of grasses in the composition of the variants with different fertilizing rates.

All variants with organic fertilizing had higher values in terms of dry matter content, crude protein, crude fiber and crude ash compared to the untreated control. The average value of crude protein in the dry matter of the studied grasslands wass 12.995% at CV: 8.80% (Table 3). The variants with the lowest dose of manure (1000 kg/da) registered the highest content of crude protein (14.39%) followed of the grasslands (14.05%) treated with the highest dose of organic fertilizer (4000 kg/da). The values of the indicator exceeded the control by 20.0%% and 17.2%, respectively. With a slight difference in the protein content are the grasslands with annual intake of 2000 and 3000 kg/da (respectively 12.03 and 12.52%), where the excess compared to the control was 0.3% and 4.4%, respectively.

Table 3. Mean values ± SE (%) and coefficient of variation (CV %) of basic quality indicators of natural grassland cover, after applied organic fertilizing.

Variants	Dry matter	Organic matter			
		Crude protein	Crude fibre	Crude fat	Crude ash
Control	89.88	11.99	38.83	2.32	6.50
1000	90.10	14.39	42.84	2.43	6.83
2000	90.01	12.03	41.26	2.00	6.65
3000	90.18	12.52	44.37	1.37	6.98
4000	90.00	14.05	42.01	2.30	7.24
Average	***90.030±0.11***	***12.995±1.14***	***41.861±2.05***	***2.082±0.43***	***6.836±0.29***
CV	***0.13***	***8.8***	***4.9***	***20.61***	***4.21***
$LSD_{0.05}$	***0.331***	***0.763***	***4.221***	***0.723***	***0.506***

The average values for the amount of minerals (6.836%) and crude fiber (41.861%) are characterized by a low coefficient of variation (CV: 4.21-4.90%). The highest fiber (44.37%) and the lowest concentration of crude fat (1.37%) is the dry biomass from grasslands that were treated by manure with a dose of 3000 kg/da. The treated variants registered with 2.3% (2000 kg/da) to 11.4% (4000 kg/da) higher content of crude ash compared to the control. The average content of crude fat (2.082%) in the dry matter of the studied grasslands was characterized by a very high coefficient of variation (20.61%). Only the grasslands with the highest protein content exceeded by 4.7% the amount of crude fat (2.43%) in the dry matter compared to the control.

Conclusion

The applied biological fertilizing led to an increase in the productivity of the degraded grassland at all levels of fertilizing. The excess in the values of the indicator compared to the control was from 49.1% (1000 кg/da) to 98.8% (2000 кg/da). Some desired changes in the individual groups and species of the botanical composition of the grassland were also observed For the study period, with the highest resistance and adaptability in mountain conditions were species: *Lotus corniculatus, Vicia sativa, Trifolium hybridum* (**from legumes**) and *Festuca arundinaceae* Scherb., *Festuca rubra, Anthoxanthum odoratum, Agrostis capillaris* (**from grasses**). Organic fertilizing variants had a higher content of dry matter, crude protein, crude fiber and crude ash compared to untreated control. Grasslands with the lowest dose of manure (1000 kg/da) registered the highest concentration of crude protein (14.39%) followed by those (14.05%) with the highest dose (4000 kg/da) of manure. The values of the indicator exceeded the control by 20.0% and 17.2%, respectively. The difference in the protein content of the grassland treated annually with a dose of 2000 and 3000 kg/da was insignificant (12.03 and 12.52%, respectively), with an excess compared to the control - 0.3% and 4.4%. The highest fiber (44.37%) and the lowest concentration of crude fat (1.37%) were found in the biomass of grasslands with an imported dose of 3000 kg/da. In the fertilizing variants, the crude ash content was 2.3% (2000 kg da) to 11.4% (4000 kg/da) higher than the control.

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Received: 28.09.2021
Accepted: 22.10.2021

ECOLOGIA BALKANICA
2021, Vol. 13, Issue 2 December 2021 pp. 211-222

Plastic Degradation by Extremophilic Microbial Communities Isolated from Bulgaria and Russia

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Abstract. This work reports the potential impact of extremophiles in resolving one of the biggest contemporary ecological problem - accumulation of huge amounts of plastic pollutants. Lye (C1) and water mud (C2) from Atanasovsko ezero, Bulgarian salterns were enriched for 2 weeks in a mineral salt medium provided with four synthetic plastics, Polycaprolactone (PCL), Polyvinyl alcohol (PVA), Polystyrene (PS), and Polypropylene (PP). Esterase activity was established for C1 on PVA and for C2 - on PCL and PVA. Scanning electron microscopy analysis of the plastics showed most explicit alterations on PCL surface. While the presence of C1 in the culture medium caused damage of the plastic surface, C2 interacted with it directly by forming a biofilm in surface breaks. Metagenomic analysis of C2, control and C2 with PCL revealed lower phylogenetic diversity in the presence of PCL and sharp rise of Gammaproteobacteria 16S rRNA sequences. The flourishing of the family *Halomonadaceae* was accompanied by a strong domination of the genus *Halomonas*, suggesting its active participation in PCL degradation. Thermophilic microorganisms in samples from Kamchatka hot springs were enriched in a medium with high- and low-density polyethylene in anaerobic conditions, at 78°C. Degradation of polyethylene after incubation was monitored by SEM. Results of 16S rRNA genes profiling compared with control variants revealed a domination of bacteria of phylum *Dictyoglomi* and family *Thermoanaerobacteraceae* in anaerobic thermophilic Kamchatka enrichments in the presence of polyethylene. These results indicate the possibility of thermophilic anaerobes to degrade the two types of polyethylene.

Key words: synthetic plastics, plastic biodegradation, saltern environment, hot springs, halophilic community, thermophilic community, esterase activity, 16S rRNA, biofilm.

Abbreviations: Polycaprolactone - PCL, Polyvinyl alcohol - PVA, Polystyrene - PS, Polypropylene - PP, Scanning electron microscopy – SEM, transmission electron microscopy – TEM, high-density polyethylene – HDPE, low-density polyethylene - LDPE.

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Introduction

Plastics are man-made polymeric hydrocarbons characterized by high durability and low cost. The excellent features and advantages of these long-chain polymers contribute to economic growth, but their difficult degradation favors their accumulation in the natural environment as solid wastes remaining intact for a very long time, with some being broken down for hundreds of years (Mohanan et al., 2020). Anthropogenic wastes from fishing, tourism, and marine industries are the main reason for coastal and marine pollution (Veiga et al., 2016). The exponentially growing scientific interest in plastic accumulation and its impact on nature reflect the public awareness to one of the biggest problems of our society. For example, the amount of plastic polymers in the oceans has exceeded six-times compared to plankton, due to which aquatic birds and fishes are in danger (Auta et al., 2017). More than 80% of the total used plastics are synthetic, derived from petrochemicals, such as polyvinyl chloride, polyethylene, polypropylene (PP), polystyrene (PS), polyethylene terephthalate, polyurethane, and polycaprolactone (PCL) (Urbanek et al., 2018). Plastics belong to two main groups according to their degradation rate: non-biodegradable that are characterized by a slow rate process and biodegradable for which biodegradation is faster. The necessity of degradation of plastic wastes is obvious, however physical and chemical methods create various environmental hazards.

The growing amount of plastic waste creates selective pressure on microorganisms for the assimilation of the new substrates. Biodegradation is a process by which microbial organisms utilize plastics as a sole carbon source and decompose large polymer molecules to oligomers. It occurs after or in parallel with abiotic (physical and chemical) environmental factors influence as abiotic degradation weakens the strength and changes the structure of the polymers. However, as the evolutionary process was not accompanied so far by plastics presence in nature, the natural adaptation of microorganisms happens with a low degradation rate that could be accompanied by the risk of irreversible environmental changes (Debroas et al. 2017). It is important to consider the microbial degradation of synthetic polymers in order to understand what microorganisms are active in the biodegradation and the enzymes and mechanisms involved. A comparatively low number of reports tried to reveal the influence of plastic pollution on marine microbial life and the impact of microorganisms in the biodegradation process.

Extremophilic microorganisms develop a remarkable diversity of metabolism, however, little is known about their ability to break down synthetic polymer substrates (Atanasova et al., 2021a). Halophilic microorganisms are adapted to grow and thrive in saline environments, such as salterns, and high salt seawaters. Halophilic microorganisms grow at NaCl concentration of 1-3% (slight halophiles), 3-15% (moderate halophiles) or above 15% (extreme halophiles) (Ventosa et al., 2015). The most abundant moderate and extreme halophiles are members of two genera: *Halomonas* and *Chromohalobacter* (family *Halomonadaceae*), Gammaproteobacteria. Their natural niches are salterns, saline lakes, oceans, and coastal areas. The oceans are the largest saline environments with an average salinity of 3.5%, while hypersaline environments are derived from the evaporation of seawater and contain salt concentrations in excess of seawater reaching up to 35%. The information for plastic degrading marine communities is very scarce (Urbanek et al., 2018), although plastics become more and more persistent in the oceans. The reports on the composition of marine communities involved in plastic degradation have referred mainly to seawaters where salt concentration is not as high as the salterns one. These investigations have showed many unique features and revealed flourishing community taxa in the presence of plastics suggesting their active role in the degradation process. The metagenome analysis could reveal the community structure and the role of different members in the biodegradation process as some of microorganisms have the capacity for degrading-enzymes production, while others could contribute to biofilm formation posing the substrate and enzyme producer in a close vicinity. The results from the metagenome analysis could also contribute metagenome screening for desired enzyme activities from the preferred microbial group.

Few reports of depolymerization of plastics by thermophilic bacteria are known, mainly related to the ability of thermophilic actinomycetes (*Thermobifida fusca, Thermomonospora curvata, Thermomyces insolens*) to degrade PET (Wei et al., 2014). The thermophilic bacterium *Brevibacillus borstelensis* reduced the molecular weight of PE by 30% in 30 days at 50 ° C (Hadad et al., 2005). Consortium of *Brevibacillus* sp. and *Aneurinibacillus* sp. degraded PE and PP at 50°C (Skariyachan et al., 2018). All the above mentioned microorganisms are aerobes; the ability of thermophilic anaerobes to degrade plastics so far has not been reported.

The aim of the current work is to investigate the potential of halophilic microbial communities isolated from Pomorie salterns, Bulgaria and thermophilic communities isolated from Kamchatka hot springs, Russia for plastic degradation.

Materials and Methods

Sample collection and culture enrichment

Two samples, lye (C1) and water mud (C2) containing plastic debris were collected from Atanasovsko ezero (33% salinity), a part of Burgas salterns (42°32'09"N 27°28'49"E), Burgas Bay, Black Sea, Bulgaria, and used for the inoculation of a mineral medium containing one of the four synthetic plastics – two biodegradable, polycaprolactone (PCL) and polyvinyl alcohol (PVA) and two non-degradable or very difficult to degrade – polystyrene (PS) and polypropylene (PP). The samples were collected in sterile glass bottles and stored at 4 °C during their transfer to the laboratory. Two grams lye or mud were used as an inoculum for enrichment in 20 mL basal medium containing (%): NH_4NO_3, 0.01; KH_2PO_4, 0.03; K_2HPO_4, 0.14; $MgSO_4$, 0.01; $FeSO_4.7H_20$, 0.002; $Na_2MoO_4.2H_2O$, 0.0005; NaCl, 15, yeast extract, 0.03, pH 7.3. The plastics were sterilized separately by three hours soaking in 96% ethanol and five minutes sonication at room temperature. They were added to the basal medium at a final concentration of 0.3%±0.02. The cultivation was performed at 30°C, 80 rpm for two weeks, then the culture medium was replaced with a fresh one and cultivation continued for another two weeks. The control flasks did not contain any plastic. Growth (OD_{660}) and esterase activity in the supernatant were monitored daily. Three flasks were used for each variant.

Samples of water and sediments from two Uzon Caldera (Kamchatka) hot springs with temperature 78-80ºC were used for the enrichment of anaerobic thermophilic prokaryotes able to degrade low density polyethylene (LDPE) and high density polyethylene (HDPE). The samples were collected in 2018 during the Extremophiles Metabolism Laboratory (Federal Research Center of Biotechnology RAS) expedition to Kamchatka. One spring was located at Eastern thermal field of Uzon Caldera, the temperature at the sampling site was 80°C and pH was 6.5-7.0. Another spring was located at the Central thermal field of Uzon Caldera, the temperature at the sampling site was 78-80°C and pH was 6.1. For the enrichment of anaerobic thermophilic prokaryotes 1 ml of each sample from two Kamchatkan hot springs was used for the inoculation with 8 ml of anaerobically prepared medium. The gas phase of the enrichments was filled with N_2. The medium composition was as follows (%): NH_4Cl, 0.1;

$MgCl_2.2H_2O$, 0,033; $CaCl_2.6H_2O$, 0.01; KCl, 0.033; KH_2PO_4, 0.05; resazurin, 0.0001; trace elements solution, 0.1; vitamin solution, 0.1 (Wolin et al., 1963); $NaHCO_3$, 0.05; Na_2S. $9H_2O$, 0.05, pH 7.0. The medium was prepared under N_2 and 8 ml of medium were poured into 15 ml vials supplemented with 100 mg of shredded LDPE or HDPE were. The headspace of the vials was filled with N_2, and after that the vials were hermetically stoppered. Inoculated tubes with and without polymeric substrates, as well as those with the same non-inoculated medium and with polymers were incubated at 78°C for 45 days.

Electron Microscopy

Plastic surface appearance after four weeks of cultivation with C1 and C2 was observed by SEM. Control samples of plastics in the absence of bacteria were processed in parallel. The samples were fixed for 2 h in 4% glutaraldehyde in 0.1M Na cacodylate buffer (pH 7.2), then washed and post-fixed in 1% OsO_4 for 1 h. Dehydration was performed in graded ethanol series. After sputter-coating with gold using Edwards sputter coater, the samples were examined by SEM (Philips scanning electron microscope), at accelerating voltage 30 kV.

In the experiments on extremely thermophilic degradation of polyethylene, the morphology of the LDPE surface after bacterial treatment was investigated by transmission electron microscopy (Philips TEM-301, Netherlands). Samples were prepared using the replica method. For better contrast, platinum (VUP-5, Russia) was thermally sprayed onto the sample surface in a vacuum at an angle of 30° and then carbon was sprayed on the surface. The Pt-C replica was removed from the sample using gelatin. Gelatin was dissolved in water, and the replica was caught on a microscopic grid. The analysis was carried out at an accelerating voltage of 60 kV.

Esterase assay

Esterase assay was used for the estimation of enzyme activity. It was measured in the supernatant after centrifugation of the culture liquid at 4,000 g for 15 min. Hydrolysis of p-nitrophenyl palmitate (p-NPP) as a substrate was determined spectrophotometrically as described previously (Gupta et al., 2002) at 30°C in 0.05 M sodium phosphate buffer, pH 7.0 at 405 nm. One unit of esterase activity was determined as the amount of enzyme needed to liberate 1 µM p-nitrophenol per one minute in the described conditions. The molar extinction coefficient for p-nitrophenol at pH 7.8 was found to be 3.39×10^3 /M.

Analysis of the Microbial Communities

The metagenomic analysis of C2 microbial community was determined after four weeks-cultivation in a basal medium without plastic (MKC-C) and in the presence of 0.3% PCL. Two hundred mL culture liquid was centrifuged and the pellet was sent to Eurofins Genomics Europe, Ebersberg, Germany for a metagenomic analysis. 16S targets were PCR amplified from sample DNA extracts using target specific NGS primers and analysed by Amplicon sequencing on the Illumina MiSeq platform. During bioinformatical analysis the sequences were sorted into sequence sets according to their similarity. Each set was represented by a master sequence. Comparison of each master sequence with entries in the nucleotide collection of the US National Center for Biotechnology Information, NCBI finally provided the taxonomical assignment and hence the species presented in the sample. If the taxonomical assignment could not be resolved on the species level, a higher taxonomic rank was reported. Taxons with a fraction of at least 0.1% of all assigned reads were reported.

High-throughput sequencing of the variable V4-region of 16S rRNA gene was applied for characterization of microbial communities in extremely thermophilic enrichments on LDPE and HDPE. DNA isolation from the samples was performed using DNeasy PowerLyzer Microbial Kit

(Cat. 12255-50, Qiagen, Germany) according to the manufacturer's instructions and included the step of bead beating on FastPrep-24™ 5G grinder and lysis system (MP Biomedicals, USA).

Amplicon libraries were prepared according to the scheme described in Gohl et al. (2016). To obtain V4 amplicons PCR was performed on a StepOne Plus Real-Time instrument (Thermo Fisher Scientific, USA) using qPCRmix-HS SYBR mixture (Evrogen, Russia) and the following primer system: forward primer 515F(5′-CAAGCAGAAGACGGCATACGAGATGT GACTGGAGTTCAGACGTGTGCTCTTCCG ATCT XXXXXXXXXXXX ZZZZ GTGBCAGCMGCCGCGGTAA-3′), consisting, respectively, of "5 ′ Illumina Linker Sequence", "Index 1", "Heterogeneity Spacer" (Fadrosh et al., 2014) and reverse primer Pro-mod-805R (5′-AATGATACGGCGACCACCGAGATCTAC ACTCTTTCCCTACACGACGCTCTTCCGA TCT XXXXXXXXXXXX ZZZZ GACTACNVGGGTMTCTAATCC-3′), consisting of "3 'Illumina Linker Sequence", "Index 2", "Heterogeneity Spacer" and Pro-mod-805R primer sequence (Merkel et al., 2019). Amplicons were purified using the Cleanup Standard kit (Cat. BC022, Evrogen, Moscow, Russia). The quality of the final libraries was assessed using electrophoresis in agarose gel. High-throughput sequencing of the libraries was performed with MiSeq Reagent Micro Kit v2 (300-cycles) MS-103-1002 (Illumina, USA) on a MiSeq sequencer (Illumina, USA) according to the manufacturer's instructions. Primary data analysis, preparation of the OTU table, and analysis of taxonomic composition were performed using the SILVAngs online data analysis service and Silva138.1 SSU database.

Results

Plastic degradation by halophiles

Samples C1 and C2 were used for inoculation of the medium with PCL, PVA, PS, or PP as a main carbon source. The comparison of the microbial growth with and without plastic revealed a higher optical density of the PP, PS and PCL media inoculated with C1, while C2 did not show better growth on any of the used plastics (Table 1).

The highest level of esterase activity was established in C1-PVA, C2-PCL and C2-PVA media. SEM analysis confirmed the higher resistance to the non-biodegradable PP and PS on the action of bacterial communities, unlike the activity against the biodegradable plastics (Fig. 1). Its data were in a good agreement with the results of esterase assay (Table 1).

The four-week cultivation in the presence of C1 of PP, PS and PCL resulted in the occurrence of different degree of alterations of the plastic surfaces. Squamous-like deformations occurred focally at the surface of PS. In the case of PP, small surface elevations occurred which locally evolved in bigger fluffy formations. The visual changes of the PCL comprised the release of surface-attached thin threads as well as the formation of fluffy elevations alike the ones observed on PP, but with bigger size. With all three plastics, no evidence of biofilm formation was obtained in the presence of C1.

Table 1. Growth and esterase activity of microbial communities from Burgas salterns.

Synthetic Plastics	**C1**		**C2**	
	Growth (OD660nm)	**Esterase activity (U/ml)**	**Growth (OD660nm)**	**Esterase activity (U/ml)**
Control	0.30	0	0.34	0
Polypropylene (PP)	0.51	0	0.10	0
Polystyrene (PS)	0.49	0	0.37	0
Polycaprolactone (PCL)	0.51	5	0.09	46
Polyvinyl alcohol (PVA)	0.14	33	0.11	50

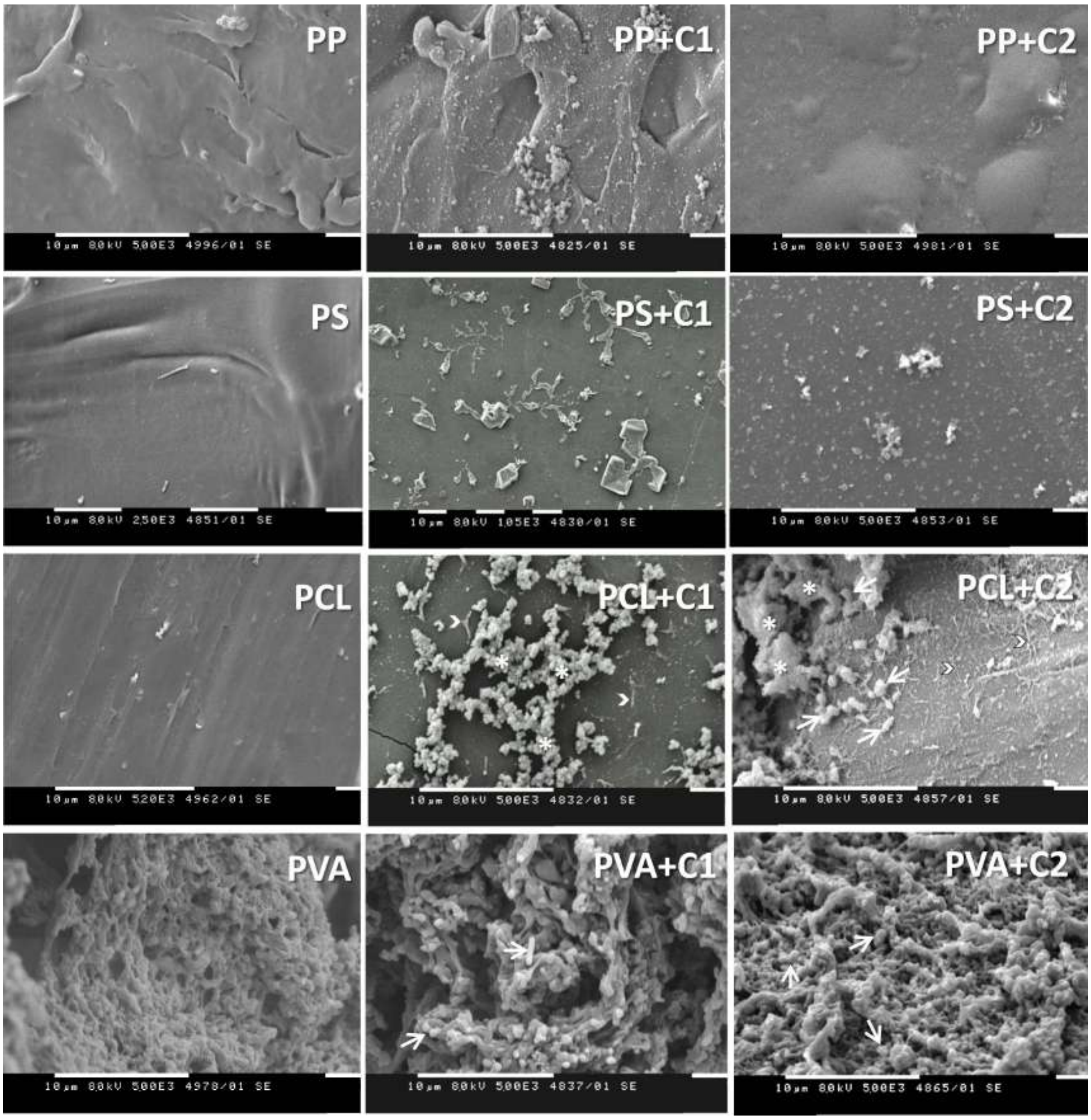

Fig. 1. Changes in the surface relief of the four examined types of plastics after four weeks incubation in the absence or presence of the bacterial communities C1 and C2. Arrows - bacterial cells. Asterisks, fluffy debris, deposited at the plastic surface. Arrowheads point to thread-like material on the plastic surface.

A comparison with the data in Table 1 implies that this community might interact with PP, PS and PCL by secreting in the culture liquid of enzymes (other than esterase) and utilizing for their growth the released degradation products. This community visually appeared to produce serious erosion of the surface of PVA where bacteria were seen to penetrate and attach to the plastic infolds. Together with the different pattern of changes in growth and esterase activity than the ones with the other three plastics, the data indicate a specific mode of interaction of C1 with PVA.

The presence of C2 in the culture medium did not apparently affect PP and caused some small deformations on PS. However, with PCL, surface-attached thread-like formations as well as cracks were formed at the plastic surface. The loci with threads and cracks served as foci of bacterial attachment, and bacteria adherent at the surface and deeper in the cracks were visualized. Often, the openings of the cracks were overlaid with fluffy debris. The interaction of C2 with PVA was alike that described for C1. Altogether, the observation of bacterial attachment on PCL and PVA, on the background of the little growth in the liquid medium, and the increased esterase activity (Table 1) evidences the role of C2-biofilms in the interaction of the community with the two plastics.

Table 2. Taxonomic groups, identified in C2-C.

Identified group	**Reads, %**	
	C2-C	**C2-PCL**
Halomonadaceae	24.1	49.7
Hyphomonadaceae	19.5	3.8
Halomonas sp.	15.7	40.2
Alcanivorax sp.	13.0	0.2
Phyllobacteriaceae	4.9	0.8
Martelella sp.	3.1	1.0
Chromohalobacter sp.	2.9	2.4
Hyphomicrobiaceae	2.3	-
Pelagibacterium sp.	2.3	-
Methylophaga sp.	1.8	0.6
Marinicauda sp.	1.6	0.2
Algiphilus aromaticivorans	1.1	-
Proteobacteria	0.9	0.4
Rhizobiales	0.3	0.1
Rhodobacterales	0.1	-
Rhodobiaceae	0.1	-

The significant changes of PCL surface structure and the high esterase activity of C2-PCL determined our interest in the comparison its community structure with that of C2-Control by 16S rRNA profiling. In both communities all identified groups belonged to Alpha- and Gammaproteobacteria, however, the fraction of the two classes, Alpha- and Gammaproteobacetria differed significantly in both samples. Alphaproteobacteria dominated in C2-C (61.3%) while it represented only 6.9% in C2-PCL. At the same time significant domination of Gammaproteobacteria was observed for the last sample (93.1%). Seventeen phylogenetic groups were represented in the control (C2-C) while in C2-PCL they were twelve (Table 2). Under the selective pressure of PCL as a carbon source the share of the family *Halomonadaceae*, order *Oceanospirillales*, increased significantly – from 24.1% in C2-C to 89.9% suggesting the essential role of its representatives in PCL degradation process and a possible activity of the bacteria of genus *Halomonas*, as the number of its which 16S rRNA sequences increased almost three-fold.

Plastic degradation by thermophiles

The sampling sites were placed in two Uzon Caldera hot springs (Kamchatka). The light microscopy of the cultures supplemented with LDPE and HDPE showed the growth of two types of cells: very short rods and long thin rods after 45 days of incubation (Fig. 2). Transmission electron microscopy of LDPE surface from the enrichment culture and from non-inoculated control demonstrated a significant difference in the surface structure. LDPE from the control medium had a very visible thread structure, while after the incubation with thermal samples the surface of the polymer became smooth, probably due to microbial activity (Fig. 3).

Fig. 2. Light microscopy of the enrichment culture obtained from Kamchatka hot spring with LDPE as a substrate.

High-throughput sequencing of 16S rRNA V3-V4 variable gene fragments showed that in the absence of the polymers microbial community contained representatives of genera *Thermodesulfobacter, Desulfovirgula, Fervidibacterium*, as well as members of phylum *Acetotermia* and uncultured Archaea (Fig. 4). Bacteria of genera *Dictyoglomus* (phylum *Dictioglomy*) and of genus *Caldanaerobacter* (phylum *Firmicutes*) represented 6 and 2%, respectively. In the enrichment culture supplemented with LDPE the bacteria of genera *Dictyoglomus* and *Caldanaerobacter* dominated, representing 64 and 23% of the whole community. The same picture was observed in the HDPE-supplemented enrichment (73 and 15%, respectively). These results are in agreement with the light microscopy of the enrichments where short rods (*Caldanaerobacter*) and long thin rods (*Dictyoglomus*) dominated.

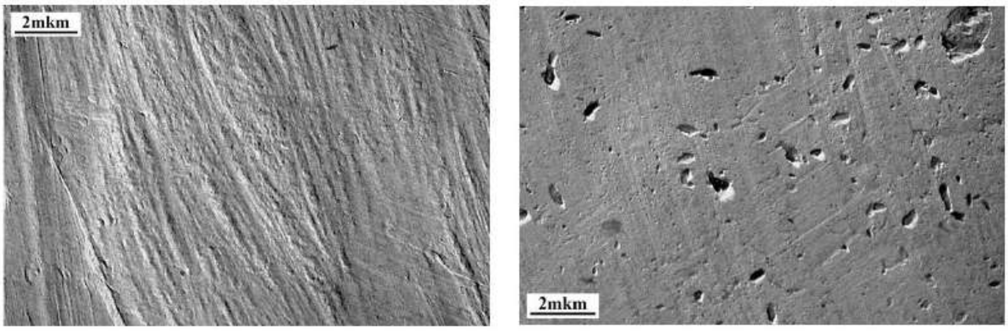

Fig. 3. TEM-images of LDPE surface after 45 days incubation at 78°C. A, Surface LDPE without inoculate; B, Surface of LDPE inoculated with thermal sample.

Discussion

Although the optical density of C1 in the presence of PP, PS and PCL was higher than in the control, no esterase activity was registered. These results could be explained by two reasons. Although some insignificant alteration of PP and PS surfaces was observed when C1 was cultured, these polymers are among difficultly degraded plastics (Pathak & Navneet, 2017; Urbanek et al., 2018) that probably required longer lasting experiments. The impossibility to register enzyme activity in our case could be due to a synthesis of very low enzyme amounts. Another possibility is that the active enzyme was not an esterase. Two groups of enzymes were identified as actively participating in PS and PP degradation, namely esterases and oxidoreductases (Mohanan et al., 2020). For example, P450 monooxygenase was involved in a saturated carbon-carbon bone cleavage reaction in the molecule of PS (Xu et al., 2019). An enzyme activity was registered in PVA culture liquid although the measured optical density was very low. This fact confirmed the opinion that the standard methods for the estimation of a bacterial growth, like the measurement of optical density or light microscopy cell counting are appropriate only in the case when the cells do not form biofilms on the plastic surface (Atanasova et al., 2021b). High levels of esterase activity in culture liquids of C2-PVA and C2-PCL were in a good correlation with the surface changes observed by SEM. Low OD of C2-PCL gives an evidence for a good biofilm formation ability of this community on PCL confirmed by SEM analysis. A good bacterial biofilm was observed after a fast colonization of plastic materials in seawater (Urbanek et al., 2018). The close vicinity between the substrate and microbial cells accelerates metabolic reactions that lead to the change

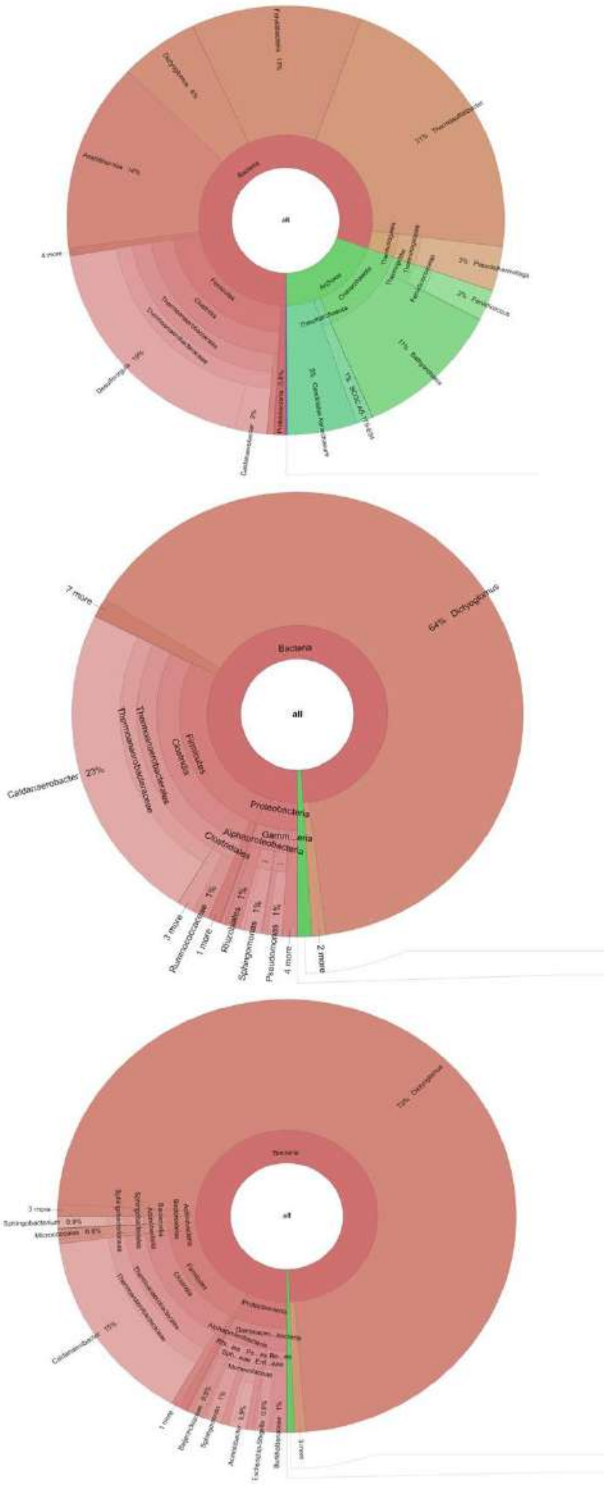

Fig. 4. Microbial diversity characterized by a high throughput sequencing of variable 16S rRNA fragments: a – initial sample from Uzon Caldera hot spring; b – microbial community after 3 weeks incubation with LDPE; c – microbial community after 3 weeks incubation with HDPE.

of plastic molecules followed by the breakdown of plastic itself (Harrison et al., 2011). Together with the nature of biofilm-forming microorganisms, the attachment at the surface and formation of biofilms depends on the surface structure and properties, like roughness, surface free energy, surface electrostatic interactions, and surface hydrophobicity (Rummel et al., 2017). Additional influence on biofilm development could be provoked by environmental conditions such as salinity, temperature, oxygen level, and limitation of light (Dash et al., 2013).

Analysis of the composition of C1 and C2 revealed the presence of 16S rRNA genes typical for halophilic environments. Higher density of bacteria attached to plastic wastes in comparison with free-living bacteria and bacteria attached to other organic particles in the natural saline environments, such as salt marshes and salt-rich industrial wastewaters was revealed by several researches. Similarly to the data in our investigations, a universal presence of representatives of the classes Gammaproteobacteria and Alphaproteobacteria, however in different proportions according to salinity, was observed in industrial water samples (Tourova et al., 2020a). A different community composition in biofilms formed on PS debris and industrial water was observed in Black Sea water at 10°C (Tourova et al., 2020b). These authors observed the family *Hyphomonadaceae* (mainly *Hyphomonas*) and the family *Erythrobacteraceae* (mainly *Erythrobacter*) as the most abundant across several stations in the coastal Baltic Sea. Dussud et al. (2018) reported the enhanced activity, higher numbers and bigger diversity of bacteria living on plastics in comparison with those living on organic particles and surrounding seawaters. Dominant microorganisms attached to plastic debris in Western Mediterranean Sea revealed a domination of *Cyanobacteria* (40.8%, mainly *Pleurocapsa* sp.) and Alphaproteobacteria (32.2%, mainly

Roseobacter sp. and *Erythrobacter* sp.). The most probable reason for the lower phylogenetic diversity observed in C2-PCL in comparison with other described marine communities and the different family and species domination (*Halomonadaceae*, 89.9%; mainly *Halomonas*, 40.2%) was the high salt concentration that restricted the number of the presented taxa. The observed dominant genus in our work differed from the genera commonly described as plastic degraders, like *Arthrobacter*, *Corynebacterium*, *Micrococcus*, *Pseudomonas*, *Rhodococcus*, and *Streptomyces* (Jackuin et al., 2019).

Most of the known microorganisms capable of polyethylene degradation are mesophilic aerobic organisms, including fungi and bacteria isolated from plastic contaminated soils, landfills and wastewater, as well as from sea water (Kotova et al., 2021). To date, only three thermophilic strains are known to decompose polyethylene at 50-60°C: *Aneurinibacillus* sp., *Brevibacillus* sp. (Skariyachan et al., 2018) and *Brevibacillus borstelensis* 707 (Hadad et al., 2005). In this work we found that anaerobic thermophilic bacteria of the genera *Dictyoglomus* and *Caldanaerobacter* are dominating in enrichment cultures with LDPE and HDPE. Representatives of both these genera are capable to hydrolyse complex organic substrates (Brumm et al., 2016; Kozina et al., 2010), however, they were never tested for the ability to degrade plastics. Bacteria of the genus *Dictyoglomus* are extreme thermophiles with the growth optimum in the temperature range from 70 to 75°C, and represent a deep phylogenetic lineage *Dictyoglomi* with only two cultured representatives. The genus *Caldanarobacter* is represented by moderate thermophiles of phylum *Firmicutes*. Further investigations are needed to prove that these two organisms are capable of using PE as the growth substrates; if confirmed it would be the first evidence on anaerobic PE degradation by thermophilic bacteria.

Conclusions

This work represents a first effort to elucidate the ability of saltern communities to degrade some synthetic plastics. Two communities isolated from Bulgarian salterns caused significant changes in the appearance of PVA after cultivation of C1 and C2, and PCL after cultivation on C2. The measured esterase activity was in a good agreement with the observed plastic surface changes. The analysis of the C2 microbial community composition with and without plastic revealed the selective pressure of the carbon source and the possible important role of the family *Halomonadaceae* representatives, and especially the species *Halomonas* in the process of PCL degradation. This result differed from other reported dominant taxa in halophilic niches. As *Halomonas* is among the most abundant genera in the seawater and salterns much hope is oriented to its active role in the development of future biodegradation processes in high salt environments.

The observed changes in polymer structure and microbial communities composition allow us to make an assumption on the ability of anaerobic organotrophic extremely thermophilic bacteria to degrade the polyethylene of low and high density.

Acknowledgements

This study was supported by the National Fund for Scientific Research, Bilateral Projects 2019 – BULGARIA – RUSSIA (Grant KP-06-Russia-17).

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Received: 07.11.2021
Accepted: 11.12.2021

ECOLOGIA BALKANICA
2021, Vol. 13, Issue 2 December 2021 pp. 223-237

Local and Invasive Species of Freshwater Turtles (Reptilia: Emydidae, Geoemydidae) in the Eastern Part of Strandzha Nature Park (Bulgaria) - Distribution and Populations Assessment

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Abstract. The current study presents data on the distribution, abundance, density, sex and age ratio of the populations of two freshwater turtle species - *Emys orbicularis* (Linnaeus, 1758) and *Mauremys rivulata* (Valenciennes,1833) in selected wetlands in the eastern part of "Strandzha" Nature Park. During the research period were recorded a total of 142 ind. of *E. orbicularis* and 65 ind. of *M. rivulata*. Two single localities of the invasive species *Trachemys scripta elegans* (Wied-Neuwied, 1839) have been documented as well. Morphometric characteristics and body condition index of the captured individuals is also presented. Unmanned aerial vehicles (drones) were used for video recording of some sections of the studied transects, testing their possible usage for monitoring purposes. The current conservation status and threats for the studied species are also discussed.

Key words: *Emys orbicularis, Mauremys rivulata, Trachemys scripta elegans*, UAVs monitoring, "Strandzha" Nature Park, South-East Bulgaria.

Introduction

There are two species of freshwater turtles native to Bulgaria - the European Pond Turtle *Emys orbicularis* (Linnaeus, 1758) and the Balkan Pond Turtle *Mauremys rivulata* (Valenciennes, 1833), but the invasive species *Trachemys scripta* is also present with three subspecies already established in the country: *T. s. elegans* (Wied-Neuwied, 1839), *T. s. scripta* (Schoepff, 1792) and *T. s. troostii* (Holbrook, 1836) (Biserkov et al., 2007; Rhodin et al., 2017). The European Pond Turtle in Bulgaria is widespread throughout the country, missing only in the highlands (Biserkov et al., 2007) - up to 1221 m a.s.l. (Kornilev et al., 2017). The habitats that *E. orbicularis* uses are: swamps, rivers, limans, micro-dams, fishponds, irrigation and drainage canals, thermal springs, standing and slow-flowing reservoirs (Biserkov et al., 2007; Kornilev et al., 2017). *M. rivulata* is found only in the southernmost, warmest and lowest habitats in Bulgaria, representing the northernmost limit of distribution of the species (Beshkov, 1987; 1993; Biserkov et al., 2007; Petrov, 2007).

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The maximum altitude at which it is registered is 474 m. (Kornilev et al., 2017). The distribution of the two native species of freshwater turtles partially overlaps only in the southern parts of the country. The Eastern part of the "Strandzha" Nature Park is one of the few places in Bulgaria where the two native species can be observed together.

There are relatively few studies on the ecology of freshwater turtles in the country. The data from this study will help to prepare an assessment of the state for their populations in the eastern part of "Strandzha" Nature Park and possibly to predict their future development.

Material and Methods

Study area

The study was conducted in the period June - August 2021 on the territory of "Strandzha" Nature Park, which overlaps with NATURA2000 protected area - "1007 Strandzha", with code BG0001007. The mouths of the rivers Silistar and Veleka were studied, as well as several smaller rivers flowing into the Black Sea - north and south of the town of Ahtopol. Also, the studied areas fall within the boundaries of several protected areas: Protected Area "Estuary of the Veleka River", Protected Area "Silistar" and Nature monument "Nakovo Kladenche". These are important habitats for local species of freshwater turtles, included in the National System for Monitoring the State of Biological Diversity (Tsankov et al., 2016).

Field surveys

The turtles were identified visually using binoculars "MINOX BF 10x25 BR" (MINOX GmbH Walter Zapp.). The field guides by Bannikov (1977) and Biserkov et al. (2007) were used for determine the native species and the CITES Identification Guide - Turtles & Tortoises (Charette, 1999) and the Global Invasive Species Database (ISSG, 2021) for determine the invasive species.

In the present work, the density and the abundance of freshwater turtle populations were studied by the linear transect method (Sutherland, 2000), following the appropriate habitat types (river banks, nearby small standing water basins). The transects we used are given in Fig. 1: Transect №1 - from the river mouth to the bridge over Veleka River; Transect №2 (upstream from the bridge of Veleka River - covered by boat; Transect №3 (Silistar River); Transect №4 (NM "Nakovo kladenche") and Transect №5 (Ahtopol Town).

The transects for Veleka and Silistar Rivers have a fixed width of 50 m (25 m on each side of the medial line of observation), and for the smaller rivers near the town of Ahtopol they have a fixed width of 10 m (5 m on each side of the medial line of observation). The density was calculated by the following formula:

$$\mathrm{D_e} = \frac{\mathrm{n}}{2\mathrm{rl}} \times 10000,$$

where:

D_e - population density (number of individuals per ha);

n - number of observed individuals;

r - transect width (in meters);

l - transect length (in meters).

To compare the data on the numbers of the individuals of the populations of the two native species, we used the abundance parameter (Ab). Abundance is defined as the total number of individuals of a given species found in a given territory (Turpie, 1995; Sutherland, 2000). Due to the difference in the widths of the transects, the following formula was used for better comparison and analysis of the data from the present thesis:

$$\mathrm{Ab} = \frac{\mathrm{n}}{\mathrm{L}}.1000,$$

where:

Ab - abundance (number of individuals per 1000 linear meters - l.m.);

n - number of observed individuals;

L - studied area in linear meters.

In some cases, the turtles are caught by hand or with the help of a fishing net, for more accurate determination; taking some morphometric parameters (SCL - straight carapace length, MPL - maximum plastron length, H - height of the shell and MCW - maximum carapace width (measured at the widest part) (following Mazzotti, 1995 and Alcayde, 2007), measured in millimeters (mm), with a caliper (with an accuracy of 0.1 mm - Fig. 2, as well as body weight (BW) in grams (g), measured with a digital scale "WeiHeng" with an accuracy of 10 g); determination of sex (where possible, following characteristics, indicated by Biserkov et al. (2007) and Zuffi & Gariboldi (1995) and age of the individuals, based on carapace length (individuals with carapace length of less than 110 mm were considered juveniles (according to Ayaz & Çiçek (2011); between 110 and 140 mm - subadults (according to Kotenko, 2000) and individuals over 140 mm were considered adults.

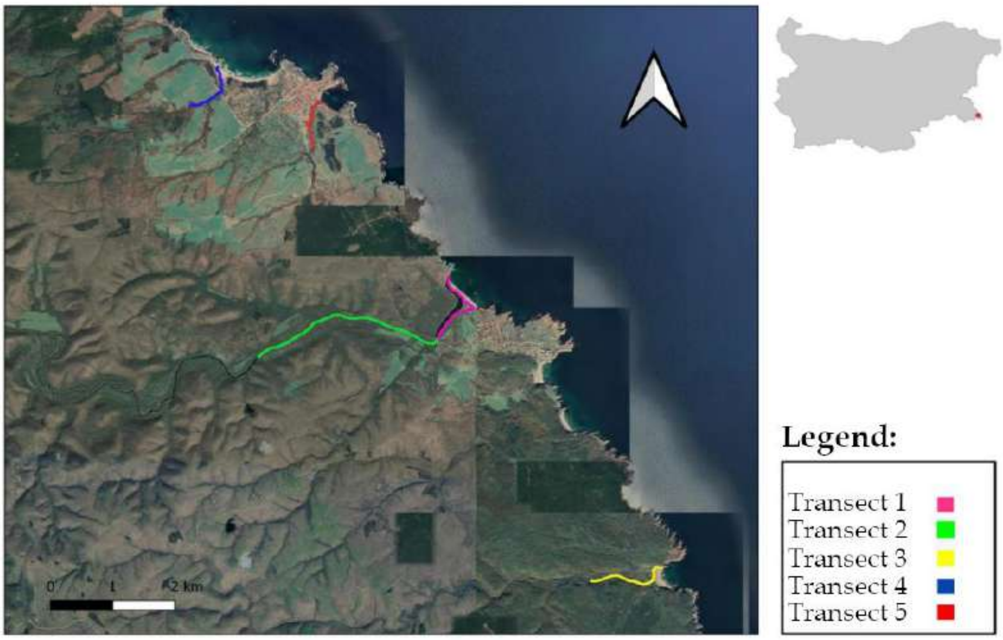

Fig. 1. Locations of the used transects for the field studies (explanations are in the text).

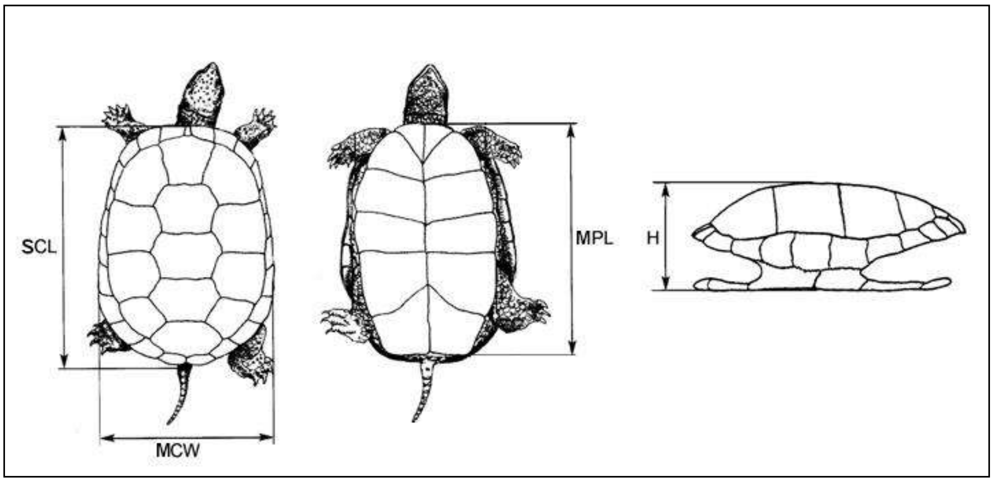

Fig. 2. Measured morphometric parameters (abbreviations according to Zuffi & Gariboldi (1995), with changes): SCL - straight carapace length, MCW - maximum carapace width, MPL - maximum plastron length, H - height of the shell (after Alcayde, 2007).

The body condition index (BCI) is used to assess the health status of individuals, which is widely used in conservation ecology, as it provides indirect data on the quality of habitat (Stevenson & Woods, 2006). The body condition index (BCI) used in the present work is defined in the sense of Willemsen & Hailey (2002), by the formula:

$$BCI = \log_{10} BW/BW',$$

where: BCI – body condition index;
BW – the actual weight of the individual;
BW' – the expected weight of the individual as a function of size.

The expected weight of the individuals is obtained according to the modified ellipsoid volume formula (after Loehr et al., 2004 and Alcayde, 2007):

$$BW' = \frac{\pi \times SCL \times H \times MCW}{6000}.$$

A BCI value = 0 indicates that the observed weight is equal to the expected weight; values above "0" indicate good health of the individual due to good environmental conditions, while negative values indicate poor physical condition as a result of poor nutrition or some kind of stress (Stevenson & Woods, 2006; Alcayde, 2007).

The team conducting the field research has an up-to-date permit issued by the MOEW (№ 870 / 29.04.2021) pursuant to Ordinance № 8 (Promulgated State Gazette No. 4 / 16.01.2004) on the terms and conditions for issuing permits for exemptions from the prohibitions introduced by the Law on the Biological Diversity of Animal and Plant Species from Annexes № 2, 3 and 4.

QGIS 3.16.6-Hannover (QGIS.org, 2021) and Garmin BaseCamp (Garmin Ltd., 1996-2021) were used for geographical visualization of the collected data. The statistical package PAST v. 4.0 was used for the statistical processing of the data and their graphic presentation (Hammer et al., 2001). The data were analyzed by descriptive statistics and presented graphically by Box & Whiskers plots. A Shapiro-Wilk test was used for testing the normal distribution of the data (Shapiro & Wilk, 1965). When comparing or looking for differences between individual variables, nonparametric tests (χ^2-test, Mann-Whitney U-test for independent pairs) were applied, in the absence of a normal distribution of data (Fowler et al. 1998). Differences with $p<0.05$ [$\alpha=5\%$] were considered statistically significant.

Results

Spatial distribution

The distribution of the three registered species of freshwater turtles (*E. orbicularis, M. rivulata and T. s. elegans*) in the study area is presented in Fig. 3, 4 and 5.

Fig. 3. Distribution of the European Pond Turtle (*Emys orbicularis*) in the eastern part of NP "Strandzha".

Fig. 4. Distribution of the Balkan Pond Turtle (*Mauremys rivulata*) in the eastern part of NP "Strandzha".

Fig. 5. Distribution of the Red-eared slider (*Trachemys scripta elegans*) in the eastern part of NP "Strandzha".

In total, in all studied areas, for the period of the study, we recorded 142 ind. *E. orbicularis,* 65 ind. *M. rivulata* and 2 ind. *T. s. elegans.* For Veleka River - a total of 89 ind. *E. orbicularis* and 16 ind. *M. rivulata,* indicating that *E. orbicularis* is the predominant species. The results of the study at the mouth of the Silistar River show a sympatric distribution of the two local species, with a total of 45 ind. *E. orbicularis* and 44 ind. *M. rivulata.* From the invasive species *T. s. elegans,* we recorded 2 ind. along the Veleka River (upstream above the bridge and in a small spillway near the river mouth).

Fig. 6 and 7 show the spatial distribution of *E. orbicularis* and *M. rivulata* along the rivers Veleka and Silistar - the two rivers with the most registered individuals of the target species. The distance from each recorded point of an observed individual or group of individuals of each species to the river mouth is measured by the actual river kilometers, and the graphs show the mean values and the standard deviation.

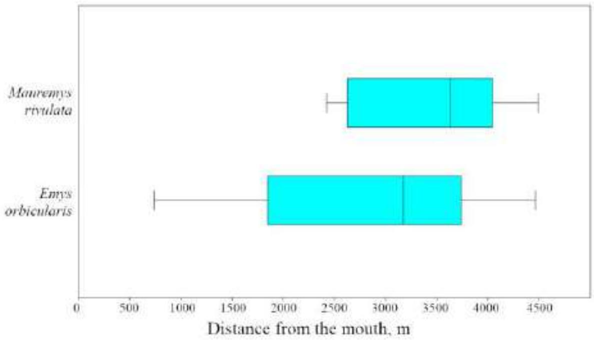

Fig. 6. Box & whiskers plots of the spatial distribution of the two native freshwater turtle species at Veleka River.

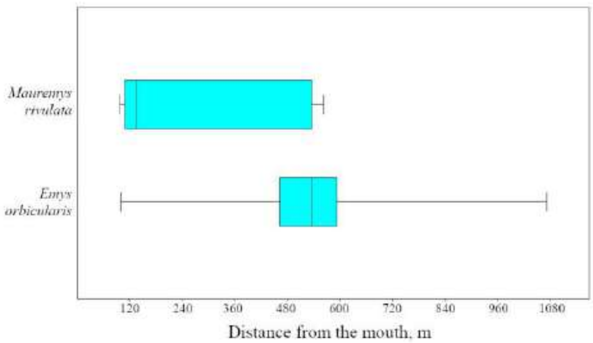

Fig. 7. Box & whiskers plots of the spatial distribution of the two native freshwater turtle species at Silistar River.

In the area of the Veleka River *E. orbicularis* is distributed mainly in the lower to middle parts of the studied area, while *M. rivulata* is observed mostly in the middle to the upper parts (Fig. 6). Although we did not find a statistically significant difference in the calculated distances from the estuary between the two species (Man-Whitney test: U=166.00, z=-1.44, p=0.15), the minimum distances are quite different (741 m for *E. orbicularis* and 2422 m for *M. rivulata*), while the maximums are similar (4463 m vs. 4491 m). Most likely, this result is due to the strong degree of disturbance by people from the estuary to about 2000 m upstream.

Similar to our results on the spatial distribution of the two species of native aquatic turtles at Veleka River from the bridge to 4.5 km upstream were reported by Popgeorgiev et al. (2017), who survey the river by boat using a slightly different methodology. However, the results they obtained also showed the prevalence of *M. rivulata* in the middle and upper parts of the study area, while *E. orbicularis* was found mainly in the lower and middle parts.

Along the Silistar River, the opposite trend was observed in the spatial distribution of the two species, as *M. rivulata* was recorded mainly closer to the river mouth, where there is a strong degree of disturbance by humans (Fig. 7). We did not record a statistically significant difference in the measured distances from the estuary to the individuals (Man-Whitney test: U=37.00, z=-1.48, p=0.14), although there is a visible difference in maximum values (1071 m for *E. orbicularis* and 563 m for *M. rivulata*), at very close minimum values. During our field work we observed a large accumulation of freshwater turtles near the bridge at the beach, at the river mouth of Silistar. The reason for this was the tourists in the area who threw food (bread) into the river, which may be the probable reason for the observed trend in the spatial distribution there.

Abundance and density of the populations

Data on the numbers, abundance and density of the studied five transects are presented in Table 1.

Table. 1. Numbers, abundance and density of the recorded species of turtles in Transect №1 (from the mouth of the river to the bridge over Veleka River). *Legend:* N - numbers, Ab - abundance (ind./1000 l.m.), D - density (ind./ha), ± - standard deviation, * - individuals registered with a drone.

Species	June			July			August			Total		
	N	Ab	D	N	Ab	D	N	Ab	D	N	Ab	D
Emys orbicularis	19	11.88	1.19	1	0.63	0.06	1	0.63	0.06	21	13.13±6.50	1.31±0.65
*Emys orbicularis**	-	-	-	0	0.00	0.00	1	0.63	0.06	1	0.63	0.06
Trachemys scripta	1	0.63	0.06	0	0.00	0.00	0	0.00	0.00	1	0.63	0.06

Table 2. Numbers, abundance and density of the recorded species of turtles in Transect №2 (Veleka River). *Legend:* N - numbers, Ab - abundance (ind./1000 l.m.), D - density (ind./ha), ± - standard deviation.

Species	June			July			August			Total		
	N	Ab	D	N	Ab	D	N	Ab	D	N	Ab	D
Emys orbicularis	30	9.09	0.91	22	6.67	0.67	16	4.85	0.48	68	20.61±2.13	2.06±0.21
Mauremys rivulata	1	0.30	0.03	1	0.30	0.30	14	4.24	0.42	16	4.85±2.27	0.48±0.23
Trachemys scripta	0	0.00	0.00	1	0.30	0.30	0	0.00	0.00	1	0.30	0.03

Table 3. Numbers, abundance and density of the recorded species of turtles in Transect №3 (Silistar River). *Legend:* N - numbers, Ab - abundance (ind./1000 l.m.), D - density (ind./ha), ± - standard deviation, * - individuals registered with a drone.

Species	June			July			August			Total		
	N	Ab	D	N	Ab	D	N	Ab	D	N	Ab	D
Emys orbicularis	2	1.43	0.06	11	7.86	0.33	32	22.86	0.97	45	32.14±11.00	1.36±0.47
*Emys orbicularis**	-	-	-	3	2.14	0.09	5	3.57	0.15	8	5.71±1.01	0.24±0.04
Mauremys rivulata	0	0.00	0.00	12	8.57	0.36	32	22.86	0.97	44	31.43±11.55	1.33±0.49
*Mauremys rivulata**	-	-	-	0	0.00	0.00	1	0.71	0.03	1	0.71	0.03

Table 4. Numbers, abundance and density of the recorded species of turtles in Transect №4 (NM "Nakovo kladenche"). *Legend:* N - numbers, Ab - abundance (ind./1000 l.m.), D - density (ind./ha), ± - standard deviation, * - individuals registered with a drone.

Species	June			July			August			Total		
	N	Ab	D	N	Ab	D	N	Ab	D	N	Ab	D
Emys orbicularis	0	0.00	0.00	1	0.91	0.45	4	3.64	1.82	5	4.55±1.89	2.27±0.95
*Emys orbicularis**	-	-	-	1	0.91	0.45	0	0.00	0.00	1	0.91	0.45
Mauremys rivulata	0	0.00	0.00	0	0.00	0.00	5	4.55	2.27	5	4.55±2.62	2.27±1.31

Table 5. Numbers, abundance and density of the recorded species of turtles in Transect №5 (Ahtopol Town). *Legend:* N - numbers, Ab - abundance (ind./1000 l.m.), D - density (ind./ha), ± - standard deviation, * - individuals registered with a drone.

Species	June			July			August			Total		
	N	Ab	D	N	Ab	D	N	Ab	D	N	Ab	D
Emys orbicularis	2	1.43	0.71	0	0.00	0.00	1	0.71	0.36	3	2.14±0.71	1.07±0.36
*Emys orbicularis**	-	-	-	-	-	-	1	0.71	0.36	1	0.71	0.36

According to Beshkov (1987), along the lower reaches of the Veleka River (up to 4 km from the mouth) "the numbers of *M. rivulata* is at least a few dozen individuals". In our study at Veleka River a total of 89 ind. *E. orbicularis* and 16 ind. *M. rivulata.* According to the same author "along the river flowing into the sea on the northern beach near Ahtopol" are registered 3 ind. *M. rivulata* and 10 ind. *E. orbicularis,* later Beshkov (1993) reported that the populations of both species from this locality were completely destroyed. Unfortunately, we also did not find any aquatic turtles at this locality during our study.

In a study by Popgeorgiev (2008) on the negative effects of fires in the Eastern Rhodopes and Sakar Mts., the author reports the following values for abundance (Ab) of the populations of *E. orbicularis* from several studied areas during the period 2004-2006. A total of 64 individuals have been identified near the village of Rogozinovo in the control sample (K) (Ab = 2.94 ind./1000 l.m.), near the village of Kolets, a total of 12 ind. (Ab = 0.11 ind./1000 l.m.) in the control sample (K), near the village of Ostar Kamak, a total of 64 ind. (Ab = 0.60 ind./1000 l.m.) in control sample (K). In the area of the village of Gorno Lukovo 1 individual of the species *M. rivulata* (Ab = 0.06 ind./1000 l.m.) was recorded in the control sample (K) and in a sample from the burned area (P), and near the village of Gorno Bryastovo *E. orbicularis* was recorded only in the dam in the burned area (P), with 7 ind. (Ab = 0.39 ind./1000 l.m.).

Mollov (2019) calculates the abundance of the *E. orbicularis* population along Maritsa River, in the city of Plovdiv (0.692 ind./1000 l.m.) and the abundance of the population of the same species from an irrigation canal in the northern part of the city (0.454 ind./1000 l.m).

Our data on the abundance of populations of the two native species of freshwater turtles from the eastern part of "Strandzha" Natural Park greatly exceed those reported from the Eastern Rhodopes and Sakar by Popgeorgiev (2008) and those from the Plovdiv City (Mollov, 2019), which is an expected result, given the status of the protected area. This is an indication of the high abundance and density of the populations of *E. orbicularis* and *M. rivulata* in "Strandzha" Nature Park.

Various authors estimate of the density of *E. orbicularis* populations from some countries in Europe: in Bardello, Italy - 7.2 ind./ha (Mazzotti, 1995); for Lake Yayla, Turkey - 81 ind./ha. (Ayaz et al., 2008); from the area to Pazaragac (Turkey) - 83 ind./ha. Ayaz et al. (2007a); in Lake Sulyuklu (Manisa, Turkey) - 83 ind./ha. (Ayaz & Çiçek, 2011a); Tisza River in Southern Hungary - 142-228 ind./ha. (Balázs & Györffy, 2006), but all are based on the Capture-Recapture method and do not allow comparison with our data. Güçlü & Türkozan (2010) report a population density of *M. rivulata* - 434 ind./ha in Izmir province, western Turkey, using the same method.

The values established by us for the density of the populations of *E. orbicularis* in the studied area, using the transect method vary from 1.07 ind./ha to 2.27 ind./ha., and for *M. rivulata* - from 0.48 ind./ha to 2.27 ind./ha.

In the present study, an attempt was made to use unmanned aerial vehicles (UAV, drones) to travel the distance along the constructed transects and photograph and video record the turtles, in order to determine and count them in laboratory conditions. This is the second study in Bulgaria that uses UAVs in the study of freshwater turtles, after the pilot study of Biserkov & Lukanov (2017) in Sofia City. Our experience has shown that the use of UAVs for monitoring freshwater turtles is possible only over water basins with less vegetation, which allows unimpeded flight over the river or reservoir. In some cases during the field work this was not possible due to the dense and low tree vegetation along the river banks - e.g. Silistar River (Transect №3). This transect was explored by drone only for the first 500 m from the river mouth, as the passage of the drone upwards was impossible due to the low branches of the trees and the loss of connection with the remote control. This is the reason for the significantly smaller number of registered turtles with a drone than those recorded when walking the line transect. It was also impossible to study Transect №2 with UAVs, due to the curves that the river makes in this section and the loss of connection with the drone. For this reason, and the densely overgrown shores, this transect was completely traversed only by boat.

Despite the indisputable advantages that UAVs gives in the monitoring of freshwater turtles, they do not provide 100% imaging of the entire transect and registration of all turtles (Fig. 8 and 9).

Improvements in the technology of UAVs (drones) and in particular the cameras (higher resolution) will allow the capture of smaller species of herpetofauna, mainly terrestrial representatives (lizards, frogs, turtles, etc.). The study by Huerta et al. (2020) assesses whether UAV technology can be used as a method for passive study of herpetofauna species, in addition to traditional research methods. It is possible that this method will be the main tool for future detection and monitoring of some species of the herpetofauna.

The UAVs equipment used in our study is a Phantom 4 Pro drone equipped with a 2.5 cm, 20-megapixel sensor capable of shooting 4K / 60 fps (ie 4000 horizontal pixel resolution at 60 fps), video with a flight time of 30 minutes (DJI Technology Inc. Shenzhen, China). Large (> 20 cm) and brightly colored objects can be recognized by video and photos taken up to 10 m altitude by the drone. Small objects (<10 cm) are indistinguishable up to about 5 m altitude. Although the UAVs recognition method may not be a significant improvement in determining the presence or relative abundance of herpetofauna in the study areas, it has the advantage of providing the researcher with a video of the present species, which can be used later.

Morphometric characteristics

The morphometric parameters measured by us for *E. orbicularis* and *M. rivulata* from the Veleka and Silistar rivers are presented in Table. 6 and 7. Only *E. orbicularis* individuals were caught from the Veleka River during the study.

The values of the main morphometric characteristics for both species of freshwater turtles presented by us fall within the ranges reported by other authors for these species from other European countries (Mazzotti, 1995; Ayaz & Çiçek, 2011a; Ayaz & Budak, 2008). However, due to the small number of individuals caught by us, we cannot make comparisons between the sexes and the two populations.

Fig. 8. *E. orbicularis,* captured with "Phantom 4 Pro" drone from 5 m altitude at Silistar River.

Fig. 9. *E. orbicularis*, captured with "MavicPro" drone from 5 m altitude at Silistar River.

The body condition index (BCI), calculated by us for *E. orbicularis* and *M. rivulata* from the two studied locations has positive values, although close to zero (Tables 6 and 7), which means that the weight of the studied aquatic turtles is close to expected one and the quality of the habitats is "good".

Sex and age ratio of the populations

The sex ratio of the populations of *Emys orbicularis* was studied at Veleka River (Transect №1 and 2) and Silistar River (Transect №3), and that of *Mauremys rivulata* only at Silistar River, due to the low number of captured individuals at Veleka River.

The population of *E. orbicularis* at Veleka River, showed a male-female ratio of 1:0.82 (n=20), and we did not record a statistically significant difference from the normal distribution - 1:1 (χ^2=0.10025, df=1, p=0.75). We recorded a similar sex ratio at Silistar River - 1:0.7 (n=17), again without a statistically significant difference from the normal expected distribution - 1:1 (χ^2=0.26, df=1, p=0.61).

Table 6. Measured morphometric parameters in *E. orbicularis* from the mouth of the Veleka River. Mean values ± standard deviation (SD) are presented. The abbreviations are indicated in the chapter "Material and Methods".

Parameter	**Males (n=2)**	**Females (n=2)**	**Juveniles (n=1)**
Body weight (BW), g	572.50±123.73	495±28.28	60
SCL, mm	146.50±9.19	132.5±1.41	65
MPL, mm	140.50±0.19	127.5±3.53	60
H, mm	62.5±9.19	55.5±0.71	28.1
MCW, mm	110.50±2.69	108.00±16.97	56
SCL/MCW	1.32±0.049	1.24±0.18	1.16
SCL/BW	0.26±0.042	0.27±0.014	1.08
BCI	0.0052±0.0007	0.0065±0.00099	0.03

Table 7. Measured morphometric parameters in *E. orbicularis* and *M. rivulata* from Silistar River. Mean values ± standard deviation (SD) are presented. The abbreviations are indicated in the chapter "Material and Methods".

Parameter	*Emys orbicularis*			*Mauremys rivulata*		
	Males (n=7)	**Females (n=3)**	**Juveniles (n=7)**	**Males (n=3)**	**Females (n=5)**	**Juveniles (n=8)**
Body weight (BW), g	382.43±86.47	346.67±102.75	131.25±17.02	515.00±265.56	494.00±137.13	134.37±36.20
SCL, mm	134.03±6.68	128.67±12.66	91.05±6.49	163.37±41.30	149.50±12.76	103.76±9.55
MPL, mm	119.10±9.03	118.33±15.57	86.27±6.15	133.90±26.42	135.50±11.14	85.47±8.57
H, mm	52.48±6.47	39.90±10.65	34.95±1.63	52.53±26.42	60.10±7.15	34.20±3.37
MCW, mm	103.34±5.22	101.00±8.89	74.17±5.38	114.83±21.03	109.10±7.60	76.09±6.58
SCL/MCW	1.30±0.030	1.27±0.023	1.23±0.015	1.41±0.11	1.37±0.077	1.36±0.033
SCL/BW	0.36±0.059	0.39±0.086	0.70±0.056	0.36±0.12	0.31±0.061	0.81±0.15
BCI	0.0069±0.0012	0.0096±0.0026	0.017±0.0022	0.0059±0.0031	0.005±0.001	0.015±0.0033

The sex ratio of the population of *M. rivulata* at Silistar River showed a male-female ratio of 1:1.25 (n=16), but again without a statistically significant difference from the expected normal distribution of 1:1 (χ^2=0.12, df=1, p=0.72).

According to Mazzotti (1995), the ratio between males and females ind. of *E. orbicularis* population in Bardello, Italy is 1:2. In a population studied by Balázs & Györffy (2006) in southern Hungary, the sex ratio of *E. orbicularis* was approximately 1:1, slightly in favor of females. According to Ayaz et al. (2007a) near Pazaragac (Turkey) the sex ratio in adult *E. orbicularis* individuals was significantly in favor of males (2.02♂:1♀; p<0.001). Ayaz et al. (2008) reported a ratio of 54% males, 42% females, and 4% juveniles for *E. orbicularis* population, and overall sex ratio of 1.31:1 (659♂:504♀, p<0.001). Güçlü & Türkozan (2010) studied a population of *M. rivulata* in Izmir province, western Turkey and reported a sex structure (females:males:juveniles) in favor of the females (3.01:1.17:1).

Rifai & Amr (2004) calculate the ratio between males and females for *M. rivulata* (1:1.3), as their data are similar to ours from Silistar River - 1:1,25, in favor for the females.

The age structure of the population of *E. orbicularis* at Veleka River has a visible, statistically significant (χ^2=31.671, df=2, p=0.0000001) predominance of adult individuals (n=53), followed by subadults (n=14) and the lowest share of juveniles (n=2). The population at Silistar River is again dominated by adults (n=23), as the share of subadults (n=13) and juveniles (n=10) is almost equal, and here we did not find a statistically significant difference from the normal expected distribution 1:1:1 (χ^2=2.81, df=2, p=0.24).

For *M. rivulata,* the age structure of the Veleka River population is with a slight dominance of adult individuals (n=9), followed by subadults (n=5) and juveniles (n=4) are almost equal (χ^2=1.09, df=2, p=0.58), while the population at Silistar River showed dominance of adults (n=24), followed by juveniles (n=12) and subadults (n=8), without a statistically significant difference from the normal expected distribution 1:1:1 (χ^2=4.38, df=2, p=0.11).

The low proportion of juveniles in three of the studied four populations of the two species of freshwater turtles does not necessarily mean that these populations are decreasing. It should be noted that the juveniles are the most difficult to register due to their small size and more discrete way of life. In the area of Silistar River, at coordinates N42° 01.366', E28° 00.506' we found 3 hatchlings of a freshwater turtle, as the number of hatched eggs in each varied from 7 to 9. Unfortunately, based only on the shells of the eggs we can not determine the species, but this is a possible proof that the two species of native freshwater turtles are breeding successfully and in all probability their populations in the area of the Silistar and Veleka rivers are stable.

Similar results were reported in study by Mollov (2019), using the same methodology, the age structure of the *E. orbicularis* population from Maritsa River in Plovdiv City, showed a ratio between the three age groups (Ad:Sub:Juv) 1:0.75.:0.5, with the largest proportion of adults (χ^2=0.67; df=2; p=0.72). In a study by Vamberger et al. (2017) in the largest swamp in Slovenia, 20% of the *E. orbicularis* caught were subadult. In a one-year observation of a population of *E. orbicularis* in the "Los Aribes del Duero" Nature Park (Zamora, Spain), Alarcos et al. (2008) calculated the percentage of adult individuals - 87.2%, using a transect method to study the population, in combination with other methods.

Conservation significance and threats

Both species of native freshwater turtles are of high importance for the conservation of the biodiversity, according to the National and European environmental legislation.

During the present study, we identified the following threats for the freshwater turtles in the study area:

- Disturbance from tourists, especially during the active summer season (July-August). A stream of tourists, using boats, kayaks and other vessels, enter the Veleka River and disturb the turtles. Near the mouth of Silistar River there is a well-developed camping area near the river itself, which also disturbs our two native species.

- Aquatic turtles are caught accidentally on the hooks of fishermen, and according to Beshkov (1993), most of them are often killed afterwards. On the Silistar River we captured a *M. rivulata* individual with a hook stuck in its mouth, as well as observations of numerous cases of illegal fishing in the mouth of Veleka River.

- We recorded two adult individuals of *Trachemys scripta elegans* - one at the mouth of Veleka River and one a little further upstream. The uncontrolled release of *T. scripta* and its subspecies into various water bodies is leading to the potential spread of this highly invasive species.

Conclusions

1. In the study area, for the period of the study, we found both native species of freshwater turtles, in all major rivers. In most of the researched localities the species are found sympatrically and *E. orbicularis* is the predominant species.

2. In the area of Veleka River *E. orbicularis* is found mainly in the lower to middle parts of the studied area, and *M. rivulata* is observed mostly in the middle to the upper sections, while in the area of the Silistar River *M. rivulata* is found mainly in the lower sections of the river, near the mouth, and *E. orbicularis* - higher upstream in the study area.

3. The populations of both species are characterized by medium to high values of abundance and density (compared to other regions in the country).

4. The sex ratio of the populations of the two species shows a ratio close to 1:1, with a slight predominance of the males. The age structure shows the predominance of adults from both species, with almost equal ratio of subadult and juvenile individuals.

5. The body condition index (BCI) shows values above zero in all studied individuals of both species, which is an indirect indication of good health status of the turtles and the good condition of the surveyed habitats.

6. Both species of freshwater turtles are characterized by a high conservation status, and the threats we recorded in the study area are disturbance by the tourist flow (especially along Veleka and Silistar rivers),

involuntary capture of individuals by fishermen, as well as the presence of the invasive species of red-eared slider (*Trachemys scripta elegans*), in the area of Veleka River. We propose to ban the import and sale in pet stores of *T. s. trostii* (*T. s. elegans* and *T. s. scripta* are already banned), as well as raising public awareness of the problem, building appropriate centers for the invasive turtle species, collected from the wild, as a more ethical and environmentally safe alternative for dealing with unwanted pets and eliminating established populations by removing individuals from the wild.

Acknowledgements

The present work was carried out with the financial support of the Bulgarian Biodiversity Foundation, through a competition for support of works under the project "Game Over? Do not let the climate change end the game.", 2021.

This research is also conducted under the project "BSB ECO MONITORING - 884" Joint Monitoring for Environmental Protection in BSB countries (2020-2022). Joint Operational Program for Cross-Border Cooperation under the European Neighborhood Instrument "Black Sea Basin 2014-2020", funded by the European Neighborhood Instrument. Association for Culture, Technology, Education and Development - Plovdiv University - Strandzha Nature Park.

The authors would like to express their sincere gratitude to Assoc. Prof. Dilian Georgiev for some of the data on the distribution of *E. orbicularis* in the researched area and to Emil Yordanov for our joint field work, piloting and video recording with unmanned aerial vehicles (UAVs).

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Received: 12.11.2021
Accepted: 13.12.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 239-244

Short note

The Longest Food Deprivation Period of a Griffon Vulture (Gyps fulvus) Recorded in the Wild and Exceptionally Long Nest Attendance

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Abstract. The Eurasian Griffon Vulture (*Gyps fulvus*) is an obligate scavenger that feeds on large carcasses and provides critical ecosystem services. Vultures evolved various ecological and physiological adaptations to cope with the unpredictability of the food resources and withstand long starvation periods. However, there is a lack of empirical data from wild individuals. Here we describe a case of a male Griffon Vulture tagged with GSM/GPS-ACC transmitter, which withstood without food for over 36 consecutive days. It is the longest food deprivation period recorded for the species in the wild. This unusual behavior occurred during the incubation period. The loss of the female and the strong parental instinct forced the male to continue with the incubation of the egg and attend the nest for 31 consecutive days.

Key words: food deprivation, lone breeding, GPS tracking, Accelerometer, remote sensing.

The Eurasian Griffon Vulture (*Gyps fulvus*) is a large Old-World vulture with a wingspan reaching 2.8 m and weight 6-11 kg (Cramp & Simmons, 1980). Its range spreads from Portugal to the Himalayas (BirdLife International, 2021). The species population on the Balkan Peninsula has significantly declined in the mid-XXth century, but in the past three decades it showed recovery in Serbia, Bulgaria and Croatia while in continental Greece and North Macedonia it still experience a decline due to various threats and increased mortality (Dobrev et al., 2021). The Griffon Vulture is an obligatory scavenger that feeds mostly on carcasses of livestock and wild ungulates (Cramp & Simmons, 1980; Margalida et al., 2011; Arkumarev et al., 2021). Vultures forage over large areas by travelling great daily distances with minimal energy expenditure (Spiegel et al., 2013; Duriez et al., 2014). Thus, vultures evolved various adaptations to cope with the food resource unpredictability e.g. high foraging efficiency, gregarious lifestyle, storage of large body reserves and mechanisms to decrease metabolic rates (Prinzinger et al., 2002; Ruxton & Houston, 2004; Spiegel et al., 2013). These ecological and physiological adaptations allow vultures to withstand long

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food deprivation periods (Houston, 1976). However, there is a lack of quantitative data from free-living vultures on how long they can sustain with no food. Here we describe the longest food deprivation period of a Griffon Vulture recorded in the wild due to a partner loss and an exceptionally long nest attendance during incubation.

In May 2017 in the Eastern Rhodopes, Bulgaria an adult male Griffon Vulture was captured and tagged with 57 g GSM/GPS and accelerometry transmitter (E-Obs GmbH; Munich, Germany) attached as backpack with 11.2 mm Teflon ribbon. The vulture was still wearing the transmitter after the end of this study. It was also marked with a color wingtag, plastic colour ring and metal ring to ease its identification in the wild. The transmitter harness, rings and wingtag did not exceed 3% of the bird's body mass in accordance to the recommended limits to avoid adverse effects (Bodey et al., 2018). The vulture was aged following Forsman (2003). Blood samples were taken and used to determine the sex of the bird. The transmitter was programmed to acquire a GPS fix and ACC data every 5 min during the day (between 03:00 – 17:00 UTC) with dormancy periods during the night. However, when the battery charge dropped below 50% the transmitter was acquiring a GPS fix every 30 min but the ACC data was kept at 5 min intervals. The ACC sensor was set to measure the acceleration in three perpendicular axes at 7 HZ frequency for 4 s each. All data were automatically downloaded and incorporated into Movebank. We used ACC-based classification of the Griffon Vulture behavioral modes (Nathan et al., 2012). We used the web application AccelaRater (Resheff et al., 2014) and a training set of ground-truthed behaviors (n = 244) collected by direct visual observations of tagged birds at a feeding station and at nests (for details see Arkumarev et al., 2021). For the aim of this study we were interested to determine vulture's feeding, flying and incubating behavior.

The Griffon Vulture was successfully breeding in Thrace, NE Greece (Evros Regional Unit) in 2017, 2018 and 2019. In 2020 the pair occupied the same breeding cliff where it raised chicks in two consecutive breeding seasons. According to transmitter's data, the first brooding behavior was recorded at 12:42 (UTC) on 30th of January. Until 01st of March the tagged vulture was incubating on average for 47h:29min which is similar to previous records for this species (Xirouchakis & Mylonas, 2007) and indicated that the female was alive and relieving the male from incubation (Table 1).

Table 1. Periods and duration of nest attendance during incubation by the male Griffon Vulture derived from the GPS and ACC data.

Nest attendance start time (UTC)	Nest attendance end time (UTC)	Duration (hours)	Duration (days and hours)
30.01.2020 12:42	03.02.2020 15:05	98h:25min	4 days 2h:25min
06.02.2020 12:20	08.02.2020 09:05	44h:45min	1 day 20h:45min
11.02.2020 10:45	13.02.2020 13:40	55h:55min	2 days 2h:55min
15.02.2020 08:45	15.02.2020 13:20	04h:35min	0 days 4h:35min
16.02.2020 07:10	17.02.2020 12:15	29h:05min	1 days 5h:05min
21.02.2020 10:55	23.02.2020 14:15	51h:20min	2 days 3h:20min
27.02.2020 09:35	29.02.2020 14:55	53h:20min	2 days 5h:20min
01.03.2020 13:30	01.04.2020 07:15	737h:45min	30 days 17h:45min
01.04.2020 13:30	03.04.2020 07:30	42h	1 day 18h

When the female was in the nest the male was searching for food and feeding at the supplementary feeding station in Dadia-Lefkimi-Soufli Forest National Park or on carcasses found in the field. On 24th of February it visited the supplementary feeding station for the last time and on 27th of February at 07:00 it attended a feeding event near the village of Ragada (Fig. 1). This was the last recorded feeding behavior based on the ACC data.

The field team visited the area on 26th of March and observed the vulture drinking water on the top of the cliff after rain and then immediately returned back to the nest. The next feeding behavior was recorded on 03rd of April at 11:40 near Plagia Village, hence the food deprivation period lasted for a record 36 days 04h:40min (Fig. 2). We suppose that this unusual behavior was caused by a loss of the breeding partner. The male had to incubate the egg alone, with no possibility of leaving the nest and searching for food. This hypothesis was confirmed by the ACC, GPS data and visual observations. The male stayed in the nest for 30 days 17h:45min. On 01st of April at 07:15 it left the nesting cliff and made a foraging flight, but the attempt was unsuccessful and it returned to the nest at 13:30. Although, it spent another 42h in the nest (lying and standing), it abandoned it at 07:30 on 3rd of April. At 11:40 the ACC data indicated feeding behavior and later the same day the vulture was observed by the field team perching on the breeding cliff with dirty head which was an evidence that it had fed.

Griffon Vulture's incubation period is 54-58 days (Shirihai, 1996; Xirouchakis, 2010). If we assume that the egg was laid at the same day when the male showed brooding behavior for the first time – 30th of January, we could expect that the chick would hatch in the period between 24th and 28th of March. The ACC data indicated that the vulture was moving more often and was spending more time standing in the nest after 26th of March which coincides with the expected hatching period. During a field visit on that day the observers noticed that the vulture is moving more than before in the nest but chick was not seen. Based on these records we can assume that a chick successfully hatched. However, the presence of a chick in the nest could not be verified. During the next field visit, three days later, the vulture was moving more often than the previous days suggesting a presence of a hatchling. Due to the COVID-19 restrictions in Greece, the field team could not conduct observations of the nest on a daily basis. During the subsequent field visit on 3rd of April the nest was already empty and we assumed that the chick had probably died.

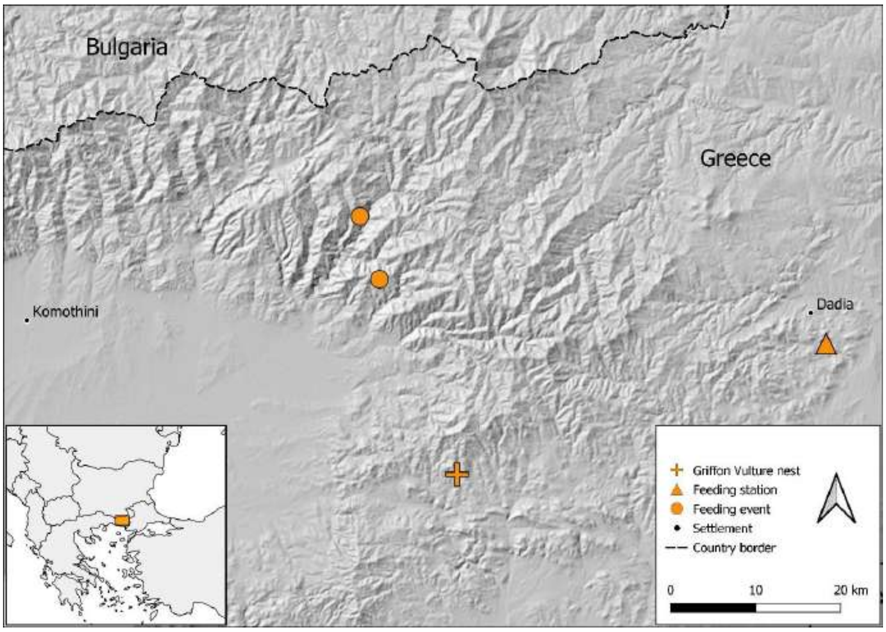

Fig. 1. Map of the study area presenting the locations of the Griffon Vulture nest, the nearest supplementary feeding station and the feeding events recorded in the wild.

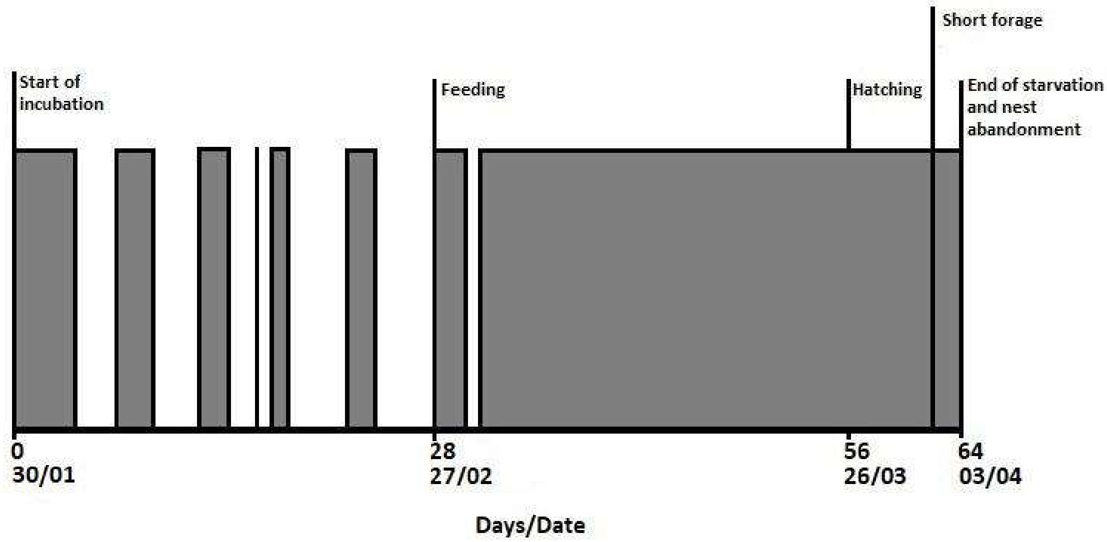

Fig. 2. Nest attendance by a male Eurasian Griffon Vulture incubating an egg without a partner from day 31 onwards and not feeding for 36 consecutive days (from day 28 until day 64) (Grey bars – days when the male is present at the nest, white gaps – days when the male is absent from the nest).

We report a case of long food deprivation experienced by a wild Griffon Vulture due to exceptionally long nest attendance during incubation. Our tracking data indicated that the vulture fully recovered from the long starvation, but did not breed in the next breeding season. As a response to starvation, Griffon Vultures can change their foraging strategies (Spiegel et al., 2013) and significantly decrease metabolic rates to minimize their energy requirements (Bahat et al., 1998). However, in our case the recorded prolonged food deprivation period was not caused by an inability for foraging. Instead, the vulture probably lost its partner and was devoted to lone-incubation. In *Gyps* species, usually both sexes are equally committed to brooding and parental care (Mundy et al., 1992). Thus, it is not clear why the male vulture continued the lone-incubation for one month without being able to forage efficiently but this could be an adaptive behavior in social species breeding in colonies. A non-breeding individual from the colony might replace the lost partner and take shifts in the incubation increasing the chances for a successful breeding attempt. Johnson (2018) describes a solo breeding attempt by a closely related White-backed Vulture (*Gyps africanus*) in South Africa. A female vulture laid an egg and incubated it alone for 30 days. However, the solo breeder has left the egg unattended in 55 occasions, ranging from 5 min to 45 hours. In one occasion, the bird was observed returning from foraging with full crop and blood on the face, which evidenced feeding. In the case we describe the vulture did not leave the breeding cliff for 31 consecutive days and did not show feeding behavior for 36 days in total. In 2017 in Kresna gorge, Bulgaria two female Griffon Vultures, which lost their partners due to poisoning (Peshev et al., 2018), showed similar behavior. The two females continued incubation for 24 and 25 days respectively after their mates were poisoned. One of them hatched a chick, which died two days later probably due to starvation (E. Stoynov pers. comm.). In another case in Spain an adult Griffon Vulture was recorded incubating alone after a partner loss for about 30 days. An inspection of the nest revealed that the egg has hatched, but the chick most probably died of starvation after 2-3 days. However, in these cases the vultures were not tagged with GPS devices and were not constantly monitored so they might have left their nests unattended and searched for food. During long deprivation periods Giffon Vultures may decrease their night body temperature requirements (Bahat et al., 1998) but there is no data what consequences such drop of the body temperature of the adults may have on the embryos during incubation. We have no direct prove that in our case the male vulture hatched a chick, but the ACC data suggested that. More studies are needed in this field to fully understand the ecological and physiological adaptations, which vultures have developed to withstand such long periods of food deprivation, which are with no doubt unique in the avian community and the adaptive value this behavior may have for the successful reproduction.

Acknowledgements

This research was financed by the National program "Young scientists and Postdoctoral candidates" funded by the Bulgarian Ministry of Education and Science. The fieldwork and data collection were conducted in the frame of the LIFE project "Conservation of Black and Griffon Vultures in the cross-border Rhodope Mountains" (LIFE14 NAT/NL/000901) funded by the European Union and supported by Rewilding Europe and Segre Foundation. We would like to express our gratitude to Atanas Delchev and Vera Alekseeva for their contribution in vulture trapping and tagging.

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Received: 08.09.2021
Accepted: 11.11.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 245-255

Synopsis

Thermal Growing Season Characteristics over Central and Southeast Europe in the Changing Climate 1950-2019

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Abstract. The ongoing climate change is profoundly important for ecosystems, for all sectors of the international economy, and on the quality of life. Managed systems as agriculture and forestry are evidently most dependent on climate and the climate changes have a direct and indirect impact on biotic and abiotic disturbances with strong implications. Among others, temperature is one of the major environmental factors affecting the growth, development, and yields of crops. The timing of the onset and termination of the vegetation growing season plays a key role in terrestrial ecosystem carbon and nutrient cycles. Based on the availability of reliable and up-to-date sources of information, which represent the temperature distribution in a pan-European context, the study exploits a set of 4 indicators with a special focus on the onset, termination, and length of the thermal growing season. It is defined by a single-value threshold of the daily mean air temperature. Two thresholds are applied: 5°C and 10°C. Among other conclusions, the study provides strong evidences for the prolongation of the thermal growing season. This lengthening is a direct consequence of the ongoing warming and is consistent with most new studies. Another key message, which confirms our recent findings is the revealed asymmetry of this prolongation: the total lengthening of the thermal growing season is caused in greater extent to the shifting to an earlier date of the onset, rather than to its later termination.

Key words: thermal growing season, climate change, seasonal shift, trend analysis.

Introduction

The ongoing climate change is the defining challenge of our time which exerts influence on the ecosystems, on all sectors of the international economy, and the quality of life. Climate change evokes an increasing frequency of climate extremes and the subsequent adverse effects are manifold (Alexander et al., 2006; Lavaysse et al., 2017).

The global warming effects and the associated regional climatic changes over Central and Southeast (CSE) Europe have been profoundly studied in the last decades using data from various sources (Bartholy & Pongracz, 2006; Birsan et al., 2014; Chervenkov & Slavov, 2020; Cheval et al., 2014; Lakatos et al., 2013, 2016). Most of these studies are focused on the second half of the 20th and the first decade of the 21st century, clearly evidencing that, similarly to the global and continental trends, the regional temperature got higher during the period.

Food security is a fundamental precondition for human well-being, and the agricultural and food sector is of major economic importance. Agriculture is arguably the sector most dependent on climate. Crops and livestock are directly impacted by adverse local weather and climate (Hatfield & Prueger, 2011; 2015; Seemann et al., 1979). Associated with climate change several factors are affecting agricultural ecosystems, which can act independently or in combination (Harkness et al., 2020). As evidenced in many recent publications, the climate changes have a direct and indirect impacts on biotic and abiotic disturbances with strong implications.

Temperature is one of the major environmental factors affecting the growth, development, and yields of crops. On one hand, crops have a basic requirement for the temperature to complete a specific phenophase or the whole life cycle. On the other hand, extremely high and low temperatures can have detrimental effects on crop growth, development, and yield, particularly at critical phenophases (Luo, 2011). Changes in thermal conditions may also have adverse effects such as the development of thermophilic weeds, pests, or the emergence of new plant diseases (Szyga-Pluta & Tomczyk, 2019). The thermal impact could be quantified by various agrometeorological (AM) indicators (Harding et al., 2015) and among the most widely used is the thermal growing season (TGS). The timing of the onset and termination of the vegetation growing season plays a key role in terrestrial ecosystem carbon and nutrient cycles (Liu et al., 2016). The precise knowledge of these dates is necessary also for the calculation of climatic indicators with evident effect on the production of many cultivars (Mesterhazy et al., 2018). During the last decades, an increasing number of studies have revealed a lengthening of the growing season across the Northern Hemisphere that is related to air temperature increases, and thus this is an important indicator of climate change (see Menzel et al, 2003 and references therein). Changes in the start and end dates as well as the length of the growing season, in addition to the consequences for ecosystems, may lead to long-term changes in the carbon dioxide cycle, and changes in vegetation will affect the climate system (Linderholm, 2006).

The purpose of the present paper is to assess the multiyear spatial patterns and as well as to estimate the trend of the temporal evolution of the onset (i.e. start day), termination (end day), and length of the TGS over CSE Europe for the 70-years long period 1950–2019. Following the traditional approach, these indicators are defined by single-value thresholds of daily mean air temperature, noted for sake of brevity tg henceforth, using the high-quality and high-resolution (0.1°×0.1°) multivariate daily gridded dataset E-OBSv21.0e (Cornes et al., 2018). Our group has previous experience of regional climatological studies with a focus on the growing season length (GSL) of the past and recent (Chervenkov & Slavov, 2019; 2020; 2021a; b) as well as projected future climate (Ivanov et al., 2020; Chervenkov & Slavov, 2022) and the presented study could be regarded in the general context of the continuation of our recent efforts.

Material and Methods

The well-known in the climatological community gridded daily dataset E-OBS was developed primarily for regional climate model evaluation, but it is also being used subsequently for various applications, including monitoring of extremes. Besides the improved quantification of the uncertainty, the ensemble version of the E-OBS (Cornes et al., 2018), represents generally better than predecessors the temperature extremes. As demonstrated in (Chervenkov & Slavov, 2019) E-OBS is a suitable product for the computation of climate indices which motivates our choice of it for a source of input data.

Unlike some other AM indices which computation could be performed in different ways, the GSL definition is agreed in frames of collaborative initiatives like the European Climate Assessment & Dataset (ECA&D) project and Expert Team on Climate Change Detection and Indices (ETCCDI) (Zhang et al., 2011). For many applications, such as the description of forest and agricultural regions or the integration of the length of the growing season in models, a plant-independent and more general definition is of interest (Menzel et al., 2003). According to the unified definition of ECA&D and ETCCDI, the GSL is the annual count between the first span of at least 6 days with tg>tb and the first span after July 1 (in Northern Hemisphere) of at least 6 days with tg<tb (Zhang et al., 2011; Birsan et al., 2014). The GSL is computed on annual basis and the units of measurement are days. Although some alternative definitions, respectively calculation methods, exists (Linderholm, 2006), we will follow strictly the ECA&D and ETCCDI one.

The threshold temperature tb is in the definition above equal to 5°C which is suitable for the cold-tolerant cultivars. This threshold is probably most frequently used in the computation of the climatological TGS (Szyga-Pluta & Tomczyk, 2019). In the present study, in particular, due to the geographical location of the region, we will estimate the GSL also for tb=10°C which is a threshold for the thermophile species (Ivanov et al., 2020; Lakatos et al., 2013).

Due to its importance, the GSL is the subject of many studies, considering the global and regional climate (see, for example, Menzel et al., 2003; Mesterhazy et al., 2018 and detailed list in Linderholm, 2006), whereas relatively smaller attention is paid on its onset day of the year (DOY) and termination DOY, noted further as in (Chervenkov & Slavov, 2021b) DOYB and DOYC respectively. Changes in the timing or the seasonality and not only in the length of the growing season, however, may have essential relevance for plant ecosystems. The essential importance of DOYB and DOYC is the main motivator to consider them in the present study.

The definition of the GSL gives the possibility for straightforward computation of the DOYB and DOYC. The time series of the tg of the first/second half of the year is inspected for the first occurrence of a continuous period of six days where the tg is above/bellow tb. The computations are performed with purpose-built software and for each gridcell of the domain individually. According to the original proposal in (Chervenkov & Slavov, 2021b, 2022), the middle day of the growing season (DOYM), where DOYM=(DOYB+DOYC)/2, is an indicator of the seasonal shift which is independent of the GSL. It is also considered in our study as will be demonstrated in the next section.

Results and Discussion

First, to assess the long-term inter-annual changes, the multiyear means (MM) of the DOYB, DOYM, DOYC, and GSL for the first 30 years, i.e. 1950-1979 are superimposed to these for the last 30 years, i.e. 1990-2019. The absolute difference of the MM for the second period in respect to the first (i.e. (1990-2019)-(1950-1979)) is applied as a measure of the long-term change. The comparison is performed for both thresholds of 5°C and 10°C separately and the results are shown in Fig. 1 and 2 correspondingly. Intending to make the comparison of both figures easier, the same color legend is used.

Many conclusions could be outdrawn from both figures. The first and most noticeable is the clear spatial structure of all of the considered quantities. As noted in the study (Birsan et al., 2014), regarding some ETCCDI-indices, the vertical gradients are better expressed than the horizontal ones. As expected, the growing season onset is earlier and its termination is later in the regions with generally warmer climate – the southern coastal areas. The GSL is shortest over the NW part of the domain and especially over the mountains, most prominent over the main Carpathian ridge (MCR).

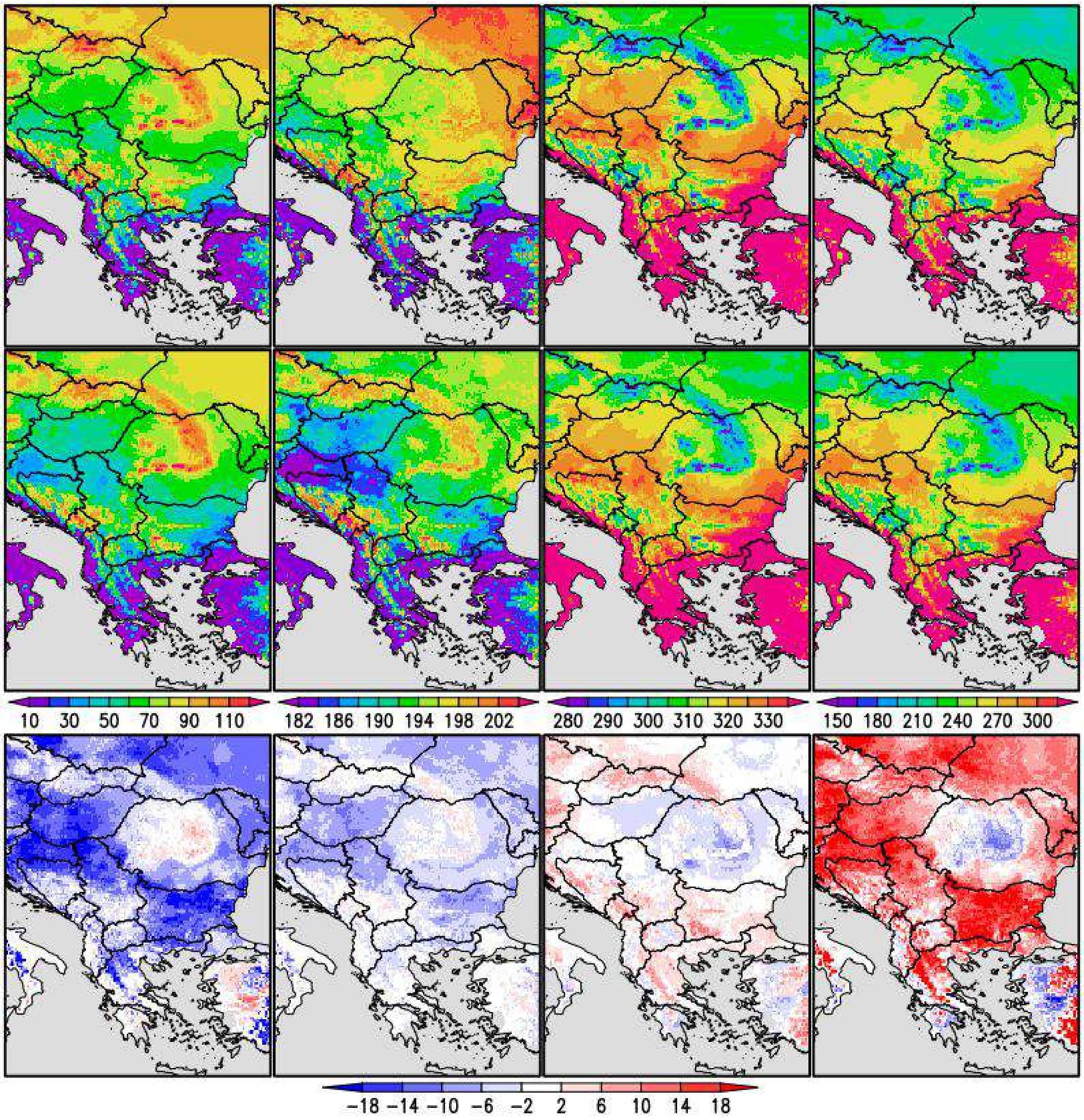

Fig. 1. From left to right: MM of the DOYB, DOYM, DOYC and GSL for the threshold of 5°C for the period 1950-1979 in the first and for 1990-2019 in the second row. The differences (1990-2019)-(1950-1979) are shown on the third row. The units are DOYs for DOYB, DOYM, DOYC and days for GSL and for the differences.

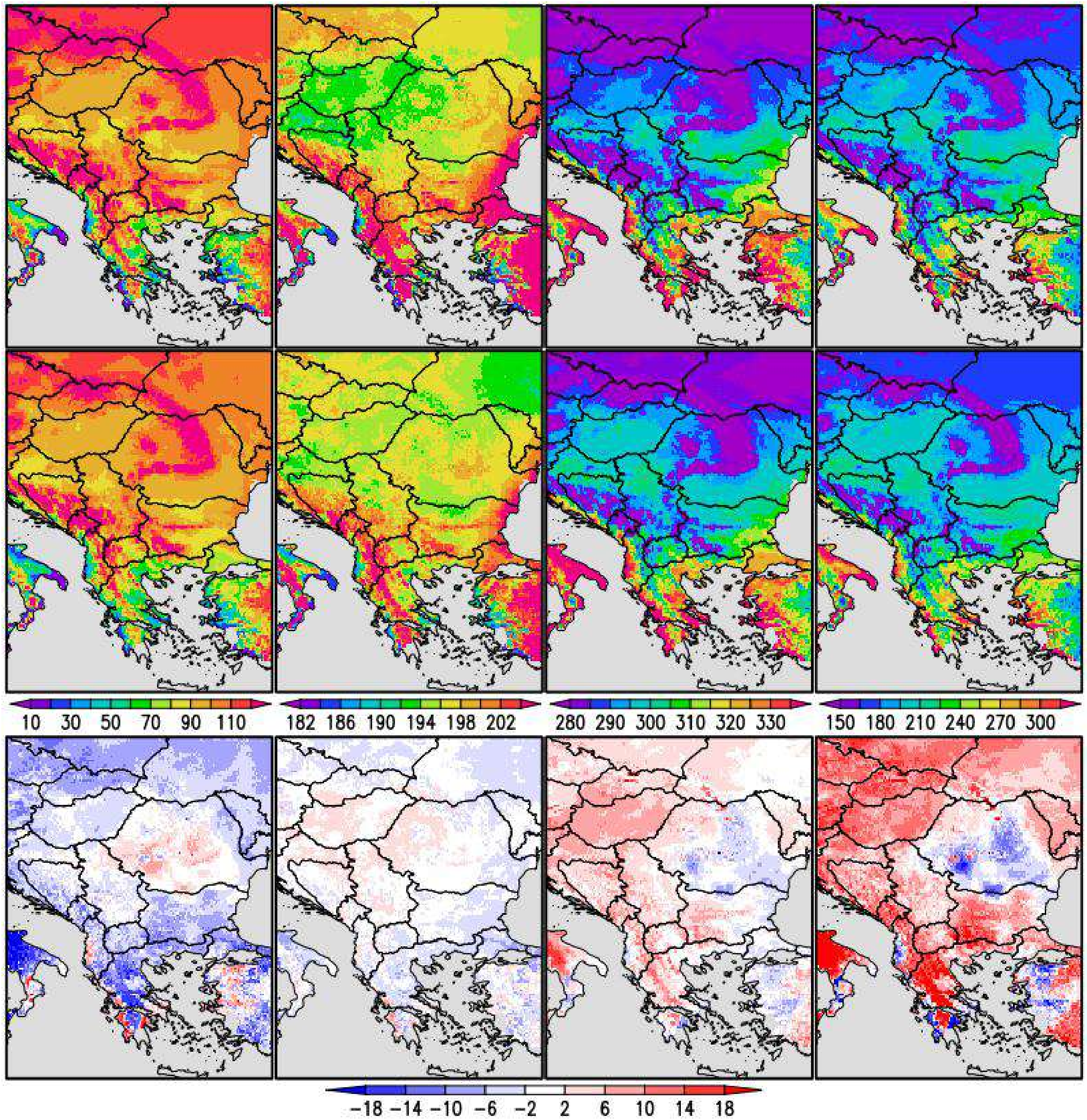

Fig. 2. Same as Fig. 1 but for the threshold of 10°C.

As a whole, the GSL varies significantly: roughly from 180 to more than 300 days for the threshold of 5°C and from 150 to 300 days for the threshold of 10°C. The differences between the MM of both periods are also remarkable: the generally warmer climate in the second period leads to spatially dominating earlier onset and later termination of the growing season. As result, the GSL is lengthened with more than two weeks over the bigger part of the considered domain. Generally, the tendencies are better expressed for the threshold of 5°C. The long-term changes are also bigger for the DOYB rather than these of DOYC. Evidently, the regional warming leads to asymmetric changes of the timing of the GSL. A direct consequence from this is that the DOYM is shifted to earlier dates, more noticeable for the threshold of 5°C.

The latter findings are in principle agreement with the results of similar studies and our recent outcomes. One of the key messages in (Menzel et al., 2003) is that the lengthening of the growing season over Germany, Switzerland, Austria, and Estonia can mostly be attributed to advances in spring phenology rather than the delay of autumn. One of the main conclusions of the study (Chervenkov & Slavov, 2021b) which considers CSE Europe and the period 1961–2018, is that the total lengthening of the GSL for the threshold of 5°C is caused in greater extent to the shifting to an earlier date of the onset, rather than to its later termination.

Hence the long-term inter-annual changes of the parameters of the TGS are in the focus of our study, their temporal evolution should be also considered. Fig. 3 shows the time series of the areal averaged (AA) over the whole domain of the DOYB, DOYM, DOYC, and GSL for both temperature thresholds.

The most important result of the analysis of Fig. 3 is the clear increasing course of the GSL for both temperature thresholds, especially noticeable after the eighties. The downward and upward tendency of the DOYB and DOYC correspondingly could be also detected, although they are not so apparent.

The importance of assessing the long-term trends is often emphasized (Alexander et al., 2006; Bartholy & Pongracz, 2006; Chervenkov & Slavov, 2019). The primary reason is to estimate the sustainability of the detected inter-annual changes. In the present study, the magnitude of the trend is estimated with the Theil-Sen Estimator (TSE) and its statistical significance is analyzed with the Mann-Kendall (MK) test. The TSE and the MK test are procedures, especially suitable for non-normally distributed data, data containing outliers and nonlinear trends. Consequently, they are widely used in many engineering and geophysical branches as hydrology. They are recommended from the World Meteorological Organisation [24] and are practically standard tools for statistical assessment of trend in the meteorology (Chervenkov & Slavov 2020; 2021a; b; Cheval et al., 2014; Lakatos et al., 2016).

The results from the trend analysis of the GSL-related measures are shown in Fig. 4. Traditionally, (Chervenkov & Slavov 2021a; b; Cheval et al., 2014; Lakatos et al., 2016) the results were evaluated at the significance levels of 5%.

According to the results of the analysis of Fig. 4, first and foremost is the prevailing positive tendency for DOYC and especially for the GSL; contrary, the overall trend for the DOYB and DOYM is negative. Generally, the trend appears statistically significant over a relatively big part of the domain for DOYB and GSL only. There is no principal disagreement of the revealed tendencies for the threshold of 5°C on the one hand and for the threshold of 10°C on the other. As a whole, the tendencies, however, are better expressed for the lower threshold.

In the present study Bulgaria is the focus of the author's interest. The estimated parameters of the trend of the AAs of the considered TGS-characteristics for the whole domain noted for sake of brevity CSE Eu, one the one hand are superimposed to their counterparts for Bulgaria ('Bg' - the area in the green box in Fig. 4) on the other hand. A measure magnitude of the trends is the slope of the regression line β_0 computed according to the TSE and of its significance - the p-value of the MK-test. The results are shown in Table 1.

The numbers in the first row of Table 1 are the values of the slope of the trend lines of the time series, shown in Fig. 3.

The results in Table 1 could be summarized in many directions. First and foremost is the positive and statistically significant trend of the most important indicator, the GSL. This is valid for both domains and for both thresholds. The trends of the DOYB and DOYM for the more recognizable threshold of 5°C of are, as expected, negative and statistically significant. Contrary, the changes of the DOYC, both positive, are not statistically significant at the chosen level.

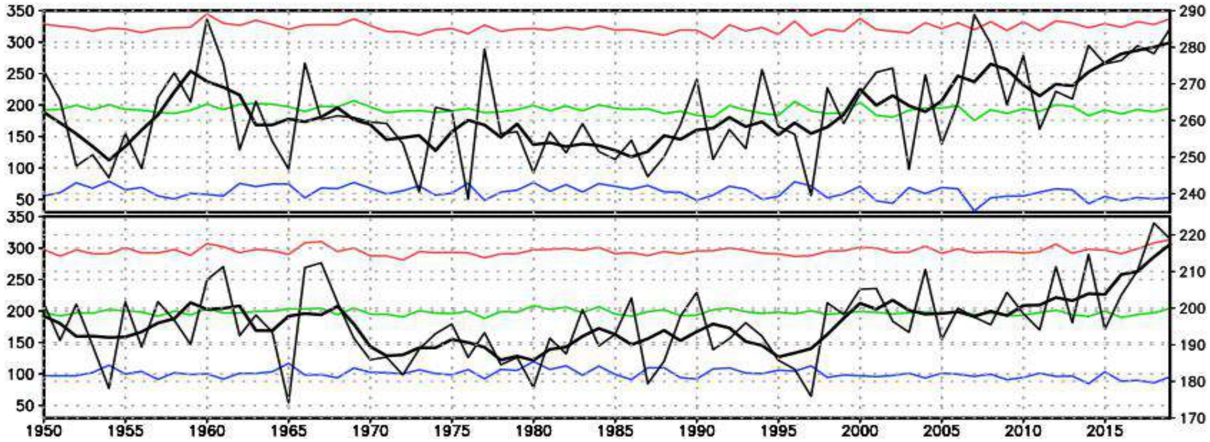

Fig. 3. Upper pane: temporal evolution of the DOYB (blue line, left ordinate), DOYC (red line, left ordinate), DOYM (green line, left ordinate) and GSL (black line, right ordinate, units: days) for the threshold of 5°C. The fat black line is the centered 5-year running mean of the GSL. Lower pane: Same as upper pane but for the threshold of 10°C.

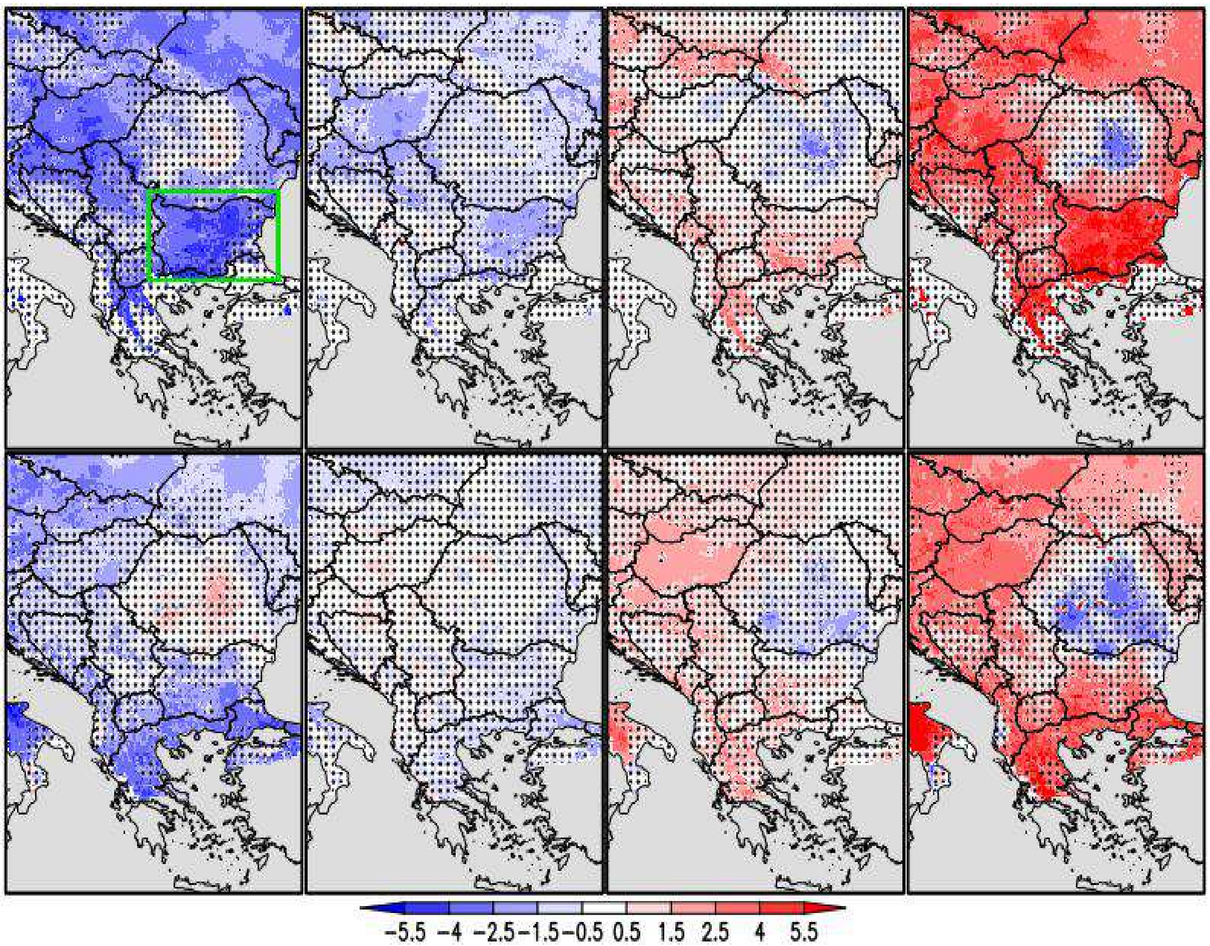

Fig. 4. Trend magnitude (unit: days per 10 years) of the DOYB, DOYM, DOYC and GSL (from left to right) for the threshold of 5°C (first row) and for the threshold of 10°C (second row). Stippling indicates grid points with changes that are **not** significant at the 5% significance level. The area in the green box in the first subplot is used in later analysis.

Table 1. Trend magnitude and significance of the AAs of the growing season indicators for the whole domain (CSE Eu) and for Bulgaria (Bg). The trends that are **not** significant at the 5% significance level are shown in bold.

Parameters/Threshold	Threshold of 5°C				Threshold of 10°C			
	DOYB	DOYM	DOYC	GSL	DOYB	DOYM	DOYC	GSL
β_0(d./10 yr), CSE Eu	-1.9	-0.7	0.17	2.3	-0.9	-0.4	0.4	1.4
p-value, CSE Eu	0.002	0.040	**0.715**	0.006	0.021	**0.205**	**0.222**	0.018
β_0(d./10 yr), Bg	-3.3	-1.2	1.06	4.4	-1.5	-0.6	0.3	1.6
p-value, Bg	0.001	0.021	**0.139**	0.002	0.027	**0.105**	**0.637**	**0.139**

The long-term tendencies of the considered indicators at the threshold of 10°C are more complex. Most generally, they are with the same sign as their counterparts for the lower threshold. According to their magnitude they are, as a whole again, weaker. The trends of DOYM and DOYC are statistically not significant for both domains; even the tendency of the GSL for Bulgaria is not significant.

The complexity of the revealed picture implies further investigation, in particular applying different methods for the determination of the TGS and other, independent basic datasets.

Conclusions

Based on the availability of up-to-date data, we present an analysis of the characteristics of the thermal (i.e. climatological) growing season over CSE Europe for the second half of the 20th and the first two decades of the 21st century. To determine the TGS, we used two single-value thresholds, based on td, namely 5°C and 10°C. Although not exhaustive, the study gives clear evidence of its lengthening which is in the principle agreement with many studies for the mid and higher latitudes of the Northern Hemisphere revealed from different data sets, such as station network measurements, satellite images, CO_2 records, and phenological 'ground truth' (Menzel et al., 2003; Szyga-Pluta & Tomczyk, 2019). This outcome confirms also our recent findings (Chervenkov & Slavov, 2021b). The increasing length of the growing season appears in a form of an earlier onset and a later termination. Alongside the other results the most important key messages could be summarized as follows:

The total lengthening of the TGS is more linked to its earlier onset rather than the later termination.

Direct consequence of the above result is the steady seasonal shift of the TGS towards the earlier dates. The conclusion is confirmed also with the revealed negative trend of the middle day of the TGS (DOYM). This trend is also statistically significant at the 5% level for the AAs values of the DOYM.

The revealed long-term tendencies are generally similar for both thresholds. The tendencies for the upper threshold, 10°C are, as a whole, weaker.

The revealed tendencies for the more recognizable threshold of 5°C are, both in magnitude and statistical significance, stronger expressed over Bulgaria, rather than over the whole domain.

Evidently, longer TGS favors a greater diversity of crops, including those with long maturation periods, and the potential for multiple harvests on the same land. This lengthening is likely to contribute to increased biomass formation, which is part of a global increase in biospheric activity. Conversely, both irrigation needs and the risk from invasive species, pests, and pathogens may increase (Harding et al., 2015). The estimated changes of the TGS-related indicators could be a prerequisite for deep ecological and economical consequences. Subsequently, similar studies could be the methodologically reliable scientific basis of the long-range policy and expert assessments for managing systems as agriculture and forestry.

Acknowledgments

We acknowledge the E-OBS dataset from the EU-FP6 project UERRA (http://www.uerra.eu) and the Copernicus Climate Change Service, and the data providers in the ECA&D project (https://www.ecad.eu). This study has been funded by the Bulgarian National Science Fund (DN14/3 13.12.2017).

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Received: 13.09.2021
Accepted: 06.12.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 257-287

Synopsis

Fluctuating Asymmetry in Pelophylax ridibundus (Anura: Ranidae) and Bufotes viridis (Anura: Bufonidae) Meristic Morphological Traits as Indicators of Ecological Stress and a Method for Assessing Environmental Quality of Their Habitats – 9 years Monitoring in Bulgaria: Systematic review

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Abstract. This paper summarizes and presents the results from our years-long *in situ* analyses of the populations of the Marsh frog *P. ridibundus* and the Green toad *B. viridis* inhabiting freshwater ecosystems in South Bulgaria with different degrees of anthropogenic transformation. It covers the period 2009-2017 with tests of the application of fluctuating asymmetry in meristic morphological traits as a marker for deviation assessment of the developmental stability of individuals of both species as well as for parallel and independent from physicochemical analyses assessment of the quality of their living environment. The analyses held on the territory of South Bulgaria are divided into three phases: first (2009-2010), second (2011-2015) and third (2016-2017). The results of our investigations conducted over a period of 9 years, show that changes in asymmetry levels in meristic morphological traits in adult and sexually mature representatives of *P. ridibundus* and *B. viridis* are a very good bioindication method.

Key words: Marsh frog, Green toad, fluctuating asymmetry, developmental stability, anthropogenic pollution, environmental health.

Introduction

Anthropogenic pollution and its role for global decline of amphibians. Anuran amphibians and their potential as indicators of ecological stress in biomonitoring in situ analyses

In the 21st century, the industrial revolution that started in the end of the 18th century became the reason for a drastic change in the relations between modern man and surrounding nature. Today, the concept of Anthropocene is defined as a new geological period (Crutzen, 2006). Currently, the role of the anthropogenic factor is key to the change of geological and climatic processes on planet Earth and involves the functioning of all ecosystems (Dirzo *et al.*, 2014; El Idrissi *et al.*, 2019). Agricultural revolution is inextricably related to industrial revolution (Green *et al.*, 2004). Accelerated agriculture in the past

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and current century is characterized not only by the use of new arable land at the expense of cutting down the last large forests on the planet but also by the increased use of various fertilizers and chemical protection preparations (Mann *et al*., 2009; Relyea, 2009). It is not by accident that the first places in the list of most significant pollutants of anthropogenic origin are taken by such related to industry and agriculture: polycyclic aromatic hydrocarbons, polychlorinated biphenyls, anticholinesterase pesticides, carbamate, pyrethroid, organophosphate and organochlorine insecticides, endocrine disruptors (estrogen mimics such as DDT, polychlorinated hydrocarbons, surfactant alkyl phenolic compounds), heavy metals, sulfur dioxide, products resulting from the oxidation of sulfur, petroleum products and wastewater from industrial enterprises (Raimondo *et al*., 2007; Selgrade, 2007; Spraling *et al*., 2010).

The negative effects of anthropization in the 20th and 21st century strongly affect different types of continental freshwater basins and the natural wetland connected with them (Czech & Parsons, 2002; Dupler *et al*., 2019). In the context of increasing anthropization, pollution of continental freshwater systems is a serious social problem because freshwater is not only a habitat of its natural biome but is also used for human agricultural activities such as irrigation, fish farming, and tourism. This requires not only knowledge of the mechanisms of functioning of these sensitive ecosystems but also development of efficient measures for their protection, restoration and efficient management (Vollmer *et al*., 2018).

Amphibians are the first vertebrates colonizing land; they survived the periods of the five top planetary extinctions (Schoch, 2014). Anthropocene is the period of the sixth mass extinction on a planetary scale (Wake & Vredenburg, 2008) and the amphibians are the most vulnerable vertebrate group with over 1800 (32%) species directly threatened with extinction (Baillie *et al*., 2004). Global changes in the planet's climate, together with local changes in temperature, precipitation and sunshine (ultraviolet radiation), combined with strong anthropogenic pressure are cited as the main reason for the global decline in amphibian populations (Stuart *et al*., 2004; Whittaker, 2013). Tailless amphibians (Anura) are an important link in the trophic chains connecting aquatic and terrestrial ecosystems (Crump, 2010; Dupler *et al*., 2019) because they can be predators feeding on insects and arthropods (Ferrie *et al*., 2014) as well as prey for many waterfowl and some mammals (Scheffer *et al*., 2006) at the same time. The larval stages of most tailless amphibians thrive entirely in water, feeding mainly on algae, and adults of many species are strongly attached to the water body in which reproduction took place, even after they move to land (Vitt & Caldwell, 2014; Navas *et al*., 2016). This complex life cycle, combined with highly permeable skin, exposes them to the direct action of a variety of toxic agents entering their habitats with anthropogenic wastewater. The chances of survival of the species under altered environmental conditions are determined by the hereditary biological characteristics of individuals (genetic limits of variation of traits) and the associated individual adaptive potential (Burraco & Gomez-Mestre, 2016), and hence the conservation opportunities of the specificity in the structure of the populations and the plasticity of their polymorphism (Vershinin, 2005, 2007). When toxic agents are present in static concentrations (albeit in sub lethal doses) for a long time in the habitat (chronic action or pulse emissions) or increase their concentrations, this leads to depletion of the organism's resistance and its death (Earl & Whiteman, 2009; Montiglio & Royauté, 2014; Saaristo *et al*., 2018). The death of amphibians in the presence of toxic substances is due to the immediate disruption of the organism’s homeostasis most often as a result of strong oxidative stress at the cellular and tissue level (Verma & Srivastava, 2003; Liendro *et al*., 2015; Prokic´ *et al*., 2017), or is a consequence of changes in the behavior of animals under the influence of toxicants (Boone *et al*., 2007; Romero & Wingfield, 2016; Zhang *et al*., 2018). Environmental stress caused by toxicants leads to immunosuppression, which weakens the organism's defenses of amphibians, and hence reduces their resistance to disease and parasites (Corn, 2000). Many species of tailless amphibians cannot live in high-toxicant environments and die, but there are species that survive and adapt to life under environmental stress (Sparling *et al*., 2010; Whittaker, 2013). These species are extremely suitable as test objects in bioindication analyses (Venturino *et al*., 2004; Gonçalves *et al*., 2019). Biomonitoring studies are a valuable risk assessment tool as they rely on biomonitoring species to understand temporal and spatial changes in the quality of the environment and its impact on wildlife (Huggett *et al*., 2018; Conan *et al*., 2021). Biomonitoring (indicator) species are organisms that provide quantitative information on habitat quality through various somatic, physiological or biochemical measures (Brodeur *et al*., 2020a). A number of important characteristics in some species of tailless amphibians (despite the global decline of amphibians in general), such as relatively large abundance and wide range (population level), clear taxonomy and low dispersal capacity increase their potential as bioindicators (Hellawell, 1986; Marques *et al*., 2009). This potential is greater in species that respond to environmental stress through changes in various morphological and physiological traits (Gondim *et al*., 2020; Brodeur *et al*., 2020b).

Analyses performed immediately after the capture of animals, often in natural conditions (*in situ*) are a powerful tool for objective and realistic assessment of the state of populations of different anuran species (health status of individuals, number, density, phenetic, sexual and demographic structure of populations) living in specific ecological conditions of the environment. The information obtained from *in situ* analyses is correct, complements the physicochemical analyses, and often reflects the state of the living environment much more objectively (Otero *et al*., 2018; Zhang *et al*., 2018). The reason for this is that physicochemical analyses take a "snapshot" of the state of the water body (reflecting the presence or absence of toxicants at the time of sampling), while changes in the biological parameters of test objects reflect the long-term effects of toxic agents. (Prokic´ *et al*., 2017; Saaristo *et al*., 2018; Pollo *et al*., 2019). The study of the effects of the chronic action of toxicants on the various functional systems of test animals allows not only to obtain an objective picture of the state of their living environment, but also to reveal the mechanisms and levels of adaptation of these animals to environmental stressors (including those of anthropogenic origin). The advantages of using bioindicator organisms and biomarkers for integrated assessment of biosystems with different levels of organization and complexity is that they respond not only to individual pollutants, but also to a whole complex of influencing substances (Mahmood *et al*., 2016; Jayawardena *et al*., 2017). Chemical analysis of water and soils is a direct evidence of the nature and extent of pollution, but sensory organisms can reveal the state of the dynamic scenario and the long-term effects. Biomarkers complement and improve the reliability of chemical monitoring data by offering an integrated assessment of the impact of pollutants on the health of organisms (Hansen, 2003; Van der Oost *et al*., 2003; Cazenave *et al*., 2009). Many amphibian species usually have the required population density for *in situ* analyses with a sufficient number of individuals, do not perform large spatial migrations (Flickinger & Nichols, 1990) and are also highly philopathic, which with a high degree of confidence means that adults analyzed in specific habitats have most probably undergone their larval development in them (Sinsch, 1990). Endangered species of tailless amphibians are difficult to study due to their low abundance, legal measures for their protection and the difficulties associated with issuing permits for their capture. However, widespread species with abundant populations can serve as biological indicators of ecosystem health (Linder *et al.*, 2003; Venturino *et al.*, 2004; Marcogliese *et al.*, 2009). However, the development of non-lethal sampling techniques is of paramount importance, especially when working with endemic and / or endangered species of tailless amphibians (Narayan, 2013; Barriga-Vallejo *et al.*, 2015).

Use of fluctuating asymmetry (FA) as a biomarker for the assessment of the effects of various anthropogenic stressors on the stability of the development of tailless amphibians and the quality of their habitat

One way to assess the impact of various toxic agents on individuals (populations) of tailless amphibians is through their developmental stability (DS) – that is, the ability to neutralize stress during individual development: tadpoles, larvae periods, adults (Van Dongen & Lens, 2000; Zakharov *et al.*, 2020). According to the classical notion, developmental stability (morphogenetic homeostasis) is considered as the ability of the genome of each organism to form a stable phenotype, with relatively constant parameters of the environment in which it develops (Zakharov & Graham, 1992; Thornill & Møller, 1997; Klingenberg, 2003). Even in the most balanced and structured genome, random stochastic phenomena can lead to disturbances in the processes at the molecular and cellular level, and hence to the impossibility of reproducing the predetermined phenotype (Palmer & Strobeck, 1986, Zakharov *et al.*, 2001). According to modern notions, each phenotypic trait and the degree of its manifestation depends on the influence of two opposing forces: developmental stability and developmental noise (ontogenetic noise) (Palmer, 1994, 1996; Zakharov *et al.*, 2001). Noise is a characteristic of a living system and is a reflection of its instability in development - a strong system is characterized by minimal noise (Zakharov *et al.*, 2020). When developmental noise is stronger than developmental stability in the developing organism, small accidental errors can occur in the pre-set parameters of cellular, biochemical and physiological processes (Mc Adams & Arkin, 1999). The result of developmental noise is the formation of a phenotype with characteristics fluctuating above the level of natural variations (Palmer & Strobeck, 1986; Polak, 2003). Ontogenetic disturbances in developmental stability can be caused by various genetic (Frankham, 2005; Garrido & Perez-Mellado, 2014) or environmental stressors (Anciães & Marini, 2000; Wright & Zamudio, 2002; Schmeller *et al.*, 2011). Since developmental stability and noise cannot be observed independently, but together lead to a certain level of asymmetry, their components of variation cannot be easily separated (Zakharov, 1992; Lens *et al.*, 2002).

As far as "ideal forms" practically do not exist in nature, the measurement and presentation in an integral form of the deviations from the strict bilateral symmetry in different morphological features of the organism is proposed as a measure of development instability (Van Valen, 1962). Fine deviations from symmetry are most often described by frequency distributions on the right and left side and can be represented in integral form (Van Valen, 1978; Palmer, 1994). After describing the three asymmetry types (Van Valen, 1962): directional asymmetry (DA), antisymmetry (AS) (in both asymmetry types the characteristics on one of the sides are more frequent i.e. the average is always different from zero), and fluctuating asymmetry (FA) that measures the fine accidental fluctuations from perfect bilateral symmetry, with average always equal to zero), the scientific community decided that FA is most suitable for describing disturbances in the stability of individuals from "wild populations" of various animal organisms (Leary & Allendorf, 1989; Parsons, 1992; Zakharov, 1992; Palmer, 1994). The main advantage of FA is that it is one of the few morphological characteristics for which the norm is known (i.e. perfect symmetry is known) and it is not under strict gene regulation (Zakharov, 1987; Palmer, 1996; Parsons, 2005; Graham *et al.*, 2010). At the same time, large morphological differences between the sides are observed in the other two asymmetry types and they are usually strictly generically determined (Palmer, 2005; Zakharov *et al.*, 2020). The other important advantage is that FA measurements do not require expensive equipment and are performed without killing the animals. It is the humanity of the method that is its strength, because it allows it to work even with endangered species. Susceptibility to a particular stressor and tendency to fluctuate from symmetry may differ among individuals and populations of the same species in different localities (Sanseverino & Nessimian, 2008), and different species may be related to the intensity of stress in different ways, both negative and positive (Piha *et al.*, 2007; Oda *et al.*, 2016; Gondim *et al.*, 2020). One of the major problems with in situ analyses assessing FA levels in wild populations is related to finding reference populations (those that are not affected by FA). In many cases, this can be problematic, as most habitats are in complex ecosystems and are subject to many major stressors (Sanseverino & Nessimian, 2008). According to Sanseverino & Nessimian (2008), the choice of control areas away from the studied stress-exposed habitat is often impractical due to the difficulty of combining environmental characteristics (same habitat types, comparable physicochemical characteristics, etc.). This requires a very careful approach in field analyses studying the manifestations of FA in order to minimize similar errors, and a possible tool for solving the problem is the use of parallel physical-chemical analyses. In a number of environmental studies, elevated levels of FA have been reported in animal populations (including tailless amphibians) subjected to various types and levels of environmental stress, including anthropogenic (Leung & Farbes, 1996; Leamy & Klingenberg, 2005; Loehr *et al.*, 2012; Trokovic *et al.*, 2012; Jumawan *et al.*, 2016; Saber *et al.*, 2016; Shadrina & Vol'pert, 2016; Gondim *et al.*, 2020). Despite these positive results, however, in the last years of the past century and at present there is evidence that FA is not the "universal stress indicator" sought by scientists and perceptions of the role of FA and its application in bioindication analyses need to be reconsidered and revised (Bjorksten *et al.*, 2000; Van Dongen & Lens, 2000; Rasmuson, 2002; Costa, & Nomura, 2016; Niemeier *et al.*, 2019). Most skeptical opinions are grouped around controversial issues regarding the choice of the appropriate test type, the type of characteristics used to analyze FA (meristic traits or metric signs), their number (single or a group of characteristics), error control in measuring asymmetry (Swain, 1987; Whitlock, 1998; Bjorksten *et al.*, 2000; Van Dongen, 2000). An additional problem is the lack of consensus on the methods of statistical processing of the obtained data (Whitlock, 1996; Van Dongen & Lens, 2000) and last but not least the presence of heterogeneity in the relationship between FA, developmental stability, stress, quality of life and fitness (Clarke, 1998; Bjorksten *et* *al*., 2000; Van Dongen & Lens, 2000). This, however, does not mean a refusal to conduct research related to the study of FA levels in natural animal populations living under stress or to address environmental issues based on the values of this indicator (Lens *et al*., 2002; Beasley *et al*., 2013; Breno *et al*., 2013). Despite the existing pessimism, recent studies convincingly show that FA is an informative indicator of environmental stress in animal populations, including tailless amphibians (Cánovas *et al*., 2015; Lajus *et al*., 2015; Matías-Ferrer & Escalante, 2015; Natividad *et al*., 2015; Eisemberg & Bertoluci, 2016; Peligro & Jumawan, 2016; Coda *et al*., 2017; Guo *et al*., 2017; Gondim *et al*., 2020; Rodríguez-Gonzáleza *et al*., 2020; Conan *et al*., 2021) and has its place in bioindication examinations along with physicochemical analyses and other bio-tests. According to Lens *et al*. (2002), researchers' efforts should focus on better clarifying why, in some cases, but not in others, FA reflects stress and fitness, and in particular, what are the factors that cause this discrepancy.

The present paper summarizes and presents the results of our long-term *in situ* analyses conducted in populations of marsh frog *Pelophylax ridibundus* (Pallas, 1771) and green toad *Bufotes viridis* (Laurenti, 1768), synonymous with *Pseudepidalea viridis* (Frost *et al*., 2006, 2013), inhabiting freshwater ecosystems in South Bulgaria with varying degrees of anthropogenic transformation. It covers the period from 2009 to 2017 and includes studies related to the application of fluctuating asymmetry in meristic morphological traits as a marker for the assessment of deviations in the stability of development of individuals of both species, as well as for parallel and independent of physicochemical analyses assessment of the quality of their living environment. The two species of tailless amphibians were not randomly selected as test subjects. They are ecologically plastic, resistant to anthropogenic stress and widespread throughout the country (Beschkov & Nanev, 2002; Stojanov *et al*., 2011). In the course of studies, different and specific working hypotheses related to the objectives of the respective studies were tested, but the common denominator was the assumption of expected differences in the values of fluctuating asymmetry in the populations of tailless amphibians living under anthropogenic stress in comparison with values for individuals from reference populations. The other main hypothesis was based on the assumption that the obtained FA values for the populations of *P. ridibundus* and *B. viridis* can be the basis of an objective, parallel and independent from physicochemical analyses assessment of the ecological status of each of the studied habitats in the short and short term.

The analyses conducted on the territory of South Bulgaria can be divided into three phases: first (2009-2010), second (2011-2015) and third (2016-2017). In each phase, goals were set in accordance with our current capabilities for conducting analyses in the populations of the two test subjects of tailless amphibians. Below we present in detail the goals set and the tasks performed for their realization, during the different phases of the research:

<u>2009-2010</u>

The main goal set in this period was to establish for the first time on the territory of Bulgaria the levels of fluctuating asymmetry (FA) in populations of *P. ridibundus* and *B. viridis*, inhabiting anthropogenically polluted habitats (different degree of pollution) and to use them as a marker to assess developmental stability disorders in these species of tailless amphibians. Another main goal of the research was, based on the obtained values of FA in the populations of *P. ridibundus* and *B. viridis* to assess the quality of the environment in their habitats, an analysis also applied for the first time in Bulgaria.

<u>2011-2015</u>

The main goals of the research during this time period were aimed at expanding the use of FA as a method for assessing developmental stability disorders in the populations of *P. ridibundus* and *B. viridis* and their use as a marker for assessing the degree of anthropogenic disturbances in their living environment. In the course of the analyses the following tasks were solved:

1) Use of FA levels as a biomarker for environmental quality assessment, in parallel and independently of the physicochemical analyses of the water, in large freshwater ecosystems (rivers and dams) on the territory of South Bulgaria.

2) Determining whether FA values differ in populations of *P. ridibundus* inhabiting relatively clean waterbodies of running (rivers) and still (dams) type.

3) Determining whether FA values differ in populations of *P. ridibundus* inhabiting water bodies with identical type of pollution of flowing and standing type.

4) Determining whether FA values differ in populations of *P. ridibundus* inhabiting water bodies with different type of toxicants.

5) Determining whether FA levels in populations of *P. ridibundus*from biotopes with different degrees and different types of pollution change over time.

6) Study of FA values in populations of *P. ridibundus* and *B. viridis* in conditions of syntopic inhabitation of freshwater ecosystems on the territory of South Bulgaria and on the basis of the obtained results to perform assessment of the ecological condition of the respective water bodies.

<u>2016-2017</u>

During this time the research was focused on the practical application of FA as a method for monitoring the ecological status of a large freshwater ecosystem on the territory of South Bulgaria - Sazliyka River. The study was conducted in the spring of 2017 (5 years after the construction of a large treatment plant after the city of Stara Zagora) and not only compared FA values obtained from river analyses performed in 2015, but FA was also directly opposed to physicochemical analyses in order to assess of its objectivity in determining the ecological quality of the environment in different locations along the river. The obtained results allowed not only to perform a parallel and independent of physicochemical analyses assessment of the living environment along the river, but also on the basis of FA values to assess the effects of the treatment plant on water quality in the Sazliyka River.

Another main task in this time period was the study of FA levels in populations of *B. viridis*, living in conditions of increased urbanization and their use as a method for assessing the living environment in their habitats – two syenite hills located in the central part of Plovdiv (places lacking permanent natural water sources, which makes it impossible to apply physicochemical analyses).

Materials and Methods

The analyzed material was collected during the spring-autumn seasons of 2009-2017 from rivers, dams, rice fields and other types of smaller water bodies (natural and artificial reservoirs) located on the territory of South Bulgaria.

Test subjects (indicator species of tailless amphibians) in in situ analyses performed on the territory of South Bulgaria

The main tested species of tailless amphibian used in the full research period (2009-2017) is the marsh frog *P. ridibundus*. This species of aquatic anurans has a wide range in Eurasia: from Denmark and Finland to the north, through Central Europe, to the southern regions of Russia, Turkmenistan, Iraq and Iran to the south, with isolated habitats in Saudi Arabia and Bahrain (Amphibia Web, 2021). In Bulgaria the species is distributed throughout the country: from the coast to 1400 m above sea level and up to 2000 m in Belasitsa (Beschkov & Nanev, 2002; Stojanov *et al*., 2011). The species is protected: BDA (IV), 92/43 (V), BERN (III), IUCN (LC) (Biserkov *et al*., 2007). According to Article 42, Article 41 and Appendix II of Article 41 of BDA, permits for capturing *P. ridibundus* are not issued for the purpose of scientific research. The other type of tailless amphibian that is a test subject in our *in situ* bioindicator analyses perform in the period 2009-2017 is green toad *B. viridis*. Only the nominal species *B. viridis* (Dufresnes *et al*., 2019) inhabits the territory of Bulgaria although the diploid complex of green toads (*B. viridis* complex) includes at least 12 evolutionary lines and at least 14 morphologically similar species distributed throughout Europe, much of Asia and North Africa (Stöck *et al*., 2008, Özdemir *et al*., 2014, Vences *et al*., 2019).

In Bulgaria *B. viridis* inhabits a wide range of habitats (including highly urbanized areas) and is distributed throughout the country (Stojanov *et al*., 2011; Mollov, 2019). The species is protected: BDA (III), 92/43 (IV), BERN (II), IUCN (LC) (Biserkov *et al*., 2007). According to Article 49, Paragraph 1 and Article 48, Paragraphs 2, 5 from BPA permits for capturing *B. viridis* for scientific purposes are issued. Analyses using *B. viridis* in the period 2009-2015 are performed in compliance with permit by Ministry of Environment and Water of Bulgaria No 637/26.05.2015, and the ones in the period 2016-2017 in compliance with permit by MOEW No 701/06.04.2017.

Note: BDA (2002): Biological Diversity Act of Bulgaria. Appendix III - species protected in the whole of the country; Appendix IV - species under the mode of protection and regulated use of nature (State Gazette, No 77/9.08.2002).

DCE'92/43 from 21.05.1992: Annex IV - animal and plant species of community interest in need of strict protection; Annex V - animal and plant species of community interest who is taking in the wild and exploitation may be subject to management measures (EO 1992).

BERN (1979) (Convention on the Conservation of European Wildlife and Natural Habitats, Bern, 19.09.1979): Appendix II - strictly protected fauna species (status in force since 1 March 2002); Appendix III - protected fauna species (status in force since 1 March 2002).

IUCN (International Union for Conservation of Nature and Natural Resources): LC - Least Concern (a taxon is Least Concern when it has been evaluated against the criteria and does not qualify for Critically Endangered, Endangered, Vulnerable or Near Threatened. Widespread and abundant taxa are included in this category.

Geographical locations and summarized data from the physicochemical monitoring of the studied aquatic ecosystems at the time of research

2009-2010

Field analyses in 2009-2010, which were the first bioindication analyses of this type in Bulgaria were held in the locations of different types of water bodies in the southern part of the country, the lands of Galabovo (Stara Zagora district), Dimitrovgrad and Harmanli (Haskovo district), Plovdiv and Stamboliyski (Plovdiv district). A summary map with exact coordinates of the locations is presented in Zhelev *et al*. (2012a).

2011-2015

In situ analyses in the period 2011-2015 were held in large freshwater ecosystems: the rivers Sazliyka (after the village of Rakitnitsa), Topolnitsa (after the village of Chavdar) and Vacha (south of the town of Krichim) and the dams Rozov Kladenets (near the village of Obruchishte), Topolnitsa (near the village of Poibrene), Vacha (near the village of Mihalkovo), Studen Kladenets (in the "tail") and Tsalapitsa rice fields on the territory of South Bulgaria. Detailed maps with exact coordinates of the locations where the analyses were performed are presented in Zhelev *et al.* (2011b, 2012b. 2013, 2014, 2015, 2016, 2017).

2016-2017

The field analyses in the period 2016-2017 were conducted in the region of Plovdiv in the central highly urbanized city area – two of the natural syenite hills in the city. The natural formations "NL "Halm Bunardzhik" Hill" (22.0 hectares, with peak altitude at 265 m a.s.l.) and "NL "Mladezki Halm" Hill" (36.2 hectares with highest point at 285.5 m a.s.l.) are located in the center of Plovdiv and are originally syenite hills formed during the Paleogene. Both hills have been declared protected areas by the Ministry of Environment and Water (MOEW) in order to protect the natural landscape and unique geomorphological formations. Despite the prohibition of public and business activities, these hills are used for recreation and tourism and, especially on holidays, they are visited by a significant number of people. In addition, the hills were built-up to a high degree before they were declared protected areas (Mollov, 2019). The hills lack permanent natural water sources. A map with the exact coordinates of the places where the frogs were caught is presented in Zhelev *et al.* (2021).

In the spring of 2017, a large-scale survey was conducted on the Sazliyka River, 5 years after the construction of a modern water treatment plant downstream the city of Stara Zagora (2011), whose wastewater is one of the main sources polluting the river. Detailed maps with the exact coordinates of the locations along the Sazliyka River (before the villages of Kazanka and Rakitnitsa, after the village of Dinya and after the towns of Radnevo and Galabovo, as well as at the mouth of the Maritsa River south of Simeonovgrad) are presented in Zhelev *et al.* (2019).

The monitoring and control over the condition of the surface waters in the Republic of Bulgaria is performed by the National System for Environmental Monitoring (NSMOS). All *in situ* analyzes performed in the period 2009-2017 are compliant and performed on the basis of basic data on the ecological status of each of the water bodies in which frogs were caught, obtained on the basis of physicochemical analyzes of water performed by order of the Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, Ministry of the Environment and Waters (2021) in the full period of research (Newsletters on the state of water in each of the studied water bodies – rivers and dams, for the period 2009-2017). Physicochemical analyses measure the values of the priority substances for each of the water bodies, determined according to the monitoring Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, in accordance with the Water Framework Directive WED 2000/60/EO (EC, 2000) and Ordinance No H-4/14.09.2012 (Ordinance, 2012) and No 256/1.11.2010 (Ordinance, 2010) for characterization of surface waters in Bulgaria. Until 2013 on the grounds of Ordinance No 7/08.07.1986 on the indicators and norms in determining the quality of running surface water and Annex No 3 to Order No RD – 272/03.05.2001 of the Minister of Environment and Water, pursuant to cl. 118 (2) of the Water Act of 1999, the waters in the Republic of Bulgaria were divided into four categories: first category (drinking water), second category (slightly polluted water), third category (moderately polluted water) and fourth category (heavily polluted water). Ordinance No 7/8.08.1986 was canceled, starting on 05.03.2013. Since 2013, the provisions of Ordinance No H-4/14.09.2012 on the characteristics of surface waters in Bulgaria (State Gazette No 22/5.03.2013) have introduced new terminology (ecological status and ecological potential) in the categorization of surface waters in Bulgaria, in accordance with annex V of the Water Framework Directive (EC, 2000).

The rivers Sazliyka (after the town of Stara Zagora) and Topolnitsa are one of the most polluted in Bulgaria. Along the Sazliyka River, two "local hotspots" have been marked nationally: the first after the town of Stara Zagora (148 887 citizens, on 15.06.2017) at the confluence of the Bedechka River and the second after the town of Radnevo (12 850 citizens on 15.03.2016) at the confluence of the Blatnitsa River. The main share of the water quantities in the rivers Bedechka and Blatnitsa falls on the waste domestic-fecal and industrial waters of the cities of Stara Zagora (12 km extremely severely affected section) and Nova Zagora – the whole course after the town of Nova Zagora (22 238 citizens on 15.12.2017). The pollution of the Rozov Kladenets Dam connected with the river is of the same type. The main polluters of the Topolnitsa River and the associated dam of the same name are the Aurubis copper plant (formerly Pirdop), the Asarel-Medet MOK and the Chelopech-Mining tailings pond. Heavy metals are the main toxicants in the Topolnitsa River and the Topolnitsa Dam. Pollution with high doses of heavy metals is also reported for the Studen Kladenets Dam. The water in Vacha Dam is clean, without data for anthropogenic pollution. The locations in the upper reaches of the Sazliyka River (near the villages of Rakintitsa and Kazanka) are also relatively clean. Water bodies without data for exceedances of the studied physicochemical parameters of water (according to the above-mentioned statutory acts in Bulgaria) are accepted as conditional (conventional) controls in the analyses. Detailed data (physicochemical analysis of water) on the status of each of the studied water bodies are presented in Zhelev *et al.* (2011b; 2012a; b; 2013; 2014; 2015; 2016; 2017; 2019).

Experimental analyses

Adult individuals of both sexes were used for all analyzes performed in the period 2009-2019 (SVL > 60.0 mm, according to Bannikov *et al.*, 1977). The distinction by sex was made on the basis of the degree of development of secondary sexual characteristics in male individuals (nuptial pads on 1 finger and vocal sacs). The catch was made in the evening by the light of Petromax lamps placed on the water body shore, or using a flashlight. In the second case, sections with length of about 1-2 km and width of 4 m along the river or along the shoreline in the water area of the water bodies were surveyed, according to Sutherland (2000). All manipulations were performed in compliance with ethical standards for working with live animals (Steven *et al.*, 2004). After the analyses, the frogs were returned in the wild.

Identification and measurement of fluctuating asymmetry (FA) in P. ridibundus and B. viridis

As a method for assessing the developmental stability in *P. ridibundus,* we used fluctuating asymmetry in 10 morphological traits, as suggested by Chubinishvili (1997) and Zakharov *et al.* (2000a) (Fig. 1). As a method for assessing the developmental stability in *B. viridis*, we used fluctuating asymmetry in 11 meristic morphological traits, as suggested by Peskova (2006) and Peskova *et al.* (2011) (Fig. 2).

The level of asymmetric manifestation for each of the ten traits was recorded for each individual; it may vary from 0 (no asymmetry) to 1 (all the traits are asymmetric) (see Fig 3). It is possible for some of the traits not to express asymmetry, but only in very rare cases it is possible for all 10 (11) traits to be bilateral (Zakharov *et al.*, 2000a; b). The advantage of the method is that it does not require killing animals and according to the methodologies proposed by Zakharov *et al.* (2000a; b), a minimum of 20 adults is sufficient for the tests. The fluctuating asymmetry was defined by the index frequency of asymmetric manifestation of an

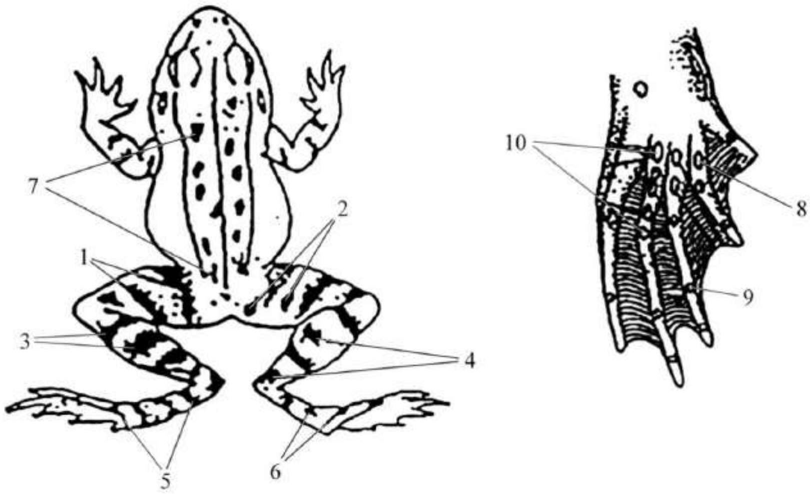

Fig. 1. Diagram of morphological features for assessing the developmental stability in *Pelophylax ridibundus*. This diagram is published in Zhelev (2012b; 2016) and Zhelev *et al*. (2015). *Legend*: 1 – number of stripes on the dorsal side of the thigh (femur), 2 – number of spots on the dorsal side of the thigh (femur), 3 – number of stripes on the dorsal side of the left shank (crus), 4 – number of spots on the dorsal side of the right shank (crus), 5 – number of stripes on the left foot (pes), 6 – number of spots on the right foot (pes), 7 – number of stripes and spots on the back (dorsum), 8 – number of white spots on the ventral side of the second finger of the hind leg, 9 – number of white spots on the ventral side of the third finger of the hind leg, 10 – number of white spots on the ventral side of the fourth finger of the hind leg.

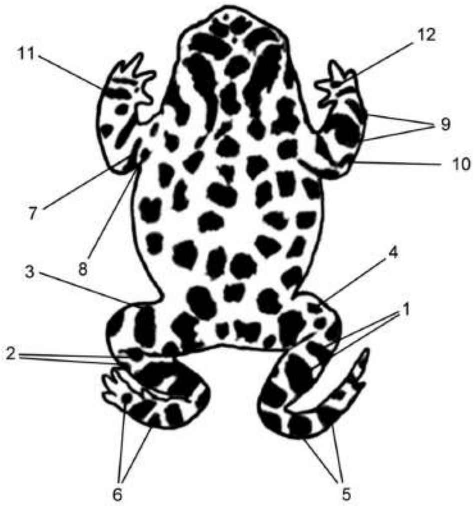

Fig. 2. Diagram of morphological features for assessing the developmental stability in *Bufotes viridis*. This diagram is published in Zhelev (2012b; 2016). *Legend*: 1 - number of spots on the dorsal side of the right shank (crus); 2 - number of spots on the dorsal side of the right shank (crus); 3 – number of stripes on the dorsal side of the thigh (femur); 4 – number of spots on the dorsal side of the thigh (femur); 5 - number of stripes on the left foot (pes); 6 – number of spots on the right foot (pes); 7 - number of stripes on the dorsal side of the right back; 8 -number of spots on the dorsal side of the right back; 9 - number of stripes on the dorsal side of the left forearm; 10 - number of spots on the dorsal side of the left forearm; 11 - number of stripes on the dorsal side of the right wrist; 12 - number of spots on the dorsal side of the right wrist.

Fig. 3. Photos of the most variable meristic morphological traits in *P. ridibundus* individuals found in our investigation in 2017. This photo is published in Zhelev *et al*. (2019). ***Legend*:** a) trait 1 – number of stripes on the dorsal side of the thigh (femur); b) trait 2 – number of spots on the dorsal side of the thigh; c) trait 3 – number of stripes on the dorsal side of the shank (crus); trait 4 – number of spots on the dorsal side of the shank; d) 5 – number of stripes on the foot (pes); trait 6 – number of spots on the foot; e) trait 7 – number of stripes and spots on the back (dorsum); f) trait 8 – number of white spots on the ventral side of the second finger of the hind leg; trait 9 – number of white spots on the ventral side of the third finger of the hind leg; trait 10 – number of white spots on the ventral side of the fourth finger of the hind leg.

individual (FAMI): FAMI = $(\sum X_i) / n$, where X_i measures the asymmetry of an individual and is the number of asymmetric characters (traits) in each specimen divided by the number of used characters and *n* is the number of individuals in the sample (Palmer, 1994; Zakharov *et al*., 2001). Based on the observed mean FAMI values the status of the populations (respectively for the corresponding biotope) was rated, using a special scale suggested for the marsh frogs (Zakharov *et al*., 2000b; Peskova & Zhukova, 2007), but also successfully applied to *Bufotes sp.* (Chikin, 2001; Peskova *et al*., 2011; Guo *et al*., 2017). The scale classifies the quality of the habitat as follows FAMI < 0.4 [grade 1: conventional rate (clean water basin)], 0.41 ≤ FAMI ≤ 0.5 [grade 2: minimal impact on organisms (slightly polluted water basin)], 0.51 ≤ FAMI ≤ 0.6 [grade 3: a satisfactory condition of organisms (average polluted water basin)], 0.61 ≤ FAMI ≤ 0.7 [grade 4: an unfavourable condition of organisms (heavily polluted water basin)] and FAMI ≥ 0.71 [grade 5: a critical condition of organisms (very heavily polluted water basin)], respectively.

Number of test animals used for the analyses in the period 2009-2017

2009-2010

For the whole period in water bodies of different type (rivers, dams, small temporary reservoirs, rice fields), in the locations specified in subsection Geographical locations and summarized data from the physicochemical monitoring of the studied aquatic ecosystems at the time of research, a total of 352 individuals of *P. ridibundus* and 115 individuals of *B. viridis* were collected and treated. Details of the exact number of individuals and the dates of the catches in each of the water bodies at the locations indicated in subsection Geographical locations and summarized data from the physicochemical monitoring of the studied aquatic ecosystems at the time of research, are presented in Zhelev & Peskova (2010) and Zhelev *et al*. (2011a; b; 2012a; b).

2011-2015

On the Sazliyka River, 7 biotopes (microhabitats) located on its left bank from village ot Rakitnitsa, to the mouth of Maritsa River south of the town of Simeonovgrad, and on the left banks of two of its large tributaries: the rivers Blatnitsa and Sokolitsa were studied. A total of 411 individuals of *P. ridibundus* were used for the analyzes. Details with the exact number of individuals and catch dates in each of the habitats are presented in Zhelev *et al*. (2013; 2015; 2016).

A total of 1020 individuals of *P.ridibundus* were used for the studies of the river ecosystems of the rivers Sazliyka, Topolnitsa and Vacha and the dams Rozov Kladenets, Topolnitsa, Vacha and Studen Kladenets. Details with the exact number of individuals and catch dates in each of the habitats are presented in Zhelev *et al*. (2012b; b; 2015; 2016).

Studies of FA levels and assessment of developmental stability in the populations of *P. ridibundus* and *B. viridis* inhabiting in sympatric and syntopic conditions the region of the Studen Kladenets Dam in the period 2009-2011 were performed with a total of 101 individuals of *P. ridibundus* and 92 individuals of *B viridis*. Details of the exact number of individuals and catch dates in each of the years of analysis are presented in Zhelev *et al*. (2014; 2016). Similar analyzes in the populations of the two species inhabiting habitats in the regions of the Rozov Kladenets and Topolnitsa Dams in spring 2010 were held with 69 individuals of *P. ridibundus* and 65 individuals of *B. viridis* (Zhelev, 2011b).

Fifty frogs (25 individuals of each sex) were used for the research held in the Tsalapitsa rice fields in spring 2013. The Vacha River, south of the town of Krichim, was used as a reference site for this study. 50 frogs were also captured there – equal number of both sexes (for details see Zhelev *et al*., 2017).

2016-2017

For the research conducted in spring 2017 on the river ecosystem of the Sazliyka River, 5 years after the construction of a treatment plant after the town of Stara Zagora (2011), a total of 300 individuals of *P. ridibundus* were used. Frogs (25 male and 25 female) were caught in 6 biotopes (microhabits) listed in subsection Geographical locations and summarized data from the physicochemical monitoring of the studied aquatic ecosystems at the time of research. Details of the exact number of individuals and catch dates in each of the habitats are presented in Zhelev *et al.* (2019).

The studies of *B. viridis* conducted in spring 2017 in the city of Plovdiv were conducted with a total of 120 individuals - 60 frogs (30♂, 30♀) caught from each of the two syenite hills located in the central part of the city (for details see Zhelev *et al.*, 2021).

Statistical analyses

The scientific community is currently debating which ways to represent the levels of asymmetry in meristic traits are most appropriate: whether to present the data in integral form and then compare them numerically, or to use mathematical models (Lens *et al.*, 2002). In the period from 2009 to 2015, in order to derive the statistical reliability of the data, we compared the FAMI values obtained for the different habitats with parametric analysis methods (at normal data distribution and homogeneous of samples): Students t-test, or one-way analysis of variance (ANOVA). In order to examine the odds for observing asymmetry in the studied traits in the individuals from each habitat (site), in the 2016-2017 period, a generalized linear model (GLM) using logit link function was fitted to the FAMI data (Molenberghs, 2003). Statistical processing of the data was performed, using the Statistica 7.0 Software (Statistica, 2004), or R language (version 3.1.2, R Development Core Team, 2015).

Results and Discussion

2009-2010

The results obtained regarding the values of the integral indicator for FA in the populations of the two species of tailless amphibians, from studies conducted in the period 2009-2010, are summarized in Table 1.

The Table 1 shows that FA values in *P. ridibundus* that inhabit 14 different habitats from 8 water bodies: small natural and artificial reservoirs, rice fields, dam lakes and three rivers – Sazliyka River (3 sites), Maritsa River (2 sites) and Topolnitsa River (2 sites) are determined in this period. In parallel, FA values in *B.viridis* in 7 different habitats (7 water bodies) in which the reproduction and larval development of the species originated) were studied (see Table 1). The results presented in Table 1 show that lowest FA levels in *P. ridibundus* were found in three water bodies (A, B and F) and on this basis these three water sources receive the minimum degree 1 according to Zakharov *et al.* (2000a) and Peskova & Zhukova (2007). The populations of *P. ridibundus* in these habitats were found to be in a stable condition, under optimal developmental environmental conditions. The anthropogenic pressure on them is far from the levels at which processes leading to disturbances in developmental stability begin. In the other species, *B. viridis*, with reproduction and early development taking place in water bodies A, B and F, FA levels are a little higher for the populations from habitats A and B and correspond to degree 2 of developmental disorder according to Zakharov *et al.* (2000a) and Peskova & Zhukova (2007). In habitat F, FA levels are identical to the ones of *P. ridibundus*. In our opinion, these facts suggest that in the early stages of its development *B. viridis* is a slightly more sensitive to disturbances in environmental factors than *P. ridibundus*. In water bodies D and G, FA levels in *P. ridibundus* also correspond to degree 2 of disorders in developmental stability. This means that these populations also develop under relatively good environmental conditions, but they have a reaction (albeit weak) to environmental stressors. In habitats J (degree 4), C, K, L, M and N (degree 5), higher FA levels were detected. This means that these populations are under conditions of severe stress caused by anthropogenic activity: introduction of pesticides into habitat J, phosphates (PO^{3-}_4) and petroleum products in habitat E, wastewater from TPP "Maritsa - East 1" in habitat C, sewage domestic pollutants and reactive forms of nitrogen (NO^-_3-N, NO^-_2-N) in habitats K and L and heavy metals in habitats M and N. The conclusion to be drawn is that at high levels of contamination, regardless of the type of toxicants in the populations of both species of anuran amphibians, high asymmetry levels are observed (Students t test > 3.0, $p < 0.001$). Details and detailed comments on the research in populations of *P. ridibundus* and *B. viridis* in the 2009-2010 period can be found in Zhelev & Peskova (2010) and Zhelev *et al*. (2011a; b; 2012a; b; c).

Most of the studies related to the analysis of the levels of FA in meristic traits of anurans are conducted on the territories of Russia, Ukraine, Georgia, Belarus, Moldova, Kazakhstan and Uzbekistan (Chubinishvili, 1998; Zakharov *et al*., 2000a; 2000b; Chikin, 2001; Loginov & Gelashvili, 2005; Ustyuzhanina & Streltsov, 2001a; b; Nikashin, 2005; Fomin, 2006; Maksimov 2007a; b; Spirina, 2009; Lada *et al*., 2012; Peskova *et al*., 2011). There are single studies conducted in China (Guo *et al*., 2017) and Turkey (Dönmaz & Şişman, 2021). In most of these studies, low FA levels were reported for populations of anurans (mainly *P. ridibundus*), living under low environmental stress conditions – in protected areas or in habitats far from settlements, agricultural lands or industrial enterprises (Zakharov,1987; Hitsova *et al*., 2004; Peskova & Vasyutina, 2005). High asymmetry levels (degree 4 and 5) are usually reported for anurans populations under severe anthropogenic stress. It is noteworthy that FA values are not influenced by the type of the toxicants, but only by their levels. At high levels of polluting agents such as chemical contaminators (Chubinishvili, 1998; Spirina, 2009), heavy metals (Loginov & Gelashvili, 2005; Nikashin, 2005; Fomin, 2006; Guo *et al*., 2017; Dönmaz & Şişman, 2021), nutrients and phosphates (Erdeneev & Zvolinski, 2002; Peskova & Zhukova, 2007; Vasilev & Vasileva, 2009) or pesticides (Ustyuzhanina & Streltsov, 2001a, 2001b), are reported to cause high levels of FA in populations of frogs.

<u>2011-2015</u>

The results from the research on FA levels in populations of the two species of tailless amphibians in various types of water bodies: running (rivers) and still (reservoirs) and with different types of anthropogenic pollution (domestic sewage pollution and heavy metal pollution) conducted in 2011-2015 period are presented in Table 2.

The Table 2 shows that in this period FA levels in the populations of *P. ridibundus* inhabiting three large river ecosystems: the rivers Sazliyka (12 different sites), Topolnitsa (two sites) and Vacha (one site) and four reservoirs (the dam lakes Vacha, Rozov Kladenets, Topolnitsa and Studen Kladenets) were determined. In parallel, FA levels were determined in the populations of *P. ridibundus* and *B. viridis*, inhabiting under syntopic conditions the region of Studen Kladenets Dam Lake for three years period.

This research shows that the highest levels of FA were found in *P. ridibundus* populations under conditions of permanent anthropogenic pollution, in rivers, at high concentrations of toxicants, regardless of their nature. The grade rating by Peskova & Zhukova's scale (2007) is 5 – the maximum for the six sites (habitats) from the two rivers (B, C, D, F, M and N), with the highest FAMI values. Similar high levels of FA (FAMI = 0.65-0.87) were found in the populations of *P. ridibundus* in the northern and central parts of the area in conditions of different types of anthropogenic pollution: the Voronezh River – metallurgic industry in the region of the town Novolipetsk (Nikashin, 2005), the Hadazhka River – a region of a pig farm in the West Caucasus (Peskova & Zhukova, 2007), the Sviyaga River, near the town of Ulyanovsk, pollution from chemical industry (Spirina, 2009) and the Karasu River in

Turkey polluted with heavy metals (Dönmaz & Şişman, 2021). In water bodies of still type (dam lakes), irrespective of the nature of toxicants, values of FA (FAMI index) in the populations of *P. ridibundus* are lower than the ones in populations inhabiting water bodies of running type (rivers) (ANOVA, $F_9 = 180.077$, $p < 0.001$). The research conducted in the 2011-2015 periods reaffirms the opportunities for assessing the developmental stability of *P. ridibundus* populations that live in conditions of anthropogenic pollution through the method of FA. It supplements the data of FAMI values in *P. ridibundus* populations from rivers and dam lakes polluted with heavy metals and domestic sewage pollution. The results provide better opportunities to use FA in *P. ridibundus* populations for bioindication and biomonitoring, and for parallel and independent analyses on the physicochemical analyses assessment of the environmental condition (for details see Zhelev *et al.*, 2013, 2014, 2015, 2017).

The levels of fluctuating asymmetry in *B. viridis* were higher than those in *P. ridibundus* when they live together in sympatric and syntopic conditions at the dam lake Studen Kladenets for the whole three years period of the research (Students t test > 2.0, $p < 0.05$) (for details see Zhelev *et al.*, 2014, 2016).

2016-2017

The results of the study on the Sazliyka River, conducted in 2017, the 5 years after the construction of a modern, wastewater treatment facility near the town of Stara Zagora are presented in Table 3.

Table 1. The values of FAMI index (Means±SEM) and values of grade scale (Zakharov *et al.*, 2000a; Peskova & Zhukova, 2007) of two anuran species from investigated habitats (sites) in South Bulgaria in 2009-2010 periods. *Legend:* *Sites: A – the Galabovo town (water body with groundwater sources); B – the Galabovo town (Sazliyka River); C – the Galabovo town (sedimentation lake of TPP "Maritsa - East 1"); D – the Stamboliyski town (tailings of a pulp and paper factory); E – the Dimitrovgrad town (Maritsa River in the industrial collectors of "Neohim"); F – the Plovdiv town (Maritsa River in the sugar factory); G – the Plovdiv town (the Chaya River flows into Maritsa River); H – the Galabovo town (Rozov Kladenets Dam lake); I – the Poybrene (Topolnitsa Dam lake); J – the Plovdiv town (Tsalapitsa rice fields); K – the Stara Zagora town (Sazliyka River); L – the Radnevo town (Sazliyka River); M – the Pirdop town (Topolnitsa River); N – the Chavdar village (Topolnitsa River); – no data.

Sites* (habitats)	*Pelophylax ridibundus* 2009							Publications
	A	B	C	D	E	F	G	
	0.22±0.03 1	0.37±0.06 1	0.73±0.03 5	0.53±0.09 2	0.57±0.06 3	0.37±0.02 1	0.53±0.06 2	Zhelev& Peskova (2010); Zhelev (2012b)
	Bufotes viridis 2009							Zhelev (2011a, 2012b)
	0.48±0.01 2	0.47±0.03 2	0.67±0.05 5	–	0.63±0.04 4	0.39±0.03 1	–	
	Pelophylax ridibundus 2010							Zhelev (2011b, 2012b, 2012c)
	H	I	J	K	L	M	N	
	0.57±0.023 3	0.54±0.01 3	0.63±0.11 4	0.82±0.02 5	0.80±0.02 5	0.73±0.02 5	0.75±0.01 5	
	Bufotes viridis 2010							Zhelev (2012a, 2012b)
	0.56±0.03 3	0.63±0.04 4	–	–	–	–	–	

Table 2. The values of FAMI index (Means±SEM) and values of grade scale (Zakharov *et al*., 2000a; Peskova & Zhukova, 2007) of two anuran species from investigated habitats (sites) in South Bulgaria in 2011-2015 periods. *Legend:* *Sites: the Sazliyka River south of village Rakitnitsa (A), downstream of the Stara Zagora town and the confluence with Bedechka River (B), downstream of the Radnevo town and the confluence with Blatnitsa River (C), downstream of the Lyubenovo village (D), north of the Galabovo town, under the sluices which block the river to discharge part of it to "TPP Brikel" (E), south of the Galabovo town, downstream of the confluence with Sokolitsa River (F), south of the Kalugerovo village until it runs into Maritsa River (G), the Blatnitsa River south of the Lyubenova Mahala village (H), the Sokolitsa River in the Rozov Kladenets Dam (I), east of the Obruchishte village (J), east of the Iskritsa village (K), east of the Orlov Dol village (L); the Topolnitsa River below the bridge on the road Panagyurishte – Pirdop (M), below the village of Chavdar (N), the Vacha Dam lake (O), the Rozov Kladenets Dam lake (P), the Topolnitsa Dam lake (Q), the Vacha River (S) and the Tsalapitsa rice fields (T).

Sites* (habitats)	*Pelophylax ridibundus* 2011-2013							Publications
Rivers: Sazliyka, Blatnitsa, Sokolitsa, Topolnitsa and Vacha; Dams: Vacha, Rozov Kladenets and Topolnitsa; Tsalapitsa rice fields	A	B	C	D	E	F	G	Zhelev *et al*. (2013, 2015, 2017); Zhelev, (2016)
	0.47±0.01 1	0.82±0.02 5	0.80±0.02 5	0.72±0.03 5	0.64±0.02 4	0.72±0.02 5	0.63±0.03 4	
	Pelophylax ridibundus 2011-2013							
	H	I	J	K	L	M	N	
	0.85±0.02 5	0.55±0.01 3	0.57±0.02 3	0.49±0.01 2	0.33±0.01 1	0.73±0.01 5	0.76±0.01 5	
	Pelophylax ridibundus 2011-2013							
	O	P	Q	S	T			
	0.38±0.01 1	0.59±0.01 3	0.54±0.01 3	0.37±0.01 1	0.71±0.01 5			

Sites* (habitats)	*Pelophylax ridibundus*		*Bufotes viridis*		Publications
Studen Kladenets Dam lake	2009	0.53±0.03 3	2009	0.57±0.03 3	Zhelev *et al*. (2014); Zhelev (2016)
	2010	0.63±0.02 4	2010	0.68±0.03 4	
	2011	0.63±0.02 5	2011	0.67±0.03 4	

Table 3. The values of FAMI index (Means±SEM) and values of grade scale (Zakharov *et al*., 2000a; Peskova & Zhukova, 2007) of two anuran species from investigated habitats (sites) in South Bulgaria in 2016-2017 periods. ***Legend:*** *Studied sites on the Sazliyka River: A – before the village of Kazanka, B – before the village of Rakitnitsa, C – below the village of Dinya, D – below the town of Radnevo, E – below the town of Galabovo and F – at the mouth of Maritsa River south of the town of Simeonovgrad; Studied sites in the Plovdiv town: G – Halm Bunardzhik Hill and H – Mladezki Halm Hill.

Sites* (habitats)	*Pelophylax ridibundus* 2017						Publications
	A	B	C	D	E	F	
	0.39±0.01 1	0.49±0.01 2	0.76±0.02 5	0.82±0.02 5	0.84±0.02 5	0.64±0.01 4	Zhelev *et al.* (2019)
	Bufotes viridis 2017						
	G	H					Zhelev *et al*. (2021)
	0.27±0.01 1	0.25±0.01 1					

The obtained FA levels in the populations of *P. ridibundus* inhabiting in 6 sites are compared with the data from the 2011 survey (see Table 2 and Zhelev *et al.*, 2015). No significant differences between years were found except for the site 5, in which there were two, times higher odds to observe asymmetries in the year of 2017 in comparison to 2011 (GLM, $Z = 4.201$, $p < 0.001$) (for details see Zhelev *et al.*, 2019).

What is the possible explanation for the established lack of positive effects on the condition of the Sazliyka River water in the section after the town of Stara Zagora, 5 years after the construction of wastewater treatment plant Stara Zagora? In our opinion, the reasons can be found in the technical characteristics of WTP Stara Zagora set in its design. Wastewater treatment plant Stara Zagora is with capacity for 256 300 citizens and has facilities for treatment (reduction of parameters)as follows: total phosphorus (TP) – until 1 mg/l, total nitrogen (TN) – until 10 mg/l, biological oxygen demand five days (BOD_5) – until 25 mg/l, chemical oxygen demand (COD) – until 125 mg/l. The quantity of treated water entering the Sazliyka River is 33 500 000 m^3/year, (Register of the issued permits for use of a water body for discharging wastewater 2010-2019, Permit №33140101/11.05.2011. This treated waste water constitutes 25% of the resource of the water body: Q_{WB} - 136 235 520 m^3/year (Final report of the National Institute of Meteorology and Hydrology - Bulgarian Academy of Sciences on implementation of the agreement with the Ministry of Environment and Water for 2015). The technically set purification degrees according to the above indicators meet the requirements of Commission Directive 98/15/EC (EC, 1998) for settlements with over 100 000 citizens but they fail to meet the standards for high water quality of inland surface water. The quantity of treated water from WTP Stara Zagora is evenly distributed throughout the year. The river flow of the Sazliyka River has been greatly altered due to the transfer of water from another river catchment (Koprinka Dam). This water enters the river before Stara Zagora through a derivation tunnel. Before entering the Sazliyka River, the water passes through two cascading hydroelectric power plants (north of Stara Zagora, before the treatment plant). The transferred water is used for irrigation. River outflow is seasonal and depends on the irrigation season. From mid-April to mid-October; the water levels in the river are approximately twice as high as those outside the irrigation season. During the winter period (low water season), the treated wastewater may exceed the water quantities measured in the section of the river before the discharge of WTP Stara Zagora. This pronounced seasonality of the river outflow is also confirmed by the large fluctuation in the measured values of TN, TP, BOD_5 and COD indicators in the waters of the Sazliyka River. They strongly correlate with the sampling time – high values outside the irrigation season and lower values in the irrigation season (for details see Zhelev *et al.*, 2019). The above facts show that with the so set water purification degree WTP Stara Zagora is unable to reach the standards for high water quality of inland surface water for the TP < 0.150 mg/l, TN < 0.07 mg/l, BOD_5< 2 mg/l, COD – 25 mg/l. The treated wastewater from WTP Stara Zagora constitute one fourth from the resource of the Sazliyka River. Due to the lack of other significant wastewater emitters, it can be said with a high degree of reliability that the main pollution of the river in this section is from domestic wastewater coming from the big city. In the section after the town of Radnevo, the Sazliyka River crosses the territory of the coal-mining complex Maritsa East. In the region of the town of Galabovo a part of river water is used for industrial purposes; it is diverted to TPS Brikel (200MW) and then it enters the

Rozov Kladenets Dam (3.6 km^2). Since 2011 there has been a newly built TPS in the region: AES Galabovo (670MW). The Rozov Kladenets Dam has been built for the purposes of the neighboring TPS Brikel and AES Galabovo, where lignite coal is burnt. Industrial effluents, that are used to cool the turbines, enter the dam; it is in direct connection with the Sazliyka River. Besides, this river section is the place of wastewater discharge from the lake-precipitator of TPS Brikel and AES Galabovo. In comparison with the data from the physicochemical analyses conducted on Sazliyka River in the period 2001-2012 (see Zhelev *et al.*, 2013, 2015), the new physicochemical analyses, in the period 2012-2017 (see Zhelev *et al.*, 2019), do not give any reason to state that the state of the river has improved with the commissioning of a wastewater treatment plant after the town of Stara Zagora.

The results of research conducted with the other species tailless anuran – the green toad *B. viridis* performed in urbanized area – the town of Plovdiv are presented in Table 3. The results of this study show low levels of fluctuating asymmetry for *B. viridis* individuals from both sites: 0.27 (the Halm"Bunardzhik" Hill) and 0.25 (the "Mladezhki Halm" Hill), respectively. Low asymmetry levels reported for *B. viridis* individuals from both sites indicate that their early stages of development have occurred under good environmental conditions with minimal risk of disturbances in their developmental stability. This means that the environmental conditions at both sites were good at the time of the study (for details see Zhelev *et al.*, 2021).

Conclusions

1. The results of our investigations conducted over a period of 9 years, show that changes in asymmetry levels in meristic morphological traits in adult and sexually mature representatives of *P. ridibundus* are a very good bioindication method, supplementing physicochemical analysis data, while providing an independent, parallel assessment of the state of the living environment. In addition, the method is easy to implement, it does not involve expensive equipment and does not require the killing of the test animals. It can be applied at any point in the area inhabited by the species, even in protected areas because frogs are returned to nature.

2. The results of the analysis of fluctuating asymmetry levels in our researches in the 2009-2017 periods support the potential for the use of green toads in bioindication analyses. This reveals prospects for the search for new indicator species and the widening of the possibilities for the practical introduction of the method into bio-indication experiments.

Acknowledgments

This research was supported by the National program "Young scientists and Postdoctoral candidates" funded by the Bulgarian Ministry of Education and Science.

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Received: 01.08.2021
Accepted: 01.09.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 | December 2021 | pp. 289-320

Use of Haematological Indicators in Anurans for Assessing Their Health Status When Inhabiting Conditions of Anthropogenic Stress. Pelophylax ridibundus (Amphibia: Ranidae) as an Example: A Review and Appraisal

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Abstract. We review evidence for and against the use of haematological parameters as indicators for assessing the health status in free-living anuran populations that live in conditions of ecological stress. We focus on the use of direct measured blood parameters, such as erythrocyte and leukocyte counts, haemoglobin concentration, haematocrit value, leukogram and calculated haematological indexes: mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and mean corpuscular volume. In addition, we evaluate the possibilities for use as biomarkers of the metric parameters of erythrocytes. This paper summarizes the results from our long-year *in situ* analyses of the populations of Marsh frog *P. ridibundus* inhabiting freshwater ecosystems in Southern Bulgaria with different degrees of anthropogenic transformation. It presents the results from using different haematological indicators as markers for the assessment of changes in their organism when inhabiting areas affected by anthropogenic stressors. Our results strongly support using blood changes in anuran amphibians as objective indicators for assessing their health status. We believe they are a reliable biodiagnostics instrument in modern in field ecotoxicological studies.

Key words: Anurans, Marsh frog, haematological parameters, anthropogenic stress, biomarkers, health status.

Introduction

Why are the haematological parameters in anuran amphibians' suitable biomarkers for assessing the effects of various anthropogenic stressors?

Blood is a highly differentiated and reactive internal environment of the body. It is a type of specialized connective tissue of mesenchymal origin, consisting of blood plasma, which includes water, nutrients, waste, enzymes, antibodies, hormones and shaped elements (blood cells) – erythrocytes, leukocytes and platelets (Seiverd, 1972). The formation of plasma and its components is generally related to tissue metabolism, starting with the organs of the gastrointestinal tract. Differentiated blood cells have a relatively short life and are formed continuously by stem cells produced in haematopoietic organs (Glomski et al., 1997). In all vertebrates, the first blood cells appear during the embryonic period, but the haematopoietic

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centers are different in different taxonomic groups and this largely depends on the age of the individuals (Jordan, 1933; Foxon, 1964; Glomski et al., 1997). Although in amphibians (including anurans) blood composition is similar to that in other vertebrates, such as heterothermic animals, they have developed a number of adaptive structures and mechanisms to deal with changes in the environment (Foxon, 1964; Wojtaszek & Adamowicz, 2003). Many of these adaptations are the result of their phylogenetic history, involving a transition from water to land life (Wei et al., 2015; Franco-Belussi et al., 2021). In all vertebrates, blood is responsible for important bodily functions such as immune response, transport of nutrients, transport of gases and metabolic waste (Wells & Baldwin, 1990; Allender & Fry, 2008; Álvarez-Mendoza et al., 2011).

All changes in environmental factors, including those related to anthropogenic stress, cause changes in functional state of the blood (Cajaraville et al., 2000; Davis et al., 2008; Gonçalves et al., 2019; Oliveira et al., 2019). This makes it very difficult and practically impossible to derive reliable reference values for different haematological parameters in different species of tailless amphibians, although in the past many studies have been directed at this. However, research from the end of the past century and the present century give clear evidence that setting reference values for haematological parameters in anurans without parallel tracing and analysis of environmental factors and the possible presence of such with anthropogenic origin in their habitats, does not give reliable results (Cabagna et al., 2005; Romanova & Egorikhina, 2006; Davis et al., 2008; Priyadarshani et al., 2015; Pollo et al., 2016, 2017; Salinas et al., 2017; Sures et al., 2017; Corduk et al., 2018; de Assis et al., 2018; Xiong et al., 2018; Davis & Golladay, 2019; Oliveira et al., 2019; Zamaletdinov et al., 2019; Brodeur et al., 2020). Most of the above tests with anuran amphibians include mainly analyses using as biomarkers quantitative and qualitative characteristics of the erythrogram and leukogram, since variations in their parameter values provide the main volume of information about the indication of changes in main physiological processes such as breathing and immune defense of the organism when inhabiting anthropogenically transformed environment (Venturino et al., 2004; Davis et al., 2008). In addition, this type of research can be performed immediately after the capture of the animals and does not require expensive laboratory equipment and consumables.

Changes in the erythrogram of anurans inhabiting under conditions of anthropogenic stress.

The erythrogram is that part of the blood picture that quantitatively and qualitatively evaluates erythrocytes. This is done by counting the total number of these cells (RBCs), measuring haemoglobin concentration (Hb) and calculating haematocrit value (Hct) or packed cell volume (PCV). In addition, haematological indices (MCH: mean corpuscular haemoglobin: MCHC: mean corpuscular haemoglobin concentration in erythrocytes and MCV: mean corpuscular volume) are also part of the erythrogram. Here we explicitly clarify that research on the values of haematological indices in "wild populations" of amphibians is very scarce (Johnstone et al., 2017).

The number of erythrocytes in amphibians is the lowest compared to all other groups of vertebrates (Hutchison & Szarski, 1965). This is due to the morphological features of their erythrocytes: relatively large nuclear cells, and it is known that the number of erythrocytes is inversely correlated with their volume (Wintrobe, 1933; Banerjee, 1979). Researchers believe that animals in better physical condition maintain higher levels of Hb and Hct, so these indicators should be positively correlated with body condition indexes, at least when mobilization of metabolic energy sources is beneficial for the body (Artacho et al., 2007; Norte et al., 2008). The benefit of higher Hb or Hct is associated with a greater ability to transport oxygen in the blood and its faster delivery to metabolizing tissues and therefore meeting high energy needs. This assumption supposes that animals in better overall physical condition have the ability to better allocate metabolic resources to maintain a higher "healthy" level of Hb and Hct. This suggests that each species should have an upper limit of Hct ("optimal haematocrit"), beyond which increased blood viscosity would lead to deterioration in blood flow velocity (de Boer et al., 2006).

When *in situ* analyzes examine changes in the erythrogram of anurans, in order to obtain reliable and objective information on the condition of individuals from populations living under stress, it is necessary to compare the data with those obtained from a reference population (such living in optimal environmental conditions, without the presence of anthropogenic stressors) (Pollo et al., 2016, 2017; Salinas et al., 2017; Sures et al., 2017; Corduk et al., 2018; Xiong et al., 2018; Zamaletdinov et al., 2019; Şişman et al., 2021). The most objective information is obtained when complex changes in the erythrogram are monitored and analyzed, and not its individual components (Johnstone et al., 2017; Brodeur et al., 2020). According to literature data, short-term stress-induced changes in the erythrogram affecting Hb and Hct values are usually due to changes in plasma volume rather than erythrocytes. Thus, morphological variables in red blood cells, such as mean corpuscular volume (MCV) or MCHC, should not be altered by short-term stress (Johnstone et al., 2017). Haemoglobin and haematocrit values may decrease in anemia, or increase (more pronounced Hct) in general dehydration (Coppo et al., 2005). In the absence of food, or in the case of unbalanced food, as well as in the absence of minerals such as Fe, Cu, Co and Se, the values of haemoglobin and haematocrit may also decrease (Jain, 1993). Johnstone et al. (2017) generally classify anemias into two types: regenerative and nonregenerative. According to Lewis et al. (2006) anemia occurs as a result of either a reduced number of erythrocytes or a reduced concentration of haemoglobin in the erythrocytes [mean cell concentration, or corpuscular concentration of haemoglobin - MCHC (g/dl)]. Regenerative anemia occurs when an individual responds to this condition by releasing immature erythrocytes (reticulocytes) from the haematopoietic centers and / or increasing erythropoiesis. Typically, the stimulus that triggers the release of immature erythrocytes is mediated by hypothalamic-pituitary-adrenal stress, such as a hemorrhage injury or parasitic infection (McGrath, 1993; Tyler & Cowell, 1996). Non-regenerative anemia occurs when an individual is unable to respond and / or compensate for the loss of red blood cells and this condition may be the result of food stress, certain chronic diseases or toxic substances (McGrath, 1993; Tyler & Cowell, 1996). Regenerative anemias, as well as those of hypoxemic type, caused by toxicants present in the habitats of anurans (leading to a decrease in tissue oxygen levels) are an expression of long-term toxic (or other type) negative effects on their organism (Johnstone et al., 2017). However, in wild populations, the differences between the two types of anemia may not be clear, but combined synergistic effects are possible as well, as a result of physiological reactions caused by short-term and long-term stress (Dhabhar et al., 1996; Romero & Romero, 2002, Romero & Reed, 2005). The presence of a high number of immature erythrocytes in the peripheral blood of tailless amphibians is considered as an indicator of a compensatory regenerative response caused by anemia or loss of circulating erythrocytes (Valenzuela et al., 2006; Prieto et al., 2008; Allender & Fry, 2008; Briggs & Bain, 2012). This thesis is supported by specific results obtained in *in situ* studies in populations of anurans inhabiting conditions of anthropogenic stress in habitats heavily polluted with different types of xenobiotics (heavy metals, fertilizers and pesticides) (Peskova & Zhukova, 2005; Barni et al., 2007; Marques et al., 2008; Spirina, 2009; Mineeva & Mineev, 2010; Pollo et al., 2017; 2019).

One of the important but very poorly studied morphological characteristic of the erythrocyte cell is the changes in its size and shape (as well as those in the nucleus) in populations of anuran amphibians inhabiting conditions of anthropogenic stress. The ability of erythrocytes to change the size and shape of their cell depends on the ratio of surface to volume, the mechanical properties of their cell membrane and their internal viscosity (Chien, 1987; Baskurt & Meiselman, 2003). According to Baskurt & Meiselman (2003), changes in cell shape depend on the critical ratio between cell size and volume (S/V).

When moving from an ellipse to a sphere, due to the smaller volume of the latter, its area increases. It should be noted that under conditions of environmental and physiological stress, changes in the shape of erythrocytes are accompanied by changes in the properties of blood flow such as osmotic balance, viscosity, flow rate, etc. (Baskurt & Meiselman, 2003; Allender & Fry, 2008; Kim et al., 2015). Rounding of erythrocyte cells (oval or spherical erythrocytes) can be considered an adaptive response in conditions of hypoxia, as it increases the contact surface of the cell (Wells & Baldwin, 1990) and ensures the diffusion of more oxygen to the tissues (Hartman & Lessler, 1964; Allender & Fry, 2008).

The opposite effects have also been reported in literature – increase of cell size and reducing cell contact surface leads to difficulties in gas metabolism (Nicol et al., 1988; Lay & Baldwin, 1999; Bondarieva et al., 2012). However, we must clarify that positive biological effects in tissue hypoxia can be expected not only from the presence of small cells with a larger relative surface, but also from accelerated haematopoiesis, accompanied by normal haemoglobin synthesis. The size of erythrocyte nuclei in tailless amphibians (and in other groups of vertebrates with erythrocytes with nuclei) depends on the size of the genome and the degree of chromatin condensation. (Macadangdang et al., 2014; Levy & Heald, 2016), but these are not the only factors determining their size. According to Kim et al. (2015) changes in the size of the nuclei may be due to changes in the osmotic pressure of the cytoplasm and the activity of the motor structural elements of their cytoskeleton. In addition, changes in nuclear shape may be due to changes in nuclear plate (Webster et al., 2009). It is known that in amphibians the rate of metabolism is inversely proportional to the size of their genome (Gregory, 2001; Vinogradov & Anatskaya, 2006), but in general the causes and mechanisms leading to changes in the size of the nuclei remain unclear and very poorly studied (Edens & Levy, 2014; Mueller, 2015). Very little work has been devoted to studies on the morphology of erythrocytes in populations of anuran amphibians inhabiting conditions of anthropogenic pollution (Zhelev et al., 2006; Omelykovets & Berezyuk, 2009; Mineeva & Mineev, 2010; Salinas et al., 2017; Dönmez & Şişman, 2021).

Although the authors of these works report changes in the size and shape of erythrocytes in the blood of anurans inhabiting anthropogenically polluted habitats, compared to populations of frogs inhabiting background areas, there are no definite opinions about the causes of the observed changes and their biological role. In such *in situ* studies, it is even more difficult to find causal relationships between changes in erythrocyte size and specific toxicants. One of the reasons is that toxic agents in wastewater are usually in the composition of complex mixtures and enter into synergistic interactions both with each other and with various abiotic environmental factors such as altitude, temperature, hydrogen index, electrical conductivity of water, dissolved oxygen, etc.

(Haden, 1940; Hartman & Lessler, 1964; Ruiz et al., 1989; Addy et al., 2004).

Changes in the leukogram of anurans inhabiting conditions of anthropogenic stress.

The leukogram is a study that determines the total number of leukocytes (WBCs) and in particular in the differential count – the number of individual types of leukocytes with their percentages.

Leukocytes in anurans are identical to those in other groups of vertebrates: agranulocyte leukocytes – lymphocytes, monocytes and granulocyte leukocytes – neutrophils, eosinophils and basophils (Davis et al., 2008; Arikan & Çiçek, 2014; Zhelev et al., 2017a). Lymphocytes are involved in immune responses and the production of haematopoietic growth factors. They are morphologically divided into small and large lymphocytes, and immunologically and functionally into B and T cells (Campbell, 2004). Monocytes (mononuclear phagocytes) are relatively large cells, of different origin from lymphocytes (myeloid stem cell line) and are an important part of the monocyte-macrophage system, providing non-specific immune protection of the organism. Together, lymphocytes and monocytes make up 80% of the leukocytes of anuran amphibians (Arikan & Çiçek, 2014). The functions of granulocytes in amphibians and anurans in particular are similar to these cells in other vertebrate species and have been identified in terms of their morphological characteristics (Wright, 2001). Neutrophils (rod-nuclear and segment-nuclear) are the largest group of granulocytes. They are associated with chemotaxis and are involved in the inflammatory process (recognizing and binding cytokines and leukotrienes released during inflammation) and the killing of bacteria by phagocytosis. Neutrophils carry out the so-called "oxidative burst" (secretion in the phagosome of toxic to pathogens oxygen compounds, such as hydrogen peroxide and hypochlorous acid as well as lysozyme and interferon), which makes them major participants in the fight against bacterial and fungal infections.

In birds and reptiles, neutrophil is replaced by heterophile that performs the same immunological function (Hawkey & Dennett, 1989; Jain, 1993). Eusinophils are also involved in phagocytosis, especially in allergic-type reactions (they absorb biologically active substances such as histamine). They are major participants in the disposal of tumor cells and parasites (Thrall et al., 2006). In most vertebrates, basophils are the smallest group of granulocytes and their functions are still unclear. However, in some species of anurans, there is evidence of a high percentage of basophils in peripheral blood (Campbell, 2004). They are thought to be involved in "delayed-type" immunobiological reactions (releasing proteolytic enzymes, heparin, but also histamine, which causes blood flow to the inflamed areas). They participate in the management of asthma attacks and other allergic conditions. In amphibians and anurans in particular, the number of leukocytes in 1 mm^3 of blood varies. Variations in leukocyte count are due to sex, age, season, diet, pregnancy in females, environmental and pathological factors (Thrall et al., 2006; Davis et al., 2008; Liu et al., 2013; Arikan & Çiçek, 2014; Bunjerdluk et al., 2021; de Gregorio et al., 2021; Franco-Belussi et al., 2021). All this makes it very difficult to define reference ranges, both for the total number and for the percentage ratio of different types of leukocytes in different types of anurans. An additional problem in this regard is the heterothermic metabolism and its strong dependence on abiotic environmental factors, among which temperature is in the first place.

Low temperatures are a major problem for amphibians, and one strategy they use to deal with low temperatures is thermal acclimatization: tuning cellular and physiological processes in response to environmental conditions (Feder, 1992;

Greenspan et al., 2017). However, thermal acclimatization requires time, which leads to suboptimal levels of immunity after temperature changes, while the immune system undergoes adjustments (Raffel et al., 2006, 2015). Therefore, it is always necessary in *in situ* analyzes, when examining the leukogram parameters in anurans inhabiting conditions of anthropogenic stress, to analyze and compare them in parallel with data from a reference population living as close as possible to the optimal environmental conditions.

The WBC profile (the number of circulating immune cells in the bloodstream) can be used to assess haematopoietic productivity and the level of activation of the immune system (Davis et al., 2008). In recent years, this type of analysis has been increasingly used in various in situ studies in "wild" populations, including populations of amphibians inhabiting conditions of anthropogenic stress (Rohr et al., 2008; Shutler & Marcogliese, 2011; Peterson et al., 2013; Das & Mahapatra, 2014; Vallejo et al., 2015). Lymphocytes and neutrophils usually make up the majority of leukocytes (Davis & Durso, 2009) and the ratio of these cells is often used alone as a reliable indicator of the state of the immune system (Davis et al., 2008; Silva et al., 2021). High relative proportions of circulating neutrophils and low relative proportions of circulating lymphocytes (high N:L ratio) are characteristic of a haematopoietic stress response or an activated immune system in amphibians (Bennett & Daigle, 1983; Cooper et al., 1992; Maniero & Carey, 1997; Davis & Maerz, 2008a; Vallejo et al., 2015; Greenspan et al., 2017; de Gregorio et al., 2021) and other vertebrates (Davis et al., 2008). The functional explanation for the high N:L ratios is that they can increase immune readiness under stressful conditions when animals are likely to face an immune challenge (Dhabhar et al., 1994, 1995; Franchimont, 2004). The increase in neutrophils in blood may intensify the inflammatory response, while the trafficking of lymphocytes out of circulation and into tissues may improve immune control or protect these cells from the inhibitory effects of stress hormones (Dhabhar et al., 1994; 1995; Rich & Romero, 2005). Interpretation of changes in leukocyte profiles (ie, attribution of stress against infection) can be problematic, especially if the state of the infection is unknown (Davis et al., 2004). This can be a problem in field analysis, although the two factors (stress and infections) are closely related and stress usually leads to infections and diseases, weakening the body's overall immunity. One of the approaches to overcome this problem is the parallel analysis of other haematological parameters (erythrogram).

In literature there are many studies, though unsystematized, reporting changes in the leukogram of different groups of vertebrates living under stress (Dhabhar et al., 1994; 1995; Davis et al., 2008; Davis & Maerz, 2008a; b) but research in populations of anurans from anthropogenically polluted habitats is generally very limited. There are studies reporting high leukocyte count in severe neutrophilia and monocytosis in the blood of *P. ridibundus* inhabiting the area of a chemical plant in Ukraine (Tarasenko, 1981), a system of rice fields in Southern Russia (Peskova & Zhukova, 2005) and of *Rhinella arenarum* (Hensel, 1867) from rice fields in Argentina (Pollo et al., 2017). The reverse changes – general leukopenia in combination with reduction of leykocyte and monocytes count is reported in populations of *P. ridibundus* inhabiting urbanized environment – in a residential area in Ekaterinburg densely built-up with residential buildings (Sils & Vershinin, 2005; Sils, 2006, 2008) and Novgorod (Romanova & Romanova, 2003; Romanova, 2005; Romanova & Egorikhina, 2006) in the Russian Federation. Peskova (2001) proposes changes in the differential formula of blood in populations of anurans inhabiting conditions of anthropogenic stress to be generally referred to two types: In the "first type of changes" (under conditions of relatively low anthropogenic load on the environment) the leukogram is characterized by leukocytosis and neutrophilia (increases of the total number of neutrophils or only that of rod-nuclear cells accompanied by a decrease in segment-nuclear neutrophils). In addition, monocytosis is almost always observed compared to controls. This general picture of changes in the leukogram of amphibians may show some variation with diverse changes in the number of lymphocytes, basophils and eosinophils.

The "second type of changes" in the leukogram in anurans is characterized by leukocytosis and neutropenia, which may be accompanied by monocytosis and eosinophilia, or monocytosis and eosinopenia. In the opinion of the author (Peskova, 2001), when toxicant concentration does not exceed any critical value, characteristic for the given polluting agent, the leukogram of the amphibians reacts according to the first type. Significant neutrophilia is an expression of a complete protective reaction of the body. In combination with monocytosis, it provides adaptation of amphibians to inhabiting conditions of pollution and elimination of toxic and / or associated bacterial agents that have entered the body. The second type of leukogram reaction is evidence not only of the body's attempts to cope with high doses of toxicants, but also a sign of depletion of its resistance in an environment with chronic presence of stressors (anthropogenic pollutants). However, severe neutropenia leaves little hope for amphibians to survive in such severe environmental conditions, despite the appearance of immature granulocyte forms, which is evidence of compensatory stimulation of neutrophilic granulocytopoiesis. The second type of changes in the leukogram of anuran amphibians is an indication for transition from adaptations to pathological changes that probably lead to the subsequent death of the animals – third phase (exhaustion of the organism), according to the theory of general adaptation syndrome (GAS) (Selye, 1956).

This paper summarizes and presents the results from our long-term *in situ* analyzes conducted in the populations of Marsh frog *Pelophylax ridibundus* (Pallas, 1771) inhabiting freshwater ecosystems in Southern Bulgaria with varying degrees of anthropogenic pollution. It covers the period from 2001 to 2017 and includes studies on the application of blood markers complex for the purpose of assessment of general health status of frogs that inhabit areas, polluted with toxicants of different types and in different concentrations. To achieve the goals of research, the values of tested blood parameters of frogs from polluted habitats were compared with those obtained from frogs inhabiting less disrupted habitats (references sites). In the course of research different and specific working hypotheses related to the objectives of the respective analyses were tested, but what they had in common was the assumption of expected differences in the values of the tested blood markers in the populations of anurans inhabiting conditions of anthropogenic stress when compared with the corresponding values obtained for the individuals from the reference populations. This makes it possible to assess how exposure to toxic agents (usually over prolonged time) in anthropogenically contaminated habitats affects marsh frogs *P. ridibundus* and what changes are caused by the toxicants in the body of these anurans (indication of stress and exhaustion of the body, or adaptation to life in a hostile environment).

Materials and Methods

The material for the analyzes was collected during the spring-autumn seasons of 2001-2017 from rivers and dams (natural and artificial water bodies) in Southern Bulgaria with test subject – Marsh frog *P. ridibundus*. This species of water frog has a large range in Eurasia (Amphibia Web, 2021) and in Bulgaria it is distributed throughout the country (Beschkov & Nanev, 2002; Stojanov et al., 2011). The species is under protection: BDA (IV), 92/43 (V), BERN (III), IUCN (LC) (Biserkov et al., 2007). According to Article 42, Article 41 and Appendix II of Article 41 of BDA, permits for catching *P. ridibundus* are not required when collecting them for scientific purposes. Frogs were caught in the evening by the light of strong lamps placed on the shore of the waterbody, or with the help of an electric lantern. In the second case, areas with length of about 1-2 km and width of 4 m along the river or along the shoreline of dams were scanned, following Sutherland's (2000) methodology. Adult individuals of both sexes (SVL > 60.0 mm, according to Bannikov et al., 1977) were used for the analyses. They were separated by sex, using secondary sexual characteristics: the presence of "marital corns" on the first finger and resonator bubbles in the corners of the mouth of the males (detailed physiographic maps with exact locations where frogs were caught are presented in Zhelev et al. (2013; 2015; 2016a; b; 2017b; 2018; 2020; 2021).

The studies in the period 2001-2017 are compliant and performed with basic data on the ecological status of each of the water bodies where frogs were caught; such data were obtained on the basis of physio-chemical analyzes of the water carried out on behalf of the Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, (Newsletters on the state of water in each studied waterbody–rivers and dams, in the period 2001-2017). Physio-chemical analyzes measure the values of priority substances for each of the water bodies; such values are determined according to the monitoring plans of the East Aegean River Basin Directorate / Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, in compliance with Water Framework Directive WED 2000/60/EO (EC, 2000), Ordinance No H-4/14.09.2012 (Ordinance, 2012) and No 256/1.11.2010 (Ordinance, 2010). Detailed data (physicochemical analysis of water) on the condition of each tested water body in the period 2001-2017 are presented in Zhelev et al. (2013; 2015; 2016a; b, 2017b; 2018; 2020; 2021).

After the catches in each of the studied habitats, in order to reduce stress, the frogs were transported to the laboratory in containers with water. The frogs were treated and the experiments were performed within 24 h of capture. The manipulations were carried out in compliance with ethical standards for working with animals (Fellers et al., 1994; Steven et al., 2004). The frogs were anesthetized with ether according to the procedure proposed by Stetter (2001). In a living condition, a 20 mm long heparinized needle was used to pierce the heart and draw up to 0.20 ml of blood according to the procedure proposed by Wright (2001). All haematological analyzes were performed according to standard clinical methods (Pavlov et al., 1980). Erythrtocyte count (RBC) and leucocyte count (WBC) were manually quantified using Hayem and Turck's solution and a Burker haemocytometer chamber. The haemoglobin concentration (Hb) was determined by the cyanmethaemoglobin spectrophotometric method and the packed cell volume (PCV), or (Hct value) by the microhaematocrit method. Leukograms (differential leukocyte counts) were performed in blood smear with Giemsa–Romanowsky staining. The derivative haematological parameters (MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, and MCV: mean corpuscular volume) were calculated by the Brown's formulas (1980). MCV was calculated by dividing haematocrit per liter of blood by total RBC count. The erythrocyte sizes were determined with an Olympus stereo microscope (SZX16, resolution 900 line pair/mm, Germany). We used the methodology proposed by Atatür et al. (1999) and Arserim & Mermer (2008) for nuclear erythrocytes: 40 randomly selected erythrocytes from each individual were measured with an ocular micrometer (MOB-1–15x). Four basic erythrocyte sizes were evaluated: cells length (EL) and width (EW) and nuclear length (NL) and width (NW). Cell and nuclear shapes were assessed with EL/EW and NL/NW ratios, and nucleus/cytoplasm with a NS/ES ratio. We used the formula: [ES = EL × EW × π / 4 and NS = NL × NW × π / 4] to calculate the erythrocyte sizes (ES μm^2) and their nuclear sizes (NS μm^2).

Statistical processing of the data was performed, using the STATISTICA 7.0 Software (Statistica, 2004) or R language (version 3.1.2, R Development Core Team, 2015). A Shapiro-Wilk test was used to evaluate the normal distribution of data, which proved to be statistically significant. For differences between individuals of *P. ridibundus* inhabiting habitats with different degrees and different types of pollution and those inhabiting relatively clean habitats (control groups) parametric Student's t-test for normally distributed independent samples and analysis of variance (one-way ANOVA) were applied, with subsequent verification of the statistical significance of the differences (depending on the homogeneity of the variances) by LSD, Tukey or Games-Howell *post-hoc* tests. Results with $p < 0.05$ [$\alpha = 5\%$] were considered significant. Data was given as Mean ± SEM and their confidence interval – 95% (or Minimum and Maximum values). Multivariate statistical analyses (a principal component analysis PCA and a standard discriminant analysis DA) were applied as an integral assessment of the health status of *P. ridibundus* from the investigated sites in Southern Bulgaria (for details see Zhelev et al., 2013; 2015; 2016a; b; 2017b; 2018; 2020; 2021).

Results and Discussion

The descriptive statistics and results from the comparisons by a one-way ANOVA analysis of the erythrogram and leukogram parameters in *P. ridibundus* individuals from investigated sites in Southern Bulgaria are presented in Table 1 and Table 2.

Table 1. The erythrogram parameters (Mean±SEM, Minimum-Maximum, or confidence interval – 95%) used for evaluation of health status of *P. ridibundus* individuals from freshwater ecosystems with different degree of anthropogenic pollution in southern Bulgaria. *Legend:* Sites: Site 1 (Sazliyka River near the village Rakitnitsa), site 2 (Sazliyka River near the town of Radnevo), site 3 (Topolnitsa River near the village Poibrene), site 4 (Studen Kladenets Dam Lake near the LZP "Kardzhali"), site 5 (Vacha River), site 6 (Tsalapitsa Rice Fields), site 7 (Vacha River) and site 8 (Chaya River). Abbreviations: Reference site (RS), Polluted site (PS), number of individuals (n), biological oxygen demand five days (BOD_5), nitrite nitrogen (NO^-_2-N), nitrate nitrogen (NO^-_3-N), ammonium nitrogen (NH^+_4-N), total nitrogen (TN), orthophosphates (PO^{3-}_4), copper (Cu), iron dissolved in water (Fe), lead (Pb), manganese (Mn), arsenic (As), cadmium (Cd), zinc (Zn), erythrocyte count (RBC), haemoglobin concentration (Hb), packed cell volume, or haematocrit value (PCV), mean corpuscular haemoglobin (MCH),: mean corpuscular haemoglobin concentration (MCHC),: mean corpuscular volume (MCV). Significance codes: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns $p > 0.05$.

Sites	Parameters						Publications
	RBC (x 10^6/µl)	Hb (g/dl)	PCV (L/l)	MCH (pg)	MCHC (g/l)	MCV (fl)	
Site 1(RS) (n=30, ♀+♂)	366.670±10.40 (270.000–490.000)	5.53±1.38 (4.57–7.10)	0.25±0.005 (0.21–0.31)	153.32±5.02 (105.17–220.17)	225.05±5.28 (149.77–298.45)	682.57±17.82 (517.44–937.50)	Zhelev et al. (2013; 2015; 2016a)
Site 2 (PS: BOD_5, NO^-_2-N, NO^-_3-N, TN, PO^{3-}_4) (n=30, ♀+♂)	482.670±12.12 (380.000–590.000	6.88±2.04 (4.87–8.64)	0.34±0.005 (0.30–0.40)	160.30±2.77 (129.02–186.25)	203.98±4.27 (152.41–245.12)	774.44±9.83 (670.00–931.25)	
Site 3 (PS:	629.670±26.84	8.28±1.02	0.37±0.006	146.08±5.09	221.18±3.74	650.91±20.82	

Cu, Fe, Pb, Mn, As) (n=30, ♀+♂)	(400.000–920.000)	(7.18–9.25)	(0.31–0.43)	(98.55–191.05)	(141.72–265.19)	(452.78–912.50)	
Site 4 (PS: Cd, Zn, Pb, Cu) (n=30, ♀+♂)	571.633±10.72 (480.000–690.000)	7.16±1.83 (5.21–8.97)	0.34±0.005 (0.30–0.41)	134.59±2.92 (110.04–172.85)	211.65±4.13 (157.97–254.53)	652.82±12.59 (540.50–806.25)	
Statistics (one way ANOVA, F, LSD tests)	F=8450.612; 1/4***; 2/4***; 3/4*	F=1.917; 1/4***; 2/4ns; 3/4***	F=3.107; 1/2***; 1/3***; 2/3***; 1/4***; 2/4ns; 3/4***	F=2.667; 1/2ns; 1/3ns; 2/3*; 1/4***; 2/4***; 3/4ns	F=2.525; 1/2***; 1/3ns; 2/3***; 1/4*; 2/4ns; 3/4ns	F=7.072; 1/2***; 1/3ns; 2/3***; 1/4ns; 2/4***; 3/4ns	
Site 5 (RS) Female (1) n=25 Male (2) n=25	♀: 610.720±25.89 (450.000–920.000) ♂: 616.400±22.13 (450.000–910.000)	♀: 7.82±1.98 (4.64–9.25) ♂: 7.61±1.99 (5.21–9.10)	♀: 0.35±0.01 (0.30–0.42) ♂: 0.35±0.01 (0.30–0.42)	♀: 144.37±5.93 (98.55–196.48) ♂: 138.03±6.36 (105.68–228.49)	♀: 218.62±4.83 (157.97–254.33) ♂: 208.05±4.57 (141.72–246.52)	♀: 644.42±20.87 (452.78–781.25) ♂: 656.33±16.43 (478.39–818.75)	Zhelev et al. (2018)
Site 6 (PS: fertilizers, pesticides) Female (3) n=25 Male (4) n=25	♀: 395.200±14.83 (270.000–600.000) ♂: 357.600±12.10 (270.000–510.000)	♀: 5.65±1.77 (4.43–7.16) ♂: 5.37±1.34 (4.49–7.24)	♀: 0.25±0.01 (0.22–0.31) ♂: 0.24±0.01 (0.21–0.30)	♀: 146.76±5.85 (107.41–198.97) ♂: 154.53±5.79 (105.17–220.70)	♀: 226.21±5.50 (156.49–298.45) ♂: 221.56±5.13 (149.77–261.12)	♀: 635.59±17.817 (517.44–810.34) ♂: 689.05±19.48 (511.31–937.50)	
Statistics (two way ANOVA, Tukey HSD)	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1/3ns; 1/4ns; 2/3ns; 2/4ns	1/2ns; 3/4ns; 1/3ns; 1/4ns; 2/3ns; 2/4ns	1/2ns; 3/4ns; 1/3ns; 1/4ns, 2/3ns; 2/4ns	
Site 7 (RS) Female (1) n=10 Male (2) n=10	♀: 543.900±23.21 (497.391–596.407 ♂: 659.000±43.47 (560.657–757.342)	♀: 8.02±1.59 (7.64–8.36) ♂: 7.92±2.73 (7.31–8.53)	♀: 0.35±0.01 (0.33–0.37) ♂: 0.36±0.01 (0.34–0.38)	♀: 160.28±7.99 (142.21–178.37) ♂: 142.86±12.56 (114.42–171.27)	♀: 219.14±9.38 (197.91–240.38) ♂: 216.25±4.92 (205.13–227.37)	♀: 712.46±23.34 (659.65–765.25) ♂: 647.66±31.13 (577.22–718.09)	
Site 8 (PS: NH^+_4-N, NO_3-N, NO_2-N, Pb, Cd, Zn, Cu, As) Female (3) n=10 Male (4) n=10	♀: 360.100±16.79 (321.977–398.003) ♂: 355.200±14.24 (322.786–387.213)	♀: 5.57±2.63 (4.97–6.17) ♂: 5.31±2.03 (4.84–5.76)	♀: 0.23±0.01 (0.21–0.24) ♂: 0.23±0.01 (0.22–0.25)	♀: 154.08±11.49 (128.08–180.09) ♂: 149.69±6.89 (134.12–165.29)	♀: 238.58±8.89 (218.46–258.71) ♂: 227.79±7.68 (210.41–245.18)	♀: 612.65±27.62 (550.17–675.14) ♂: 651.11±29.42 (584.53–717.67)	Zhelev et al. (2020)
Statistics (one way ANOVA, F, LSD tests)	F=30.27, 1<2***, 1>3***, 1>4***, 2>3***, 2>4***, 3/4$_{ns}$	F=40.69, 1/2$_{ns}$, 1>3***, 1>4***, 2>3***, 2>4***, 3/4$_{ns}$	F=81.47, 1/2$_{ns}$, 1>3***, 1>4***, 2>3***, 2>4***, 3/4$_{ns}$	F=0.54, 1/2$_{ns}$, 1/3$_{ns}$, 1/4$_{ns}$, 2/3$_{ns}$, 2/4$_{ns}$, 3/4$_{ns}$	F=1.61, 1/2$_{ns}$, 1/3$_{ns}$, 1/4$_{ns}$, 2/3$_{ns}$, 2/4$_{ns}$, 3/4$_{ns}$	F=2.19, 1/2$_{ns}$, 1>3**, 1/4$_{ns}$, 2/3$_{ns}$, 2/4$_{ns}$, 3/4$_{ns}$	

Table 2. The leukogram parameters (Mean±SEM, Minimum-Maximum, or confidence interval – 95%) used for evaluation of health status of *P. ridibundus* individuals from freshwater ecosystems with different degree of anthropogenic pollution in southern Bulgaria. *Legend:* Sites: Site 1 (Sazliyka River near the village Rakitnitsa), site 2 (Sazliyka River near the town Radnevo), site 3 (Topolnitsa River near the village Poibrene), site 4 (Dam Lake Studen Kladenets near the LZP "Kardzhali"), site 5 (Vacha River), site 6 (Tsalapitsa Rice Fields), site 7 (Vacha River) and site 8 (Chaya River). Abbreviations: Reference site (RS), Polluted site (PS), number of individuals (n), biological oxygen demand five days (BOD_5), nitrite nitrogen (NO^-_2-N), nitrate nitrogen (NO^-_3-N), ammonium nitrogen NH^+_4-N, total nitrogen (TN), orthophosphates (PO^{3-}_4), copper (Cu), iron dissolved in water (Fe), lead (Pb), manganese (Mn), arsenic (As), cadmium (Cd), zinc (Zn), leukocyte count (WBC), stab neutrophils (St), segmented nuclei neutrophils, total neutrophils (Ne), basophils (Ba), eosinophils (Eo), monocytes (Mo), lymphocytes (Ly). Significance codes: *p < 0.05; **p < 0.01; ***p < 0.001; ns p > 0.05.

Sites	Parameters							Publications
	WBC (x10^6/µl)	St	Sg	Ba	Eo	Mo	Ly	
Site 1(RS) (n=30,	2.396±0.08 (1.500–3.400)	2.40±0.23 (1–6)	7.20±0.29 (4–10)	2.13±0.18 (1–4)	1.83±0.14 (1–3)	3.40±0.35 (1–9)	83.04±0.80 (72–90)	Zhelev et al. (2013; 2015;

♀+♂)								2016a)
Site 2 (PS: BOD_5, NO^-_2-N, NO_3-N, TN, PO^{3-}_4) (n=30, ♀+♂)	3.400±0.09 (2.600–4.400)	3.03±0.30 (1–8)	11.10±0.38 (5–15)	5.60±0.52 (2–10)	4.70±0.53 (1–11)	7.17±0.49 (3–13)	68.40±1.03 (57–78)	
Site 3 (PS: Cu, Fe, Pb, Mn, As) (n=30, ♀+♂)	4.123±0.09 (3.100–4.850)	4.80±0.26 (3–8)	3.46±0.29 (1–7)	0.77±0.08 (0–3)	0.37±0.10 (0–2)	12.30±0.62 (6–19)	78.30±0.74 (72–87)	
Site 4 (PS: Cd, Zn, Pb, Cu) (n=30, ♀+♂)	3.647±0.06 (3.200–4.300)	3.43±0.21 (2–6)	13.47±0.32 (10–17)	2.57±0.17 (2–6)	0.87±0.16 (0–3)	9.67±0.44 (5–16)	70.00±0.60 (65–75)	
Statistics (one way ANOVA, F, LSD tests)	F=48.9989, 1/4***; 2/4ns; 3/4***	F=73.3599, 1/4**; 2/4ns; 3/4***	F=92.3850, 1/4***; 2/4***; 3/4***	F=7.1650, 1/4ns; 2/4***; 3/4***	F=4.6820, 1/4*; 2/4***; 3/4ns	F=13.3772, 1/4***; 2/4***; 3/4***	F=78.2500, 1/4***; 2/4ns; 3/4***	
Site 5 (RS) Female (1) n = 25 Male (2) n=25	♀: 4.028±0.91 (3.200–4.800) ♂: 3.972±0.96 (3.200–4.900)	♀: 2.60±0.21 (1–6) ♂:2.20±0.15 (1–4)	♀: 7.68±0.23 (6–10) ♂:6.48±0.23 (5–9)	♀: 4.24±0.38 (1–6) ♂:4.20±0.26 (1–6)	♀:1.16±0.20 (0–3) ♂: 1.08±0.1 (0–3)	♀: 3.84±0.31 (1–9) ♂: 3.92±0.18 (2–5)	♀: 80.48±0.69 (71–88) ♂: 80.48±0.69 (71–88)	
Site 6 (PS: fertilizers, pesticides) Female (3) n = 25 Male (4) n = 25	♀: 2.332±0.81 (1.600–3.400) ♂: 2.340±0.10 (1.600–3.300)	♀: 5.16±0.19 (4–8) ♂:4.96±0.24 (3–8)	♀: 3.12±0.10 (2–4) ♂:3.44±0.23 (2–6)	♀: 1.04±0.18 (0–3) ♂:1.32±0.19 (0–3)	♀: 4.72±0.32 (0–6) ♂:4.72±0.32 (0–6)	♀: 14.80±0.36 (12–19) ♂: 12.80±0.44 (9–16)	♀: 71.16±0.49 (67–76) ♂: 72.28±0.49 (68–77)	Zhelev et al. (2018)
Statistics (two way ANOVA, Tukey HSD)	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1<3**, 1<4***; 2<3***; 2<4***	1>2***; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1<3***; 1<4***; 2<3***; 2<4***	1/2ns; 3>4***; 1<3***; 1<4***; 2<3***; 2<4***	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	
Site 7 (RS) Female (1) n = 10 Male (2) n = 10	♀: 4.680±1.23 (4.400–4.959) ♂: 4.290±1.25 (4.006–4.573)	Ne ♀: 9.10±0.31 (8.39–9.81) ♂: 9.41±0.34 (8.63–10.17)		♀: 4.50±0.56 (3.23–5.77) ♂: 3.20±0.29 (2.54–3.85)	♀: 1.0±0.33 (0.24–1.75) ♂: 0.70±0.26 (0.11–1.29)	♀: 3.60±0.41 (2.69–4.51) ♂: 4.50±0.22 (3.99–5.01)	♀: 81.80±0.80 (79.99–83.61) ♂: 82.20±0.51 (81.04–83.34)	
Site 8 (PS: NH^+_4-N, NO^-_3-N, NO_2-N, Pb, Cd, Zn, Cu, As) Female (3) n = 10 Male (4) n = 10	♀: 2.130±0.63 (1.986–2.273) ♂: 2.128±1.29 (1.837–2.422)	Ne ♀: 19.60±0.42 (18.57–20.62) ♂: 20.21±0.51 (19.04–21.35)		♀: 0.70±0.21 (0.22–1.18) ♂: .50±0.17 (0.12–0.89)	♀: 4.40±0.39 (3.63–5.17) ♂: .50±0.27 (4.89–6.11)	♀: 11.80±0.42 (10.86–12.75) ♂: 11.30±0.36 (10.47–12.13)	♀: 63.50±0.65 (62.02–64.97) ♂: 62.50±0.73 (60.84–64.16)	Zhelev et al. (2020)
Statistics (one way ANOVA, F, LSD tests)	F=30.27, 1<2***, 1>3***, 1>4***, 2>3***, 2>4***, 3/4ns	F=145.18, 1>2**, 1>3***, 1>4***, 2>3***, 2>4***, 3/4 ns		F=32.11, 1/2ns, 1>3***, 1>4***, 2>3***, 2>4***, 3/4ns	F=63.49, 1/2ns, 1<3***, 1<4***, 2<3***, 2<4***, 3/4ns	F=146.21, 1/2ns, 1<3***, 1<4***, 2<3***, 2<4***, 3/4ns	F=257.96, 1/2ns, 1>3***, 1>4***, 2>3***, 2>4***, 3/4ns	

In the study performed in the Sazliyka and Topolnitsa Rivers as well as of Studen Kladenets Dam Lake, the analyses were made with pooled samples – 30 individuals of both sexes from each population. In this study, the data obtained found the following statistically significant changes in the blood of frogs from anthropogenically contaminated sites compared to these inhabiting less disrupted habitats (site 1: reference group).

• in population 2 (site 2: domestic sewage pollution and nutrients) - erythrocytosis, hypohromia, leukocytosis, neutrophilia (with increasing Sg cells), basophilia, eosinophilia, monocytosis, lymphopenia.

• in population 3 (site 3: heavy metal pollution) - erythrocytosis, hyperchromia, leukocytosis, neutropenia, monocytosis, basopenia, eosinopenia, lymphopenia.

• in population 4 (site 4: heavy metal pollution) - erythrocytosis, hyperchromia, leukocytosis, neutrophilia (increasing St and Sg cells), monocytosis, eozinopeniya and lymphopenia.

Analyzing PCV (Hct) and the three derivative parameters: MCH, MCHC and MCV, we found increasing PCV values in each population that inhabit conditions of anthropogenic pollution, decreasing MCH values (in population 4) and MCHC (in populations 2 and 4) and an increase of MCV in population 2.

Since establishing reference values for haematological parameters in heterothermic animals is a difficult task, comparing the literature data is the only way to assess the fluctuations in their values and their dependence on environmental factors and anthropogenic pollutants. The most common cause of erythrocytosis in conditions of prolonged living in anthropogenic pollution is tissue hypoxia (Thrall et al., 2004; Allender & Fry, 2008). The increase in erythrocyte count leads to an increase in the oxygen capacity of blood and is considered a compensatory reaction to the general decrease of oxygen – hypoxemia (Randall, 1982; Randall & Perry, 1992; Lai et al., 2006). There is evidence in literature that a short stay of *P. ridibundus* in polluted industrial wastewater reduces the number of erythrocytes and the amount of haemoglobin (Romanova & Egorikhina, 2006; Vafis & Peskova, 2009). However, in long-term habitation of the species of *Rana (Pelophylax)* genus (*P. ridibundus* in particular) in an environment with persistent toxicants, the opposite effects are observed – increase of RBC counts and Hb values (Tarasenko & Tarasenko, 1988; Peskova & Zhukova, 2005; Sedalishtev, 2005; Toktamysova, 2005). In some anuran amphibians there is suppression of erythropoiesis as a result of the accumulation of heavy metals in the liver (Egorov et al., 2002; Akulenko, 2005; Chiesa et al., 2006). The results in our study for the population of *P. ridibundus* inhabiting Topolnitsa River (site 3), where pollutants are mainly heavy metals, however, show the opposite direction of change – erythrocytosis in combination with hyperchromia, a reaction that is adaptive rather than pathological. There is scarce and unsystematized data in literature on the status of the indicators PCV (Ht), MCH, MCHC and MCV in Anura, and most of the works is aimed at establishing reference values for different species (Arserim & Mermer, 2008; Gül et al., 2011; Mahapatra et al., 2012). It should be noted, however, that no environmental factor values were monitored in these works. Data on changes in PCV and the three derivative indicators in amphibian populations inhabiting conditions of anthropogenic pollution are scarce. In our previous paper (Zhelev et al., 2005) we found a statistically significant increase of PCV in populations of *P. ridibundus* inhabiting an area with well developed energy industry – TPP "Maritsa Iztok - 1" near Galabovo Town and we did not report differences in the values of the indicators MCH, MCHC and MCV in comparison with the reference group from the area of Harmanli Town (relatively clean biotope). At the same time, in the populations of *P. ridibundus* inhabiting another anthropogenically polluted region – the town of Dimitrovgrad, in the area of the chemical plant "Neochim Inc." we found a statistically significant decrease in the values of all four indicators (PCV, MCH, MCHC and MCV) in comparison with the animals from the control group. The analysis of differential leukocyte counts in *P. ridibundus* populations from four sites in Southern Bulgaria, shows that regardless of the living environment parameters in the four habitats the blood of frogs has a lymphoid character, but the quantity of the largest cell group is with highest statistical significance in the relatively non-polluted habitat (site 1) and lowest in the population from site 2 (Table 2). The found significant lymphopenia in the populations of anthropogenically polluted habitats is present on the background of statistically significant changes in the numerical ratio of all other mature white blood cells in the peripheral blood of the animals. This shows the general mobilization of the body's defenses. The changes in the differential leukocyte count of animal blood from population 2 (site 2) were referred to the so-called first type of reaction (adaptive), according to the classification proposed by Peskova (2001). Similar changes in the differential leukocyte count in Anura, in the presence of toxins of anthropogenic origin, have been found by other authors as well (Romanova & Romanova, 2003; Cabagna et al., 2005; Romanova & Egorikhina, 2006; Peskova & Sharpan, 2007; Peskova & Vafis, 2007; Zhelev, 2007; Mineeva & Mineev, 2011; Shutler & Marcogliese, 2011). The changes in the differential leukocyte count in population 3 (site 3) were referred to the second type of reaction by the terminology of Peskova (2001), according to which these changes are a result of serious violations in the physiological functions of the body (even these of pathological nature) under the influence of toxins. Similar changes in the differential formula in Anura, inhabiting conditions of toxicosis have also been reported by (Isaeva & Vyazov, 1997; Zhykova & Peskova, 1999; Cabagna et al., 2005; Davis et al. 2008). The changes in the leukogram in *P. ridibundus* individuals that inhabit in site 4 (Studen Kladenets Dam Lake) rather, they could be attributed to the so-called first type of reaction according to Pescova's (2001) classification. The results from the discriminant analysis (see Fig. 1) group together populations 2 and 4 and at the same time clearly separate them not only from population 1 but also from population 3. This is undoubtedly an interesting fact considering the nature of the toxicants present in Studen Kladenets Dam Lake – heavy metals (for details see Zhelev et al., 2015). The same type of pollutants is present in the habitat inhabited by population 3 (for details see Zhelev et al., 2013). The conclusion to be drawn is that with the same nature of toxicants, the disturbances in the physiological condition of the animals from the respective populations of *P. ridibundus* are different – in the Topolnitsa River they are more serious. The reason for the reported differences in the state of *P. ridibundus* populations can be traced to the type of water body and the concentration of toxicants. In closed water bodies (such as Studen Kladenets Dam Lake) the lack of currents and larger sizes create conditions for the accumulation of heavy metals in the bottom sediments, while in shallower and faster rivers this is not always the case. It is quite possible that the changes in the blood of *P. ridibundus* from the population inhabiting the Topolnitsa River (population 3) were preceded by changes similar to those found in the populations of the anthropogenically transformed habitats of the Sazliyka River (population 2) and Studen Kladenets Dam Lake (population 4). This makes it extremely important to establish a thin borderline between the changes characterizing the unique "battle phase" in the blood of amphibians, expressed in the mobilization of the body's defenses, and the threshold of toxicants leading to the predominance of degenerative changes of pathological nature. Of course, this task is complicated by the fact that in natural reservoirs of different types, along with the action of the anthropogenic factor, the effect of the combination of a large number of environmental factors must be taken into account. Such a direction outlines prospects for further research not only of fundamental, but also of strictly applied nature for the needs of bioindication and biomonitoring.

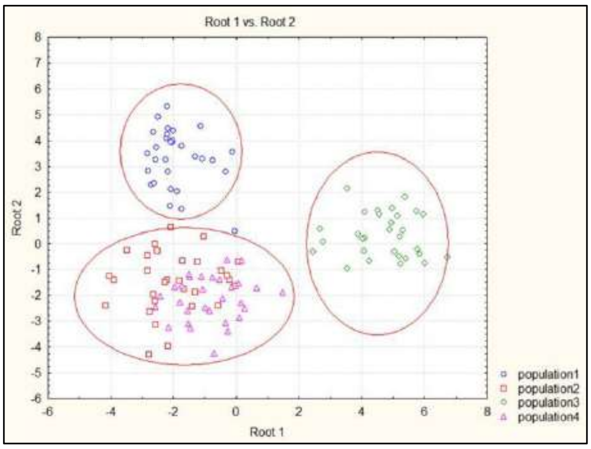

Fig. 1. Discriminatory coordinates by six most informative parameters (RBC, PCV, WBC, Sg, Ba, and Eo) of individuals from the four compared *Pelophylax ridibundus* populations from the sites with different levels of anthropogenic pollution in Southern Bulgaria. This diagram is published in Zhelev et al. (2015).

Studies conducted in anthropogenically polluted habitats of Tsalapitsa Rice Fields (pesticides and fertilizers) and the Chaya River (heavy metals), were carried out taking into account the sex of the frogs. The values of the studied haematological markers were compared with those obtained for the respective number of individuals of both sexes inhabiting the reference site (the Vacha River). Significant differences in the values of tested haematological markers were established in the blood of the frogs (in individuals of both sexes) inhabiting the Tsalapitsa Rice Fields compared to such values in the blood of frogs from the reference site. In our view, the erythropenia, leucopenia, hypochromia, lower values of PCV, St-neutrophilia, Sg-neutropenia, basopenia, eosinophilia, monocytosis and lymphopenia that were found in *Pelophylax ridibundus* individuals inhabiting the Tsalapitsa Rice Fields were probably caused by the pesticides and fertilizers that enter the paddy cages during the rice production process. These results are an indication of severely deteriorated health status of frogs inhabiting in the Tsalapitsa Rice Fields. This conclusion is also confirmed by the results of the discriminant analysis (Fig. 2). The spatial distribution of factor loadings separates in two clearly differentiated "clouds": the individuals of *P. ridibundus* from Tsalapitsa Rice Fields and also these from the Vacha River (for details see Zhelev et al., 2018).

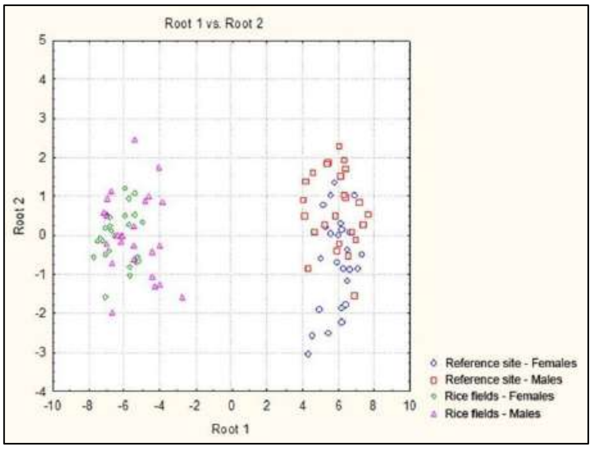

Fig. 2. Plot (discriminatory coordinates for haematological parameters of the importance of canonical discriminant function of the *Pelophylax ridibundus* individuals from the investigated sites (Tsalapitsa Rice Fields – polluted site and the Vacha River – reference site). Root 1 – the first canonical function Root 2 – the second canonical function. This diagram is published in Zhelev et al. (2018).

The studies of the populations' of *P. ridibundus*, inhabiting the industrial zone of the city of Plovdiv City and the Chaya River which flows through it, were conducted with a smaller number of test animals (10 individuals of both sexes). This number was consistent with analyzes for the bioaccumulation of heavy metals in the tissues of these frogs (muscle and liver). The values of the tested haematological markers were compared to these obtained for the same number of individuals inhabiting in the reference site: the Vacha River (see Tables 1 and 2). In addition to haematological parameters, a set of other morphophysiological markers were used in this study, namely: body condition index and organ indexes: hepatosomatic index, spleenosomatic index and renosomatic index.

The general adaptation syndrome theory (Selye, 1956), considers the physiological response of each organism to stress as a process involving three stages: During the first stage, the body's defenses are mobilized (alarm stage). The second stage is characterized by general adaptation of the body's defenses and developing mechanisms in response to stressors (resistance stage). In the third stage, as a result of the chronic action of toxicants (or an increase in their concentration over time), the adaptive potential of the organism is depleted, its energy resources are reduced and death occurs (exhaustion stage). The results of this research showed that the exhaustion stage was reached for the frogs inhabiting an area of industrial pollution in Southern Bulgaria, and an impossibility to cope with the force and enduring action of the toxicants present in the environment was observed as well. Low body weight combined with values of the body condition index is observed in the frogs of both sexes inhabiting the Chaya River and they are anemic (erythropenia and hypochromia), their immunity is weakened (leucopenia, neutrophilia, monocytosis, lymphopenia and lower values of the spleenosomatic index) and the functions of their livers and kidneys are deteriorated (for details see Zhelev et al., 2020). The results of the discriminant analysis clearly differentiate and separate the *P. ridibundus* individuals in the Chaya River from these inhabiting the reference site (see Fig. 3).

Variations in the size and shape of erythrocytes (and their nuclei) in anurans have been very poorly studied. Single studies have focused on changes in erythrocyte morphology in anurans populations inhabiting conditions of anthropogenic stress (Zhelev et al., 2006; Omelykovets & Berezyuk, 2009; Bondarieva et al., 2012; Salinas et al., 2017; Dönmez & Şişman, 2021).

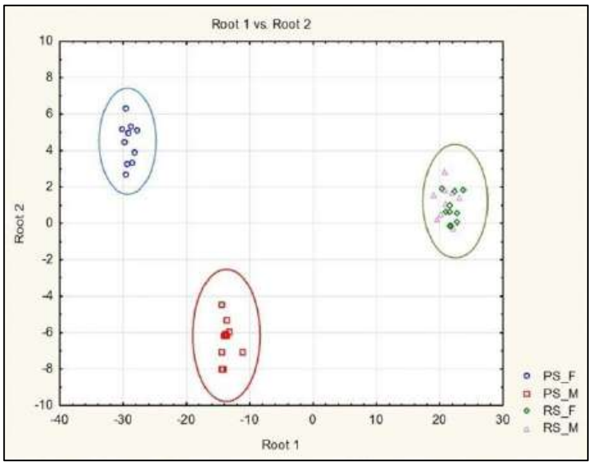

Fig. 3. Canonical discriminant functions scatter plot of tested biological parameters of *Pelophylax ridibundus* specimens from the sites studied. This diagram is published in Zhelev *et al.* (2020). *Legend:* Polluted siete (PS): the Chaya River, Reference site (RS): the Vacha River, F: females, M: males.

Although the authors of those works report changes in the size and shape of erythrocytes in anurans from polluted ecosystems, when comparing them to populations of frogs inhabiting background territories, there are no categorical opinions regarding the causes of the observed changes or their biological role.

Tables 3 and 4 present the results of our research, conducted in *P. ridibundus* populations that inhabit five water bodies in Southern Bulgaria (2 rivers and 3 reservoirs) with different degrees and types of anthropogenic pollution (less disrupted water bodies, domestic sewage pollution and heavy metal pollution).

Table 3. Erythrocyte cells measurements (Mean ± SEM values and their confidence interval – 95%) in *Pelophylax ridibundus* individuals from investigated sites in Southern Bulgaria and results from their comparisons. The signs > and <are used to compare the mean values of the parameters. *Legend*: Sites: Site 1 (Sazliyka River below the village of Rakitnitsa), site 2 (Sazliyka River below the town of Radnevo), site 3 (Topolnitsa River below the village of Chavdar), site 4 (Topolnitsa River below the village of Poibrene), site 5 (Vacha dam lake), site 6 (Rozov Kladenets dam lake), site 7 (Studen Kladenets dam lake), site 8 (Vacha River), site 9 (Chaya River). Abbreviations: Reference site (RS), Polluted site (PS), number of individuals (n), biological oxygen demand five days (BOD_5), nitrite nitrogen (NO^-_2-N), nitrate nitrogen (NO^-_3-N), ammonium nitrogen (NH^+_4-N), total nitrogen (TN), orthophosphates (PO^{3-}_4), copper (Cu), iron dissolved in water (Fe), lead (Pb), manganese (Mn), arsenic (As), cadmium (Cd), zinc (Zn), erythrocyte length (EL), erythrocyte width (EW), erythrocyte size (ES). Significance codes: *p < 0.05; **p < 0.01; ***p < 0.001; ns p > 0.05.

Sites	**Parameters**				**Publications**
	EL (μm)	**EW (μm)**	**EL/EW ratio**	**ES (μm²)**	
Site 1 (RS) (n=15, ♀+♂)	24.01±0.07 (23.87–24.15)	13.90±0.05 (13.79–14.00)	1.73±0.01 (1.72–1.74)	263.21±1.75 (259.78–266.63)	
Site 2 (PS: BOD_5, NO^-_2-N, NO^-_3-N, TN, PO^{3-}_4) (n=15, ♀+♂)	24.98±0.08 (24.81–25.14)	14.50±0.07 (14.37–14.63)	1.73±0.01 (1.72–1.74)	286.57±2.32 (282.00–291.13)	
Site 3 (PS: Cu, Fe, Pb, Mn, As) (n=15, ♀+♂)	22.61±0.05 (22.51–22.71)	13.76±0.03 (13.69–13.82	1.64±0.01 (1.63–1.65)	244.63±1.02 (242.63–246.63)	
Site 4 (PS: Cu, Fe, Pb, Mn, As) (n=15, ♀+♂)	22.28±0.06 (22.15–22.41)	13.65±0.05 (13.55–13.74)	1.63±0.01 (1.63–1.64)	239.79±1.49 (236.87–242.72)	
Site 5 (RS) (n=15, ♀+♂)	24.45±0.09 (24.27–24.62)	14.15±0.06 (14.03–14.27)	1.73±0.01 (1.72–1.74)	273.71±2.24 (269.32–278.11)	Zhelev et al. (2017b)
Site 6 (PS: BOD_5, NO^-_2-N, NO^-_3-N, TN, PO^{3-}_4) (n=15, ♀+♂)	24.79±0.08 (24.63–24.95)	14.32±0.06 (14.20–14.44)	1.74±0.01 (1.73–1.75)	280.81±2.19 (276.51–285.12)	
Site 7 (PS: Cd, Zn, Pb, Cu) (n=15, ♀+♂)	22.50±0.06 (22.39–22.61)	13.59±0.05 (13.49–13.67)	1.66±0.01 (1.65–1.67)	240.98±1.35 (238.33–243.63)	
Statistics (one way ANOVA, F, LSD tests)	F=254.322, (1.2 = 4.1) > 1.1 > (2.1 = 5.1) > 2.2, (1.2 = 4.1) > 3.1 > (2.1 = 5.1) > 2.2, 1.2 > (2.1 = 5.1) > 2.2, 4.1 > (2.1 = 5.1) > 2.2	$F_{6, 4193}$ =42.262, 1.2 > 4.1 > 1.1 > (2.1 = 2.2) > 5.1, 1.2 > 4.1 > 3.1 > (2.1 = 2.2) > 5.1, 1.2 > (2.1 = 2.2) > 5.1, 4.1 > (2.1 = 2.2) > 5.1	F=136.312, (1.1 = 1.2 = 4.1) > 5.1 > (2.1 = 2.2), (3.1 = 1.2 = 4.1) > 5.1 > (2.1 = 2.2), 1.2 > 5.1 > (2.1 = 2.2), 4.1 > 5.1 > (2.1 = 2.2)	F=116.513, 1.2 > 4.1 > 1.1 > (2.1 = 2.2 = 5.1), 1.2 > 4.1 > 3.1 > (2.1 = 2.2 = 5.1), 1.2 > (2.1 = 2.2 = 5.1), 4.1 > (2.1 = 2.2 = 5.1)	
Site 8 (RS) Female (1) n = 10 Male (2) n = 10	♀: 24.35±0.09 (24.15–24.55) ♂: 24.23±0.10 (24.03–24.42)	♀: 14.15±0.07 (14.01–14.29) ♂: 14.14±0.07 (13.99–14.29)	♀: 1.73±0.01 (1.71–1.74) ♂: 1.72±0.01 (1.71–1.73)	♀: 272.22±2.46 (267.39–277.05) ♂: 270.89±2.59 (265.78–275.99)	
Site 9 (PS: NH^+_4-N, NO_3-N, NO_2-N, Pb, Cd, Zn, Cu, As) Female (3) n = 10 Male (4) n = 10	♀: 22.47±0.06 (22.35–22.59) ♂: 22.54±0.07 (22.40–22.67)	♀: 13.52±0.04 (13.52–13.69) ♂: 13.65±0.05 (13.55–13.75)	♀: 1.65±0.01 (1.64–1.66) ♂: 1.65±0.01 (1.64–1.66)	♀: 240.56±1.29 (238.02–243.09) ♂: 242.31±1.49 (239.37–245.25)	Zhelev et al. (2021)
Statistics (one way ANOVA, F, LSD tests)	F=148.091, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	F=24.074, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	F=65.079, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	F=72.719, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	

Table 4. Erythrocyte nuclei measurements (Means±SEM and their Confidence – 95%) in *Pelophylax ridibundus* individuals from investigated sites in Southern Bulgaria and results from their comparisons. The signs > and <are used to compare the mean values of the parameters. *Legend:* Sites: Site 1 (Sazliyka River below the village of Rakitnitsa), site 2 (Sazliyka River below the town of Radnevo), site 3 (Topolnitsa River below the village of Chavdar), site 4 (Topolnitsa River below the village of Poibrene), site 5 (Vacha dam lake), site 6 (Rozov Kladenets Dam Lake), site 7 (Studen Kladenets Dam Lake), site 8 (Vacha River), site 9 (Chaya River). Abbreviations: Reference site (RS), Polluted site (PS), number of individuals (n), biological oxygen demand five days (BOD_5), nitrite nitrogen (NO^-_2-N), nitrate nitrogen (NO^-_3-N), ammonium nitrogen (NH^+_4-N), total nitrogen (TN), orthophosphates (PO^{3-}_4), copper (Cu), iron dissolved in water (Fe), lead (Pb), manganese (Mn), arsenic (As), cadmium (Cd), zinc (Zn), nucleus length (NL), nucleus width (NW), nucleus size (NS). Significance codes: *p < 0.05; **p < 0.01; ***p < 0.001; ns p > 0.05.

Sites	Parameters					Publications
	NL (μm)	NW (μm)	NL/NW ratio	NS (μm^2)	NS/ES ratio (μm^2)	
Site 1 (RS) (n=15, ♀+♂)	9.67±0.05 (9.56–9.77)	5.65±0.03 (5.58–5.71)	1.73±0.01 (1.71–1.74)	43.39±0.44 (42.53–44.24)	0.17±0.002 (0.16–0.17)	Zhelev et al. (2017b)
Site 2 (PS: BOD_5, NO^-_2-N, NO^-_3-N, TN, PO^{3-}_4) (n=15, ♀+♂)	9.78±0.05 (9.68–9.89)	5.64±0.03 (5.59–5.70)	1.74±0.01 (1.73–1.76)	43.73±0.40 (42.94–44.51)	0.16±0.002 (0.15–0.16)	
Site 3 (PS: Cu, Fe, Pb, Mn, As) (n=15, ♀+♂)	8.08±0.05 (7.99–8.17)	5.14±0.03 (5.09–5.19)	1.58±0.01 (1.56–1.59)	33.01±0.34 (32.35–33.67)	0.13±0.001 (0.13–0.14)	
Site 4 (PS: Cu, Fe, Pb, Mn, As) (n=15, ♀+♂)	8.28±0.05 (8.18–8.37)	5.20±0.03 (5.15–5.27)	1.60±0.01 (1.58–1.61)	34.22±0.34 (33.54–34.90)	0.14±0.001 (0.14–0.15)	
Site 5 (RS) (n=15, ♀+♂)	9.62±0.05 (9.53–9.72)	5.39±0.03 (5.33–5.44)	1.80±0.01 (1.78–1.82)	41.07±0.37 (40.35–41.79)	0.15±0.001 (0.15–0.16)	
Site 6 (PS: BOD_5, NO^-_2-N, NO^-_3-N, TN, PO^{3-}_4) (n=15, ♀+♂)	9.79±0.05 (9.70–9.88)	5.53±0.03 (5.47–5.58)	1.79±0.01 (1.77–1.81)	42.71±0.34 (42.04–43.39)	0.16±0.001 (0.15–0.16)	
Site 7 (PS: Cd, Zn, Pb, Cu) (n=15, ♀+♂)	8.43±0.05 (8.34–8.53)	5.31±0.03 (5.26–5.36)	1.59±0.01 (1.58–1.61)	35.58±0.35 (34.89–36.27)	0.14±0.001 (0.14–0.15)	
Statistics (one way ANOVA, F, LSD tests)	F=252.757, (1.1 = 1.2 = 4.1) > 5.1 > 2.2 > 2.1, (1.2 = 4.1) > 3.1 > 5.1 > 2.2 > 2.1, 1.2 > 5.1 > 2.2 > 2.1, 4.1 > 5.1 > 2.2 > 2.1	F=54.115, (1.1 = 1.2) > 4.1 > 5. 1 > (2.1 = 2.2), 1.2 > 4.1 > 3.1 > 5. 1 > (2.1 = 2.2), 1.2 > 5. 1 > (2.1 = 2.2), 4.1 > 5.1 > (2.1 = 2.2)	F=145.099, 4.1 > (1.1 = 1.2) > (2.1 = 2.2 = 5.1), (3.1 = 4.1) > 1.2 > (2.1 = 2.2 = 5.1), 1.2 > (2.1 = 2.2 = 5.1), 4.1 > (2.1 = 2.2 = 5.1)	F=158.168, (1.1 = 1.2 = 4.1) > 5.1 > 2.2 > 2.1, (1.2 = 4.1) > 3.1 > 5.1 > 2.2 > 2.1, 1.2 > 5.1 > 2.2 > 2.1, 4.1 > 5.1 > 2.2 > 2.1	F=38.608, 1.1 > (1.2 = 1.4) > (2.2 = 5.1) > 2.1, (1.2 = 4.1) > 3.1 > (2.2 = 5.1) > 2.1, 1.2 > (2.2 = 5.1) > 2.1, 4.1 > (2 = 5.1) > 2.1	
Site 8 (RS) Female (1) n=10 Male (2) n = 10	♀: 9.58±0.06 (9.44–9.71) ♂: 9.65±0.06 (9.53–9.78)	♀: 5.49±0.04 (5.41–5.57) ♂: 5.54±0.04 (5.47–5.62)	♀: 1.76±0.01 (1.73–1.78) ♂: 1.75±0.01 (1.73–1.77)	♀: 41.76±0.51 (40.75–42.78) ♂: 42.51±0.51 (41.51–43.52)	♀: 0.16±0.002 (0.15–0.17) ♂: 0.16±0.002 (0.15–0.17)	Zhelev et al. (2021)
Site 9 (PS: NH^+_4-N, NO_3-N, NO_2-N, Pb, Cd, Zn, Cu, As) Female (3) n=10 Male (4) n=10	♀: 8.14±0.05 (8.02–8.25) ♂: 8.48±0.06 (8.35–8.61)	♀: 5.20±0.03 (5.14–5.26) ♂: 5.35±0.03 (5.28–5.41)	♀: 1.57±0.01 (1.55–1.59) ♂: 1.59±0.01 (1.57–1.60)	♀: 33.64±0.41 (32.82–34.45) ♂: 36.13±0.45 (35.23–37.02)	♀: 0.14±0.001 (0.13–0.15) ♂: 0.15±0.002 (0.14–0.16)	
Statistics (one way ANOVA, F, LSD tests)	F=148.181, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3<4^{***}$	F=18.807, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3<4^{**}$	F=114.870, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	F=82.438, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3<4^{***}$	F=18.219, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3<4^{**}$	

The analysis of the data showed that the changes in metric parameters of erythrocytes depend on the concentration of toxic substances and their type, and to a lesser extent show dependence on the type of water body. We found a statistically significant increase in the parameters: erythrocyte length (EL), erythrocyte width (EW) and erythrocyte size (ES) in populations of *P. ridibundus* inhabiting water bodies with domestic sewage pollution, compared to those of control groups from reference sites. This leads to a change in the shape of erythrocyte cells in these frogs which takes an elongated-elliptical shape (see Fig. 4). At the same time, in these populations of *P. ridibundus* the basic nuclear parameters (NL: nucleus length; NW: nucleus width and NS: nucleus size) are unchanged compared to the control groups. The nuclei retain their typical elliptical shape. In *P. ridibundus* populations inhabiting water bodies contaminated with heavy metals, the parameters EL, EW, ES, NL, NW and NS decrease compared to the control groups. The cells and nuclei were rounded (oval or spherical shape). In the populations of anthropogenically polluted water bodies the NS / ES decreases in comparison with the control groups, regardless of the type of toxicants (for details see Zhelev et al., 2018).

Our studies in the populations of *P. ridibundus* inhabiting habitats (sites) 1, 2 and 3 (see Tables 3 and 4) were continued in a seasonal aspect: analyses were performed in these habitats not only in the spring but also in the summer and autumn. The results of these studies are published in Zhelev et al. (2016b). Although some fluctuations in the metric parameters of erythrocyte cells and their nuclei were observed in the populations of *P. ridibundus* from the two anthropogenically contaminated habitats (sites 2 and 3), the changes observed in the spring persisted during the other two seasons. In the population from the habitat with domestic sewage pollution (site 2) the cells had a typically elliptical shape, but the ellipse became a little more elongated in summer and autumn.

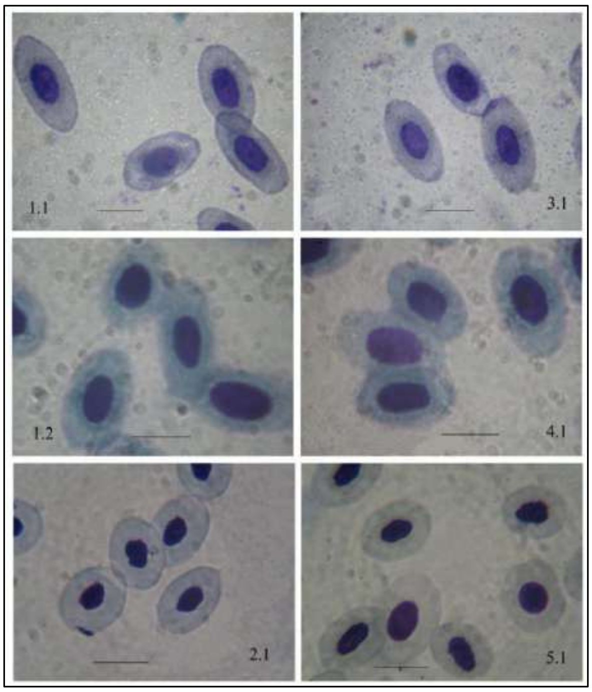

Fig. 4. Photomicrographs of erythrocytes of *Pelophylax ridibundus* populations from the investigated water bodies (*rivers and **reservoirs) in Southern Bulgaria: (1.1*, 3.1**) - less disrupted, (1.2*, 4.1**) - domestic sewage polluted and (2.1*, 5.1**) - heavy metal polluted water basins. Scale lines = 10 µm. Legend: 1.1 – the river Sazliyka below the village of Rakitnitsa, 1.2 – the river Sazliyka below the town of Radnevo, 2.1 – the river Topolnitsa below the village of Chavdar, 2.2 – the river Topolnitsa below the village of Poibrene, 3.1 – the Vacha reservoir, 4.1 – the Rozov Kladenets reservoir and 5.1 – the Studen Kladenets reservoir. This photos is published in Zhelev et al. (2018).

The nuclei had a typical oval shape, but some elongation of the ellipse also occurred (NW values were close to those from the less disrupted one, NL/NW became significantly higher in the population during summer and autumn). Throughout spring to autumn, NS/ES decreased in comparison with the less disrupted group (site 1). In the population from the habitat with heavy metal pollution (site 2), the values of all 9 cellular and nuclear parameters were the lowest throughout the investigation. EL, EW, NL, NW and ES experienced a progressive and statistically significant decrease during the seasonal transitions. EL/EW significantly increased throughout summer and showed a statistically significant decrease again in autumn. NL/NW experienced no changes in spring and declined in autumn. NS/ES reached its peak in spring, and significantly decreased during the course of the other two seasons. The shape of cells and nuclei became slightly more rounded; this was most pronounced during spring (for details see Zhelev et al., 2016b). The changes in the sizes of erythrocyte cells and their nuclei, found in the populations of *P. ridibundus* inhabiting the anthropogenically polluted sites (2 and 3), were confirmed in the study conducted in the populations of *P. ridibundus,* inhabiting the industrial zone of Plovdiv City (see Tables 3 and 4). In the blood of frogs from the anthropogenically polluted habitat of Chaya River (heavy metals, nitrates, nitrites and ammonium), oval and spherical erythrocytes circulate. Also, a significant reduction was found in the nucleus / cytoplasm ratio of the blood of these frogs (for details see Zhelev et al., 2021). The results from the PCA analysis confirmed that the differences between frogs from the polluted site (the Chaya River) and the reference site (the Vacha River) are mainly due to changes affecting the two main parameters of erythrocyte cells and their nuclei (length and width), and also to changes in nucleus sizes and nucleocytoplasmic ratio (Fig. 5).

We believe that the changes in the morphology of erythrocyte cells and their nuclei, found in the populations of *P. ridibundus* inhabiting anthropogenically polluted habitats in Southern Bulgaria (sites, 3, 4, 7 and 9 – see tables 3 and 4), can be considered adaptations increasing the contact surface and oxygen capacity of erythrocytes in conditions of hypoxia. Although these changes are essentially aimed at adaptation and a better life in an environment with deteriorating parameters, we cannot say for sure that this is actually taking place in these populations.

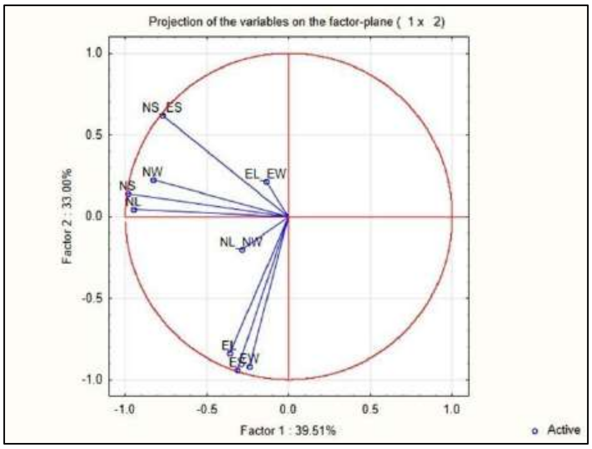

Fig. 5. Ordination on the two canonical variables (Factor 1) and (Factor 2) for tested erythrocyte-metric parameters in *Pelophylax ridibundus* specimens. The distinguishing force of the parameter is indicated by the arrow length; a large importance is shown by the long arrow and it is strongly correlated with the ordination axes. This diagram is published in Zhelev et al. (2021).

We believe that in order to be able to adequately assess the effects of morphological changes in the erythrocytes of these animals, they must be combined with analyzes of other physiological (in particular haematological) parameters. This thesis is supported by the results obtained in the analysis of a complex of morphophysiological parameters in the study conducted in the populations of *P. ridibundus* from the Chaya River (site 9). In these frogs, we found anemic changes (erythropenia and hypochromia) and weakened immunity (neutrophilia, eosinophilia, monocytosis, basopenia, and lymphopenia) compared to the frogs from the control group (site 8). The changes indicate a severe deterioration of the health status of these frogs (for details see Zhelev et al., 2020). On the other hand, it is very difficult to unambiguously assess the effects of changes in erythrocyte sizes in the polluted sites 2 and 5 (large erythrocytes with an elongated elliptical shape of the cell). There are interesting studies on birds in literature (Nadolski et al., 2006; Banbura et al., 2007; Janiga et al., 2017) and deep-sea marine mammals (Hedrick & Duffield, 1991; Promislow, 1991; Debey & Pyenson, 2013) where it is reported that large erythrocytes, combined with high haemoglobin and haematocrit values, may also be adaptive. In birds, they give a better chance of survival to young chicks, and in marine mammals, they supply cells slower and longer with oxygen when diving to great depths. Despite these examples, we believe that the effects of changes in erythrocyte size and shape found in homothermic animals should not be unambiguously interpreted and extrapolated to heterothermic animals (such as anurans). This would not be correct, at least because of the differences in the mechanisms of metabolism and the levels of its regulation in these different groups of animals (Kozłowski et al., 2010; Adrian et al., 2016).

The results of our studies provide conclusive evidence that erythrocyte measurements can be successfully used as biomarkers of physiological stress. They support the view of Davis & Maerz (2008a; 2008b), that erythrocyte sizes combined with other haematological parameters may be an objective marker for assessing environmental stress levels in amphibian populations living in anthropogenically contaminated habitats. However, we believe that the specific lifestyle of different groups of amphibians, as well as the characteristics of their habitat, should be taken into account when the morphology of blood cells (in combination with other blood parameters) is used to diagnose their health (physical fitness). The results of our studies once again emphasize the importance of haematological studies, including the morphology of blood cells as reliable biomarkers in the field of ecotoxicological studies. This necessitates rethinking of studies aimed at presenting "reference values" for quantitative (RBC and WBC counts), qualitative blood parameters (haemoglobin concentration haematocrit value and derivative parameters such as haematological indexes: MCH, MCHC and MCV), and for erythrocyte sizes. In our opinion, these studies would not be correct without providing data on the state of physio-chemical characteristics of the environment as well as tracing the possible presence of anthropogenic stressors in the specific habitat. Based on our results from studies with *P. ridibundus* test-subjects, we believe that the analysis of environmental factors is particularly necessary for analyses performed with water frogs, which spend a significant portion of their lives in aquatic habitats.

Conclusions

As a result of our long-term *in situ* studies in populations of marsh frog *P. ridibundus* inhabiting anthropogenically polluted water ecosystems in Southern Bulgaria, we find that the combination of erythrogram and leucogram parameters provides a powerful and objective tool for diagnosing their health status (physical fitness). Changes in the size of erythrocyte cells and their nuclei is also a promising biomarker for assessing the effects of environmental stress, but for a better understanding of the physiological changes occurring in the body of amphibians inhabiting conditions of chronic anthropogenic pollution, in our opinion these analyses should be combined with studies of other blood parameters, such as RBC, WBC count, Hb concentration, Hct value and differential leukocyte count. Of course, this type of analysis cannot completely replace the routine physio-chemical analyses used in the monitoring of ecological quality of water ecosystems, but they can provide sufficiently reliable supplementary information. In some cases, especially when looking for the long-term effects of toxic agents on biota, this information may be not only useful but also much more plausible than the one obtained from physio-chemical monitoring. Physio-chemical analysis gives a "snapshot" of the state of the waterbody – at the time of sampling, and haematological analysis assesses the general health status of frogs which is a total result of the long-term effects of contaminants on their body.

Acknowledgments

This research was supported by the National program "Young scientists and Postdoctoral candidates" funded by the Bulgarian Ministry of Education and Science.

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Received: 08.10.2021
Accepted: 20.12.2021

ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 321-330

Synopsis

A Review on Multi-Biomarkers in Fish for the Assessment of Aquatic Ecosystem Contamination with Organic Pollutants

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Abstract. The aim of the current review is to identify the most suitable biomarkers for evaluating chemical stress due to organic contamination in aquatic environments, as well as possible ways to reduce or limit this contamination. To safeguard the environmental status, the European Union has implemented the Water Framework Directive (WFD; 2000/60/EC) and the Marine Strategy Framework Directive (MSFD; 2008/56/EC) legislations, which encourage the use of biological tools to detect the quality of aquatic systems. Therefore, monitoring of sentinel fish species is widely used to assess the level of health status. Fishes have been found to be good biomonitors of water pollution because they occupy different trophic levels; they are of different sizes and ages and in comparison with invertebrates, are also more sensitive to different toxicants, including persistent organic pollutants, such as pesticides. Biomarkers are defined as responses to any exposure evidenced in histological, physiological, biochemical, genetic and behavioral modification. The application of multi-biomarker approach is necessary in the development of a protocol for the aquatic assessment of organic pollutants. This protocol can be applied in risk assessment and water monitoring programs in order to provide an adequate legal basis for the presence of organic pollutants in aquatic ecosystems and biological responses under concentrations equal or lower to those permitted under the European and Bulgarian legislation.

Key words: water contamination, organic pollutants, histology, enzymatic activity, biomonitors.

Introduction

Correlations between concentrations of environmental contaminants, bioaccumulation, and biological responses in organisms should be integrated for a better understanding of the environmental risks and thus, the use of multiple biomarkers at different levels of biological organization is an appropriate approach for the detection of biological responses triggered by chemical stress and should be incorporated into aquatic monitoring studies, providing an ecotoxicological diagnosis needed for environmental management (van der Oost et al., 2003; Vieira et al., 2019).

In the countries of the European Union (EU), national water agencies, which follow

EU policy and the requirements of the EU Water Framework and its Daughter Directives (Directive 2000/60/EC; Directive 2008/105/EC; Directive 2008/56/EC; European communities environmental objectives 272/2009; European communities technical report 2010), implement regular water monitoring with the aim to control and prevent pollution (Milačič et al., 2017). According to Directive 2013/39/EC the maximum permissible concentration for a single compound must not exceed 0.1 μg/L, and in the case of a larger number of residues, it must not exceed a concentration of 0.5 μg/L.

The toxicity of mixtures will depend on the bioavailability and chemical reactivity of the compounds. To gain greater insight into the risks posed by environmental contaminants, it is beneficial to understand their mode of action (MoA). The MoA is basically the process initiated by the interaction of the toxicant with the receptor, which progresses through molecular, biochemical, physiological and/or anatomical changes in the organism to result in sub-lethal and lethal effects. Such effects/response can be detected by means of biomarkers, which are broadly defined as indicators signaling events at individual level (Napierska et al., 2018).

As stated by Nataraj et al. (2017) and Ghayyur et al. (2021) fishes are very sensitive to the presence of pollutants in water, so they can be used as biomonitors and in comparison to other aquatic biomonitors, fishes occupy the top position of the aquatic trophic chain, therefore they offer an integrated image of the whole aquatic ecosystem. Moreover, fishes have the ability to accumulate toxic substances from the surrounding environment in different body organs and tissues (Ghayyur et al., 2021), which is essential it terms of multi-analyses, such as chemical, histological or biochemical.

The impact of organic pollutants, their toxicity and resistance to degradation demand the development of new and innovative materials, processes, and technologies to remove them from the aquatic environment (Nizzetto et al., 2010; Pariatamby & Kee, 2016; Negrete-Bolagay et al. 2021). In this regards according to Rani et al. (2021) remediation techniques can be divided into 4 groups: 1. Removal (contaminates or contaminate medium are eliminated from the spot with no requirement to separate from the host medium); 2. Separation (elimination of contaminants from the host medium such as water or soil); 3. Destruction (more toxic chemicals are transformed into less toxic products by degradation or neutralized chemically or biologically); 4. Containment (interference or inactivate surface and subsurface passage of the contaminates). As stated by Sarker et al. (2021) pesticide bioremediation is a greener and ecofriendly approach for the complete degradation or transformation of pesticides into nontoxic metabolites using living agents as potential degraders. According to Gavrilescu (2005) bioremediation, as one of the most environmentally-sound and cost-effective methods for the decontamination and detoxification of a pesticide-contaminated environment, is discussed especially considering the factors affecting the biodegradability of pesticides, such as biological factors and the characteristics of the chemical compounds.

Ecological monitoring on contaminated with organic substances aquatic ecosystems

Different organic pollutants, such as pesticides, pharmaceuticals, etc., are found in industrial and urban regions all over the world (Odabasi et al., 2016; Negrete-Bolagay et al., 2021). Fuoco & Giannarelli (2019) and Negrete-Bolagay et al. (2021) divided the organic xenobiotics into two main groups - anthropogenic and natural. As explained by Georgieva et al. (2021) anthropogenic sources are considered as the major pollutants of aquatic ecosystems. In addition, humans are exposed directly to different organic contaminants, when they are working in the agricultural and industrial sectors and indirectly - through the consumption of contaminated water and food, including fishes (Saleh et al., 2020; Negrete-Bolagay et al., 2021).

Pesticides

Natural ecosystem can be contaminated by different sources of persistent organic pollutants and priority substances, such as pesticides in two groups based on their solubility. The first group include the pesticides, which dissolved in surface waters enter into the ground water, and may cause severe negative effects on the non-target organisms. On the other hand, the fat-soluble pesticides enter in the organism via the process of bioamplification, which results in their longer existence in the food chain (Katagi & Tanaka, 2016). According to Rani et al. (2021) the bioamplification disturbs the entire ecosystem, particularly at higher trophic levels, the species will die because of higher toxicity in their bodies due to processes, such as biomagnification.

Schuijt et al. (2021) explained that chemical monitoring is often not performed alone, but in combination with ecological monitoring. In this regards, an integrated assessment can improve the ability to describe the effect of contamination for areas with decreased or poor environmental status detected during monitoring programs (Vethaak et al., 2017). According to Schuijt et al. (2021) the major benefit of ecological monitoring is the high ecological relevance since it provides important information on the ecosystem and integrates the overall effect of chemical stressors including mixtures' effects and bioavailability. One of the main aspects of the European Union Marine Strategy Framework Directive (MSFD), a wide-ranging Framework Directive (2008/56/EC) with the overall objective of achieving or maintaining Good Environmental Status (GES) in Europe's seas by 2020 (MSFD, 2008), is the development of common criteria and methodological standards, which will ensure consistency and comparability in the determination of Good Environmental Status (GES) across Europe (Lyons et al., 2010).

Biomonitors on contaminated with pesticides aquatic ecosystems

Biomonitors are sentinel organisms that respond to changes at various structural levels, such as cellular, physiological, biochemical, genetic and histological factors to variations in patterns of behavior, which may affect the population structure of the species as a response to stressors present in the environment (Rodrigues et al., 2010; Velusamy et al., 2014; Lima et al., 2018). Ahmad & Ahmad (2016), Shukla & Trivedi (2017) and Trivedi et al. (2021) recommended fishes as the "early warning biological model" for risk assessment associated with aquatic toxicants. In addition, in aquatic ecosystems, fishes are considered as good indicators of environmental health because of their different positions in the food chain (Adams & Greeley, 2000; Lima et al., 2018). Thus, fishes are used as suitable biomonitors of the health status of aquatic ecosystems (Rodrigues et al., 2010; Iwanowicz et al., 2012; Lima et al., 2018; Georgieva et al., 2021).

Biomarkers for contaminated with pesticides aquatic ecosystems

The term "biomarker" include a measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological (WHO, 1993; van der Oost et al., 2003). The approach given by biological tools has the advantage to provide information on the exposure and the effects of chemicals (even short-lived chemicals) on living organisms, while chemical analyses provide information about the presence and/or concentrations of the substances; it means that only chemical analysis does not reflect the response of aquatic organisms to harmful effects of pollutants (Martinez-Haro et al., 2015; Wan et al., 2018; Lomartire et al., 2021).

According to Franco-Martínez et al. (2020) biomarkers could be divided into five groups: 1. Spectrophotometric assays, based on light absorbance changes due to the presence or activity of the biomarker after the reaction with a chemical compound that is added as a reagent in the assay; 2. Immunoassays, based on the recognition of the biomarker by a specific antibody that provides a selective determination of a sample component, these immunoassays can be performed by westernblotting, ELISA or immunoturbidimetry; 3. Genome based biomarkers are usually identified from genome-wide association studies (GWAS); and use DNA markers such as single nucleotide polymorphisms (SNPs) that affect complex phenotypic traits; 4. Transcriptomic analysis of RNA samples from tissues and body fluids reveal differential gene expression patterns and potential biomarker for exposure to stress and environmental pollutants; 5. Chromatography and mass spectrometry, different pollutants can be quantified by gas chromatography (GC) and mass spectrometry (GC–MS). In addition to these methods, histopathological and histochemical assays could also serve as a sensitive biomarker for aquatic contamination (Fig. 1) (Georgieva et al., 2021).

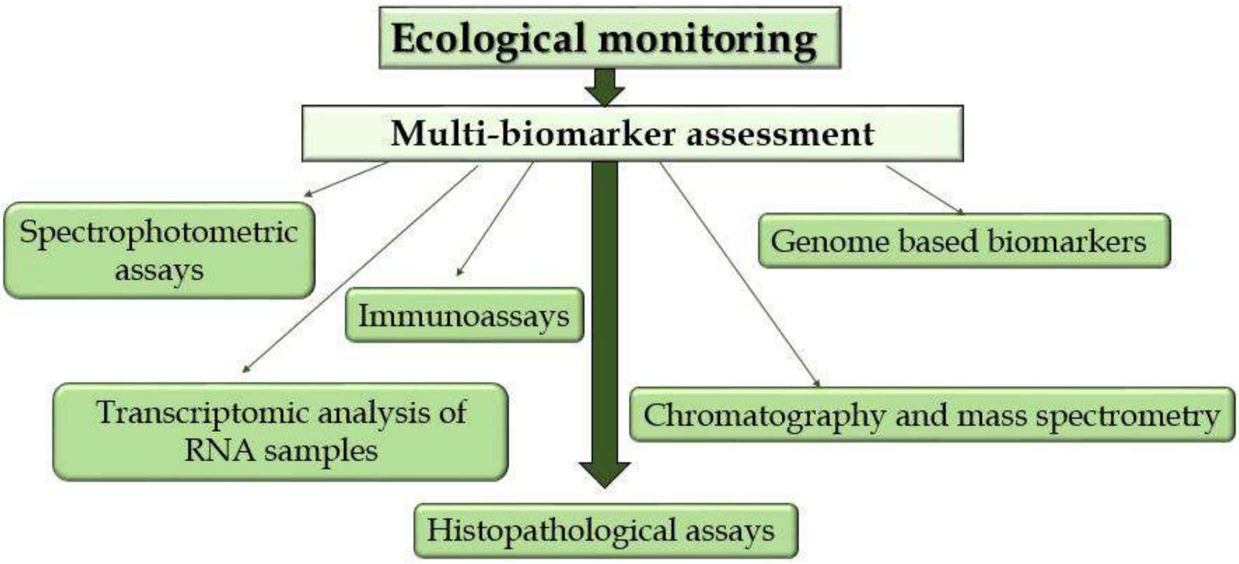

Fig. 1. Applied biomarkers in ecological monitoring.

The use of biological effects techniques (bioassays and biomarkers) offer the required tools to help define Descriptor 8 of GES according to MSFD, specifically in relation to monitoring the actual pollution effects of anthropogenic chemicals in the aquatic environment. As stated by Thain et al. (2008) a biological effect could be defined as the response of an organism, a population, or a community to changes in its environment. One of the clear advantages of the application of biological effect techniques is the link between contaminant exposure and ecological endpoints, as well as detecting the impact of substances (or combination of substances) that may not be analyzed as part of routine chemical monitoring programs (van der Oost et al., 2003; Thain et al., 2008; Lyons et al., 2010).

The multi-level biomarker approach is widely used from researchers to obtain a more complete and integrative overview of what affects marine organisms' health and, consequentially, the environment (Samanta et al., 2018; Lomartire et al., 2021). As stated by Lomartire et al. (2021) it is necessary to well define the baseline data of biomarkers to have a correspondent distinction between "noise" (natural variability) and "signal" (stress caused by contaminant). Moreover, the integrated biomarker response (IBR) that employs biochemical, morphological and physiological features is a must in regular monitoring of aquatic ecosystems (Bignell et al., 2011; Lomartire et al., 2021).

Likewise, Hedayati (2018), we consider that the application of biomarkers for aquatic environment monitoring has become widespread and it is essential in terms of ecotoxicological research. Furthermore, we agree that biomarkers have been largely used for the assessment of effects induced by several classes of chemical contaminants on fishes, for example the assessment of alterations on key enzymatic activities of biomonitor species following exposure to natural and experimental contaminated waters has been one of the major uses of biomarkers in different studies.

On one hand, as described by Yancheva et al. (2019a) the histopathological effects produced in different tissues of non-target organisms are usually used for monitoring of polluted areas and environmental conditions in aquaculture systems, and the use of histopathological biomarkers in biomonitoring has many advantages. According to the authors histopathological biomarkers can be used as an early warning system for potential effects at the level of the individual and the histopathological lesions are easily recognizable alterations that ideally indicate alone a pathological condition (Fig. 2). The increasing number of studies related to the use of histological biomarkers is based on the fact that they better reflect the health status of fish compared to other used methods. In addition, histochemical changes associated with a change in the amount of glycogen and lipids in the hepatocytes, serves as an indicator of the change in carbohydrate metabolism due to pesticide contamination in agrarian countries, such as Bulgaria (Stoyanova et al., 2019; Yancheva et al., 2019b).

On the other hand, oxidative stress induced by different organic pollutants, through an increase in the formation of reactive oxygen species (ROS) may lead to biochemical, cellular and physiological changes in the exposed organisms. Free radicals may lead to lipid peroxidation, changing the constitution of the biological membranes as well (Gonçalves et al., 2021). We agree that the exposure to organic toxicants may affect fatty acid composition of organisms by potentiating lipid peroxidation, as previously mentioned, or interfering with lipid metabolism, which can be evaluated through the assessment of fatty acid profiles. The presence of xenobiotics in water may affect the lipid metabolism and fatty acid and phospholipid composition (Olivares-Rubio & Vega-López, 2016; Gonçalves et al., 2021). According to Gonçalves et al. (2021) organic pollutants may impact the activity of a number of enzymes, from the stimulation of antioxidant enzymes in an effort to reestablish redox balance and preserve cells integrity, to the disruption of enzyme-mediated processes (Fig. 2). As the authors stated the use of enzymes as biomarkers of contaminant impacts in non-target species has proven to be a useful tool to assess their impacts. Moreover, oxidative stress plays an important role in the toxicity of pesticides and fishes have a defense system, which includes antioxidant enzymes, such as catalase (CAT), the glutathione system itself and superoxide dismutase (SOD) (Georgieva et al., 2021). Yancheva et al. (2015) described that the alterations in different fish enzymes represent a sensitive biochemical indicator for the negative pesticide effects. The authors considered that changes in other metabolic enzymes, such as lactate dehydrogenase (LDH), aspartate and alanine aminotransferase (ASAT and ALAT) are also sensitive indicators for hepatotoxicity, particularly in the fish liver, which is the main depot for bioaccumulation of xenobiotics and also a main detoxification organ, along with the kidney (Fig. 2). In addition to antioxidant and metabolic enzymes, the neurotransmitter cholinesterase (ChE) is also analyzed very often. One of the important elements in environmental analysis to be considered is the direct relationship between ChE inhibition and the behavioral, biochemical, and physiological alterations that occur in exposed organisms (Basirun et al., 2017). Moreover, ChE extracted from different organs of the same fish species or from different species possess different sensitivity towards anticholinesterastic agents exposed to it. This would make the extracted ChE as a specific biomarker for each possible toxicant.

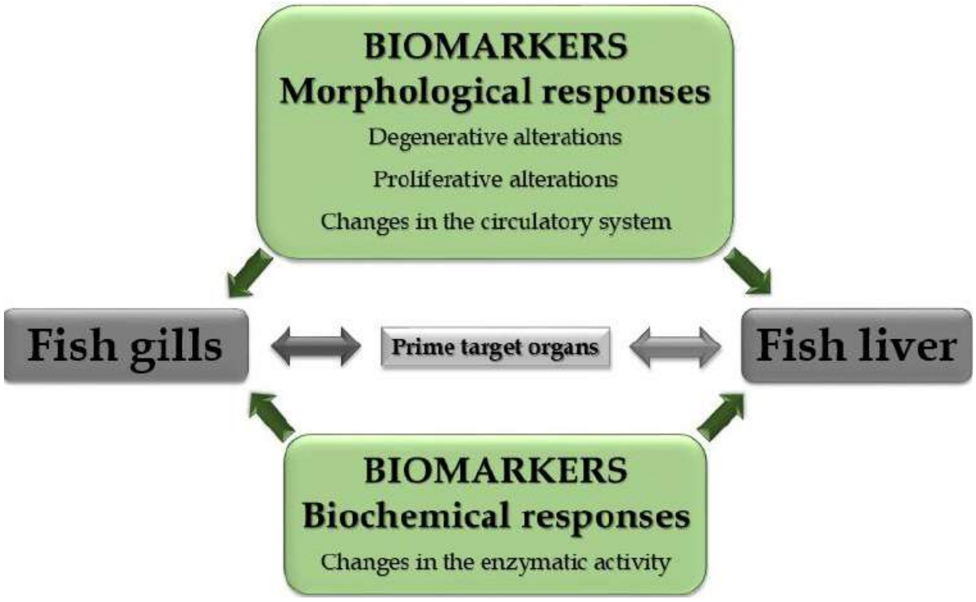

Fig. 2. Biomarkers in fish prime target organs (gills and liver).

Conclusions

The application of such complex research is necessary in the development of a protocol for the assessment of pollution of aquatic ecosystems with organic pollutants. This protocol, which includes histopathological and biochemical biomarkers can be applied in both, water monitoring and agricultural practices, to provide an adequate legal basis for the presence of organic pollutants and their effect in aquatic ecosystems. Thus, collaboration between different institutions is crucial to reduce the risks of pesticide toxicity on the environment and modifications under concentrations equal to those permitted under European legislation.

Acknowledgments

The "Vita Plus" project financed by Science Research Fund at the University of Plovdiv Paisii Hilendarski under the "Green Technologies" project line, is highly appreciated. The authors also thank the Science Research Fund at the University of Plovdiv Paisii Hilendarski for the financial support for project FP21 BF008.

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Received: 21.10.2021
Accepted: 22.11.2021

Reviewers Acknowledgment

Peer reviewers are key to advancing scholarship and contributing to the quality of scholarly journals. We would like to thank the following reviewers who have taken part in the peer-review process for vol. 13, iss. 2, 2021 of Ecologia Balkanica.

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Example: Management plan for the protected area for birds BG 0002086, "Rice Fields Tsalapitsa". (2013). Retrieved from https://plovdiv.riosv.com (In Bulgarian)

Proceedings or book chapter:

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Author, A., Author, B. & Author, C. (Year of Publication). Title of work. In A. Author, B. Author, & C. Author (Eds.). *Title of the book or proceedings.* (Edition, pp. XX-XX). Publisher City, Country: Publisher.

Software:

Author, A. (Year of Publication). *Name of software*. Vers. XX. Retrieved from http://xxxx

Example:

StatSoft Inc. (2004). *STATISTICA (Data analysis software system)*, Vers. 7. Retrieved from http://www.statsoft.com

Website:

Author, A. (Year of Publication). *Title of page*. Retrieved from http://xxxx

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Official European directives, issued from the European parliament and of the Council (EC) should be cited as follows (example):

EC. (2010). Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. *Official Journal of the European Union,* *L276,* 33-79. Retrieved from https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF

Legislation:
Official laws, orders etc. should be cited as follows (see examples).
Biological Diversity Act. (2002). *State Gazzette, 77*, 09.08.2002. (In Bulgarian).
Medicinal Plants Act. (2000). *State Gazette, 29,* 07.04.2000. (In Bulgarian).
Protected Areas Act. (1998). *State Gazette, 133,* 11.11.1998 (In Bulgarian).

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Korovin, V. (2004). Golden Eagle (*Aquila heliaca*). Birds in agricultural landscapes of the Ural. Ekaterinburg, Russia: Published by Ural University. (In Russian).

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