ECOLOGIA BALKANICA

International Scientific Research Journal of Ecology

Volume 12, Issue 2
December 2020

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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Published by the Union of Scientists in Bulgaria – Plovdiv and the University of Plovdiv Publishing house – twice a year. Language: English.

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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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Research Articles

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Plant Composition in Grasslands and Field Margins in Agroecosystems of South Bulgaria

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Abstract. The flora of the Thracian Lowland region was analyzed in 13 target localities (semi-natural grasslands) near rapeseed fields for a 3-year period. A total of 245 flowering species were registered in the experimental fields, 178 of them were enthomophyllic plant species. Eleven adventitious and invasive species were recorded in representative experimental fields and their margin. The share of adventive species are small percent of the species composition, but pose a future threat to biodiversity. Maintaining the relative naturalness of these areas has a big role in the nutrition for the pollinators. This study provides data about the identification, observation in the field margins. The collected data could be a base for evaluation of the ecosystem services provided by agrocoenoses and neighboring semi-natural grasslands in the Thracian Lowland.

Key words: field margins, plant diversity, Thracian Lowland.

Introduction

The global biodiversity is threatened by the increased anthropogenic activities (Darkoh, 2003; Wessels et al., 2003). The recent studies have shown the link between the functioning of grassland ecosystems and the provision of a range of services that are essential to each community and its development (Altieri & Nicholls, 1999; Forest et al., 2017). The range of functions that an agroecosystem can support is closely linked to its biodiversity (Altieri, 1987, 1994). This large-scale transformation of natural vegetation presents a serious and growing threat to the biodiversity (Darkoh, 2003; Wessels et al., 2003).

One of the main priorities of the Common Agricultural Policy after 2003 in Bulgaria is the preservation of permanent pastures as a resource that has a positive impact on the environment. Most of the permanent pastures in Bulgaria are predominantly semi-natural, i.e. have not been sown and fertilized in recent years. They are one of the most valuable ecosystems in the country's agricultural landscape, on the one hand because of their exceptional role in biodiversity conservation compared to other land uses, and on the other, they are particularly important for the soil conservation.

The semi-natural permanent grasslands consist of different types of vegetation - some with mostly grassland communities, while in others shrubs predominate, even in some cases tree species. In many cases, shrubs and trees are an integral part of permanent grassland, providing additional opportunities for grazing. In 2012, the permanent grasslands occupied 32.15% of

the utilized agricultural area in Bulgaria. Compared to the last years, there is a significant decrease in the area of permanent grassland, as compared to 2006 the permanent grassland decreased by 12.2%, at the expense mainly of the increased area of arable land, which increased by 6.6%. The most important plant species in the natural and renovated pastures are legumes and herbs (data from European Environment Information and Observation Network).

The ecological management of agroecosystems in Bulgaria is still poorly developed, so this study is a part of the ecosystem assessment that reflects the links between habitat management and plant diversity in rapeseed fields in the Thracian Lowland as a model approach aiming at sustainable management of agroecosystems, and maintaining semi-natural areas adjacent to permanent cropland.

The objective of the present study was to assess the plant species and their importance for biodiversity in representative agricultural fields in the Thracian Lowlands and their periphery.

Material and Methods

The subject of study are 13 pre-selected target localities in southern Bulgaria, visited 4 times during the growing season (March-July), in the period 2016-2018 (Table 1). In addition to the composition of the plant species, the following information was reported for each plot: number of registered species, number of insect pollinating species and number of adventitious species. The origin of the adventive species is according to Assyov & Petrova (2012). Several species with cosmopolitan distribution were considered invasive, according to Petrova et al. (2012).

The data for the species diversity were done in squares of 1 m^2. In each field or pasture were selected 10 squares, located at equal intervals in the fields and grasslands. The sampling plots were located consistently 10 meters from the border inside the fields, and in the margin. The plots were marked as GPS coordinates and with pegs in each angle of the square. For each plot was prepared list of the species.

The sampling plots in the grassland were 10, with distance between them at least 10 meters.

The locations of the investigated fields are represented by MGRS coordinates (1x1 km). The data of each location is a result of the summation of the determinated species in the sampling squares.

Table 1. Investigated localities in Southern Bulgaria.

Municipality	Settlement	MGRS coordinates	Altitude	Pastures
Brezovo	Zelenikovo	35TLG4095	340 m	5-10%
		35TLG3995	318 m	
		35TLG4194	292 m	
Maritsa	Kostievo	35TLG0373	175 m	1-5%
		35TLG0271	172 m	
		35TLG0470	156 m	
	Radinovo	35TLG0573	178 m	100 %
Saedinenie	Malak Chardak	35TLG0583	210 m	20%
		35TLG0483	205 m	
Rakovski	Stryama	35TLG2279	182 m	10%
	Momino Selo	35TLG2584	183 m	20%
Dimitrovgrad	Dobrich	35TLG7853	122 m	20%
	Stalevo	35TLG6556	170 m	5%

Result and Discussion

In the floristic analysis, total 245 species from 40 families were identified in the experimental plots (Table 3), and were classified 65 anemophilous (26,5%), 178 enthomophilous (72,6%), and 2 hydrophilous (0,8%) species. Of these species 11 (4.5%) are considered as adventive and invasive.

The studied localities are with sporadic participation of bush vegetation, represented by: *Quercus cerris, Q. pedunculiflora, Q. robur, Carpinus orientalis, Crataegus monogyna, Pyrus pyraster, Prunus cerasifera, Prunus vulgaris, Paliurus spina-christi, Malus domestica, Rosa canina*, and the invasive species *Robinia pseudoacacia, Amorpha fruticosa, Ailanthus altissima*. The field margins are habitats of anthropogenic origin. For this reason, they do not show the appearance of a specific type of the habitat.

Like most agricultural landscapes, the studied territories are an interacting complex of arable fields, sporadic semi-natural habitats and anthropogenic infrastructure (roads, canals, substations, etc.). The large number of settlements in the investigated area is a prerequisite for the presence of a large number of adventive and invasive species. Eleven invasive species have been identified (Table 2) – *Ailanthus altissima*, *Datura stramonium*, *Erigeron annuus*, *E. bonariensis*, *E. canadensis*, *Oxalis corniculata*, *Phytolacca americana*, *Robinia pseudoacacia*, *Sorghum halepensis*, *Xanthium orientale* var. *italicum*, *X. spinosum*. Adventive non-invasive species are *Chenopodium album, Eleusine indica, Juglans regia, Medicago sativa, Portulaca oleracea, Solanum nigrum.* Along with them, single plants or groups of cultivated species, wintering as seeds from previous or adjacent agricultural productions, are found in the orchards: *Brassica napus*, *Helianthus annuus, Triticum durum, Zea mays*. The present survey stresses the necessity of studies focused on the invasive plants and processes of plant invasions in the agricultural lands in different regions of Bulgaria.

A small part of the surveyed plots located at equal intervals along the route was permanently flooded by permanent irrigation canals (adjacent to villages of Dobrich and Stalevo villages). Aquatic macrophytes were found there: *Alisma plantago-aquatica, Echinochloa crus-gallii, Gratiola officialis, Mentha aquatica, Phragmites australis, Typha angustifolia.*

Due to the rugged terrain, the highest floral diversity and the presence of semi-natural habitats of conservation importance are observed in the land of Zelenikovo. Accordingly, the intensively managed pastures in urbanized areas, such as fields and pastures in the villages of Kostievo and Radinovo, displayed lower levels of vegetation coverage. The floristic diversity in agroecosystems of the Upper Thracian Lowland is distinguished by the presence of species such as *Papaver rhoeas, Cynosurus cristatus* or *Achillea millefolium* and rich involvement of legumes, for example, with 15 to 25% coverage of *Trifolium sp.* and *Vicia sp.* species compared to 5% in conventional intensively exploited grasslands. The predominant species in the semi-natural grasslands are entomophilic, mainly Mediterranean, sub-Mediterranean and Eurocarpathian geoelements, whereas the boreal flora is mainly represented by anemophilous plants. This fact corresponds to an increase in habitats for major pollinators and supports the need to maintain more natural grasslands, since the decrease poses a threat to the services provided by the pollinators. A more complete understanding of the effect of grassland loss and species diversity could be obtained from studies on the behavior and numbers of pollinators (Wardle et al. 1999a; b).

Table 2. List of the adventive and invasive plants recorded on the whole studied territory.

Species	Origin	Status	Occurrence
Ailanthus altissima (Mill.) Swingle	China	Invasive	3
Chenopodium album L.	North America	Adventive	5
Datura stramonium L.	Central America	Invasive	1
Eleusine indica (L.) Gaertn.	Asia	Adventive	2
Erigeron annuus (L.) Pers.	North America	Invasive	7
Erigeron bonariensis L.	Central America	Invasive	2
Erigeron canadensis L.	North America	Invasive	4
Fallopia convolvulus (L.) A.Loeve	North America	Adventive?	2
Helianthus annuus L.	North America	Adventive	**1**
Juglans regia L.	Central Asia	Adventive	**1**
Medicago sativa L.	Central Asia	Adventive	**3**
Oxalis corniculata L.	Central and South America	Invasive	**1**
Phytolacca americana L.	North America	Invasive	**1**
Portulaca oleracea L.	North Africa and South Europe	Invasive	**4**
Robinia pseudoacacia L.		Invasive	**1**
Solanum nigrum L.		Adventive	**1**
Sorghum halepensis (L.) Pers.	Syria and Turkey	Invasive	**2**
Xanthium orientale subsp. *italicum* (Moretti) Greuter	South and North America	Invasive	**1**
Xanthium spinosum L.	South America	Invasive	**1**

Conclusions

The semi-natural grasslands in the Upper Thracian Lowland are anthropogenic habitats. The study confirms the existence of an expected link between floral diversity and the intensity of land management. The lowest floristic diversity and predominance of ruderal and weed elements were found in fragmented and highly urbanized habitats, such as in the region of villages of M. Chardak, Zelenikovo and Kostievo. This corresponds to the fragmentation of agricultural land and the dynamics of land use, which increases weed and ruderal plant species. The study provides data for the identification and monitoring of plant diversity, and is the basis for a future evaluation of ecosystem services provided by agrocoenoses and adjacent semi-natural grasslands in the Upper Thracian Lowland.

Acknowledgements

We acknowledge funding through Grant STACCATO, BiodivERsA-FACCE2014-47. The authors would like to thank to the anonymous reviewers for their comments on the earlier versions of the manuscript.

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Received: 31.01.2020
Accepted: 30.07.2020

Table 3. Identified species in the study plots represented by the MGRS coordinates (see Table 1 for descripton of the MGRS quadrants): ■ – enthomophilous, ○ - anemophilous.

35TLG	**0271**	**0373**	**0470**	**0483**	**0573**	**2279**	**2584**	**3995**	**4095**	**4194**	**0583**	**6556**	**7853**
Achillea cf. millefolium L.		■	■	■	■	■		■		■	■	■	■
Aegilops cylindrica Host					○	○		○					○
Aegilops geniculata Roth.	○				○								
Agrimonia eupatoria L.										■			■
Agropyron cristatum (L.) Gaertn.		○				○							
Agrostis capillaris L.	○				○								
Ailanthus altissima (Mill.) Swingle			■	■		■							
Alopecurus myosuroides Huds.													○
Alopecurus pratensis L.		○					○					○	
Alyssum alyssoides (L.) L.								■					
Alyssum desertorum Stapf	■												
Anthemis arvensis L.						■							
Anthemis cotula L.						■				■			
Anthemis ruthenica M.Bieb.					■	■		■		■		■	■
Anthoxanthum aristatum Boiss.								○				○	
Anthoxanthum odoratum L.		○				○							
Arctium lappa L.												■	
Aristolochia clematitis L.	■				■								
Arrhenatherum elatius (L.) P. Beauv. ex J. & C. Presl.	○	○				○		○				○	○
Artemisia absinthium L.				○							○		
Artemisia vulgaris L.							■						
Asparagus officinalis L.										■			
Asperugo procumbens L.		■											
Astragalus monspessulanus L.		■											
Avena fatua L.	○		○		○	○	○	○					
Avena sativa L.						○				○			
Ballota nigra L.	■				■								
Bituminaria bituminosa (L.) Stirt.								■					
Brachypodium distachyon (L.) P.Beauv.					○	○							
Brachypodium pinnatum (L.) P.Beauv.						○	○						
Briza media L.										○			
Bromus arvensis L.					○	○		○		○	○		
Bromus commutatus Schrad.						○		○		○			
Bromus hordeaceus L.		○		○	○		○	○			○	○	○
Bromus inermis Leyss.		○			○	○		○			○		○
Bromus madritensis L.										○	○		

35TLG	0271	0373	0470	0483	0573	2279	2584	3995	4095	4194	0583	6556	7853
Bromus racemosus Huds.		○								○			
Bromus squarrosus L.		○											
Bromus sterilis L.	○	○				○				○			○
Bromus tectorum L.	○	○			○	○	○	○		○			○
Calamagrostis arundinacea (L.) Roth.											○		
Calamagrostis epigeios (L.) Roth.								○					
Calepina irregularis (Asso) Thell.			■										
Campanula sparsa Friv.										■			
Capsella bursa-pastoris (L.) Medicus	■		■	■				■		■	■	■	■
Carduus acanthoides L.	■					■							
Centaurea alba L.		■		■							■		
Centaurea diffusa Lam.		■											
Centaurea solstitialis L.												■	■
Chenopodium album L.	○					○		○		○		○	
Cichorium intybus L.	■											■	■
Cirsium arvense (L.) Scop.						■	■	■		■	■		■
Cirsium vulgare (Savi) Ten.		■											
Conium maculatum L.			■									■	
Consolida regalis S. F. Gray		■											
Convolvulus arvensis L.	■	■	■			■	■	■		■	■	■	■
Cota altissima (L.) J. Gay								■					
Cota austriaca (Jacq.) Sch.Bip.							■					■	■
Cota tinctoria (L.) J. Gay										■		■	
Crepis foetida L.		■											
Crepis setosa Hall. f.											■		
Cruciata glabra (L.) Ehrend.												■	
Cruciata laevipes Opiz						■		■		■			
Cyanus segetum Hill							■	■				■	
Cynodon dactylon (L.) Pers.		○				○				○	○	○	○
Cynoglossum montanum L.							■						
Cynosurus echinatus L.								○		○			
Dactylis glomerata L.		○											
Dasypyrum villosum (L.) Borb.	○					○	○	○					
Deschampsia caespitosa (L.) P.Beauv.		○											
Descurainia sophia (L.) Webb.	■				■	■							
Dianthus corymbosus Sm.							■			■			■
Eleusine indica (L.) Gaertn.								○		○			
Equisetum arvense L.								+					
Equisetum palustre L.								+		+			
Erigeron annuus (L.) Pers.	■	■	■	■	■	■			■				

35TLG	0271	0373	0470	0483	0573	2279	2584	3995	4095	4194	0583	6556	7853
Erigeron bonariensis L.			○	○							○		
Erigeron canadensis L.	○				○	○							○
Erodium cicutarium (L.) L'Her.						■	■	■		■			■
Eruca vesicaria (L.) Cav.										■			
Eryngium campestre L.						■	■	■	■	■	■	■	
Erysimum diffusum Ehrh.							■						
Euphorbia cyparissias L.							■	■	■	■			
Euphorbia helioscopia L.	■			■			■					■	
Fallopia convolvulus (L.) A.Loeve		■								■			
Festuca arundinacea Schreb.								○					
Festuca pratensis Huds.							○					○	
Filago arvensis L.	■										■		
Filago lutescens Jord.				■							■		
Filipendula vulgaris Moench								■		■		■	■
Foeniculum vulgare Mill.							■			■			
Fritillaria pontica Wahlenb.									■				
Galium album Mill.						■		■					
Galium aparine L.								■		■		■	■
Gallium verum L.		■						■			■		■
Geranium columbinum L.												■	
Geranium dissectum L.	■				■	■	■	■			■		■
Geranium molle L.	■	■			■	■	■	■			■		
Geranium rotundifolium L.						■				■		■	
Gratiola officinalis L.											■	■	
Helianthus annuus L.		■											
Helminthotheca echioides (L.) Holub.		■											
Hieracium spp.									■			■	
Hordeum marinum Huds.						○	○						
Hordeum murinum L.	○	○	○		○			○		○	○	○	
Hypecoum pendulum L.							■						
Hypericum perforatum L.										■			
Inula aspera Poir.		■											
Lactuca hispida DC.						■		■					
Lactuca serriola L.	■	■			■	■	■	■		■	■	■	■
Lamium amplexicaule L.												■	
Lamium purpureum L.	■								■				
Lathyrus aphaca L.													■
Lathyrus hirsutus L.												■	
Lathyrus latifolius L.												■	
Lathyrus nissolia L.													■
Lathyrus sativus L.													■

35TLG	0271	0373	0470	0483	0573	2279	2584	3995	4095	4194	0583	6556	7853
Lepidium campestre (L.) R.Br.								■		■			
Lepidium draba L.	■		■				■				■		
Linaria dalmatica (L.) Mill.											■		
Linaria genistifolia (L.) Mill.							■			■			
Linaria vulgaris Mill.								■					
Lolium multiflorum Lam.								○			○		
Lolium perenne L.	○	○	○		○	○	○	○		○			
Lolium remotum Schrank					○	○							
Lolium rigidum Gaudin				○		○		○		○	○		
Lolium temulentum L.				○		○					○		
Lotus corniculatus L.		■		■			■						
Malva sylvestris L.	■		■		■		■						
Marrubium vulgare L.							■						
Matricaria chamomilla L.	■		■		■	■	■	■		■			
Medicago arabica (L.) Huds.						■							
Medicago minima (L.) Bartal.		■		■		■						■	■
Medicago orbicularis (L.) Bartal												■	■
Medicago rigidula (L.) All.				■		■	■					■	■
Medicago sativa L.	■				■							■	
Melilotus officinalis (L.) Pall.												■	
Mentha arvensis L.		■											
Moehringia trinervia (L.) Clairv.				■									
Moenchia mantica (L.) Bartl.							■					■	
Muscari botryoides (L.) Mill.									■				
Nigella arvensis L.		■											
Nonea atra Griseb.													■
Ononis spinosa L.		■											
Orchis purpurea Huds.									■				
Orlaya daucorlaya Murb.												■	
Orlaya grandiflora (L.) Hoffm.										■			
Ornithogalum boucheanum Asch.									■				
Ornithogalum umbellatum L.												■	
Paliurus spina-christi Mill.										■			
Papaver dubium L.												■	
Papaver hybridum L.			■			■							
Papaver rhoeas L.	■		■		■	■	■	■		■	■	■	■
Parentucellia latifolia (L.) Caruel							■					■	
Phleum exaratum Griseb.		○											
Pilosella caespitosa (Dumort.) P.D.Sell & C.West											■		
Pisum sativum subsp. *elatius*													■

35TLG	0271	0373	0470	0483	0573	2279	2584	3995	4095	4194	0583	6556	7853
(M. Bieb.) Asch. & Graebn.													
Plantago lanceolata L.	○	○	○			○		○		○	○	○	○
Poa annua L.		○			○	○					○	○	○
Poa bulbosa L.					○	○			○			○	
Poa pratensis L.	○	○			○	○					○	○	
Podospermum laciniatum (L.) DC.	■		■					■		■	■	■	
Polygonum aviculare L.	○	○	○	○	○		○			○	○		
Portulaca oleracea L.			■	■					■				■
Potentilla argentea L.								■		■	■		
Potentilla inclinata Vill.										■		■	■
Potentilla reptans L.		■				■	■						
Prunus cerasifera Ehrh.	■	■	■		■								
Ranunculus acris L.													■
Ranunculus arvensis L.									■	■		■	■
Ranunculus illyricus L.									■				
Ranunculus pedatus Waldst. & Kit.												■	
Ranunculus repens L.												■	
Raphanus raphanistrum L.	■						■						
Rorippa lippizensis (Wulfen) Rchb.										■			
Rorippa pyrenaica (L.) Rchb.												■	
Rorippa sylvestris (L.) Besser	■									■			
Rosa spinosissima L.													■
Rumex acetosa L.						○						○	
Rumex acetosella L.								○				○	
Rumex conglomeratus Murray		○											
Rumex crispus L.	○	○	○				○					○	
Rumex crispus × *R. pulcher*	○		○										
Rumex palustris Sm.			○										
Rumex pulcher L.			○			○	○			○	○		
Salix alba L.												■	
Sambucus ebulus L.							■						■
Sanguisorba minor Scop.							○		○				○
Scleranthus annuus L.								■	■				
Sclerochloa dura (L.) P. Beauv.						○							
Scolymus hispanicus L.	■		■	■	■	■				■	■	■	
Scolymus maculatus L.				■						■	■		
Serratula tinctoria L.	■												
Sherardia arvensis L.												■	■
Sisymbrium officinale (L.) Scop.	■		■		■					■	■		
Solanum nigrum L.		■											

35TLG	0271	0373	0470	0483	0573	2279	2584	3995	4095	4194	0583	6556	7853
Sonchus oleraceus L.	■											■	
Sorghum halepensks (L.) Pers.		■										■	
Stellaria media (L.) Vill.							■					■	
Taeniatherum caput-medusae (L.) Nevski											■		
Taraxacum cf. officinale F. H. Wigg						■						■	■
Thlaspi alliaceum L.												■	
Thlaspi arvense L.								■				■	■
Thymus striatus Vahl										■			
Tragopogon dubius Scop.	■		■			■	■	■		■	■		
Trifolium arvense L.	■	■						■			■		
Trifolium bocconei Savi											■		
Trifolium campestre Schreb.											■		
Trifolium cherleri L.												■	
Trifolium echinatum M. Bieb.												■	
Trifolium hybridum L.	■		■	■	■						■	■	
Trifolium incarnatum L.										■			
Trifolium medium L.											■		■
Trifolium nigrescens Viv.	■					■		■		■	■	■	■
Trifolium pratense L.	■					■							
Trifolium repens L.	■	■	■	■		■	■				■	■	■
Trifolium striatum L.	■			■	■	■	■			■	■	■	
Trifolium vesiculosum Savi						■							■
Trigonella caerulea (L.) Ser.					■								
Tripleurospermum inodorum (L.) Sch.Bip.					■		■	■		■	■	■	■
Triticum durum Desf.											○		
Valerianella dentata (L.) Pollich											■		
Verbascum phoeniceum L.											■		
Verbena officinalis L.		■											
Veronica hederifolia L.							■						
Vicia cassubuca L.												■	
Vicia cracca L.			■		■	■	■				■	■	
Vicia grandiflora Scop.	■				■		■	■		■	■	■	
Vicia hirsuta (L.) Gay	■			■	■		■	■		■	■	■	■
Vicia narbonensis L.									■			■	■
Vicia pannonica Crantz subsp. *pannonica*	■		■					■				■	■
Vicia pannonica subsp. *striata* (M.Bieb.) Ponert								■	■				
Vicia sativa L.	■			■	■						■		■
Vicia tenuifolia subsp. *dalmatica* (A. Kern.) Greuter									■				
Vicia tenuifolia Roth. subsp.									■				

	35TLG	**0271**	**0373**	**0470**	**0483**	**0573**	**2279**	**2584**	**3995**	**4095**	**4194**	**0583**	**6556**	**7853**
tenuifolia														
Vicia villosa Roth.							■		■				■	
Vinca minor L.										■				
Viola arvensis Murray					■							■		
Viola tricolor L.											■			
Vitis vinifera L.														■
Vulpia myuros (L.) C.C.Gmel.								○	○			○		
Xanthium orientale subsp. *italicum* (Moretti) Greuter			■											
Xanthium spinosum L.			■											
Total number of species		**58**	**54**	**27**	**26**	**44**	**71**	**52**	**63**	**20**	**69**	**60**	**78**	**59**

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 13-20

On the Maximum Sizes in Snake Species (Reptilia: Serpentes) from Bulgaria

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To Vladimir Beshkov, our mentor and friend

Abstract. The maximum known size for all currently living snake species from Bulgaria was estimated based on a combination of literature and new data. A comparison between the maximum size documented in Bulgaria and that in the rest of the respective species range was made. Results revealed that the maximum known size of *Platyceps najadum* was registered in Bulgaria. Up to date across all species, only three specimens (all belonging to *Dolichophis caspius*) have a reported size in excess of 2 meters. The "record" sizes of the largest species are discussed.

Key words: Colubridae, Viperidae, Typhlopidae, Boidae, morphometry, Platyceps, Balkans.

Introduction

The size of snakes has long been of interest to the scientific community and the general public around the world. In the popular press, as well as in some popular science publications, there is a wealth of information about "giant snakes" without specifying the origin of this information. A number of scientific publications have also indicated maximum size for some snake species, without reference to documented measurements that could be verified. The green anaconda (*Eunectes murinus* (Linnaeus, 1758)) could be used as a typical example, with maximum size established by reliable measurements standing at 8.3 m, and a number of unconfirmed reports of specimens exceeding 12 and even 14 m (Murphy, 1997; Murphy & Henderson, 1997). In Bulgaria, there are many superstitions related not only to snake behaviour, but also to their size, the latter being significantly overestimated. In the mass media (newspapers, TV shows, internet sites, etc.), there are frequent publications that promote such misconceptions but lack any scientific justification. In this regard, the need for a scientifically sound review of the size of snakes in Bulgaria is evident.

Eighteen species of snakes are known from the territory of Bulgaria, two of which have not been found in the last 80 years (Stojanov et al., 2011). A number of

publications on Bulgarian herpetofauna (e.g. Beshkov, 1964; Beshkov & Nanev, 2002; Biserkov et al., 2007; Buresch & Zonkow, 1934; Kovachev, 1912; Stojanov et al., 2011) have stated that the Caspian Whip snake (*Dolichophis caspius*) is the largest snake in the country. In many cases, the maximum size that is known for other species of snakes is also indicated, but it is often unclear whether this size is registered in Bulgaria or applies to other parts of the range of the respective species. Targeted morphometric studies on snakes at national level have not yet been conducted.

The aim of the present work is to summarize the available data (new and published) for the maximum size of the different snake species in Bulgaria.

Material and Methods

For the purpose of this work we used all documented snake measurements (a total of 2914 measured specimens from 16 species; see Table 1), done by the authors (during field trips across Bulgaria in the period 1965-2019), as well as similar data, provided by colleagues, or derived from the mobile application SmartBirds Pro (Popgeorgiev et al., 2015). All available scientific publications, containing original data on snake size from Bulgaria, were reviewed (publications which did not provide specific data sources, were excluded). The species *Vipera aspis* (Linnaeus, 1758) and *Vipera ursinii* (Bonaparte, 1835) were not examined, because there are only four adult specimens known from Bulgaria, and currently both species are considered extinct (see Beschkov, 2015a, b).

Data on the three largest specimens of each species from Bulgaria are presented as a total length (from the snout tip to the tail tip) in centimetres. The cited maximum size for each species (in its entire range) was derived after reviewing a great number of literary sources, but most probably this review is not complete.

Abbreviations used: Mts. = mountains; vill. = village; geographic directions were denoted by their initials; in cases of presenting original data, the author names are given as initials. The Latin names of the species are given according to Speybroeck et al. (2020).

Table 1. Origin of data and number of measured specimens by species.

Species	Author's data	SmartBirds	Total
Xerotyphlops vermicularis	270	31	301
Eryx jaculus	15	2	17
Coronella austriaca	99	7	106
Dolichophis caspius	238	126	364
Elaphe quatuorlineata	29	-	29
Elaphe sauromates	9	46	55
Malpolon insignitus	74	28	102
Natrix natrix	119	16	135
Natrix tessellata	84	6	90
Platyceps collaris	3	5	8
Platyceps najadum	54	5	59
Telescopus fallax	10	-	10
Zamenis longissimus	233	31	264
Zamenis situla	40	16	56
Vipera ammodytes	922	28	950
Vipera berus	370	-	370
Total	2567	347	2916

Results and Discussion

Data on the maximum size of snakes are presented following a catalogue principle (families in systematic order, species in alphabetical order), by first giving the largest documented sizes for the respective species in Bulgaria, and then for the species in its entire range.

Family Typhlopidae

***Xerotyphlops vermicularis* (Merrem, 1820)**

Bulgaria: 33.2 cm (this study: Maleshevska Mts., SW Bulgaria, V.B.), 32 cm (Buresch & Zonkow, 1934), and 30.5 cm (this study: Maleshevska Mts., SW Bulgaria, V.B.).

Maximum for the species: 47 cm from the Cyprus Island (Grillitsch & Grillitsch, 1993 after Jan, 1864), but it should be borne in mind that the populations from Cyprus may represent a separate taxon in the *X. vermicularis* complex (see Kornilios, 2017).

Family Boidae

***Eryx jaculus* (Linnaeus, 1758)**

Bulgaria: 56.5 cm (this study: near Sandanski, SW Bulgaria, 15.05.2017, M. Stanchev pers. comm. to B.N.), 51.5 cm (this study: near Srem vill., SE Bulgaria, 12.08.2004, G.P.), and 46.5 cm (Müller, 1939).

Maximum for the species: 83.8 cm from Egypt (Flower, 1933) or 87 cm (Ananjeva et al., 1998 without providing precise locality or data source, and therefore it should be taken with caution).

Family Colubridae (sensu lato)

***Coronella austriaca* Laurenti, 1768**

Bulgaria: 76 cm (this study: near Ezeroto vill., Stara Planina Mts., 29.04.2012, N. Tzankov pers. comm. to G.P.), 75.6 cm (this study: near Balsha vill., Stara Planina Mts., 27.10.2016, A.D.), and 73.4 cm (this study: Maleshevska Mts., SW Bulgaria, 1973, V.B.).

Maximum for the species: 92 cm from Gyugavar, Azerbaijan (Nikolsky, 1916).

***Dolichophis caspius* (Gmelin, 1789)**

Bulgaria: 204 cm (Beshkov, 1981), 203 cm (Telenchev et al., 2019), and 202 cm (Stojanov et al., 2011). The size "2080 mm", given by Stojanov et al., 2011 (after Petrov et al., 2006) is based on misinterpretation (and incorrect summation) of data for a specimen from Gospodintsi (see page 896 in Petrov et al., 2006), so the following clarification is required (B. Petrov pers. comm. to B.N.): the measure "L_{body} = 168 cm" refers to the entire length of the snake, and not only to the body, despite that the tail length is given separately.

Maximum for the species: about 215 cm (injured tail) from the Samos Island, Greece (Cattaneo, 2003); without providing the data source, Arnold & Burton (1978) mention that in Europe, the species reaches to 250 cm, and this size is given later as maximal in many other publications (also without providing the data source), e.g. Engelmann et al. (1985), Ananjeva et al. (1998), Arnold & Ovenden (2002), Valakos et al. (2008), and Speybroeck et al. (2016).

***Elaphe quatuorlineata* (Bonnaterre, 1790)**

Bulgaria: 176 cm (Beshkov, 1981), 168.1 cm (this study: near Kresna, SW Bulgaria, 01.10.2018, A.D.) and 166 cm (this study: Pirin Mts., SW Bulgaria, 1971, V.B).

Maximum for the species: 180 cm from the Skiathos Island, Greece (Cattaneo, 1999); larger sizes are given by Speybroeck et al. (2016): 250 cm, and Böhme & Ščerbak (1993, after Bruno, 1966): 240 cm, but without providing any other information.

***Elaphe sauromates* (Pallas, 1811)**

Bulgaria: 175 cm (Buresch & Zonkow, 1934), 173.5 cm (this study: near Mladinovo vill., SE Bulgaria, 11.06.2012, N. Tzankov pers. comm. to G.P.), and 160 cm (this study: near Rechitsa vill., SE Bulgaria, 04.06.2012, D. Dobrev pers. comm. to G.P.).

Maximum for the species: about 180 cm (injured tail) from Cernavodă, Romania (Kiritzescu, 1901); without providing the data source, Fuhn & Vancea (1961) mention

260 cm, Speybroeck et al. (2016) – 250 cm, and Ananjeva et al. (1998) – 200 cm.

***Malpolon insignitus* (Geoffroy de St-Hilaire, 1827)**

Bulgaria: 167 cm (Pulev et al., 2018), 156.8 cm (Beshkov & Nanev, 2002), and 154 cm (this study: near Arkutino, Southern Black Sea coast, 24.06.2017, R. Georgieva pers. comm. to G.P.).

Maximum for the species: 200 cm from Jordan (Amr & Disi, 2011) and 182 cm from Egypt (Anderson, 1898); the larger sizes, mentioned in the literature, refer to the western part of the range of the genus *Malpolon*, which is occupied by *M. monspessulanus* (see De Haan, 1999).

***Natrix natrix* (Linnaeus, 1758)**

Bulgaria: 163 cm and 148 cm (this study: near Shipka vill., Stara Planina Mts., 11.06.2017, A. Sokolov and D. Alfrey pers. comm. to B.N.), about 145 cm (this study: near Konstantinovo vill., Thracian Lowland, 27.06.2012, G.P.; a part of the tail of this specimen was torn off, and the body length without the tail was 118 cm, so the total length given here, was derived by calculating the possible tail length according to published mean values for the proportion body/tail in females (see Kabisch, 1999)).

Maximum for the species: 205 cm from the Veglia (= Krk) Island, Croatia (Schreiber, 1912).

***Natrix tessellata* (Laurenti, 1768)**

Bulgaria: 114 cm (this study: near Kresna, SW Bulgaria, 1973, V.B.), 112 cm (Buresch & Zonkow, 1934; this study: near Karlukovo vill., Western Predbalkan Mts., 11.05.2019, A.D., M. Slavchev and N. Stanchev), and 111 cm (this study: near Kresna, SW Bulgaria, V.B.).

Maximum for the species: up to 200 cm in the Cyclades, Greece (Schreiber, 1912).

***Platyceps collaris* (Müller, 1878)**

Bulgaria: 74.4 cm (this study: near Primorsko, Black Sea coast, 2014, N. Tzankov pers. comm. to G.P.), 71 cm (Rehák, 1986), and 67 cm (Bartosik et al., 1981).

Maximum for the species: 112 cm and 111.9 cm from Jerusalem, Israel (respectively Rehak & Obst, 1993 and Rehák, 1986).

***Platyceps najadum* (Eichwald, 1831)**

Bulgaria: 152 cm (Stojanov et al., 2011), 150.3 cm (this study: Kresna Gorge, SW Bulgaria, 01.06.2014, A.D.), and 149 cm (Stojanov et al., 2011).

Maximum for the species: we could not find published data for larger size of this species, than the above mentioned (for example: 135 cm as a maximum length in Darewskij & Ščerbak, 1993, and 140 cm – in Speybroeck et al. 2016).

***Telescopus fallax* Fleischmann, 1831**

Bulgaria: 95 cm (this study: Maleshevska Mts., SW Bulgaria, 1971, V.B.), 90 cm (this study: Kresna Gorge, SW Bulgaria, 25.08.1991, B.N.), and 88.7 cm (this study: Kozhuh hill, SW Bulgaria, 1976, S. Vamporov pers. comm. to V.B.).

Maximum for the species: 120 cm from the former Yugoslavia (Grillitsch & Grillitsch, 1999 after Bruno & Maugeri, 1990) or 130 cm (Chmelík, 1992 without providing the data source).

***Zamenis longissimus* (Laurenti, 1768)**

Bulgaria: 176 cm (this study: near Dazhdovnitsa vill., Eastern Rhodopes Mts., 13.06.2018, G.P. and V. Vergilov), 162.6 cm (this study: near Chuypetlovo vill., Vitosha Mts., 28.04.2017, A.D.), and 154 cm (this study: near Konush vill., Eastern Rhodopes Mts., 2012, G.P.).

Maximum for the species: 225 cm from Krems, Austria (Luttenberger, 1978).

***Zamenis situla* (Linnaeus, 1758)**

Bulgaria: 104.6 cm (Moravec & Böhme, 2005), 103 cm (this study: Maleshevska Mts., SW Bulgaria, 1971, V.B.), and 99.5 cm (this study: Kresna Gorge, SW Bulgaria, 11.05.1998, B.N.).

Maximum for the species: about 116 cm (injured tail) and 112 cm from Italy (Bruno,

1969); without providing the data source, Speybroeck et al. (2016) give 120 cm as a maximal length.

Family Viperidae

***Vipera ammodytes* (Linnaeus, 1758)**

Bulgaria: 87 cm (Stojanov et al., 2011), 86 cm (this study: near Rupite vill., SW Bulgaria, 2003, G. Krastev pers. comm. to B.N.), 85 cm (Beschkov, 1977 and Stojanov et al., 2011).

Maximum for the species: 110 cm from Carinthia, Austria (Sochurek, 1974).

***Vipera berus* (Linnaeus, 1758)**

Bulgaria: 64 cm (this study: near the Ray hut, Stara Planina Mts., 1970, V.B.), 63 cm (this study: near the Mutorok peak, Pirin Mts., 1972, V.B.), and 62 cm (Buresch & Zonkow, 1932).

Maximum for the species: 104 cm from Sweden (Smith, 1919).

Conclusions

Our results indicate that presently in Bulgaria only three snake specimens have a confirmed length of slightly over 200 cm and they all belong to the species *Dolichophis caspius*. The other Bulgarian species could be grouped as follows: species with a maximum registered length of over 170 cm but below 200 cm (*Zamenis longissimus*, *Elaphe quatuorlineata*, and *E. sauromates*), species with a maximum length over 140 cm but below 170 cm (*Malpolon insignitus*, *Platyceps najadum*, and *Natrix natrix*), and species with a maximum length below 120 cm (the rest 11 species, including the two extinct vipers).

The maximum length for the three largest species across their whole ranges is given only in publications with scientific-popular character (i.e. not presenting original data or direct citations of other publications). Accordingly, the length of 250 cm for *D. caspius*, 260 cm for *E. sauromates* and 250 cm for *E. quatuorlineata* should be considered doubtful, at least until the specific sources of these data are identified. Our efforts in this regard were unsuccessful.

When comparing the maximum documented size of Bulgarian specimens with the published ones for the respective species in their entire ranges, it is evident that in Bulgaria most species do not reach a length close to the known maximum. This may be due to both the influence of specific environmental factors and the lack of detailed morphometric studies in the country. The only exception is the species *Platyceps najadum*, the largest known specimens of which are registered in Bulgaria.

Acknowledgements

We are grateful to the following colleagues who provided personal datasets of snake measurements: Anton Sokolov, Dylan Alfrey, Dimitar Plachiyski, Georgi Krastev, Krasimir Donchev, Martin Stanchev, Miroslav Slavchev, Nikola Stanchev, Ralitsa Georgieva, Vladimir Mladenov, Vladislav Vergilov, and to the late colleagues Nikolay Tzankov, Dobrin Dobrev, and Boyan Petrov. We thank Simeon Lukanov for the helpful suggestions and language corrections on the manuscript.

This study was partially supported by the Bulgarian National Science Fund under Grant contract "KP-06-N21/11" from 14.12.2018.

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Received: 26.05.2020
Accepted: 05.08.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 21-30

Innovative Bioremediation Technology of Lands Polluted with Chlororganic Pesticides

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Abstract. A complex of *ex situ* bioremediation technology proposed for lands contaminated with organochlorine pesticides, which differs from the existing ones by significantly lower material and time costs and accelerated return of polluted territories to the economy. To establish the contour of the pollution area it is proposed to use mathematical modeling of the pollution spread. The calculated dependencies for point and linear surface sources of pollution are established. The technology based on the use of humus as an affordable and inexpensive carrier of microflora, carrying out the metabolic decomposition of pesticides by microorganisms and accelerated low-cost determination of the volumetric configuration of land contaminated with organochlorine pesticides more of their standard concentration. It is established that the best bio-stimulation of autochthonous natural microflora in the studied process is 45% humus in a mixture contaminated with organochlorine pesticides.

Key words: ex situ bioremediation, organochlorine pesticides, low time and material costs, microflora carriers, pollution.

Introduction

The increasing global demand for food products has required intensification of their production (Royal Society, 2009). One of its directions was the development and use of pesticides in agriculture (Aktar et al., 2009). Their use increased the number of food products but caused an acute, ever-increasing and difficult to solve the problem of chemical pollution of land (Zhang et al., 2011), which, in turn, led to a deterioration in the quality of agricultural products and a decrease in food safety (Carvalho, 2017). The solution to this problem has long been recognized as an urgent task, but, nevertheless, the area of pesticide-contaminated land around the world is constantly growing. It is due to the lack of fairly simple, low-cost technologies that can quickly stop the migration of pesticides in the environment and quickly restore the contaminated area (FAO, 2011). This work is devoted to the development of just such a technology.

Organochlorine pesticides (OCPs) are among the most dangerous pollutants of

agricultural land because they have high toxicity, stability, long migration in ecosystems (Cernansky, 2015), and high cumulative effect (Aichner et al., 2013; Solovyanov et al., 2017).

For any content of OCPs in agricultural lands, it is necessary to remediate them, but urgent attention should be paid to plots of land contaminated with OCPs above their normative level since over time the area of dangerous pollution and all kinds of damage from it increase significantly (Yankevich et al., 2015).

We will conduct a brief critical analysis of the most common methods for soils, groundwater, and surface water treatment that are worldwide used:

- adsorption methods (Sakalova et al., 2019; Zelenko et al., 2019). These methods enable to achieve a high degree of purification but require a large number of adsorbents (the most perspective is the use of natural adsorbents: zeolites, bentonites, glauconites). Further utilization of spent sorbents is also a problem;
- reagent methods (Tulaydan et al., 2017). These methods enable to achieve almost complete purification but often require special equipment and conditions for the implementation of chemical reactions and require the use of special, often expensive reagents. Quite often there is a danger of secondary contamination by reaction products;
- biological methods (Malovanyy et al., 2016; Malovanyy et al., 2019) allow achieving the required degree of purification at a minimum cost. In the case of anaerobic methods, the decomposition of biomass is accompanied by the synthesis of biogas which is a valuable energy source (Nykyforov V. et al., 2016). The disadvantage is the significant duration of biological processes.

Numerous ex situ, in situ and on situ remediation technologies for contaminated land based on physicochemical, chemical, thermal, extraction and separation processes have been proposed, but their implementation limited by many factors, that reduces their effectiveness and use. Among these key factors are the technological complexity of the processes, significant material costs of implementation, and the likelihood of negative impacts on local natural processes (Yurchenko et al., 2014). Therefore, these technologies are not widely used in the practice of bioremediation of lands contaminated with OCPs and were used mainly on experimental sites.

During bioremediation of lands contaminated with OCPs, technologies based on the use of pesticide decomposition processes by various microorganisms in their metabolism are most prevalent. The main advantage of these technologies in ex situ, in situ and on situ implementations is the natural course of the processes and their low cost, and the common disadvantage is a long time of the destruction of OCPs, during which large areas of the contaminated area do not return to economic circulation. Also, with in situ and on situ technologies, prolonged migration and accumulation of OCPs in the components of the environment and the various negative consequences associated with this (Yurchenko et al., 2014) remain. In this regard, ex situ technologies are preferable.

The process of destruction of OCPs during bioremediation can be enhanced by using various biological products containing artificially obtained microorganisms destructors (Yurchenko et al., 2014). In this work, this direction, as a possible modern technology, was not considered, since it is in the stage of experimental research. The most promising at the present is a simple and inexpensive technology in which bio-stimulation of autochthonous microflora destructors carried out. It should be noted that this process in each case requires the development of its technological parameters since they directly depend on the type of land, microflora contained in it, types of OCPs and their concentration.

The most promising direction in the bioremediation of lands contaminated with OCPs is a simple and inexpensive composting

technology in which an environmentally friendly process of decomposition of environmentally hazardous products into non-toxic products occurs. A compost mixture prepared by mixing contaminated soil with biological mass. Livestock manure, bird droppings, activated sludge from municipal sewage treatment plants, alfalfa, hay, straw, peat, grass, etc. can be used as a biological mass (Yurchenko et al., 2014).

It should be noted that this process in each case requires the development of the composition of the compost mixture since it directly depends on the type of land, its level of pollution and the applied biological mass.

One of the key problems in the remediation of contaminated lands is the determination of their spatial configuration and volume. Existing approaches are based on the collection of a large number (up to several hundred) of samples by area and depth for contouring the pollution zone. At the same time, sampling from depths of more than 2 m is technologically complicated and highly expensive, although according to available data, soil pollution above standard values can be observed at depths of up to 6 m (Ukrainian Research Institute for Environmental Issues, 2013). Therefore, field studies, as a rule, make it possible to determine with sufficient accuracy only the area, but not the spatial configuration and the amount of pollution.

Therefore, the development of ex situ bioremediation technology for contaminated lands, devoid of these shortcomings, is an urgent and practically important task.

The study aimed to create a new ex situ bioremediation technology for lands contaminated with OCP, which differs from the existing ones by significantly lower material and time costs.

To achieve this goal, it was necessary to solve the following tasks:

– to create a way of the accelerated, low-cost determination of the spatial configuration of land contaminated with OCPs above the normative value;

– to develop a methodology for determining the composition of the mixture for composting;

– to develop a low-cost technological scheme of isolated bioremediation of lands with the accelerated return of contaminated territories to economic circulation.

Materials and Methods

Determination of the spatial configuration of the land contaminated with OCPs above the standard value. The need for the extraction and relocation of significant volumes of land, which is characteristic of ex situ technology, makes it important to reduce the time and money costs associated with determining the spatial configuration of the pollution area. Existing approaches are based on the collection of a large number (up to several hundred) of samples by area and depth for contouring the pollution zone. At the same time, due to technological and financial limitations, full-scale studies at depths exceeding 2 meters are rather rare.

In this paper, we propose a method based on the use of a mathematical model. In this case, full-scale studies are necessary to identify the parameters of the model, to verify it, and to carry out refinement analyzes on the calculated contour of the pollution area, which can significantly reduce the volume of full-scale studies and, therefore, the associated costs.

The standard concentration of OCP adopted as a criterion for determining the boundary of the pollution zone.

The proposed methodology developed for two types of surface sources of pollution - point and linear.

As a calculation basis in the case of a point source based on preliminary studies (Ukrainian Research Institute for Environmental Issues, 2013), an exponential regression model of the form was chosen:

$$C = C_{max} e^{\beta h + \alpha r}, \qquad (1)$$

where C – is the concentration of OCP at the calculated point, mcg/kg; C_{max} – the concentration of OCP in a point source, mcg/kg; r – the distance from a point source of pollution, m; h – depth, m, a and b – model parameters.

Taking into account that the boundary of the pollution zone is the achievement of the standard concentration of OCP Cs, based on equation (1), it is possible to determine the boundaries of the pollution zone. For this, we take the logarithm (1) and obtain:

$$\ln C_s = \ln C_{max} + \alpha r_s + \beta h_s, \quad (2)$$

where C_s – is the standard concentration, g/m^3; r_s – the radius of the area of pollution on the surface of the earth, m; h_s –the depth of the pollution zone, m.

Equation (2) shows that the pollution zone has the configuration of an inverted cone with an axis perpendicular to the surface of the earth and passing through the point of the pollution source (Fig. 1).

The characteristics of the pollution cone – the radius $r_{s\ max}$ and the height $h_{s\ max}$, , which determine the maximum depth and diameter of the pollution zone, can be determined from equation (2) with $h_s = 0$ and $r_s = 0$, respectively, and $C = C_s$:

$$r_{smax} = \frac{1}{\alpha} \ln \frac{C_s}{C_{max}}; \; h_{smax} = \frac{1}{\beta} \ln \frac{C_s}{C_{max}}. \quad (3)$$

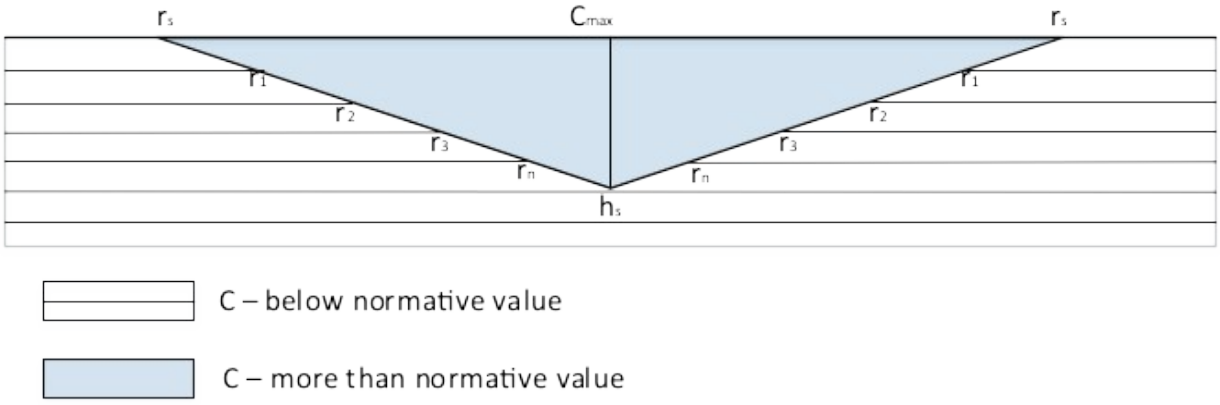

Fig. 1. Configuration of the pollution zone.

Determining the configuration of the pollution zone using the proposed model involves the following sequence of actions:

- based on a limited arbitrary set of field data of the measurements OCP concentrations are determined using the least square method and the parameters of the linear regression equation (2). Theoretically, four measurements are enough for this, but in practice, there should be more measurements (about 10-15). It is mandatory to have samples from various depths, although the depths themselves are not fundamental and may be limited to relatively small values (up to 1 m);

- an assessment made of the adequacy of the model based on one of the statistical criteria. As the latter, in particular, the coefficient of determination or the Fisher criterion can be used. In the absence of adequacy, an additional set of field data and the repetition of this stage are necessary;

- based on independent field data, the reliability of the model evaluated;

- based on the model, the boundaries of the pollution zone calculated.

The calculated values used to determine the control sampling points confirming the correctness or inaccuracy of the calculated data. In the latter case, the obtained data used to refine the model parameters and conduct repeated calculations with their subsequent control.

Thus, it is possible to use a small number of sampling points of soil components at an arbitrary distance, for example, from 1 m to 100 m from the center of pollution and, at a shallow depth, for example, from 0.1 m to 1 m, which significantly reduces the time of sampling and analysis samples and reduces the cost of this work.

The process of determining the spatial configuration of the area of contamination of soil components by a linear source of pollution is similar to the procedure for a point source of pollution. The difference lies in the use of a regression model, in which the coordinates of the calculated point will be characterized by three parameters measured relative to the center of the pollution line: depth and distance along the surface of the earth in directions parallel and perpendicular to the direction of placement of the linear source on the ground.

In this case, the regression equation represented as:

$$C = C_{max} e^{\beta h + \alpha f(x,y)}, \quad (4)$$

where x and y – are the distances along the surface of the earth from the center of the linear source in the directions parallel and perpendicular to the direction of its placement, respectively, m.

$$f(x,y) = \begin{cases} y & x \le d \\ \sqrt{(x-d)^2 + y^2} & x > d \end{cases}, \quad (5)$$

where d – is the distance from the center of the linear source to its boundary, m.

Then, after linearization, the equation for calculating the boundaries of the pollution zone will take the form:

$$h_s = -\frac{\alpha}{\beta} f(x_s, y_s) + \frac{1}{\beta} \ln \frac{C_s}{C_{max}}. \quad (6)$$

where x_s and y_s – are the coordinates of the boundary of the pollution zone on the surface of the earth, m.

The maximum depth of the contamination zone $h_{s\,max}$ was reached on the axis of the source $y_s = 0$ within its length $x_s = 0$ (for $x_s < d$):

$$h_{s\,max} = \frac{1}{\beta} \ln \frac{C_s}{C_{max}}; \quad (7)$$

The depth of the contamination zone within the length of the source decreases linearly in the direction perpendicular to the source. On the axis of the source outside, the depth of the contamination zone decreases linearly with distance from the source.

The boundary of the pollution zone on the earth's surface is determined from (6) at $h_s = 0$:

$$f(x_{s\,max}, y_{s\,max}) = \frac{1}{\alpha} \ln \left(\frac{C_s}{C_{max}} \right). \quad (8)$$

The contamination area in the plan limited by straight lines parallel to the source within its length and semicircles with centers at the ends of the source - outside its length.

Method for determining the composition of the mixture for composting. OCP biodegradation is the result of the inclusion of many organisms in the metabolic decomposition processes, but bacteria play a key role. The intensity of bioremediation processes depends on several factors. First of all, it is the presence of bacteria adapted to this type of OCP, and environmental factors - soil structure, temperature, pH, oxygen regime. Bioremediation is a technology aimed at creating favorable conditions for the growth of microorganisms that transform and decompose OCPs. In general, this technology may include the addition of microorganisms (bio-augmentation), the use of nutrients to stimulate natural microorganisms (bio-stimulation), the ventilation of the soil to saturate it with oxygen, and the removal of gases generated during the decomposition of OCP (bioventilation).

Preliminary studies of the authors showed that contaminated sites contain microorganisms adapted to OCP. However, usually, the use of OCPs in the metabolism of microorganisms cannot serve as a complete source of energy for them. Therefore, it is critically important to create conditions for bacterial cometabolism by introducing a substrate suitable for microorganisms with the formation of a compost mixture. Given the large volumes of land being restored, such a substrate should have accessibility and low cost. Therefore, for these purposes, affordable and cheap materials of natural origin were selected - peat, humus, and straw.

The method of determining the composition of the mixture for composting was developed by us (Yurchenko et al., 2014) and adapted to the conditions of the study. When developing a methodology for determining the composition of a mixture for composting, a compost mixture was prepared by mixing contaminated DDT soil with a biological mass in a quantitative ratio of 10 - 95% of contaminated soil to 30 - 70% of the biological mass. As a biological mass, cattle manure, straw, and peat were used.

Composting was carried out for seven weeks. The soil contaminated with DDT (180 mg/kg) was mixed with the biomass of the above species and placed in laboratory composters, 65 g of the mixture in each composter.

During composting, the mixture in test composters was saturated with oxygen (within 5 days) and nitrogen (within 2 days). To maintain the temperature in the range of 25-50 °C, the mixture was placed in an incubator. The humidity of the mixture was maintained at a level of 60 - 80% by adding water twice a week, and a pH of 5.25 - 9.0. After seven weeks, the samples were treated with methylene chloride and the resulting extract was analyzed on a chromatograph with an ECD detector to determine the residual content of DDT.

Development of a method for isolated bioremediation of contaminated OCP land above standard values. The authors (Yurchenko et al., 2014) developed and adapted to the research conditions a method of determining the composition of the composting mixture. The destruction of OCPs contained in the earth is proposed to be carried out in isolation from uncontaminated land, which will prevent the migration of pollutants during the process. Initially, it extracts the soil contaminated with OCPs above their standard value and mixes it with humus in the ratio of 55% and 45%, respectively. At the same time, on a non-economic territory, a corresponding depression is created with a waterproof bottom and walls and a mixture of contaminated land with humus placed in it. In the resulting isolated volume of the mixture, the destruction of OCP by microflora will occur. Isolation of the remediation volume of the earth prevents the penetration of OCPs with various natural aqueous solutions into unpolluted lands and interrupts their movement along trophic chains. Uncontaminated soil components that removed during the creation of the recess filled with a foundation pit formed after removal of soil components contaminated with OCP. The stages of the process of this remediation:

1. Determine the volumetric configuration of the land contaminated with OCP above the standard value.
2. Dig up the soil contaminated with OCP above the standard value, forming a depression. The excavated land moved to the non-economic territory.
3. Mix the displaced soil contaminated with OCP above the standard value with humus
4. On a non-economic territory, a recess is created for bioremediation of land contaminated with OCPs above the normative value, and its bottom and walls are insulated to stop the migration of OCPs.
5. Fill the isolated recess with a mixture of soil contaminated with OCP above the standard value, and humus, and fill the recess obtained during the excavation of the

soil contaminated with OCP above the standard value, not contaminated land removed when creating the recess with insulation.

Results and Discussion

Determination of the volumetric configuration of the OCP contaminated soil. During development of a method for fast, low-cost determination of the volumetric configuration of the OCP contaminated soil by a point source the plot studies data were obtained on agricultural territories of former pesticide storage warehouses in the Kharkiv Region, characterized by a small area which allows us to consider them as the main pollution sources. At present warehouses have been liquidated but the residual pollution of OCPs according to the monitoring data is many times higher than the standard values.

The use of the methodology is illustrated by the example of field data on the content of OCP in the soil in the vicinity of the liquidated warehouse in s. Dobropolie Valkovsky District of Kharkiv Region (Table 1).

Sample 1 - 4 and 6 were used to identify model parameters and evaluate its adequacy. Sample 5 was used to verify the model.

For all three indicators, dependences of the form (1) were obtained. An assessment of the adequacy by Fisher's criteria and determination coefficient confirmed the adequacy of the model. The values of the determination coefficient for a-HCH, b-HCH and g-HCH were 0,99, 0,96 and 0,9, respectively, and the values of the Fisher criterion are 88,9, 28,3 and 9,8, which exceeds the critical value of 9,3. The verification of the model showed that the predicted value calculated for control point 5 does not go beyond the boundaries of the confidence corridor. This made possible to use the model to determine the configuration of the pollution cone, in particular, to determine the diameter and depth of the pollution zone from relation (4) for each OCP. It allows us to determine the maximum boundaries of the contaminated area and the amount of land requiring bioremediation (Table 3).

The volume of land requiring bioremediation determined by the ratio:

$$V = \frac{\pi r_{s\max}^2 h_{s\max}}{3}, \qquad (12)$$

where V – is the volume of contaminated land, m^3.

Similarly, the configuration and volume of pollution zones were determined by DDT and its metabolites and HCH isomers for some of the sites in the Kharkiv Region. The results are shown in Table 2.

Field studies using the method of accelerated, low-cost determination of the volumetric configuration of the OCP contaminated soil by a linear source were obtained on the territory of the station warehouse of their former storage in the city of Khorol, Poltava Region. (Table 3). The dimensions of this warehouse (length 30 m) make it possible to consider it a linear source of pollution. Most of the warehouse building was destroyed, all pesticides were removed, but the excess of the standard value of OCPs continues to be recorded by field observations.

Sample 5 was used to verify model (5), and the rest to identify its parameters and evaluate adequacy. The obtained adequate model was used to determine the boundaries of the pollution zone for each OCP.

The choice of composition for composting. The data of the experiment to determine the composition of the composting mixture, carried out for seven weeks, are presented in Table 4.

Based on these data we can conclude that the best biostimulator of autochthonous natural microflora for the research is 45% humus in an OCP contaminated mixture.

Thus, a method for determining the volumetric configuration of OCP contaminated soil based on the development of a mathematical model for the case of point and linear surface sources of pollution is proposed and tested in practice. Its use

allows to reduce the cost and technically facilitate the process in comparison with the known methods (Ukrainian Research Institute for Environmental Issues, 2013), which provide for sampling at depths up to 6 m.

Table 1. Data from field studies of OCP (village of Dobropolye, Valkovsky District, Kharkiv Region).

Sample number	The distance from the center of the warehouse, m	Depth, m	OCP concentration, mcg/kg		
			α-HCH	β-HCH	γ-HCH
1	7	0.1	285.820	4188.594	142.334
2	7	0.55	216.593	1805.032	103.590
3	15	0.1	-	1383.032	135.505
4	15	0.55	77.873	-	-
5	17	0.1	102.889	1101.135	58.411
6	17	0.55	74.143	163.330	29.013

Table 2. Pollution zones from the former warehouses of OCP (Kharkiv Region).

Site	Pollution zone	Name of OCP					
		DDT	DDD (Derivatives of DDT)	DDE (Derivatives of DDT)	HCH		
					a	b	g
Spodobovka, Shevchenko District	Radius, m	23.59	21.37	19.47	17.48	26.19	13.14
	Depth, m	1.32	2.23	1.35	0.23	0.51	0.37
	Volume, m^3	769	1077	536	74	363	67
Dobropolye, Valkovsky District	Radius, m	30.98	22.77	30.71	16.9	28.3	23.0
	Depth, m	3.77	6.72	2.08	2.9	2	0.7
	Volume, m^3	3790	3648	2054	861	1660	396
Melnikovo, Valkovsky District	Radius, m	47.30	28.28	32.62	21.81	11.12	13.59
	Depth, m	0.63	0.75	0.62	0.36	5.09	0.09
	Volume, m^3	1476	627	694	177	660	18

Table 3. The content of OCP in the soil in the vicinity of the warehouse of their former storage (Khorol City, Poltava Region).

Sample number	The distance from the center of the pollution line, m			The content in the soil, mcg/kg		
	parallel to the source line	perpendicular to the source line	depth	α – HCH	γ- HCH	DDE
1	7.5	5	0.2	1282316.7	446169.8	194140.0
2	0	15	0.2	1838381.1	112432.1	65229.4
3	5	17	0.2	93878.4	3366.9	7031.9
4	0	7.5	0.2	1387507.7	272928.1	522536.1
5	0	21	0.2	24338.7	23921.2	40059.4
6	12	61	0.2	317.9	18.3	152.0
6	12	61	0.55	80.3	7.9	22.3
7	19	52	0.2	5456.8	2364.7	766.2

Table 4. The efficiency of using various types of biological mass in the composting mixture for bioremediation of contaminated OCP land.

The composition of the mixtures, %				OCP mass reduction,%
OCP contaminated soil	Peat	Humus	Straw	
25	1	37	37	91,0
35	0	65	0	56,9
45	0	45	10	96,1
50	0	20	30	96,3
55	0	45	0	98,3
60	0	35	5	93,0
60	0	40	0	98,0
60	0	40	0	98,0

The best available, inexpensive carrier of microflora that feeds on OCP: peat, humus and a straw carrier of natural microflora are defined as 45% humus in an OCP contaminated mixture. This exceeds the cost and technical performance of known compost methods (Chabanyuk et al., 2016.). The developed ex-situ method with isolated bioremediation of contaminated OCP land above their maximum permissible concentrations differs from the existing ones by significantly lower material and time costs and accelerated return of formerly contaminated lands to economic circulation.

Conclusions

A method has been developed for the accelerated, low-cost determination of the volumetric configuration of the land contaminated with OCPs over their normative value, by point and linear sources of pollution. A technique for determining the composition of the compost mixture is proposed. A method of remediation of lands contaminated with OCPs is presented, which is distinguished by its simplicity and quick return to the economic circulation of contaminated lands.

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Received: 19.03.2020
Accepted: 18.08.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 31-41

Individually Marked Eurasian Griffon Vulture (Gyps fulvus) Occurrence Phenology in the Eastern Rhodopes, Bulgaria

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Abstract. The Eurasian Griffon Vulture is a large raptor species with a vast range. The species is breeding in Bulgaria and its population is estimated at more than 100 pairs distributed in several sites in the country nowadays. The aim of the current study was to track the resighting frequency, age and phenology of presence of marked Griffon Vultures in the Eastern Rhodopes, Bulgaria and possibly to record the origin of the tagged individuals. During the study (n=10 years) we recorded 1888 observations of 231 marked Griffon Vultures in total. The number of vulture resightings was 42 ± 8.4 marked individuals per year. The age of 80% of the recorded Griffon Vultures was established. Adult vultures had the highest resighting rate (0.35 ± 0.03). Most of the observed marked Griffon Vultures were recorded during the autumn season, especially the immature birds. The largest portion (n = 1343 resightings) were of Griffon Vultures marked in different parts of Bulgaria. Our results can be easily complemented with GPS telemetry data to reveal entirely movement ecology of Griffon Vulture in the Eastern Rhodopes.

Key words: resighting, *Gyps fulvus*, wing tag, resighting frequency, tagging origin.

Introduction

The Eurasian Griffon Vulture, *Gyps fulvus* (Hablizl, 1783), is a large Palearctic, Indohimalyan and Afrotropical Old-World vulture, classified as 'Least concern' spreading over 18M km^2 globally (Birdlife international, 2020). Its distribution is land locked between Portugal and Nepal (Grubach, 2014). The global population of the species is estimated at 648,000-688,000 mature individuals (Birdlife International, 2020) unevenly distributed among species' populations within the range (Grubach, 2014). The European population of the Griffon Vulture is estimated at 32 – 34 thousands of pairs. The species population marked a nearly 200% increase in its size during last few decades (Birdlife international, 2020). The largest population of the species both in Europe and worldwide is the one in Spain (Botha et al., 2017). The historical range and size of the Griffon Vulture population in the Balkans are well studied. The species has fragmented distribution and size in Bulgaria, Croatia, Greece, North Macedonia and Serbia (Andevski, 2013). Following the rediscovery of the species in Bulgaria in 1978 and the long-term conservation program in the area, more than 90 pairs are breeding in the Eastern Rhodopes, Bulgaria nowadays (Dobrev et al., 2019). The national population is estimated to

number 105 – 118 pairs in 2018 with 25 pairs in 3 of the release sites and over 80 pairs in the Eastern Rhodopes (Stoynov, 2019). The species is listed as „Endangered" in the Red Data Book of the Republic of Bulgaria, in Annex I of the Birds directive and is also strictly protected by the National Law (Annex 2 and 3 of the Biodiversity Act) (Golemanski, 2015).

Individual marking is a specific approach widely used to estimate birds' survival, breeding biology, behaviour, age related mortalities, longevity of birds and their phenology (Sweeney et al., 1985). Wing tags and rings are often used in studies for number of bird species. Based on markings and capture re-capture techniques survival rates of vultures can be obtained (Monadjem et al., 2012; Monadjem et al., 2014). Such methods are further applied in reintroduction projects to explore vultures' dispersal (Mendelssohn & Leshem, 1983; Le Gouar et al., 2008; Stoynov & Bonchev, 2012; Peshev et al., 2018). Markings are an useful tool to study migration (Young & Kochert 1987), foraging movements and occurrence phenology of different vulture species (Charalambou, 2003; Botha, 2007; Zuberogoitia et al., 2013).

The aim of the current study was to survey the frequency of resighting rates and occurrence phenology of the marked Griffon Vultures in the Eastern Rhodopes, Bulgaria, their age and origin. Marked Griffon Vultures can bear not only biological information, but also to reveal data on different threats. Therefore, our results can support future conservation studies and actions in the Eastern Rhodopes and elsewhere.

Materials and methods

Study area. The study was conducted in the Eastern Rhodope Mountains, Bulgaria (Fig.1). This low mountainous area is approximately 5,900 km^2 in range and is characterized by high diversity of natural habitats, rich biodiversity and human population with a low density (Kopralev, 2002). The mean daily temperatures in winter vary between + 1^{0}C - + 2^{0}C, while the summers can be relatively hot and dry with mean daily temperatures of 24 - 25^{0}C. The yearly temperature amplitude is small with an annual rainfall in the area between 450 and 900 mm. Days with permanent snow cover reach 20 annually (Kopralev, 2002). The area retains the highest diversity of breeding raptor species in Bulgaria (Stoychev et al., 2004).

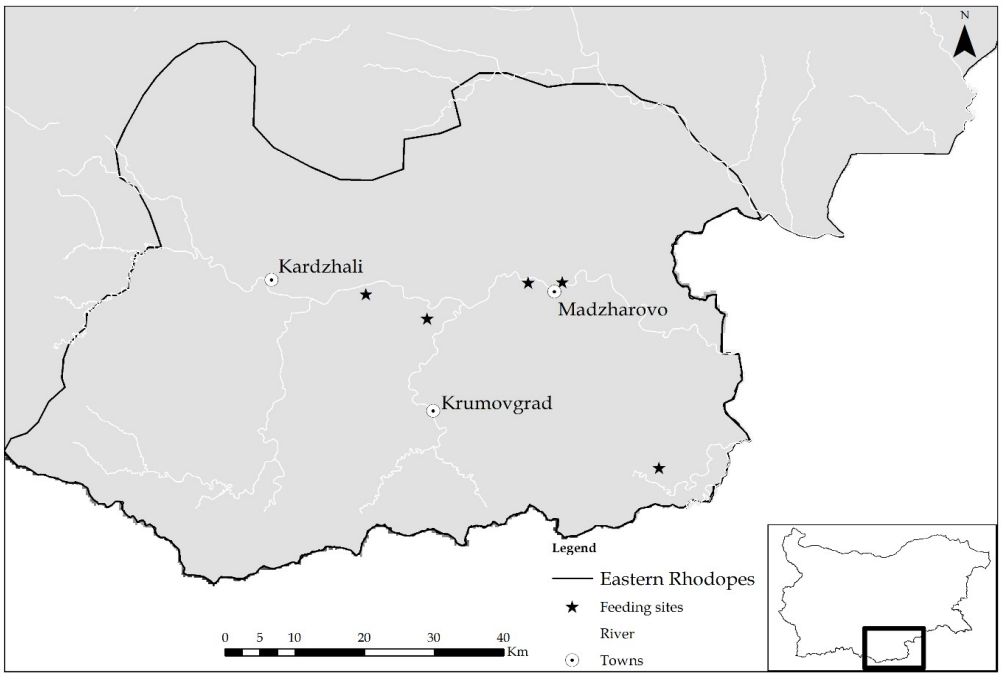

Fig. 1. Map of the study area and the feeding stations.

Data collection methods. The study was implemented in the period 2009 – 2018. All observations were conducted during supplementary feedings with a frequency of minimum of 2 feeding events per month. The observations were conducted at four of the feeding sites (Fig.1) with different frequency (42 ± 11.3 observation events per year; range 2 - 108). The observations and data collection that followed the feedings were carried out up to the entire consumption of the delivered food. The observations were done from a stationary view point, distanced far enough to avoid disturbance (Zuberogoitia et al., 2008) and with spotting scopes. The observer recorded the start of the count and at any round hour the number, age and markings of the vultures. Griffon Vultures were divided into 4 age classes (adult, subadult, immature, juvenile) (Blanco et al., 1997). Information on the type of the marking (wing tag, ring, transmitter), the color, digits, and its position (left – right; wing - leg) was recorded. Observations were held only in days with favorable weather conditions. In order to improve the data collection trail cameras were used since 2016. They were deployed to record the number and details of the marked vultures and ease the monitoring whenever the conditions were not suitable or supporting view point monitoring was absent. The trail cameras (n = 1 to 3) were deployed at distance about 5 - 10 m from the carcass so that they could photograph vultures without causing disturbance. The data on the Griffon Vulture age and markings were collected in blank sheets up to 2016 and via the SmartBirds Pro mobile application after that (Popgeorgiev et al., 2015).

Data analysis. The mean values were retrieved through descriptive statistics. The resighting rate was calculated as a proportion between the numbers of the observations of a given individual or age group, divided by the total number of observations in the given year/period. The seasonal distribution was set as follows: winter (December, January, February), spring (March, April, May), summer (June, July, August) and autumn (September, October, November). All vultures' resightings with rings and wing tags that were not entirely read were excluded from the sample analysis.

The significance values were set up to $p < 0.05$ for all tests. Mean values were presented with standard error (SE). Statistical analysis was performed using the Past software package (Hammer et al., 2001). Spatial data were mapped and processed using QGIS software (QGIS Development Team, 2016).

Results

Count and occurrence phenology. During the study we recorded 1888 observations of 231 marked Griffon Vultures during 424 monitoring events. The least number of marked birds was registered in 2010 (n=2) and the highest (n=79) was recorded in 2018 (Fig.2). The number of birds marked with rings was 36 and those marked with wing tags was 195. Some of the Griffon Vultures were both tagged with rings and wing tags.

We recorded 42 ± 8.4 marked Griffon Vultures per year. The mean resighting rate in the period was 0.09 ± 0.004. The resighting rates of marked Griffon Vultures had the highest values in 2010 (0.5 ± 0.001) and the lowest in 2016 (0.06 ± 0.01).

Age structure and phenology. The age of 80% of the recorded Griffon Vultures was established. Adult vultures had the highest resighting rate (0.35 ± 0.03), followed by the immatures (0.3 ± 0.05), subadults (0.06 ± 0.01) and the juveniles (0.04 ± 0.01). The age of 20 % of the Griffon Vultures could not be determined (Fig. 3).

Most of the observed marked Griffon Vultures were recorded during the autumn season. However, adult birds were relatively constant throughout the year, whereas non-adult (juveniles, immatures and subadults) birds were mainly recorded in the autumn (Table 1).

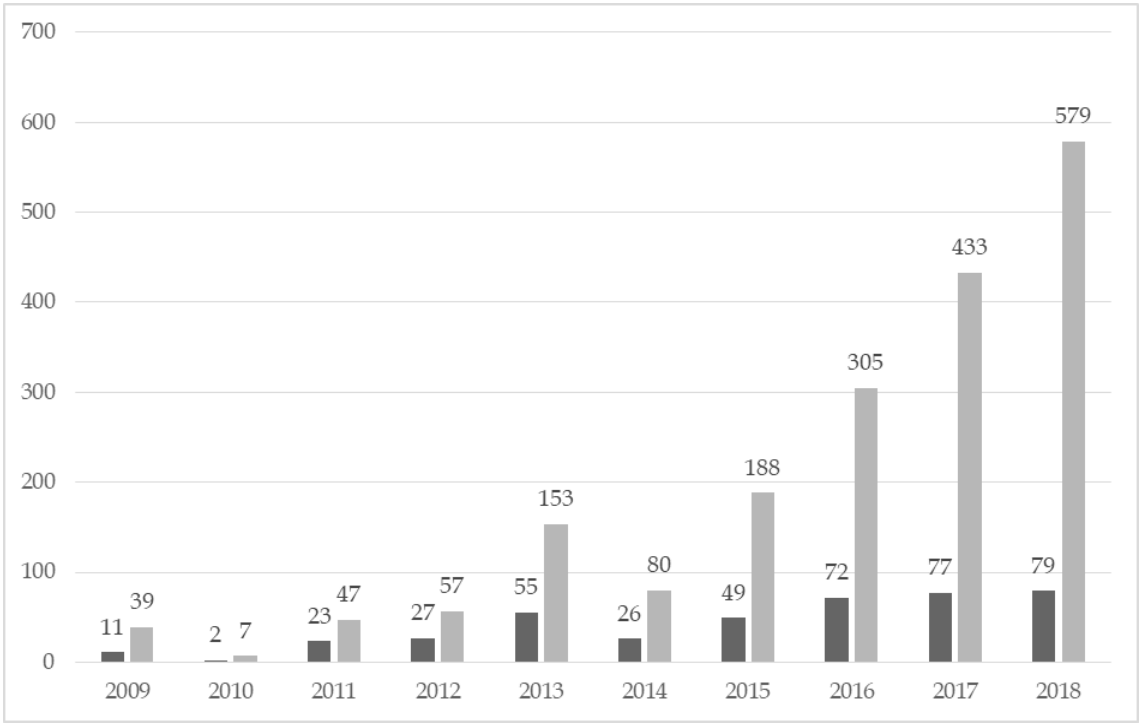

Fig. 2. Number of the registered marked individuals in the study area (black bars) and the number of resightings per year (grey bars).

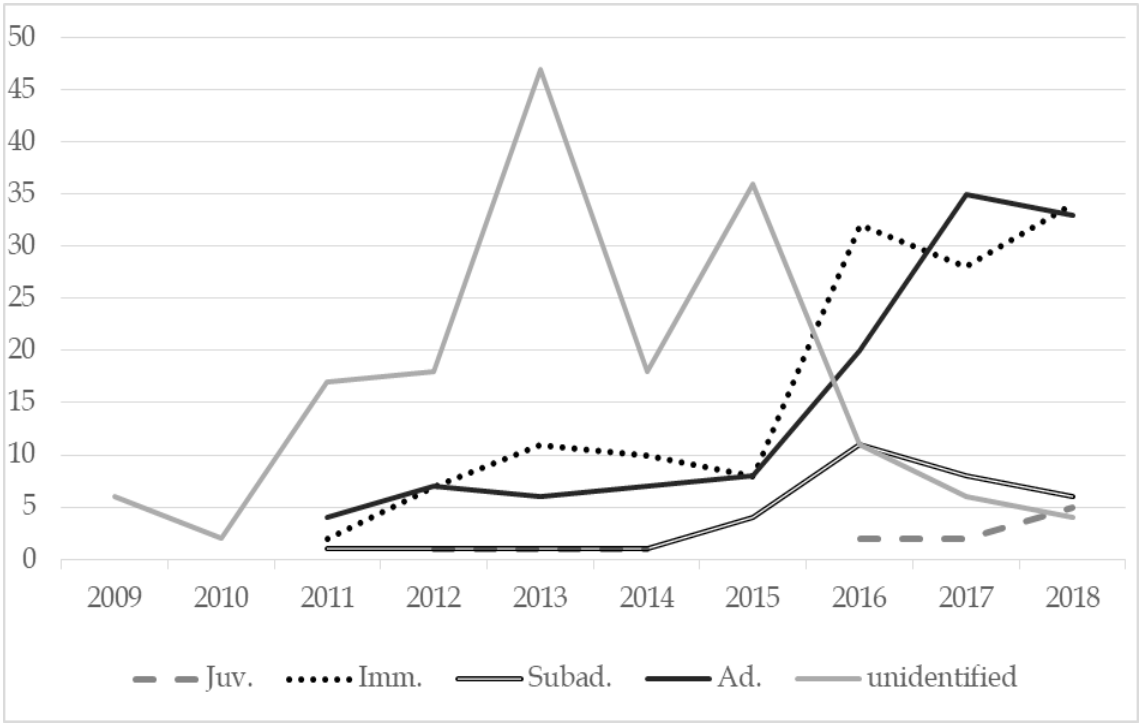

Fig. 3. Number of the observed marked Griffon Vultures by age classes during the study period.

Table 1. Percentage of marked vultures in the different seasons.

Season	Winter	Spring	Summer	Autumn
Non-adults	22.09%	26.72%	18.17%	33.02%
Adults	21.90%	26.59%	22.36%	29.15%
Total	22.01%	26.66%	20.01%	31.32%

Table 2. Number of the resightings by the place of marking of the Griffon Vultures.

Site of marking	Count of resightings	Percentage	Number of individuals
Eastern Rhodopes	589	31.2	40
Central Balkan	128	6.8	12
Vrachanski Balkan	112	5.9	13
Sinite Kamani	83	4.4	12
Kresna	80	4.2	8
Kotel	77	4.1	8
Reintroduced not specified	274	14.5	59
Bulgaria	**1343**	**71.1**	**93**
Dadia	180	9.2	23
Nestos	1	0.1	1
Greece	**181**	**9.6**	**24**
Hai Bar Carmel NP	65	3.4	5
Sde Boker	47	2.5	11
Gamla NP	29	1.5	4
Israel not specified	37	1.8	16
Israel	**178**	**9.4**	**33**
Serbia – Uvac	**103**	**5.5**	**25**
Unknown	**83**	**4.4**	**10**

Fig. 4. Locations (black dots) where Griffon Vulture marking schemes were applied regularly.

Marked birds origin. The largest portion of the resightings (n = 1343, 71.1%) were of Griffon Vultures marked in various parts of Bulgaria. We recorded 181 resightings of birds marked in Greece, 178 resightings of vultures marked in Israel and 103 resightings of birds marked in Serbia. We were not able to track and find information on the origin and sampling site for 10 of the Griffon Vultures (4.4%) recorded during the study (Fig. 4).

The resighting rate of Griffon Vultures in the Eastern Rhodopes marked in Bulgaria was 0.7 ± 0.1, Greece – 0.1 ± 0.01, Israel – 0.09 ± 0.02 and from Serbia – 0.05 ± 0.01.

Discussion

The increased number of observations of marked Griffon Vultures is a result of the increased species population, the increased monitoring effort and the larger number of marked individuals during the last years. Nearly 350 birds were released in the reintroduction program for the species in 4 former breeding sites in Balkan Mountains and Kresna gorge in Bulgaria since 2009 and all were individually marked (Stoynov, 2019). This increased the chances of observing marked birds as some of the released vultures moved into the natal colonies in the Eastern Rhodopes (Stoynov & Peshev, 2011, 2012, 2013, 2014; Peshev & Stoynov, 2015; Stoynov et al., 2015). The population in the Eastern Rhodopes has grown considerably in the last 25 years (Demerdzhiev et al., 2014) and 52 individuals have been marked and tagged until 2019 (authors unpublished data). Adult birds from the native population rarely wander outside their natal areas as revealed by GPS telemetry (BSPB unpublished data). Normally, such birds have much higher resighting rates. Furthermore, we have registered Griffon Vultures, marked in Greece and breeding in the Bulgarian side of the mountain. Such shifts were registered before and prove that vultures can exchange between the Bulgarian and the Greek side of the Eastern Rhodopes (Demerdzhiev, 2007; Skartsi, 2009). On the other hand, juveniles and immatures can roam for several years before start breeding (Zuberogoitia et al., 2013) reaching as far south as Africa (Arkumarev et al., 2019) and the Middle East. Our results confirm that some immature birds observed in our area are marked in other areas of the species range from Bulgaria and the Balkans – Greece, Serbia and reintroduced birds considerable part of the juvenile Griffon Vultures from the Balkans, take yearly roamings. They start in September – November and explore sites further away from the natal colonies in Asia Minor, Arabian Peninsula, Northern Africa and Sahel region (Griesinger, 1998; Susic, 2000; Grubach, 2014; Tsiakiris et al., 2018; Arkumarev et al., 2019). Similarly, over 90% of the juvenile birds in Spain leave their natal colonies in October and minimum of 30 % of them migrate to Africa (Griesinger, 1998). This stage of vultures' life is essential in gaining experience, sexual maturity and enriching social interactions (McGrady & Gavashelishvili, 2006; Xirouchakis & Andreou, 2009; Garcia-Ripolless et al., 2011). Moreover, long-term stability of the population could be strongly affected by the juvenile survival (Van Beest et al., 2008). In spring some juveniles, can return back to the natal colonies where they stay mostly around the feeding sites and breeding colonies. However, as those individuals are not breeders they can roam over vast areas and thus become more vulnerable to different threats. Our data showed that large groups of vultures, especially immatures and juveniles can congregate at the feeding sites from September to November. Hence, birds arriving in the Eastern Rhodopes in this period might stay wintering in Bulgaria where they find abundant food and conspecific presence. Therefore, the Eastern Rhodopes may be of great importance for the survival of juvenile and immature birds wintering there and thus support the high demography rates of their natal colonies. Our study showed that some juveniles from Serbia remain to winter in the Eastern Rhodopes. Griffon vultures from the

reintroduced population were also registered wintering, but also breeding either alone or in a mixed pairs with birds from the natal colonies in the Eastern Rhodopes. A Croatian study showed that marked immature Griffon Vulture was registered in Bulgaria, travelling almost 1,000 km from its natal colony in Croatia (Susic, 1994). In our study we did not register any birds of Croatian origin. Probably due to the fact that the birds are marked with rings which are far more difficult to record than wing tags. Being in small numbers they may have remained unrecorded.

Subadult vultures in our study area are an important part of the population although only 19% of the resighted vultures belonged to this age group. However, this might be a consequence of the sample size, species demography and the survival particularly. According to García-Ripollés et al. (2011) subadult birds are able to roam over large areas. This may play a crucial role in their survival and gaining experience. Therefore, age structure and survival of the population are obligatory to understand population demography and development (Newton, 1979; Steenhof & Newton, 2007).

We demonstrated that significant part of the immature vultures observed in our study area in spring were marked in Israel. We hypothesize these are mostly birds of Balkan origin, wintering or migrating through Israel and the Middle East. Immatures return back to their natal colonies in spring. This was confirmed by the current results and our long-term monitoring, but also data from Israel where less Griffon Vultures were established in spring in comparison to the autumn and winter seasons (Choresh et al., 2019). For example, Griffon Vultures marked in Israel are currently breeding in the Eastern Rhodopes. The same phenomena were observed in other countries as well and may be attributed to the spring migration of some of the Griffon Vultures due north (Xirouchakis, 2007; Boshoff et al., 2009). The autumn migration of the Griffon Vultures from Serbia and Croatia pass through Bulgaria over a wide front towards the Bosphorus (Fulop et al., 2014). Some of these vultures are most likely heading Greece to the south to winter as well. We can argue those are mainly Croatian birds as such were not established in the Eastern Rhodopes in our study. They might be passing in lower numbers and thus are difficult to record outside the feeding sites, though. However, our study underlines the importance of the Eastern Rhodopes as a hot spot for the Griffon Vultures. The high availability of free-ranging domestic livestock and abundant game species there determines the presence of the Griffon Vulture (Boev & Michev 1980; Arkumarev et al., 2019.). Furthermore, supplementary feeding sites in the area supply regular and safe food for the vultures. Because of the spatial and temporal unpredictability of the carcass, it is very difficult to record marked Griffon Vultures outside feeding sites. Therefore, feeding sites can be used to manage and collect important information on the vulture age structure and numbers and act as main monitoring and conservation tool (Terrasse, 1985; Houston, 1987; Piper, 2005). The supply of safe and high-quality food can reduce some negative factors causing mortality of scavengers e.g. poisoning (Robb et al., 2008; Moreno-Opo et al., 2015). Supplementary feeding stations can maintain higher breeding rates of vultures (Gonzalez et al., 2006), increase the important nutrients level such as calcium (Piper, 2005) and decrease the chance of poisoning (Grande, 2006; Oro et al., 2008).

Results of the current study prove the importance of the marking methods to reveal the phenology of the Griffon Vulture. It complements other studies from Europe, the Middle East and Africa (Mendelssohn & Leshem, 1983; Botha, 2007; Monadjem et al., 2012; Monadjem et al., 2014; Peshev et al., 2018) and provides good insight for processing resighting type of data. Our results can be easily combined with GPS

telemetry data to create comprehensive spatial study for the Griffon Vulture movement ecology in the Eastern Rhodopes. This will reveal important demography rates such as survival and population recruitment. Such knowledge will establish a sound base for further population viability analysis to project the Griffon Vulture population trend and outline respective conservation measures.

Conclusions

Monitoring of feeding sites is important tool to trace Griffon Vulture population dynamics and to record resighting rates of marked individuals (231 marked vultures in our study with various origin). Wing tagged birds were easier to record in comparison to birds marked with only rings, however, the use of trail cameras can significantly improve the monitoring in this regard. The rate of observations differed among age classes and was seasonally determined. Therefore, a combination of monitoring methods to record marked vultures and GPS technologies can bring knowledge on the population status, spatial ecology and phenology, vultures origin, threats and also help to calculate mortality rates and survival within different age classes and the entire population.

Acknowledgments

The current research was financed by National program "Young scientists and Postdoctoral candidates" funded by the Bulgarian Ministry of Education and Science. This work was supported by the LIFE project "Conservation of Black and Griffon Vultures in the cross-border Rhodopes mountains" (LIFE14 NAT/NL/000901) funded by the European Union and co-funded by Rewilding Europe and by the project "Conservation of large vultures in the Eastern Rhodopes", ran under operational program "Environment". We are grateful to the following colleagues without whom the current study would not be possible: Dimitar Demerdzhiev, Hristo Hristov, Ivaylo Angelov, Marin Kurtev, Nedko Nedyalkov, Nikolay Terziev, Vladimir Dobrev, Vladimir Trifonov, Vera Dyulgerska. We wish to express our gratitude to the colleagues from Green Balkans, Fund for wild Flora and Fauna, Birds of Prey Protection Society, Management Body of Dadia-Lefkimi-Soufli Forest National Park, Greece, WWF Greece, Rigas Tsiakiris – Greece, Birds of prey protection foundation – Serbia, Israel Nature and Parks Authority and Goran Susic – Croatia for the information on the vulture resightings, Bulgarian society for the protection of birds.

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Received: 10.05.2020
Accepted: 18.08.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 43-52

Ancient Burial Mounds – Stepping Stones for Semi-Natural Habitats in Agricultural Landscape

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Abstract. Recent agricultural intensification followed by land homogenization require for connectivity of remaining semi-natural habitats for biodiversity preservation. This study is the first attempt to assess the importance of Bulgarian ancient burial mounds (kurgans) as refuges for semi-natural vegetation and for the connectivity to other semi-natural lands based on remote sensing data. A set of 509 kurgans were selected and their main vegetation cover and the landscape within 200 m surrounding buffer were studied. The general kurgan characteristics as height and diameter of the base resemble dimensions reported for Ukraine and Hungary. Our results show the high level of kurgan isolation in Bulgaria. More than the half of the studied kurgans surroundings are occupied by over 75% of agricultural and urban or other artificial landscape. This finding emphasizes the kurgans' role for preservation of semi-natural habitats and their inhabitants. We highlight the importance of Bulgarian kurgans that save a cumulative area of semi-natural type equal to the 0.09% of the whole country territory. Kurgans could be treated as relictual landscape in highly modified matrix. The importance of kurgans for biodiversity conservation should encourage the local community to increase the education and activities for proper management ensuring their further protection.

Key words: fragmentation, habitat islands, isolation, kurgans, landscape heterogeneity, small natural features.

Introduction

Current tendency for creating large continuous agricultural lands unifies the landscape, resulting in structural and functional homogenization (Benton et al., 2003; Gámez-Virués et al., 2015; Buhk et al., 2017), fragmentation of natural and semi-natural areas (Saunders et al., 1991), and habitat and biodiversity loss (Tilman, 1999; Williams et al., 2009). This fragmentation imposes difficulties for the species' populations to sustain and disperse through the surrounding homogenized matrix and to a pronounced edge effect, which suggests

intrusion of more weeds from the adjacent arable fields (Hobbs & Yates 2003). The importance of small natural features has been emphasized for increasing heterogeneity (Tscharntke et al., 2002; Hunter, 2017). Yet, small natural features, especially when taken collectively, can also play an important role in biodiversity conservation and the provision of ecosystem services (Poschlod & Braun-Reichert, 2017). Semi-natural landscape elements in or around the crop fields such as hedges and vegetation strips serve as refuges for native species, as safe feeding areas for the fauna, and corridors for the movement of animals and dispersal of plants across a predominantly non-natural landscape (Benton et al., 2003). They also have the potential to positively influence the crop pollination as they attract pollinators and host them outside the crop flowering period (Wezel et al., 2014).

The ancient burial mounds (called also tumuli or more commonly kurgans) should be considered small semi-natural landscape elements as they are earth or earth-stone formations built from a local substrate. In the territory of Bulgaria numerous ancient burial mounds have been created as a result of the Yamna culture and Thracian civilization. The total number of kurgans in Bulgaria exceeds 50,000 (Kitov 1993) which rates the country at the forefront of kurgan numerosity in Europe together with Hungary and Ukraine (Deák et al., 2016b). The appearance of kurgans was dated back to the end of 4th millennium BC and their creation kept being a common practice in Thracian funeral rituals up to the 4th century AD, when the Christianity became a leading religion. These landscape features have been preserved for millennia both due to their historical, sacred and cultural significance and due to their steep slopes, being inappropriate for tillage (Fig.1). Recently there is growing interest about the conservation importance of kurgans for general plant diversity and steppe specialists preservation (Moysiyenko et al., 2014; Sudnik-Wójcikowska & Moysiyenko, 2012 ; Deák et al., 2016b, 2018, 2020). Several parameters of kurgans may affect their potential for maintaining semi-natural vegetation: size, degree of isolation from other semi-natural vegetation types and degree of woody plants encroachment (Deák et al., 2016a; Dembicz et al., 2016).

Fig. 1. Examples of Bulgarian kurgans, surrounded by agricultural land. Photos by I. Apostolova and N.Velev.

This study is the first attempt to evaluate the importance of kurgans as small landscape features for the conservation of semi-natural habitats in Bulgaria. The objectives of this study are: (i) to provide general characteristics of Bulgarian kurgans, (ii) to assess the degree of their isolation and (iii) to assess to what extend their existence enriches the local semi-natural habitats.

Materials and Methods

Out of 50,000 kurgans reported by Kitov (1993) nearly 11,000 are included in the National Archaeological map with database (naim-bas.com/akb). We randomly selected a set of kurgans from this map occurring throughout the country and that have not been subjects of archaeological research (i.e. their vegetation was not destructed in excavation procedure) (n = 577). The geographic location and current presence of all selected kurgans was verified both by aerial photos using digital orthophoto map of Bulgaria (with accuracy 1.5 m, provided by Ministry of Agriculture, Food and Forestry) and topographic maps (in scale 1:5,000 and 1:10,000 elaborated by Geodesy, Cartography and Cadaster Agency). During this verification procedure, 68 kurgans were excluded from further analyses because their existence was not confirmed.

We created a GIS layer for remaining 509 , using ArcGIS version 10.3 (ESRI, 2014) in which they are represented as polygon features. Attributes such as geographic coordinates, altitude, area, vegetation cover and land cover type of surrounding areas were associated with each of them. The height of each kurgan was estimated as a difference between the altitude of their base and top isohypses. Their dimensions were calculated as a 2D areas in ArcGIS. We estimated visually the relative cover (%) of grassland and woody vegetation on the kurgans by satellite images available at Google Earth (google.com/earth). Kurgans with woody cover ≤ 10% were considered grassy (n = 176); kurgans with grassy cover ≤ 10% were considered woody (n = 115), and those with intermediate woody/grassy cover were considered mixed (n = 218). The 10% threshold was chosen to facilitate visual determination on Google Earth images.

We created a buffer area with a radius of 200 m bigger than that of the kurgan base (Fig. 2) and calculated the amount of natural versus non-natural habitats in the land cover within the buffer to estimate the degree of kurgans' isolation. A radius of 200 m was chosen to reflect the dispersal limitations of vascular plants considering the statement of Cain et al. (2000) that 100 m are a 'long distance' for seed dispersal. The land cover types in the buffer are taken from the database of the Land Parcel Identification System (LPIS), maintained by the Ministry of Agriculture, Food and Forestry. The LPIS is part of the Integrated Administration and Control System (IACS), which has been developed in all EU Member Countries in accordance with the main European Union (EU) and European Commission (EC) regulations. This database is kept up-to-date, because it is used to ensure that EU agricultural subsidy procedures are properly implemented. The land cover layer is with good spatial resolution, with minimum mapping unit 0.1 ha. The layer is digitized on the basis of deciphering a digital orthophoto map of Bulgaria. LPIS uses nomenclature of 37 land cover types, which we grouped into 6 categories: (1) urban and artificial areas, (2) annual crops, (3) perennial cultivation (perennial crops, orchards, vineyards), (4) forests, (5) grasslands, (6) other semi-natural land. For the purpose of this study, we defined categories 1–3 as non-natural and categories 4–6 as natural. The link between the original 37 LPIS land cover types and our six categories is available in the Appendix.

We then checked to what extent the kurgan is isolated from the surrounding habitat types. We gave different weight to each LPIS land cover type based on its potential to serve as source of propagules for

the predominant habitat on the kurgan: 1 – LPIS land cover represents a habitat similar to the predominant habitat of the kurgan, and thus is a good potential source of propagules (e.g. pastures for the grassy kurgans, forests for the woody kurgans); 0 – the surrounding LPIS land cover types are very different habitats, or are not natural, and thus are not considered as a potential source of propagules for the predominant habitat on the kurgan (e.g. grasslands in the buffer get 0 for woody kurgans, and forests in the buffer get 0 for grassy kurgans; non-natural land cover gets 0 for both grassy and woody kurgans); 0.5 – the LPIS land cover type is a semi-natural habitat that has some potential to serve as a source of propagules for the predominant type of habitat on the kurgan (e.g. river banks in the buffer get 0.5 for woody kurgans, and wetlands in the buffer get 0.5 for grassy kurgans). Weights given to each LPIS land use cover are available in the Appendix. Then, the area of each LPIS land cover type in the buffer was multiplied by its weight and the resulting values were summed up and divided by the total area of the buffer. Resulting value we consider as a criteria of the propagule transferring ability of the surrounding buffer towards each particular type of vegetation on the kurgan. Since this ability is reversely proportional to the isolation, extracted from 100% the propagule transferring ability generates a value, which hereinafter we will call "degree of isolation".

Fig. 2. A buffer was created around each kurgan to assess the surrounding land cover types.

To test the importance of the kurgans as stepping stones, we reasoned whether and how much they increase the area of semi-natural vegetation cover in the buffer. We assumed that: (1) all analyzed kurgans are covered by semi-natural vegetation; (2) if they were not present, their 2D area would have been occupied by the same share of semi-natural vegetation (perceived also as habitats that are potential sources of grassy/ woody plant species) as the share in the buffer. Based on these assumptions, we calculated the area of three cover types in the territory of the buffer plus kurgans: (a) the area of semi-natural vegetation cover; (b) area of habitats that have the potential to provide diaspores of grassland plant species; and (c) area of habitats that have potential to provide diaspores of woody plant species. This calculation was conducted once with kurgan's data and once assuming no kurgan was there. We then used paired t-test to check whether the presence of the kurgan increase significantly those three areas.

Results

Majority of the studied kurgans (89%) is located in the lowlands and hilly-plains up to 600 m a.s.l. The average kurgans' height is 5.3 m ± 2.6 SD (min 1.9 m, max 18.6 m). Estimated 2D area of the kurgan foot ranges between 400 m^2 and 246 000 m^2 (average 2000 m^2 ± 500 SD). The territory around them is suitable for agriculture and the surrounding area of 270 kurgans (53%) is occupied by over 75% of agricultural, urban or other artificial areas (up to 25% naturalness) (Fig. 3).

Considering all studied kurgans, the prevailing number of them (i.e. 345 out of 509) have over 70% degree of isolation (Fig. 4a). Similar patterns are shown when we look at the subsets of grassy, woody and mixed cover kurgans (Fig. 4 b, c, d). On Fig. 4 it is clearly visible, that regardless of the vegetation type of the kurgans, the degree of their isolation from the buffer is high.

The presence of kurgans in a landscape increased the area of semi-natural vegetation

by an average of ca. 1503 m^2 compared to a landscape of the same size but without kurgan ($t = 3.158$, $p < 0.001$, $df = 508$). Kurgans provided on average ca. 489 m^2 more forests in the landscape than in an area of the same size but without kurgans ($t = 10.157$, $p<0.001$, $df= 508$). The increase of the area of grasslands was about 1060 m^2 compared to a landscape of the same size without kurgans ($t = 2.363$, $p = 0.009$, $df = 508$).

When we multiplied the calculated average 2D area of the sampled kurgan set (2000 m^2) by the number of currently known kurgans in Bulgaria (Kitov, 1993) (n = 50 000), the resulting area became 100 km^2. According to the recent national statistics (Ministry of Agriculture, Food and Forestry , 2019), the total arable land with annual crops covers 34,616.15 km^2. Therefore we assumed that the kurgans in Bulgaria served for saving cumulative semi-natural area equal to 0.29% of this category or 0.09% of the whole country territory.

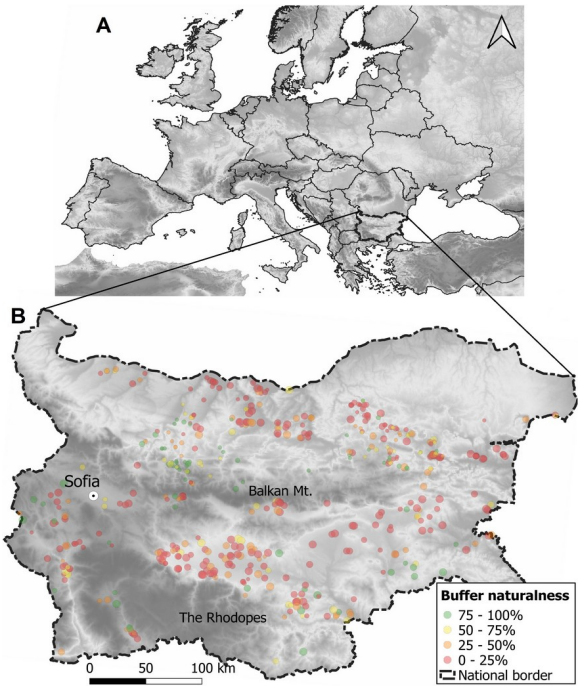

Fig. 3. (A) Study area. **(B)** Locations of the studied kurgans (n = 509). Point colors correspond to the share of non-natural habitats in the buffer, used as indicator of kurgan's isolation. Point symbol size indicates the 2D area size.

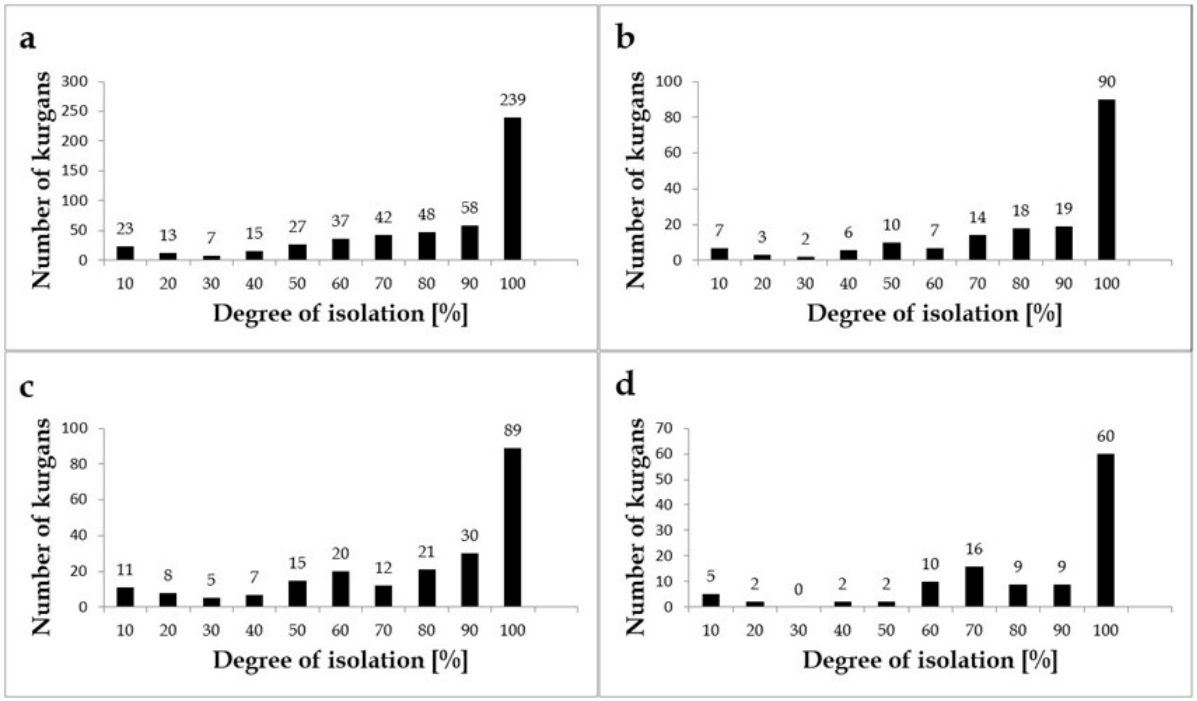

Fig. 4. Degree of kurgan isolation: **(a)** entire dataset (n=509); **(b)** subset of kurgans covered predominantly by grassy vegetation (grass cover greater than or equal to 90%; n=176); **(c)** subset of kurgans covered by mixture of woody and grassy vegetation (grassy cover greater than 10 and less than 90%; n=218); **(d)** subset of kurgans with a predominantly woody vegetation (grass cover less than or equal to 10%; n=115).

Discussion

Bulgarian kurgans have similar characteristics to Ukrainian and Hungarian ones regarding height and diameter (Sudnik-Wójcikowska et al., 2011, Deák et al., 2020). Our study revealed that the kurgan's presence within the landscape increases the share of semi-natural vegetation either grassland or woody. Their isolation from the neighboring natural or semi-natural habitats is significant and in many cases they are the only remnants of semi-natural habitats within the human modified areas. This supports the findings about the role of kurgans for maintaining natural species populations (Moysiyenko et al., 2014; Dembicz et al., 2016; Deák et al., 2016a, 2020). The cumulative importance of Bulgarian kurgans, regardless of their size but accounting their high frequency, is notable for the preservation of semi-natural landscape in the agricultural matrix. Where kurgans are surrounded by more than 95% agricultural and urban or artificial areas, they are the only territories which offer habitats for the native species (e.g. *Quercus pubescens*, *Ulmus minor*, *Stipa capillata*, *Adonis vernalis*, *Salvia nemorosa*, *Trifolium subterraneum* and many others). Therefore in the heavily homogenized agricultural landscape, same as other fragments of semi-natural character, the kurgans have a potential to serve as important sites for landscape diversification, biodiversity preservation (Tscharntke et al., 2002; Fahrig, 2003) and ecosystems functioning (Lindgren et al. 2018). In a predominantly non-natural landscape kurgans could also play an important role as stepping stones for plants and animals providing connectivity to remaining natural habitats (Dembicz et al., 2016). We consider here as stepping stone a semi-natural area, providing refuge for species survival and reproduction and facilitating their ability for dispersal in an inhospitable environment (Saura et al., 2013). The higher the share of non-natural areas in the kurgan surroundings, the higher its degree of isolation, and the higher the kurgan's importance as a stepping stone and its nature conservation importance.

Our observation showed that the vegetation cover of kurgans is diverse ranging from pure grasslands to forest. We don't have data about the management of Bulgarian kurgans, but considering that the potential natural vegetation of Bulgaria is forest (Bohn et al., 2003), the wooded kurgans exhibit a successional development in case of lacking management. The woody vegetation encroachment is traditionally considered as negative for the quality of grasslands (Eldridge et al., 2011; Deák et al., 2016a; Valkó et al., 2018). The established woody vegetation cover of some kurgans however, could be interpreted as favorable for overall habitat diversification in the agricultural matrix.

Considering the terminology introduced by McIntyre and Hobbs (1999), the kurgans could be regarded as 'relictual' habitats within destroyed matrix. Relictual habitats are the most threatened from further exogenous disturbances. Their conservation is pointed by McIntyre and Hobbs (1999) as an important goal for conservation activities and especially in the case of high levels of isolation these habitats should obtain a priority. The availability of small semi-natural landscape features within agricultural fields has been suggested as a promising solution for landscape preservation with a particular interest for biodiversity conservation also by Pe'er et al. (2017).

In Bulgaria historical monuments are protected by the Cultural Heritage Act, but the protection is not extended to their natural heritage, as it has been done for all kurgans in Hungary (Deák et al., 2016b). There is a deficiency of understanding in the society that kurgans are not only historical monuments but also important relictual habitats and this needs to be changed. Ratification of the European Landscape Convention (ETS No.176) by Bulgaria in 2004 obliges the country to apply appropriate procedures for local people and

regional authorities' involvement in activities concerning kurgan existence (such as education programs, shrub clearing, alien plants removal) to preserve them as an integral part of the traditional landscape (Jones, 2007). Raising the knowledge about the natural value of kurgans, followed by adequate legal regulation will certainly improve the efficiency of their protection and preservation of the provisional, maintaining and cultural ecosystem services delivered by them.

Acknowledgements

This work was supported by the Bulgarian National Science Fund (grant numbers КП-06-Н21/2, 2018). Authors are grateful to the two anonymous reviewers for the constructive comments on the manuscript.

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Received: 22.05.2020
Accepted: 23.08.2020

Appendix. Original land cover categories used in the national Land Parcels Information System (LPIS) and their interpretation for the current analysis.

LPIS code	LPIS description	Land cover category	Naturalness	Delivery propagules for kurgans:		
				woody	grassy	mixed
010	permanently cultivated land; arable land	annual crops	non natural	0	0	0
020	permanently cultivated land; permanent crops	perennial cultivation	non natural	0	0	0
021	permanently cultivated land; vineyards	perennial cultivation	non natural	0	0.5	0.5
022	permanently cultivated land; orchards with herbaceous vegetation among plantations	perennial cultivation	non natural	0	0.5	0.5
023	permanently cultivated land; aromatic plants plantations	perennial cultivation	non natural	0	0.5	0.5
031	kitchen garden	urban and artificial areas	non natural	0	0	0
032	urban territory near settlements	urban and artificial areas	non natural	0	0	0
040	permanent grasslands	grasslands	natural	0	1	1
041	natural meadows and pastures	grasslands	natural	0	1	1
043	grasslands situated within the forest territories usually in the mountain areas	grasslands	natural	0	1	1
050	agricultural lands with mixed land use	perennial cultivation	non natural	0	0	0
100	non cultivated land; mostly shrubland	other semi-natural land	natural	0.5	0.5	0.5
101	shrubland or grassland with scattered trees	other semi-natural land	natural	0.5	0.5	0.5
102	gullies and ravines	other semi-natural land	natural	0	0.5	0.5
103	non paved roads	other semi-natural land	non natural	0	0.5	0.5
200	forests	forests	natural	1	0	0.5
302	buildings outside the urban areas	urban and artificial areas	non natural	0	0	0
303	sport and leisure facilities	urban and artificial areas	non natural	0	0	0
400	water bodies and wetlands	other semi-natural land	natural	0	0.5	0.5
401	rivers and river beds	other semi-natural land	natural	0	0.5	0.5
402	lakes, dams and fens	other semi-natural land	natural	0	0.5	0.5
403	irrigation and drainage channels and associated land	other semi-natural land	non natural	0	0	0
404	water body near state border, icl. Danube river and Black sea)	other semi-natural land	natural	0	0	0
405	wetlands including mires	other semi-natural land	natural	0	0.5	0.5
500	disturbed lands	urban and artificial areas	non natural	0	0	0
501	mineral extraction sites	urban and artificial areas	non natural	0	0	0
502	dump site and tailing pond	urban and artificial areas	non natural	0	0	0
601	road with permanent pavement and associated land, including their transport facilities	urban and artificial areas	non natural	0	0	0
602	rail network and associated land, including railway facilities	urban and artificial areas	non natural	0	0	0
700	bare and eroded land	other semi-natural land	natural	0	0.5	0.5
701	sand, gravel and bare rock	other semi-natural land	natural	0	0.5	0.5
702	sparsely vegetated lands	other semi-natural land	natural	0	0.5	0.5
800	other territories	urban and artificial areas	non natural	0	0	0
801	small plot of non-arable land, with an area between 100 and 1000 square meters with non-agricultural permanent use.	other semi-natural land	natural	0.5	0.5	0.5
802	gorge – this type of territories are located in narrow river gorges when a river or railway passes along the river line or both. The territories include all sites, namely rivers and river beds, roads and/or railway lines.	urban and artificial areas	non natural	0	0	0
900	other areas forbidden for agricultural use - reserves, national security sites, etc.	other semi-natural land	natural	0.5	0.5	0.5

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 53-63

Occurences of the Cinereous Vulture (Aegypius monachus) in the Eastern Rhodopes, Bulgaria

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Abstract. The Cinereous Vulture (*Aegypius monachus*) is the largest vulture species in Europe. It is currently extinct in Bulgaria, but individuals from the only Balkan population of the species located in northern Greece frequently forage in the Bulgarian side of the Eastern Rhodope Mountains. We studied the patterns of occurrences, numbers and age structure of Cinereous Vultures in the Eastern Rhodopes, Bulgaria. During 2016 – 2019 we recorded 215 Cinereous Vulture occurrences. The majority of the observed individuals (72.7%) were immature while adults totaled 23.7%. The highest number of vultures were recorded in spring and summer. Cinereous Vultures visited local supplementary feeding stations singularly or in groups of up to eight individuals. Our study shows that Eastern Rhodopes and specifically SPAs 'Studen kladenets', 'Byala reka' and 'Krumovitsa' are important foraging areas and hence high priority conservation areas for the species in Bulgaria.

Key words: *Aegypius monachus*, foraging, age structure, viewpoint monitoring, feeding station.

Introduction

The Cinereous Vulture *Aegypius monachus* is a large scavenger with a wide distribution spanning from Portugal to China (Ferguson-Lees & Christie, 2001). The global population of the species is estimated to comprise 7 800 – 10 500 pairs, which equals 15 600 – 21 000 mature individuals (BirdLife International, 2020). Europe holds 2 536 – 2 838 breeding pairs distributed across five countries – Spain, Portugal, France, Greece and Ukraine (Andevski et al., 2017). The Cinereous Vulture is considered "Near threatened" according to the IUCN Red List (BirdLife International, 2020). The only breeding population of the species on the Balkan Peninsula occurs in Greece (Skartsi et al., 2008). This population was at the edge of extinction with only 4 – 5 breeding pairs and 26 individuals in 1979 (Hallman, 1979). Over the next 10 years the population started to slowly recover. Two decades later the number of breeding pairs reached 21 (Vlachos et al., 1999) but

remained stable over the next decade (Skartsi et al., 2008). Nowadays, it is confined to a colony of about 30 breeding pairs in the Dadia-Lefkimi-Soufli Forest National Park (Zakkak & Babakas, 2015). However, small populations restricted to confined areas are fragile and face higher extinction risk due to persistent threats, such as poisoning or stochastic events. Despite applied large-scale conservation measures, the population still suffers increased mortality due to poisoning (Pantovic & Andevski, 2018; Vavylis et al., 2020) and other threats, such as collision with energy infrastructure, such as wind turbines (Vasilakis et al., 2016, 2017).

In Bulgaria the species had wide distribution until the beginning of the 20th century. In some areas it was considered more abundant than the Griffon Vulture *Gyps fulvus*. It occurred in the Rhodopes, Balkan Mountains, Rila, Pirin, Dobrudzha, Danubian Plain, Provadia-Royak Plateus etc. (Farman, 1869; Reiser, 1894). However, in the middle of the 20th century the population has undergone a dramatic decline, mainly due to mass poisoning campaigns against terrestrial predators (Patev, 1950). Around 1960 it hardly occurred in Bulgaria, although it is possible that single pairs were breeding in the Eastern Rhodopes and the Eastern Balkan Mountain until the 1980s (Michev, 1985). The last confirmed breeding record in Bulgaria dates back to 1993, when a single pair bred near Studen kladenets reservoir in the Eastern Rhodopes (Marin et al., 1998). In subsequent years there were numerous observations of pairs with courtship behavior, but breeding was not confirmed (Iankov et al., 1998; Tewes et al., 2004). Cinereous Vultures frequently forage far from their breeding sites and especially over areas with high food availability (Carrete & Donazar, 2005; Moreno-Opo et al., 2010). The large number of free ranging livestock (mainly cattle) and wild ungulates in Bulgaria creates favorable feeding opportunities for vultures. High food availability along with long-term implementation of conservation measures resulted in the significant increase of the Griffon Vulture population in the area (Demerdzhiev et al., 2014; Dobrev et al., 2019) and probably had a positive impact on the Cinereous Vulture population as well. Through radio tracking and GPS telemetry it has been confirmed that individuals from the breeding population in Greece are foraging in the Bulgarian side of the Eastern Rhodopes (Vasilakis et al., 2008, 2016). Furthermore, a study on the diet of the species confirms that it is feeding on carcasses found in Bulgaria (Skartsi et al., 2015). However, there is a lack of knowledge on the phenology and age structure of the Cinereous Vultures foraging in Bulgaria. Information on the main foraging areas and seasonal dynamics of vulture occurrences is crucial to inform species conservation programs and plan the implementation of adequate conservation actions.

Here we present data on the pattern of occurences, numbers, age structure and the main foraging areas of the Cinereous Vultures in the Eastern Rhodopes, Bulgaria. This study aims to contribute to the overall knowledge on the distribution and movements of the last breeding colony of the Cinereous Vulture in the Balkan Peninsula and to inform the running conservation programs for the species.

Material and Methods

Pattern of Cinereous Vulture occurences in the Eastern Rhodopes, Bulgaria were studied in 2016 – 2019 with visual observations from stationary viewpoints and regular observations at two supplementary feeding stations.

Observations from viewpoints

Visual observations from 19 stationary viewpoints in 7 SPAs (Special Protected Areas) forming part of the Natura 2000 network were conducted twice per month between December and May in 2016 and 2017. Observations from the stationary

viewpoints were held for a total of 1114 hours. Viewpoints were chosen at higher open spots on hills with wide view in all directions to improve visibility. Observations were conducted simultaneously from 3 – 4 viewpoints with constant communication among the observers, in order to avoid double-counting, and follow the movements of the recorded vultures in the area.

Observations at vulture feeding stations

Observations were performed during organized supplementary feedings at least twice a month between 2016 and 2019, at a supplementary feeding station in SPA 'Studen kladenets'. Observations were also performed less regularly, at a feeding station in SPA 'Most Arda' due to food availability. We observed from a distance greater than 500 m from the feeding stations in order to avoid disturbance. Data collection started right after carcass disposal and lasted until darkness, or until the carcass was fully consumed. The observed Cinereous Vultures were aged following Forsman (2003): individuals in first plumage were considered as juveniles, from second to fifth plumage as immatures and from sixth plumage as adults. For every marked individual the type of tag (wingtag, ring or transmitter), position, colour and inscription were recorded. Observations were made with spotting scopes (20×60) and binoculars (10×60) during daytime, under suitable weather conditions by experienced observers. Data was collected using the SmartBirds Pro app (Popgeorgiev et al., 2015). Spatial data were mapped and processed using QGIS software (QGIS Development Team, 2016).

Results

We observed 215 Cinereous Vulture occurrences during observations from stationary viewpoints and regular observations at supplementary feeding stations. The age was determined for 78.6% of the observed individuals with immatures representing 72.7% (n = 123), followed by adults 23.7% (n = 40). Juveniles comprised only 3.6% (n=6) of the observed individuals. In total, 27 marked Cinereous Vultures were observed, of which 37% were adults, 59.3% were immatures and 3.7% were juveniles. In total, 45.5% of tagged vultures were recorded in Bulgaria more than once (between 2 and 5 times) during the study. One immature individual was observed on 44 occasions. It was recorded during every month of the year with the highest number of observations in spring and summer. It was a regular visitor of the feeding station and frequently roosting nearby. In 2018 and 2019 we observed courtship behavior with another untagged immature vulture, but no breeding attempts were recorded.

The highest numbers of Cinereous vultures were recorded in April and May (Fig. 1). In January and February only one and two vultures were observed, respectively.

Vultures observed from viewpoints

We conducted 1 114 hours of observations throughout a period of 68 days and recorded 47 Cinereous Vultures. Overall, we spotted vultures in 37% of the days with field observations. The age was determined for 72.3% of the observed individuals. Immatures were the best represented age class comprising 55.9%, followed by adults – 38.2%. Only two juveniles were observed during the study.

The main flight corridor of the species between Bulgaria and Greece was defined by observations from stationary viewpoints located at elevated spots in all SPAs in the area. We observed 34% (n=16) of the Cinereous Vultures in SPA 'Byala reka'. In SPA 'Krumovitsa' and SPA 'Studen kladenets' an equal numbers of vultures were recorded – 29.8% (n=14). 'Dobrostan' was the only SPA where no vultures were recorded (Fig. 2).

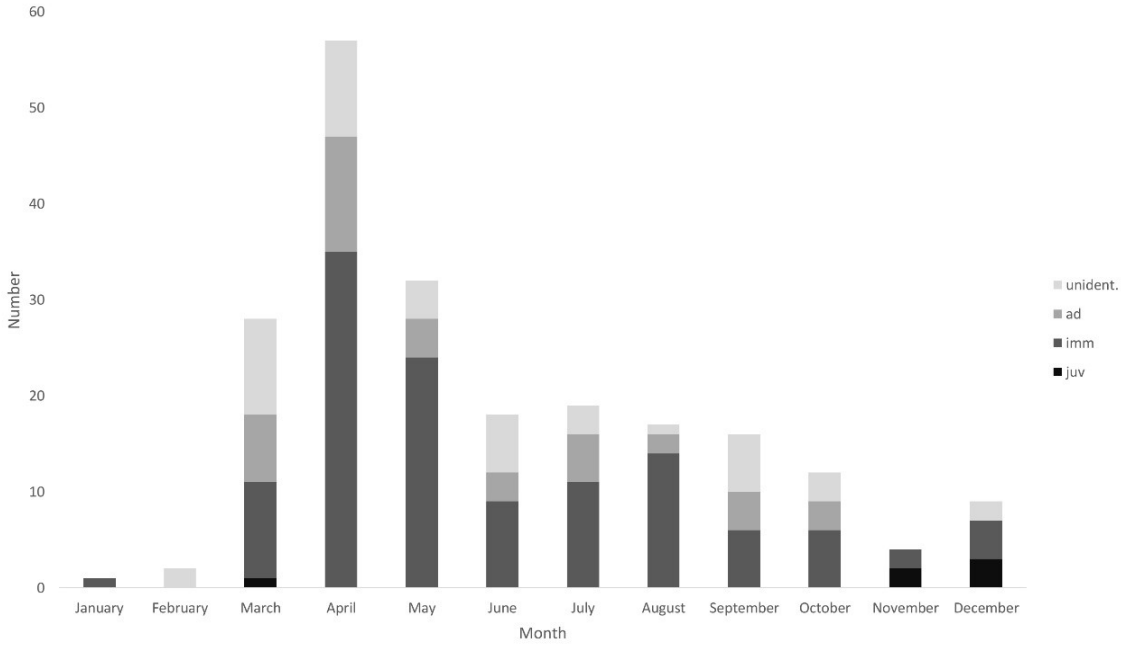

Fig. 1. Monthly dynamic of the observations of Cinereous Vultures in the Eastern Rhodopes, Bulgaria presented per age classes.

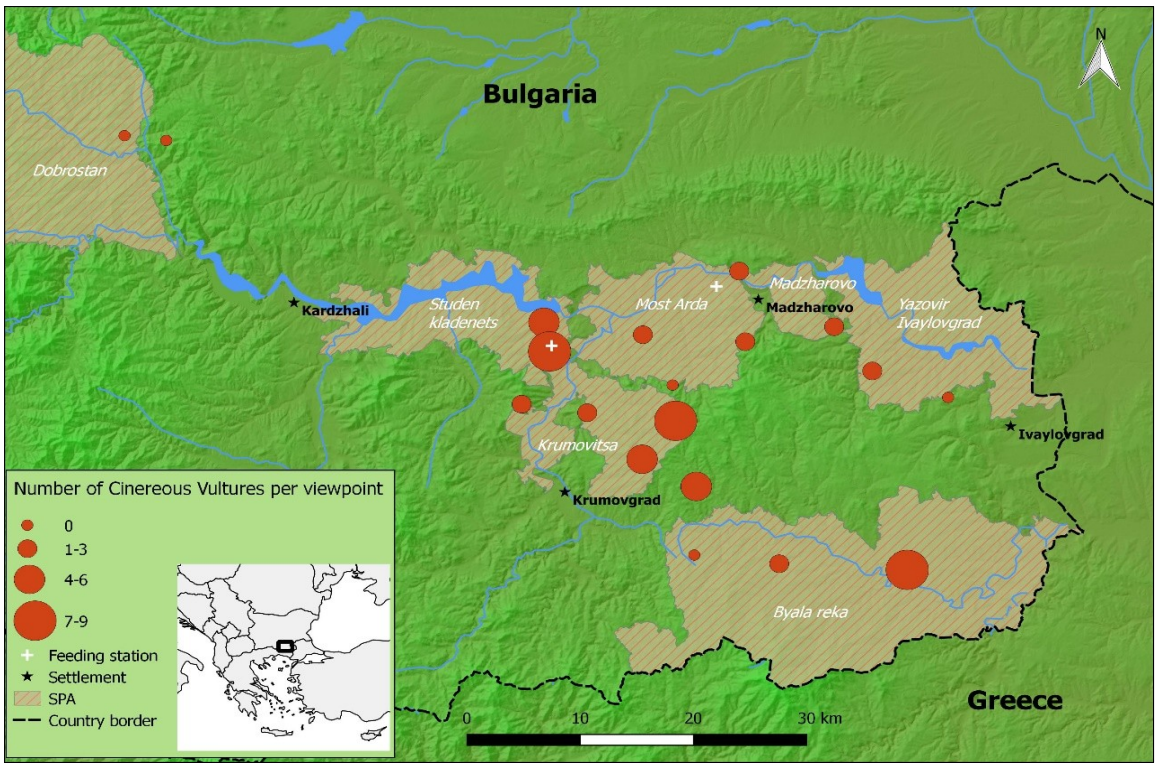

Fig. 2. Distribution and number of Cinereous Vultures observed per viewpoints.

Vultures observed at supplementary feeding stations

On 106 occasions, 168 Cinereous Vultures were observed taking advantage of the supplementary feedings. The majority of the vultures were observed at the supplementary feeding station in SPA 'Studen kladenets' (95.8%), while only 4.2% (n = 7) of the birds visited the feeding station in SPA 'Most Arda'. The age of 80.4% of the Cinereous Vultures visiting the feeding stations was identified. Adults comprised only 20% of observed individuals (Table 1).

Table. 1. Age structure of Cinereous Vultures observed at supplementary feeding stations in Bulgaria during 2016 to 2019.

Age class	Number of individuals	Number of observations	Number of ind. per observation	Age class %
Adult	27	22	1.22	20%
Immature	104	89	1.17	77.1%
Juvenile	4	3	1.33	2.9%

Cinereous Vultures visited the feeding stations mostly singularly or in small groups of up to three individuals. However, larger groups were also observed. The highest number observed simultaneously at the feeding station in SPA 'Studen kladenets' was eight.

Discussion

Our study confirms that Cinereous Vultures frequently occur in the Bulgarian part of the Eastern Rhodopes throughout most of the year, except for winter. The observed individuals originated from the only breeding colony in the Balkans which is found in the Dadia-Lefkimi-Soufli Forest National Park in Greece (Skartsi et al., 2008). One Cinereous Vulture released as part of a reintroduction program in Kotel, Bulgaria was recorded as well in 2019 (Stoynov, 2019, Blagoevgrad, pers. comm.). These observations are in line with results obtained by GPS tracking of Cinereous Vultures from the colony in Greece (Vasilakis et al., 2016) and previous observations of marked individuals. The majority of vultures were found to fly over SPA 'Byala reka' and SPA 'Krumovitsa' following the valleys of the Byala Reka river and Dushundere stream. This is the shortest route between the breeding colony in Greece and the supplementary feeding station in SPA 'Studen kladenets' in Bulgaria which is used as a main movement corridor by the species (Noidoi & Vasilakis, 2011). This finding was confirmed by data obtained from 27 Cinereous Vultures tracked with GPS transmitters during the same period (authors' unpublished data) and a study on the feeding pattern and diet of the Griffon Vulture in the Eastern Rhodopes which revealed that vultures find most of their food in SPA 'Studen kladenets' and SPA 'Krumovitsa'. The density of livestock and wild herbivores in this area is high creating favorable feeding conditions for vultures. We found that SPAs 'Madzharovo' and 'Ivaylovgrad' which are mostly covered by forests and dense vegetation were less frequently visited by Cinereous Vultures. The species prefers to forage in open areas where food detection probability is higher (Carrete & Donazar, 2005; Garcia-Baron et al., 2018) which explains the lower visitation rate recorded for these two SPAs. No vultures were recorded in SPA 'Dobrostan', which is located farther from Greece. Despite the number of livestock extensively grazing in this area being high, it is more remote from the breeding colony and is located at higher altitudes making the foraging trips more energetically costly therefore vultures rarely visit it.

The Cinereous Vultures were observed flying singularly or in small groups of up to

three individuals, in rare cases in mixed groups with Griffon Vultures. The results from the viewpoint monitoring indicated that Griffon Vultures are using the same flight corridors during foraging. Both vulture species gained height over the bare hills of the Irantepe ridge located in SPA 'Krumovitsa' which is the highest ridge in the area and then glided southeast or northwest. This area is remote and far from human settlements, but with significant numbers of livestock. About 1 000 cattle are extensively grazed along Irantepe ridge (data obtained from the BFSA www.babh.government.bg) providing good feeding opportunities for vultures which explains the regular presence of both species.

At the supplementary feeding stations Cinereous Vultures were frequently outnumbered by the Griffon Vultures which monopolize the carcasses. However, higher numbers of Cinereous Vultures were observed when offal was disposed. The disposal of offal allows more vultures to feed simultaneously and reduces the competition within the vulture guild (Cortés-Avizanda et al., 2010; Moreno-Opo et al., 2015, 2020). In 2017 the proportion of offal disposed at both feeding stations in Bulgaria was higher which might explain the observed increase in the number of Cinereous Vultures visiting the feeding sites. Thus, in order to increase the species visitation rate and the attractiveness of the feeding stations in Bulgaria we recommend the adoption of feeding protocols which envisage regular provisions with smaller pieces of meat and bones scattered around (Cortés-Avizanda et al., 2010).

The highest numbers of Cinereous Vultures in our study area were recorded in spring. The number of vultures observed from the stationary viewpoints or at the vulture feeding stations was low in winter (December – February) and gradually increased towards the summer. Large vultures, being soaring raptors, heavily rely on thermal updrafts to travel long daily distances with low energy expenditures (Ruxton and Houston 2004; Duriez et al. 2014). The adverse weather in winter impedes thermal creation and determines suboptimal foraging conditions. Rain or snow may impede flights for entire days, especially in winter (Hiraldo and Donazar 1990). Furthermore, in this period, the days are shorter and vultures have less time for foraging. Studies on Cinereous and Griffon Vultures show that in winter they spent significantly less time foraging than during the rest of the year (Hiraldo & Donazar, 1990; Xirouchakis & Andreou, 2009). Juvenile Cinereous Vultures in Spain also have larger home ranges in spring and summer but restrict their movements around the breeding colony during autumn and winter (Castaño et al., 2015). Cinereous Vultures confine their movements to smaller areas in winter and avoid long energetically expensive foraging trips. In January and February 2017, the weather conditions in Bulgaria and northern Greece were harsh with heavy snowfalls and low temperatures over a long period. Under such unfavorable conditions, Cinereous Vultures probably preferred to stay closer to the breeding colony and the supplementary feeding station in the Dadia-Lefkimi-Soufli Forest National Park which was supplied with food on a weekly basis. In spring, when flight conditions are improved, vultures make longer foraging trips (Hiraldo & Donazar, 1990) which can explain the increased number of Cinereous Vultures observed in March and April in the Bulgarian side of the Eastern Rhodopes. On the other hand, the winter months coincide with the onset of the breeding season, when pairs are building and defending their nests and start incubation (Ferguson-Lees & Christie, 2001). During this period breeding vultures have smaller home ranges and exclude long distance movements (Vasilakis et al. 2008). On the contrary, during the chick rearing period vultures increase their home ranges since they have to satisfy food provisioning for their chicks and themselves. This is confirmed by our observations of a marked

adult vulture breeding in Greece, which was observed twice feeding in Bulgaria during the chick rearing period. This shows that under suitable weather conditions some breeding adults may undertake long distance movements in search of food. Furthermore, food availability in the Eastern Rhodopes is high due to the large number of extensively grazing livestock and wild ungulates. However, in winter livestock is kept near the villages or in shelters which leads to a reduction in food availability but in spring and summer livestock suffers increased mortality which creates favorable feeding conditions for the vultures. During this period Griffon Vultures in the Eastern Rhodopes rely mostly on carcasses found in the wild rather than the food provided at the supplementary feeding stations. The combination of the unfavorable flight conditions in winter, the limited food resources available and the start of the breeding season with the associated changes in vulture behavior probably explain the low number of Cinereous Vultures observed in the Eastern Rhodopes during this season.

The majority of the observed individuals in our study were immature individuals. We must note that since not all individuals were marked we were not able to estimate how many different vultures were actually observed. Our results indicated that some individuals visit Bulgaria more frequently than others and this could have biased the determination of the age structure. However, if we consider only the marked individuals it is still evident that most of the recorded Cinereous Vultures were immatures. They wander further away from the breeding colonies compared to adults (Yamaç & Bilgin, 2012), also observed in other vulture species (Monsarrat et al., 2013; Peshev et al., 2018). In Turkey, Caucasus and Asia large proportions of juvenile and immature Cinereous Vultures are migrant and spent the winter in more southern latitudes (Gavashelishvili et al., 2012; Yamaç & Bilgin, 2012; Kang et al., 2019; Reading et al., 2020). However, young vultures from Spain and Greece does not migrate but alternatively make long distance movements far from the breeding colonies (Vasilakis et al., 2008; Castaño et al., 2015). Similar to other raptors, immature vultures disperse and explore new areas gaining knowledge on the availability of other suitable foraging and breeding sites. This behavior also helps to reduce the competition with adults near the breeding colony (Watson 2010; Gil et al., 2014). Vasilakis et al. (2008) did not find significant differences in the home range size of adult and immature Cinereous Vultures from the breeding colony in Greece. Both age classes had larger home ranges during the non-breeding season with immatures inhabiting slightly larger territories. However, the frequency of long distance movements has not been studied. Adult vultures need to find the balance between parental care for the offspring and foraging which affects daily travelling distances (Moran-Lopez et al., 2006). They probably spend more time in the vicinity of the nesting sites and less frequently undertake long distance movements compared to immature individuals. This could explain the low number of adults observed in the Eastern Rhodopes but more research is needed to confirm this hypothesis.

However, we recorded some adults during our observations, including individuals breeding in Greece which were making one-day foraging trips to Bulgaria. The relatively low number of adults permanently present in the area suggests that the presence of breeding pairs in the Bulgarian side of the mountain is unlikely. The prevalence of immature vultures and the observed courtship behavior are positive signs for future colonization of the former breeding grounds. Similar processes were observed in Portugal where the colonization of the border areas with Spain was preceded by regular occurences of immature and adult Cinereous Vultures (Lourenço, 2011; Lourenço et al., 2013; ICNB, 2017). However, the steady increase of the Spanish

population has facilitated this process (Moreno-Opo & Margalida, 2014; ICNB, 2017) while the population in Greece despite increasing over the past 30 years, suffers high mortality and reduced breeding success (Skartsi et al., 2019), thus, is not expanding outside Dadia-Lefkimi-Soufli Forest National Park (Zakkak & Babakas, 2015).

Our study confirms that the Bulgarian side of the Eastern Rhodopes is an important part of the foraging range of the Cinereous Vulture population, hence a high priority conservation area for the species. Cinereous Vultures frequently use this area for foraging and feeding throughout most of the year, except in winter. The supplementary feeding site in SPA 'Studen kladenets' is regularly visited by groups of up to eight individuals and serves as a safe and predictable food source for the species. The high number of immature individuals and the observed courtship behavior suggest that if the population in Greece increases some pairs may settle and breed in Bulgaria creating a second breeding nucleus on the Balkans. The implementation of a reintroduction program in this area must also be considered for enhancing the recovery of the species. Therefore, various conservation measures should be implemented to improve the survival of the vultures in the area e.g. insulation of hazardous powerlines in areas frequently visited by vultures, anti-poisoning and anti-poaching activities, use of lead-free ammunitions in hunting areas, improvement of the feeding conditions by increasing the number of wild animals and supporting traditional livestock husbandry practices.

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 Ioannis Tziambazis, Athanasios Chalivelentzios and Petros Babakas from the Management body of Dadia-Lefkimi-Soufli Forest National Park for their contribution in vulture tagging and data collection. We are grateful to Connor Panter for proofreading the manuscript and to two anonymous reviewers for providing helpful comments.

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Received: 25.05.2020
Accepted: 24.08.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 65-75

Impact of Mineral and Organic Foliar Fertilizing on Some Productivity Factors of a Natural Grassland of Chrysopogon gryllus L. Type and a Natural Pasture of Nardus stricta L.

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Abstract. During the period 2011-2013, the impact of some factors on the productivity of natural grassland under the conditions of mineral and organic foliar fertilization was studied in the Central Balkan Mountain region. The summarized multifactor analysis shows that in the Central Balkan Mountains, the productivity of natural grasslands (a meadow of *Chrysopogon gryllus* L. type and a pasture of *Nardus stricta* L. type) was influenced by the type of grassland (63.42%) at first place, followed by the agroecological conditions during the year (20.32%) and the fertilizing method (mineral with N and P or foliar with Biostim) - 8.82%. The level of fertilization (7.43%) had the least effect on the yield of the studied grassland. The data obtained through the summarized RSM analysis indicated a slightly higher efficiency of foliar fertilizing than the variable mineral fertilization for both types of grassland. A relatively high regressional dependence between productivity and moisture supply of the natural grass biomass has been established, which allows for an approximate prediction of the yields of natural grasslands with applied mineral and foliar fertilizing. The coefficients of determination (R = 0.7-0.8) were high enough for practical determination of productivity, by the precipitation amount during the critical months for Bulgaria (April - July). When using the annual or seasonal precipitation amount, the accuracy is less.

Key words: *Chrysopogon gryllus* L., *Nardus stricta* L., mineral fertilization, organic foliar fertilizing.

Introduction

The natural grasslands in Bulgaria occupy a considerable part of the mountain and hilly ranges of the Central Balkan Mountains, therefore they have a great economic significance. The botanical composition and dynamics of grassland community development are related to environmental conditions and affect the actual productivity of the forage mass formed (Naydenova et al., 2015). The proper use of technology combined with the implementation of appropriate measures (resowing, irrigation, nourishing, etc.) reduce the percentage of low-productive and harmful species, respectively improve the quality and nutritional value of the grass cover.

Mineral fertilization with N and P is an effective measure. It favors the floristic composition (Jankowska-Huflejt, 2012; Koukoura et al., 2005; Popescu and Churkova, 2015), increases the productivity and concentration of crude protein in natural

grasslands (Budakli et al., 2012; Maruşca et al., 2014; Min et al., 2002; Vintu et al., 2015). The annual fertilization of natural meadow of *Chrysopogon gryllus* L., with the combination - N_6P_6 leads to a proven increase in the amount of crude protein (100.42 g kg^{-1}), crude fat (22.01 g kg^{-1}), minerals (78.10 g kg^{-1}) and phosphorus (3.37 g kg^{-1}) in dry matter. The values exceed the nontreated control by 52.3% (CP), 31.1% (CF), 3.1% (Ash) and 81.2% (P), respectively (Iliev et al., 2019).

The treatment of natural grasslands with foliar organic fertilizers, which include humic and fulvoacid acids, combined with basic macro and microelements, stimulate root system growth and increase the nutrient intake (including more inaccessible phosphorus) by plants species (Abdullah, 2010; Datta et al., 2011; Sengalevich, 2007). The application of humate fertilizers increases the protein fraction and improves the nutritional value of feed (Klimas et al., 2010). Foliar fertilizing with the organic fertilizer Biostim affects the botanical composition and increases the dry matter productivity of the natural grassland with dominant species of *Chrysopogon gryllus* L., and *Nardus stricta* L., (Iliev et al., 2017; Iliev, 2018).

The agrotechnical events improve the yield and quality of the forage mass, respectively the physiological condition and productivity of livestock.

The purpose of the present study was to investigate the impact of some factors on the productivity of natural meadows and pastures, at different fertilization levels with N and P and the organic foliar fertilizer Biostim.

Material and Methods

The study was conducted in 2011-2013 at the Research Institute of Mountain Stockbreeding and Agriculture - Troyan, on:

- a natural meadow of *Chrysopogon gryllu*s L. type, located in Makaravets locality at an altitude of 460 m.

- a natural pasture of *Nardus stricta* L. type, located in Chuchul locality at an altitude of 1400 m.

The impact of mineral and organic fertilization on the productivity of natural herbs has been studied.

Combinations of alternative nitrogen and phosphorus fertilizing were investigated in the following variants of mineral fertilizing: 1. Nontreated (K_1); 2. Annual fertilizer application kg/ha: $N_{60}P_{60}$ (K_2); 3. First fertilizing year with N_{60} (N_{60}/I); Second and third fertilizing year with P_{60} (P_{60}/II and P_{60}/III); 4. First and second year fertilizing year with P_{60} (N_{60}/I; second and third fertilizn year with N_{60} (N_{60}/II and N_{60}/III); 5. First and second year fertilization with N_{60} (N_{60}/I and N_{60}/II); Third year fertilization with P_{60} (P_{60}/III); 6. First and second fertilizing year with P_{60} (P_{60}/I and P_{60}/II); Third year fertilization with N_{60} (N_{60}/III); 7. First fertilizing year with N_{60} (N_{60}/I); Second fertilizng year with P_{60} (P_{60}/II); Third fertiling year with N_{60} (N_{60}/III); 8. First fertilizng year with P_{60} (P_{60}/I); Second fertiling year N_{60} (N_{60}/II); Third fertiling year with P_{60} (P_{60}/III).

Foliar feeding with organic fertilizer was applied once a year, and the working solution was introduced with a backpack sprayer during active grass vegetation. The reaction, salt concentration and composition of foliar fertilizer Biostim were as follows: reaction (pH) - 6.8, salt concentration 20.15, nitrogen (N) - 2.1%, phosphorus (P) - 1.54%, potassium (K) - 11.2%, calcium (Ca) - 0.15%, magnesium (Mg) - 0.01%, iron (Fe) - 0.024%, zinc (Zn) - 0.037%. Organic content - 2.25, humic acids - up to 14%, fulvic acids up to 7%.

The variants of the experiment were: 1. Nontreated (Control); 2. Foliar application with Biostim 1000 ml/ha; 3. Foliar application with Biostim 2000 ml/ha; 4. Foliar application with Biostim 3000 ml/ha; 5. Foliar application with Biostim 4000 ml/ha.

The harvesting of the test areas is carried out during the shedding prior to

flowering of the seedling. The production of aboveground biomass was determined by mowing as follows: in the meadow of *Chrysopogon gryllus* L. type in the period from tasseling to the beginning of flowering period of the dominant species, and in the pasture of *Nardus stricta* L. type until the matgrass reached ear formation phase.

The territory of experiment occupies the southernmost parts of the Pre-Balkans (Lovech Mountain) in humid continental climate subregion (Sabev & Stanev, 1963). The ridges are sharp, and the slopes steep and represent a natural barrier to the main atmospheric circulation in the area. The climate is characterized by great diversity due to the physical and geographical conditions of the Troyan region assigned to the Balkan Mountain region, including the Balkan Mountain and the Pre-Balkans (Georgiev, 1979). Warm and dry winds (from the south and southwestern air currents) are common in spring and autumn, which greatly reduces air humidity and temperatures have a continental influence. The average annual temperature (10-11°C) is characterized by territorial differentiation from north to south and an increase in altitude (Ninov, 1997). Precipitation is unevenly distributed with maximum in summer (309 mm) and minimum (168 mm) in winter. In the spring and autumn the precipitation recorded is 242 mm and 209 mm, respectively. Annual precipitation amount in the Pre-Balkans ranges from 567 to 1200 mm.

For a more complete description of the area, data are presented from a weather station in Troyan, located at an altitude of 384 m (Tables 1 and 2). Temperature is an important element of climate that characterizes the climatic type of a particular area. Its spatial and temporal distribution influences plant metabolism - photosynthesis, respiration and transpiration. Forage grasses have different requirements for heat, which determines their range and their sustainability. Cheshmedzhiev (1980) reports that high temperatures during summer not only reduce the yield of natural grasslands, but impair the feed quality due to the increase in fiber and decrease in the weight ratio of leaves to the stem. The steady retention of the average air temperature above 4°C, which begins the vegetation period of the pastures, is around March 20th, with the end of the vegetation for the year around November 20th, i.e. the average duration of the vegetation season is about 245 days. The temperature in the vegetation season (April-September) for the experimental period (2011-2013) was higher than the average in the region, registered for 25 years ahead (16.8°C) by an average of 1°C. It was almost 2°C in the second year.

Meteorological conditions during the study show variation in temperature and precipitation, which specifically affects the development and productivity of grass species.

This variation can be seen both in the different years of the study and when compared with the multiannual averages (climate norms) for the period 1988-2013.

The soil on which the experiments are based is light gray, forest, with shallow - A horizon and deep, heavily charred - B horizon. The experiment was set on a slope with an inclination of 7° (near a forest), southeastern exposure, using the block method in 4 replications, with a plot size of 5 m^2.

The results were statistically processed by ANOVA 10. LSD test was conducted for statistical significance of differences, standard deviation and coefficient of variation, as well as using variational-statistical method (Lidanski, 1988). RSM- Responsible Surface Method - summary profile of variable characteristics (productivity of grassland type, fertilizing method, years).

Results and Discussion

Regressional dependences between the dry matter yield from a meadow of Chrysopogon gryllus L. *type and the amount of precipitation in mineral and organic foliar fertilization.*

In the case of variable fertilization of a grassland of *Chrysopogon gryllus* L. type with

mineral nitrogen and phosphorus, the highest yield of biomass was obtained in the variants N_{60} kg/ha (first year), P_{60} kg/ha (second year) and N_{60} kg/ha (third year). The highest biomass amount was registered in foliar fertilizing with Biostim at a dose of 3000 ml/ha.

In the third experimental year, mineral fertilization registered the highest effect on the productivity of a meadow of *Chrysopogon gryllus* L. type. The amount of dry matter in the mineral fertilization variants was from 24.47 to 66.49% ($P < 1.0$ and $P < 0.1$) higher than the nontreated control.

According to the correlation analysis, the dry matter yield correlates very well with the amount of precipitation. On this basis, regression equations have been developed to estimate the yield by precipitation as an independent variable. Dry matter yield can be precisely determined by using a second degree polynomial. The coefficient of determination expressing the relationship between dry matter yield and precipitation during the vegetation (monthly) R = 0.734 is sufficiently high (Table 3). The standard error in the estimate ranges from 0.93 to 8.05.

The error is smallest when precipitation is used as an independent variable in August. The accuracy of the equations is higher when they include precipitation in the months of April, June, July and August, and less when is used the sum of precipitation during the vegetation season (April - September). The least accurate equations are obtained using the annual precipitation sum as an independent variable.

The average precipitation increased by 79.3 mm in the third experimental year only (2013) during the vegetation season (April-September) compared to a long period (476.5 mm). A deficit of 64.5 mm was reported in 2011 and a deficit of 137.6 mm in 2012 (Table 2). Drought has adversely affected the complex biochemical processes of plant growth and development.

Particularly significant were the precipitation data in 2012, when the drought during the summer months was extreme. During the vegetation season (April-September), the registered precipitation of 338.9 mm was 137.6 mm (28.88%) less than for a 25-year period (476.5 mm). In 2013, the amount of vegetation precipitation (555.8 mm) was 79.3 mm (16.64%) more than the long-term period (476.5 mm).

Foliar fertilizing with Biostim during the first year did not significantly affect the dry matter yield as the probable cause was the specific interaction: foliar fertilizer - type of grassland - climatic conditions. In the second and third years, the excess over control was 102.1-122.9% (at 3000 ml/ha).

The relationship between dry matter yield (obtained by *Chrysopogon gryllus* L. meadow type during foliar fertilizing) and precipitation is relatively good and is proven by a high determination coefficient R = 0.801 (Table 4). There is a high dependence between the yield and precipitation in April, June, July and August, where the average error is smaller. The reliability of equations is statistically significant at $P < 0.00207$. The accuracy is less when precipitation is used in the equations in May, as well as the precipitation amount during the vegetation period or the annual precipitation amount.

Table 1. Air temperature (°C).

Month / Year	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	I –XII	IV-IX
2011	-0.2	-0.3	4.9	9.7	14.7	18.5	21.0	20.2	18.2	8.8	1.8	2.6	9.99	17.1
2012	-2.1	-5.4	5.6	12.2	15.1	20.8	24.2	21.7	17.8	13.2	7.6	-0.4	10.86	18.6
2013	1.1	3.8	5.4	12.0	17.5	18.7	19.4	22.7	15.4	12.1	7.7	0.8	11.38	17.6
Average 2011-2013	-0.4	-0.6	5.3	11.3	15.8	19.3	21.5	21.5	17.1	11.4	5.7	1.0	10.74	17.8
Average 1988-2013	-0.6	2.1	5.4	10.3	15.2	18.6	20.8	20.5	15.6	10.8	5.7	1.8	10.52	16.8

Table 2. Monthly and annual precipitation (mm).

Month Year	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	I –XII	IV-IX
2011	22.5	24.8	41.7	68.0	69.1	98.4	72.9	96.8	6.8	109.0	2.0	34.3	646.3	412.0
2012	124.5	45.6	35.5	36.0	174.0	51.8	7.2	39.1	30.8	43.6	11.8	79.1	679.0	338.9
2013	43.3	62.6	49.7	92.1	90.3	274.6	61.2	14.9	22.7	53.2	27.7	15.0	807.3	555.8
Average 2011-2013	63.4	44.3	42.3	65.4	111.1	141.6	47.1	50.3	20.1	68.6	13.8	42.8	710.9	435.6
Average 1988-2013	37.5	39.1	56.2	61.7	93.0	104.3	83.5	69.7	64.1	51.8	40.4	44.8	734.2	476.5

Table 3. Regressional equations of the dependence between dry matter yield and precipitation in a meadow of *Chrysopogon gryllus* L. type, using mineral fertilizing with N and P. Legend: Determination coefficient (R); Standard Estimation Error (*SEE); Dependent and Independent Dimensions (F); Statistical significance of the equation (P<).

Equation	**R**	**SEE***	**F**	**P<**
Y = 200900.5-543.5W I-XII+0.4W I-XII2	0.734	8.05	12.26	0.00029
Y = -26707.2+134.8W IV- IX-0.1W IV-IX2	0.734	2.48	12.26	0.00029
Y = -4337.6+243.14W IV-1.81W IV2	0.734	1.23	12.26	0.00029
Y = 10076.55-119.2W V+0.42W V2	0.734	2.11	12.26	0.00029
Y = -909.077+67.9W VI-0.199W VI2	0.734	1.32	12.26	0.00029
Y = 2537.4-73.07W VII+1.25W VII2	0.734	1.17	12.26	0.00029
Y = 3570.7-66.08W VIII+0.712W VIII2	0.734	0.93	12.26	0.00029

Table 4. Regression equations for the dependence of dry matter yield - precipitation, from a meadow of *Chrysopogon gryllus* L. type with foliar fertilizing with Biostim. Legend: Determination coefficient (R); Standard Estimation Error (*SEE); Dependent and Independent Relation (F); Statistical significance of the equation (P<).

Equation	**R**	**SEE***	**F**	**P<**
Y = 18376.74-485.2W I-XII+0.3W I-XII2	0.801	9.37	10.8	0.00207
Y = -30295.1+163.4W IV-IX-0.2W IV-IX2	0.801	2.89	10.8	0.00207
Y = -4611.46+330.79W IV-2.64W IV2	0.801	1.39	10.8	0.00207
Y = 19334.46-269.08W V+1.04W V2	0.801	2.46	10.8	0.00207
Y = 694.98+73.39W VI-0.23W VI2	0.801	1.53	10.8	0.00207
Y = 5244.1-211.04W VII+2.97W VII2	0.801	1.36	10.8	0.00207
Y = 3347.06+6.44W VIII+0.183W VIII2	0.801	1.08	10.8	0.00207

The precipitation regime of the region is continental. Most precipitation falls in summer (309 mm) and spring (242 mm), less in autumn (209 mm) and at least in winter - 168 mm (Ninov, 1997). Precipitation amount in months with temperatures higher than 0°C ranges from 596 to 600 mm.

The agroecological conditions of the experimental area, as well as the applied agrotechnical events, have a significant impact on the productivity and development of natural grassland communities.

The efficiency in supplying plants with the optimal amount of soil moisture is determined by: the depth of the root system and the weight of its total mass; the length of life of roots, their total length and surface; season, phase of development and mode of plant use.

Droughts are observed in all seasons of the year, which influences the physiological processes during the different phenophases and stages of the individual development of crops. Under these conditions, natural grasslands manage to form one harvest and very rarely autumn hay (aftergrass).

The moisture content and precipitation distribution during the vegetation season affect productivity and determine to a varying degree the effects of testing other factors, such as fertilizing method (mineral or foliar) and specific morphological structure, and species potential (a meadow of *Chrysopogon gryllus* L. type and a natural pasture of *Nardus stricta* L. type) of the grasslands.

Factor analysis of the impact of fertilizing and climatic conditions during the year on the productivity of a meadow of Chrysopogon gryllus L. *and natural pasture of Nardus stricta* L. *grassland.*

The type of grassland and the conditions of the year have a greater impact (η^2 = 0.55-0.56%) on the productivity of the studied grasslands after applied fertilization (N and P) compared to the fertilizer level factor. The difference in the strength of the influence of the two factors on the studied trait is insignificant, respectively, 21.89% and 22.70% (Table 5). The strength of their combined action (21.81%) is equal to the independent influence of the type of grassland. The private determination coefficient (η^2) confirms the power of influence of these factors.

The level of fertilization has a weaker effect (7.59%) on the yield of the natural grassland of *Chrysopogon gryllus* L. type and a pasture with *Nardus stricta* L.

For the experimental period, fertilizing with P_{60} (first year), N_{60} (second year) and P_{60} (third year) recorded the lowest values regarding the productivity of grassland. In contrast, the introduction of: P_{60} (first and second year) + N_{60} (third year) and N_{60} (first year) + P_{60} (second year) + N_{60} (third year) increased optimally (2316.0 kg/ha) the yield of the biomass.

In the variants with bio-fertilization, yields in the first and second experimental years were almost identical. In the third vegetation season, the productivity of natural biomass was significantly higher (3062.7 kg/ha), mainly in the variants with foliar fertilizing with 4000 ml/ha Biostim.

A significant difference was observed in the strength of the factorial influence in both types of grassland treated with humic fertilizer Biostim. The type of grassland had the main and strongest impact (62.81%) followed by the combination of type x year (14.04%) - Table 6. The level of fertilization had the slightest impact (2.45%) on the aboveground mass production both with the mineral fertilization and the foliar bio-fertilization.

The RSM Combined profile clearly shows that, after the application of nitrogen and phosphorus mineral fertilization, the grassland of *Chrysopogon gryllus* L. type has significantly higher productivity than that of the pasture of *Nardus stricta* L. type (Fig. 1).

The year has a much slighter impact here - only 7.84%. Fertilization has a very low impact - only 2.45%.

The interaction type in relation to year - 14.04% is higher than the year as a separate factor - 7.84%.

The RSM combined foliar fertilization profile shows that the meadow yields are higher than the pasture (Fig. 2).

The four factors tested have different effects on the productivity of both natural grasslands: a meadow of *Chrysopogon gryllus* L. and a pasture of *Nardus stricta* L. type.

The multifactor analysis of the impact of the factors, performed on 312 results from the four attempts in four replications, shows that for the conditions of the Central Balkan Mountain the productivity of the grasslands is most strongly influenced by the type of the grassland - 63.42% at the highest private determination coefficient η^2 = 0.35% (Table 7).

The year with its specific climatic conditions - rainfall - 20.32%, is in second place in terms of impact.

In third place is the fertilizing method - mineral with N and P or foliar fertilizing with Biostim - 8.82%.

The fourth place in terms of the impact is for the level of fertilization (applied doses of fertilizers and the way of their alternation) - 7.43%.

The RSM method for summarizing the variation of individual factors shows that the productivity of a meadow of *Chrysopogon gryllus* L. type on average is significantly higher than that of a pasture of *Nardus stricta* L. type (Fig. 3).

Two types of fertilization were tested - mineral with alternate application of N and P fertilizers and foliar fertilizing with organic fertilizer. Higher yields were obtained from foliar fertilizing.

The lowest yields were obtained for both types of weeds and fertilizers in 2012. In the first and third years, yields were almost identical. This is explained by the unfavorable climatic conditions this year, especially during the vegetation season.

From the first to the fifth variant of fertilization, yields increased and then decreased.

Table 5. Degree of the impact of factors in mineral fertilization with N and P. Legend: Degree of Freedom (df); Relationship between analyzed quantities (F); Reliability of the difference between indicators (P<); Partial eta-squared (η^2).

Factors	SS	df	F	P<	η^2(%)	Degree of impact (%)
Grassland type (meadow or pasture)	63241461.5	1	176.701	0.000000	0.55	21.89
Year	65586200.8	2	91.626	0.000000	0.56	22.70
Level of fertilizing	21920207.2	7	8.750	0.000000	0.30	7.59
Type*Year	63020976.6	2	88.043	0.000000	0.55	21.81
Type*Fertilizing	27508610.3	7	10.980	0.000000	0.35	9.52
Year*Fertilizing	21951145.1	14	4.381	0.000002	0.30	7.60
Type*Year*Fertilizing	25679106.1	14	5.125	0.000000	0.33	8.89
Mistakes	51537725.6	144				

Table 6. Degree of factor impacts on foliar fertilizing with Biostim. Legend: Degree of Freedom (df); Relationship between analyzed quantities (F); Reliability of the difference between indicators (P<); Partial eta-squared (η^2).

Factors	SS	df	F	P<	η^2(%)	Degree of impact (%)
Type of grassland meadow or pasture	185867120.6	1	246.87	0.000000	0.73	62.81
Year	23211291.0	2	15.414	0.000002	0.26	7.84
Level of fertilizing	7245288.5	4	2.406	0.055310	0.10	2.45
Type*Year	41534890.8	2	27.583	0.000000	0.38	14.04
Type*Fertilizing	10769864.5	4	3.576	0.009395	0.14	3.64
Year*Fertilizing	16470303.7	8	2.734	0.009545	0.20	5.57
Type*Year* Fertilizing	10827459.5	8	1.798	0.087779	0.14	3.66
Mistakes	67761628.1	90				

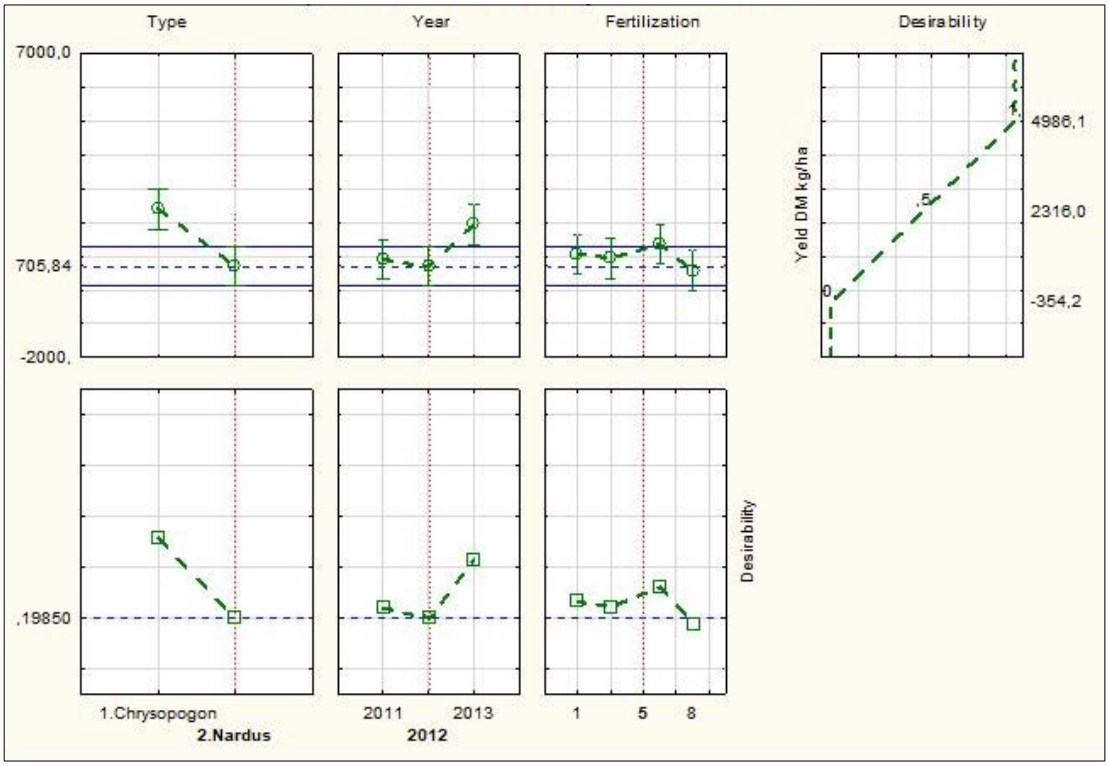

Fig. 1. RSM profiles for predicted values and desirability of factors in meadow *Chrysopogon* and pasture *Nardus* fertilized by mineral fertilization.

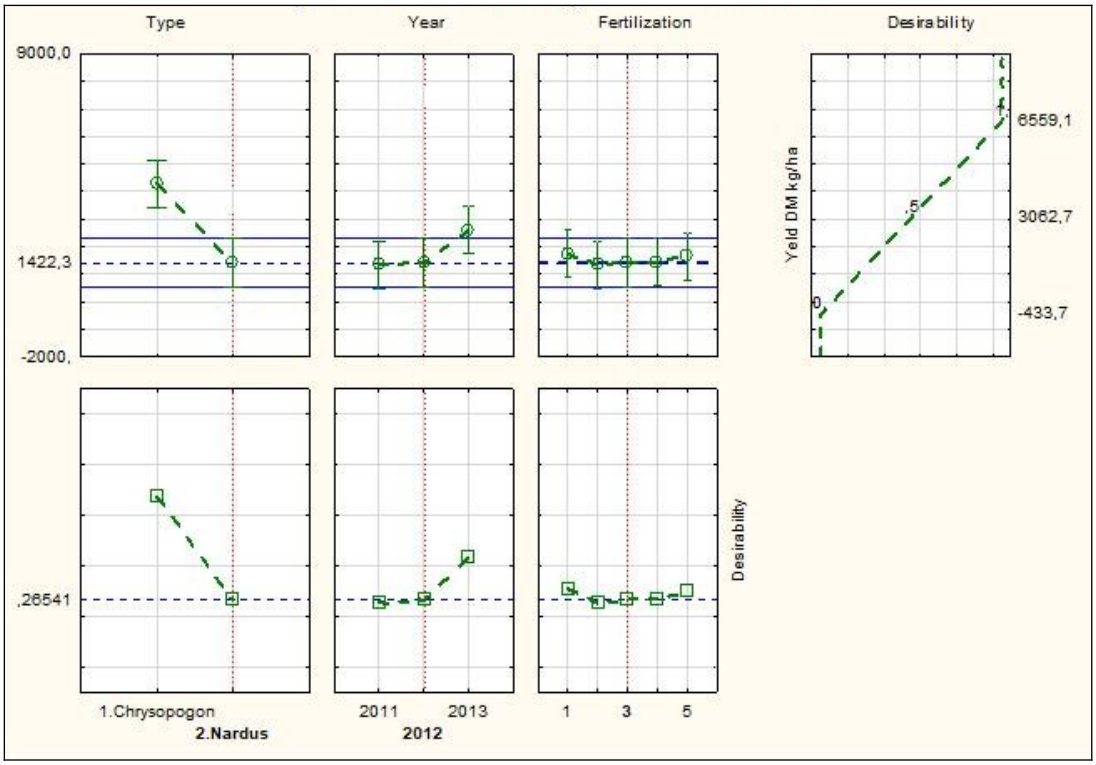

Fig. 2. RSM profiles for predicted values and desirability of factors in meadow *Chrysopogon* and pasture *Nardus* fertilized by leaf fertilized Biostim.

Table 7. Degree of impact of factors on dry matter yield (n = 312). Legend: Degree of Freedom (df); Relationship between analyzed quantities (F); Reliability of the difference between indicators (P <); Partial eta-squared (η^2).

Factors	SS	df	F	P<	η^2(%)	Degree of impact (%)
Type of grassland (meadow or pasture)	215897018.0	1	164.489	0.000000	0.35	63.42
Fertilizing method (mineral or foliar organic)	30039261.6	1	22.887	0.000003	0.07	8.82
Year	69187543.2	2	26.357	0.000000	0.15	20.32
Level of fertilizing	25289245.7	7	2.753	0.008742	0.06	7.43
Mistakes	393759473.6	300				

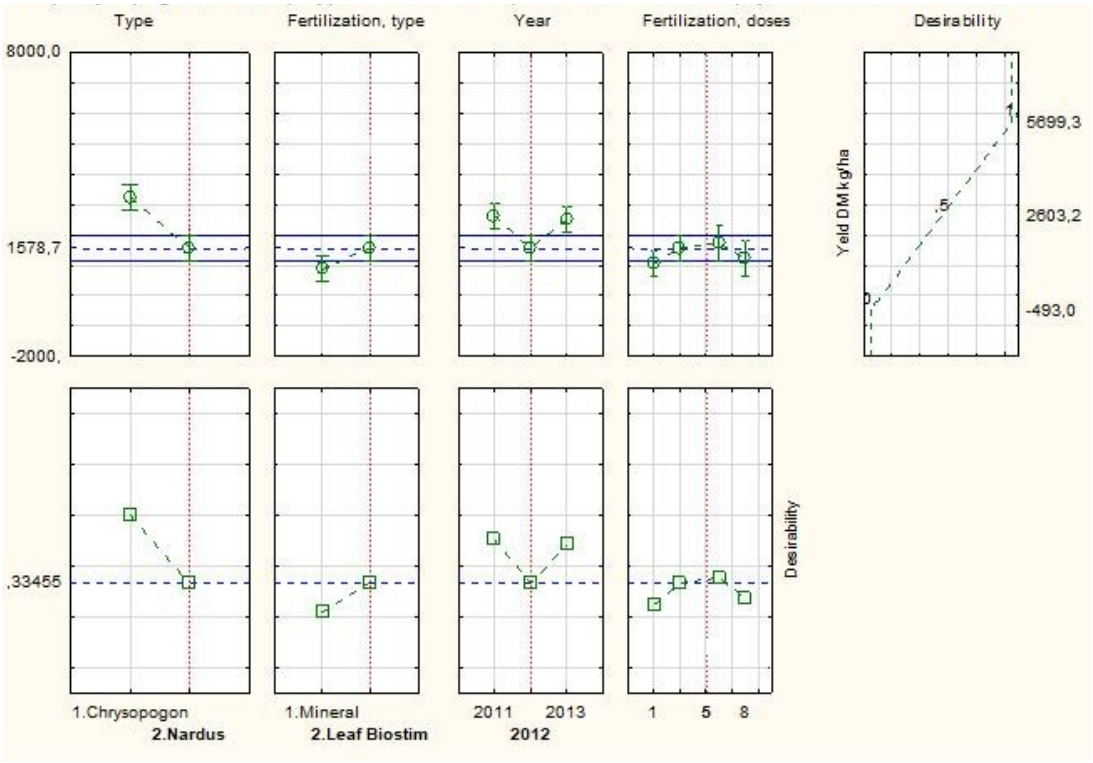

Fig. 3. RSM profiles for predicted values and desirability of factors the type pf the grass (*Chrysopogon, Nardus*), type of fertizilation (Mineral, Leaf Biostim), year and fertilization doses.

Conclusions

In the case of variable fertilization of a meadow of *Chrysopogon gryllus* L. type with mineral nitrogen and phosphorus, the highest yield of biomass is obtained in the variants N_{60} kg/ha (first year), P_{60} kg/ha (second year) and N_{60} kg/ha (third year). The highest amount of biomass was registered in the foliar fertilizing with Biostim at a dose of 3000 ml/ha. The highest yield was registered in a grassland with *Nardus stricta* L. with annual fertilization with N_{60} and P_{60} kg/ha, and with foliar fertilizing with Biostim with a dose of 1000 ml/ha.

There is good regression dependence between productivity and moisture supply, which enables, by means of regression

equations, an approximate prediction of biomass yields from natural grassland of *Chrysopogon gryllus* L. type, fertilized with mineral and foliar fertilizers. The determination coefficient R - 0.7 - 0.8 is sufficiently high for the practical determination of productivity by the sum of precipitation during the critical months of April to July. When using the annual or seasonal precipitation, the accuracy is less.

The summarized multifactorial analysis of the impact of factors, such as methods (mineral and foliar organic), fertilization levels, type of grassland and climatic conditions over the years, shows that the type of grassland has the strongest impact on the conditions of the area on the productivity of the grasslands – 63.42%. The year with its specific climatic conditions - rainfall - 20.32%, is in second place in terms of the impact. The fertilizing method, such as mineral fertilizing with N and P or foliar fertilizing with Biostim - 8.82%, is in third place in terms of impact. The level of fertilization (the applied doses of fertilizers and the way of their alternation) - 7.43% has the slightest impact.

The summary RSM analysis shows a slightly higher efficiency of foliar fertilizing for both types of grasses.

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Received: 29.05.2020
Accepted: 24.08.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 77-86

Study on Carrion Sharing Between the Golden Jackal (Canis aureus Linnaeus, 1758) and Sympatric Scavengers Over the Winter Period in Central Bulgaria using Camera Trapping

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Abstract. The position of the Golden jackal (*Canis aureus* Linnaeus, 1758) within scavenger society in three Central Bulgaria habitats of different anthropogenic impact was investigated using camera trapping during the winter period of 2019-2020. Three different types of carrion (jackal carcass, wild boar and cattle skin) were used as lures. The scavenger society was represented by 15 vertebrates, including 8 bird and 7 mammal species, in these habitats. The study found that avian scavengers had an advantage over mammals in detecting carrion in open habitats due to their greater visibility. In mountainous forest habitats the scavenger community consisted mainly of mammals, with the jackal reliably detecting and using carrion as a food source. The study also found that an observational period greater than 25 days is required to attract all potential scavengers.

Key words: carrion, avian scavenger, lures, Red fox, Wild boar, habitats.

Introduction

Soft tissues, as well as bones of animal carcasses, contain energy and nutrients that in one way or another are involved in the cycle of the ecosystem. This process is critical for the function of the ecosystem (Barton et al., 2013; Moore et al., 2004; Parmentes & MacMahon, 2009). Dead large ungulates represent an abundant nutritional resource for medium-sized predators that would otherwise not be able to hunt them. No matter what the cause of their death (road accidents, illnesses, victims of larger predators or poaching), they represent a valuable nutritional resource that supports the survival of all predators during periods of food shortage. The wild boars, red deers and roe deers serve as such a resource in Bulgaria. Occasionally corpses of farm animals such as horses, cattle, sheep and goats are left in the wild. Almost all mammalian carnivores use to scavange frequently. Upon detecting a carcass, they take advantage of it by switching from active predation to carrion eating (Moleon et al., 2014). On this basis, commensal communities consisting mainly of birds and mammals are formed in different latitudes across the land. Their composition, as well as the relationships between the species, have been the subject of numerous studies (Inagaki et al., 2020; Kane & Kendall, 2017; Sebastian–Gonzales et al., 2013; Selva & Fortuna, 2007; Selva et al., 2005; Turner et al., 2017; Wilson & Wolkovich, 2011).

In Bulgaria, the scavenger guild has not been studied yet. The Golden jackal is well known as a scavenger species. Its diet includes remains of wild ungulates and farm animals (Raichev et al., 2013; Stoyanov, 2012). The position of the Golden jackal in the scavenging community has not been studied in the country.

Material and Methods

In order to reveal the potential specification of the facultative scavenger community according to habitat type and to get a full picture of its composition in Central Bulgaria, three different habitats were selected. One of the studied areas is located on the southern slopes of the central part of the Balkan Mountains. It is a steep and wooded, inaccessible terrain with low anthropogenic influence (mainly hunting), with an average altitude of 750-800 m (Fig. 1).

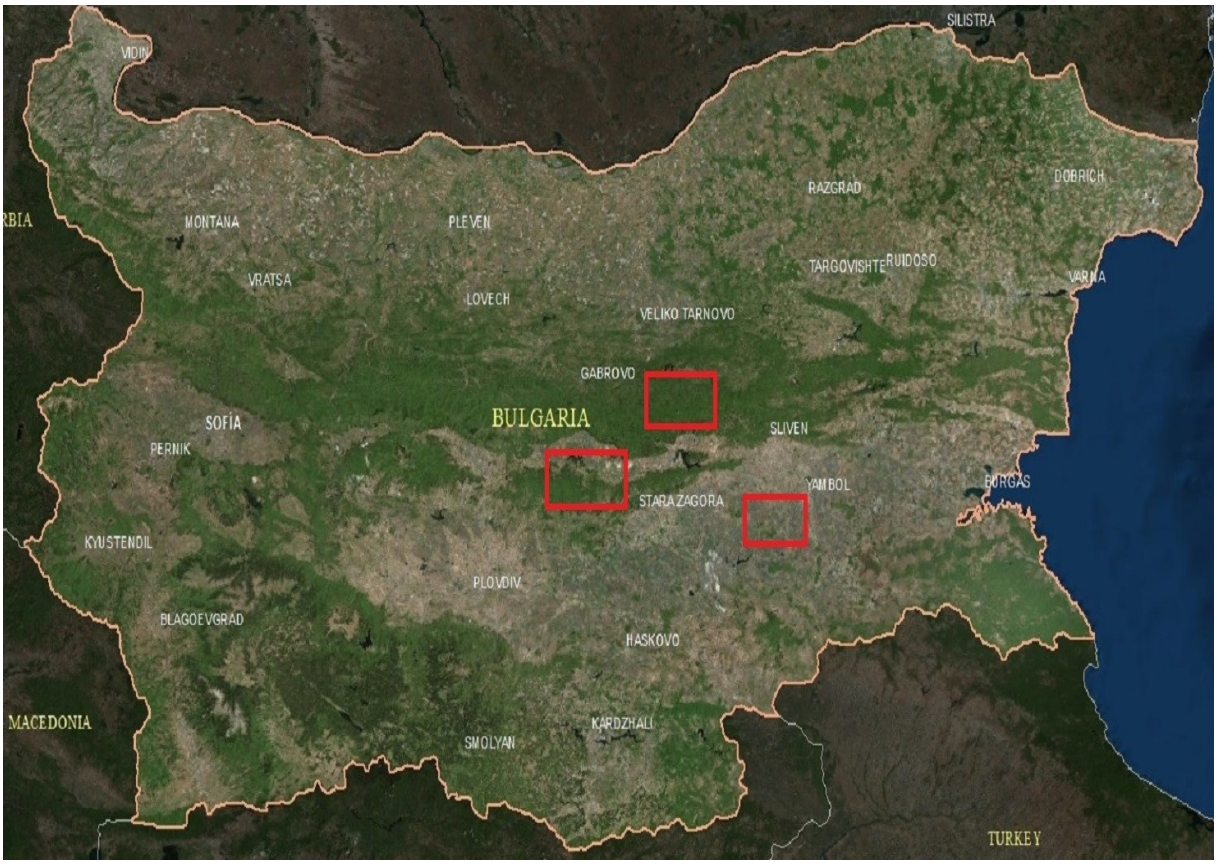

Fig. 1. The location of the three studied areas in Central Bulgaria.

The second area is a part of the territory of Sarnena Sredna gora Mts. The terrain is hilly with villages located at a distance of 5-6 km from each other which leads to stronger anthropogenic influence (agricultural activities, hunting, tourism) compared to the Balkan Mountains. The third study area is a part of the Upper Thracian plain, an open landscape with many arable lands, deserted lands and ponds. The lowland forests occupy insignificant areas and the anthropogenic impact is stronger than in the previous 2 areas. Agricultural activity is intensive, although in some places the area has become depopulated.

With the assistance of forest officials, as well as local hunters, some of the wild boar and jackal shooting sites were found in the study areas. After processing a wild boar carcass, a piece of skin weighing about 10 kg was placed in the area while a whole jackal carcass was left in the jackal shooting

location. This simulated the most common practice in our country. With the same idea, a piece of bovine leather, again weighing about 10 kg, was placed in the forest or in the field. Three types of lures were placed of equal weight to eliminate the influence of carcass size on the composition of the scavenger community (Turner, 2017). The bait was fixed to the base of a tree or shrub. The three types of lures were laid out in the three regions consecutively, in December, January and February of 2019-2020. The places were chosen according to the following rules:

- The lure must be situated away from animal paths so that passing animals are not captured by the cameras. The purpose was to capture only those animals that were attracted to the bait.
- The sites must be away from hunter or tourist trails (minimum 100 m) to minimize the impact of the anthropogenic factor.
- Each month, 9 new different carrion sites were selected to prevent creating a habit in wild animals to look for food in the same place. The goal was the bait to be found again every time. Thus 27 different sites were observed (9 cameras in three consecutive trials).

Three different models of infrared cameras were used: Keep guard Cam (KG690NV), Ltl Acorn (6310 – 3G) and HD camera with Black IR (SG56OK – 14 m). They were all set to take 2 pictures after triggering with followed one-minute interval. The devices were attached to trees at a height of 1.5 - 1.8 m from the ground, at a distance of 5 - 8 m from the laid bait. In this way, the lure was at the center of the resulting photo. Photos from a period of 25 days after the bait laying were used for the study. During this time the sites were not visited by the researcher to avoid the modification of wild animals' behavior. When reporting the results, the photos from consecutive and prolonged capturing of an individual within an hour, were counted as one event. A list of all species detected (birds and mammals altogether and separately for the three regions) was made. The relative order of their appearance was calculated; as well as the number of places visited by each species.

Results and Discussion

In winter, the scavenger community included 15 vertebrates (8 bird and 7 mammal species), throughout the study area. During the 25 monitored days, most of the mammals and birds attracted to the bait were detected by the cameras prior to the 5th day (Fig. 2).

However, the whole 25-day period is not sufficient to establish the full composition of the probable compatriots. In the three study areas, representing a diversity of habitats: forest (semi-mountainous and mountainous) and open (lowland), some differences in the composition of scavenging communities were found (Table 1). The least numerous community consisting of one bird (Eurasian jay) and 7 mammal species (Domestic dog, Golden jackal, Red fox, European wildcat, Eurasian badger, Stone marten and Wild boar) was found in the Balkan Mountains.

Despite the presence of the Grey wolf (*Canis lupus*) in the area, it was not detected by the cameras. The probable reasons for its absence were: its population density was low; it was suspicious to human smell and the investigated winter season was unusually warm. What is striking, was the low presence of birds, even though the golden eagle and the common raven were typical inhabitants of the mountainous area. In previous years, however, they took advantage of the wild boar carcass provided (personal observation).

The scavenger guild in the semi-mountainous region of Sarnena Sredna gora Mts. was represented by 2 bird species (Common buzzard, Eurasian jay) and 7 mammal species (Golden jackal, Red fox, Domestic dog, European wildcat, Stone marten, Eurasian badger and Wild boar). The

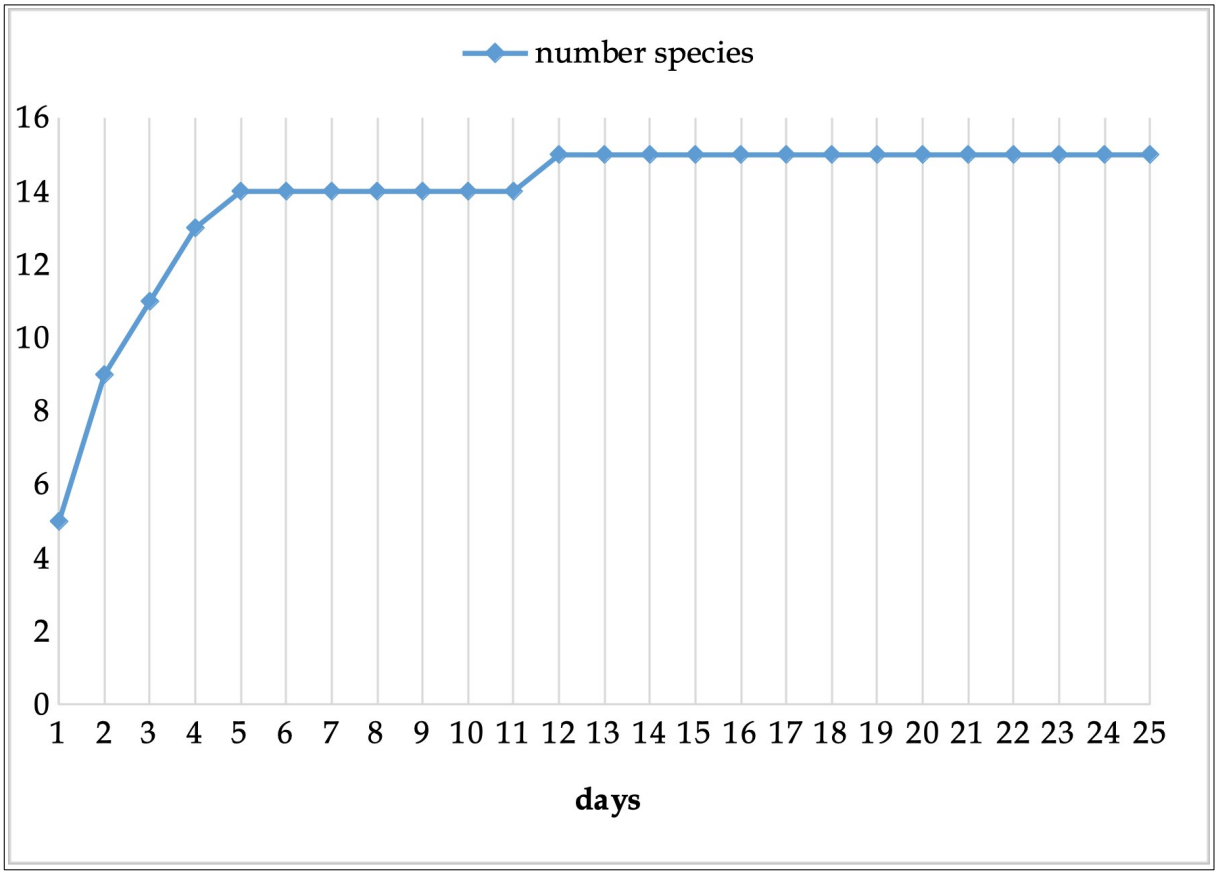

Fig. 2. Accumulation of the detected species number during investigated period.

Table 1. The list of scavenger community established in three different habitats in Central Bulgaria.

№	Species	Balkan Mountains	Sarnena Sredna gora Mts.	Upper Thrakian Plain	Mean value of order of appearance (MOA)
1	Magpie *Pica pica*			+	1.5
2	Common buzzard *Buteo buteo*		+	+	1.8
3	Eurasian jay *Garrulus glandarius*	+	+	+	1.86
4	Eastern Imperial Eagle *Acuila heliaca*			+	2
5	Golden jackal *Canis aureus*	+	+	+	2.68
6	Stone marten *Martes foina*	+	+	+	2.8
7	Eurasian Badger *Meles meles*	+	+		2.83
8	Domestic dog *Canis lupus familiaris*	+	+	+	2.83

9	Grey heron *Ardea cinerea*			+	3
10	Wild boar *Sus scrofa*	+	+	+	3.44
11	Red fox *Vulpes vulpes*	+	+	+	3.54
12	Western Marsh harrier *Circus aeruginosus*			+	4
13	Hooded crow *Corvus cornix*			+	4
14	Common raven *Corvus corax*		+		4
15	European wildcat *Felis silvestris*	+	+	+	4.5

birds contributed to the largest number of established species, detected in the Upper Thracian plain. This high detectability of birds can probably be due to good visibility in the open habitat (Turner, 2017), which facilitates the detection of carcasses from the air (Selva et al., 2005). The lures were visited by 6 species of mammals and 7 species of birds, which is the almost complete list presented in Table 1. Thus, the jackal's competition for carrion in open habitat is higher than in the other studied areas. However, in the lowland the jackal attracted by the presence and sounds of the Corvids could reach the carrion faster. The presence of the Eastern Imperial eagle and birds of the wetlands, such as Western Marsh harrier and Grey heron, is noteworthy. Vultures (*Gyps fulvus*) were absent in all three surveyed areas. After the extinction of the species from the territory of the country in the 60's, nowadays it is found in a small area along the Arda River (Stoyanov et al., 2018). It could be assumed that if the vultures were present, their role in the scavengers' guild would be palpable. The vultures play a crucial role in the ecosystem as the best cleaners (Mann & Banks, 2017). In the present study, their role is distributed to other species of birds and mammals found in the study areas.

According to the order of detection of the carcass calculated by the Mean value of the Order of Appearance (MOA) in front of the camera traps, the species can be divided into several groups. Part of the birds respond rapidly (MOA - 1.5-2) to the bait: Magpie, Common buzzard, Eurasian jay, Eastern Imperial eagle (Table 1). Although magpies hunt close to their resting places (Vines, 1981), in most of the cases they found the lure first. This is probably due to their large number and the detection of human presence when placing the carrion. Most of the mammals formed the second group of visitors (MOA - 2.68-3.54) as follow as: Golden jackal, Stone marten, Domestic dog, Eurasian badger, Wild boar and Red fox. The accidental appearance of a grey heron disrupted this sequence. Since this species appeared only once, it can be considered as a result of curiosity. However, it has been described as an element of the avian scavenger society in Spain (Hiraldo et al., 1991).

The Hooded crow, the Common raven, and the Western Marsh harrier appeared later (MOA - 4). The delay in the appearance of typical carrion-eating species such as the Common raven and the Hooded crow (Hiraldo et al., 1991) was probably due to their suspicion towards human presence. The European wildcat was the last (MOA – 4.5; Table 1), which is consistent with the claim that it rarely feeds on carrion (Hewson, 1983; Moleon & Gill-Sanchez, 2003).

The number of visited baited sites indicates the importance of the carrion for the species' diet (Selva & Fortuna, 2007). The

presence of jackals was reported in 22 of the 27 baited places. In India, the jackal scavenges on cattle carcasses because of the taboo to eat calves (Yunman et al., 2015). In Israel, the jackal not only scavenges on cattle carcasses but also attacks newborn calves (Yom-Tov et al., 1995). In the Ngorongoro Crater, the jackal scavenges opportunistically (Temu et al., 2016). In Serbia, this species is estimated to remove about 3,700 tons of animal waste per year, and in Europe, this amount consists of more than 13,000 tons per year (Cirovic et al., 2016). The jackal's dependency on carrion consumption is documented in this study. Other authors in Bulgaria found a significant presence of remains from domestic and wild mammals, as well as closely related species (Domestic dog, Golden jackal, Red fox) in the jackal's diet in the areas of this study (Vlasseva et al., 2013; Raichev et al., 2013; Tsunoda et al., 2017) and in other regions of the country (Atanasov, 1953; Genov, 1989; Stoyanov, 2012). Founding a large ungulate carcass, the jackals may form clusters of up to 14 individuals (Moehlman & Hayssen, 2018; Van Lawick & Van Lawick-Goodal, 1970). In the present study, no more than 3 individuals were captured together at one baited place. In these cases, the jackals were standing next to each other feeding in succession.

The Red fox was observed at 14 sites and the Stone marten at 10. In Spain, these two species are defined as meso-facultative scavengers. Together with the vultures, they are the main contributors for the nestedness of scavenging society (Sebastian-Gonzales et al., 2016). The Red fox is one of the major facultative scavengers in the world (Henry, 1977; Mateo-Tomas et al., 2015; Young et al., 2014). The presence of the Eurasian badger at 9 sites was observed but without consumption. That fact doesn't exclude it from the scavenger guild in the studied area, as it is a proven competitor to the Red fox for this nutritional resource (Young et al., 2014). The Wild boar, the same as an Eurasian badger, ranked fourth according to the number of visited places with bait (9 sites). As an omnivorous species, it consumes carrion (Wilson & Wolkovich, 2011) and also exhibits cannibalism (Cukor et al., 2019; Taylor & Helgren, 1997). The group nature of Wild boar visits was documented in this study.

The photographed dogs were difficult to define as feral or stray. The first group is defined as dogs gone wild, which hunt their prey and live away from humans. The second group consists of animals that live in dumps and hunt for fun, rather than passion, but not for food (Scot & Causey, 1973). Among the dogs captured in this study (6 sites), there were individuals with ear tags, i.e. neutered and fled far from settlements. Whichever category they belonged to, it was obvious that they took advantage of the carrion, which was their typical behavioral feature. (Macdonald & Carr, 1995; Selva et al., 2005). Stray dogs are less afraid of humans and can be active both during the day and at night (Zanin et al., 2019), which gives them some advantage over wild carnivores in detecting and consuming carrion.

The Magpie and the European wildcat exhibited the same level of visitation (6 sites each), and 5 places were visited by Common buzzard. The Eastern Imperial eagle, the Hooded crow, the Common raven, the Western Marsh harrier and the Grey heron visited each by one place out of the possible 27. Under the conditions of the experiment: warm and short winter period, low amount of carrion and intense competition among predatory mammals, these species behaved as sporadic scavengers. It could be assumed that their role as carrion consumers would be changed under different conditions. For example, the Common raven could be a companion of the Grey wolf, willing to take advantage of its prey (Stahler et al., 2002; Vucetich et al., 2004). In addition, Corvids are known to be mostly looking for fresh food avoiding eating poor quality carcasses. It matters to them how fresh the carrion is and what nutritional value it brings (Gomo

et al., 2017). In the present study, wild boar and cattle skins had almost no nutritional value for these bird species and this should be taken into account.

Conclusions

During winter in Central Bulgaria, the Golden jackal shares carrion as a food resource with 14 other vertebrate species. A 25-day study period is not sufficient to attract all potential compatriots. Open areas provide some advantage to birds over mammals in detecting a dead animal. In the highlands, the facultative scavenger guild consists mainly of mammals. The Golden jackal is the main species that reliably detects and certainly uses carrion as a food resource.

Acknowledgements

I am thankful to my scientific supervisor, Dr. E. Raichev from Trakia University as well as to the hunters and forestry officers from Stara Zagora region for the support at the time of hunting season in finding proper places for setting cameras. I further appreciate Dr. H. Tsunoda from Center for Environmental Science in Saitama and Dr. Y. Kaneko from Tokyo University of Agriculture and Technology for the technical support and also Ms Christina Mihaylova for her help in the English expression. The study was partly supported by a Grant-in-Aid for Scientific Research (No. 22405003 and 26257404) from the Japan Society for Promotion of Science.

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Received: 30.05.2020
Accepted: 24.08.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 87-96

The Role of Seed Zinc Priming on Tolerance and Ionic Ratios of Green Bean under Salinity

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Abstract. The effects of salt applications of different concentrations (50, 100, and 150 mM NaCl) on two green bean genotypes ['Şeker Fasulye' (salt-tolerant) and 'Local Genotype' (salt-sensitive)] grown from seeds with zinc (Zn) and without Zn priming were investigated. In order to determine which part of the green bean plant involved in salt tolerance of genotypes, the tolerance ratio (TR) were calculated on the basis of root, stem, first true leaf (FTL) and trifolia leaflet (TL) dry weights. Also, the ability of maintain in equilibrium for potassium (K)/sodium (Na) and calcium (Ca)/Na ratio in the root, stem, FTL and TL parts of plants were considered. According to TR values based on dry weight; it is thought that stem is the organ of green bean genotypes which plays an important role in salt tolerance. When considering all parts of both genotypes, K/Na ratio of 'Şeker Fasulye' was determined to be affected positively in plants from Zn primed seeds. Additionally, Zn application decreased the Ca/Na ratio in all parts of both green beans. In conclusion, the results of the current study suggest that the use of Zn priming of seeds could be an effective strategy to increase the salt tolerance of green bean genotypes.

Key words: dry weight, *Phaseolus vulgaris* L., salt tolerance, K/Na, Ca/Na.

Introduction

Salt stress prevents the growth and development of the plant as a result of osmotic and ion stress (Zhao et al., 2020). Osmotic stress is the first stress that occurs when a plant is exposed to soil salinity, and it affects plant growth instantly (Horie et al., 2012). The decrease in the amount of useable water causes the cell expansion to diminish and sprout development to slow down (Dadkhah & Grrifiths, 2006). Salinity negatively affects all development periods of the plant and promotes premature leaf fall, causing severe decreases in yield (Flowers et al., 2010). Ion toxicity then occurs when the salt level reaches a certain threshold, and after that point the plant is unable to maintain ion homeostasis and growth balance (Munns & Tester, 2008). Ion toxicity and osmotic stress are primary stresses that can cause oxidative stress and a number of secondary stresses (Liang et al., 2018). During the ion stress phase that occurs subsequent to the osmotic stress, denutrition or nutritional imbalance takes place in plants as the increasing sodium (Na^+) and chlorine (Cl^-) ions in the environment compete with necessary nutrition elements such as

potassium (K^+), calcium (Ca^{2+}) and nitrate (NO_3^-) (Bazihizina et al., 2019).

Studies of plant tolerance to salt stress cover many aspects of the influences of salinity on plant behavior, including alterations at the morphological, physiological and molecular levels (Dajic, 2006). Since salt stress has very different effects on plants, many mechanisms can tolerate this stress in plants. Plants are able to reduce ionic toxicity by reducing the accumulation of toxic ions (Na^+ and Cl^- exclusion) in leaf blade and/or by increasing their ability to tolerate salts they cannot exclude from shoots (Roy et al., 2014). There are many strategies to reduce the negative effects of salt stress. Seed coating (Song et al., 2017) and seed priming is an effective strategy for decreasing the impacts of salt stress on plants, particularly at seedling stage (Ghassemi-Golezani and Nikpour-Rashidabad, 2017). Effects of priming or pre-treatment of seed persist under sub-optimum field conditions, such as salinity (Wahid et al., 2007).

One of the protective or assisting applications used in cultivation on salty fields is zinc (Zn) application to the soil or the seed (Saleh et al., 2009; Gulmezoglu et al., 2016). Alpaslan et al. (1999) informs that Zn application on salty soil diminishes the negative effects of Na and Cl on plants. Zinc is taken in by plants in the form of Zn^{+2} and becomes a part of various metabolic occurrences in plants (Gautam & Dubey, 2018). In addition to the plant variety, Zn intake of plants are affected by many conditions such as soil pH, organic substance of the soil, useful phosphorus substance, water substance, soil temperature and salinization by other elements. Soil salinization causes the Zn concentration in the soil compound to decrease (Khoshgoftar et al., 2004). This occurrence decreases the Zn intake as a result of a strong competition of salt ions with Zn^{+2} on the root's surface (AbdEl-Hady, 2007). In a case of Zn deficiency, transmissivity of the stem cell membrane increases (Gautam & Dubey, 2018) and consequently intake amount of other nutrition elements drops with the increasing Na^+ and Cl^- amounts in the roots of plants that grow in saline areas, therefore, Zn application decreases the negative impacts of salinization (Eker et al., 2013).

As in many other plants, the effects of salt stress on green bean were examined in terms of morphological, physiological and biochemical properties (Taïbi et al., 2016). Although various authors have described green bean as sensitive to salinity (Levitt, 1980), the differences between varieties also were determined in green bean as in many other crops (Assimakopoulou et al., 2015; Gulmezoglu et al., 2016; Torche et al., 2018). For instance, according to dry weights and andioxidative response, Tema showed better protection against salinity than Djadida genotype (Taïbi et al., 2016). Torche et al. (2018) also showed that there was a genetic variability between four green bean genotypes for salinity tolerance based on yield traits, total phenolic and flavonoid content. Even though there are considerable studies on the effects of salinity on green bean plants, there is a lack of information about the strategies to cope with salt stress. Therefore, this study, aims to determine the alleviative effects of seed Zn priming on variations in tolerance, K/Na and Ca/Na ratios of green bean genotypes at different salt concentrations.

Material and Methods

Zinc priming: Seeds of two green bean genotypes ('Şeker Fasulye' and 'Local Genotype') were used in the experiment. For Zn priming, the seeds were soaked in a solution of zinc sulphate ($ZnSO_4.7H_2O$) prepared to contain 0.05% zinc (Zn) before planting (Harris et al., 2008). After the seeds were soaked in the Zn solution, they were washed with deionized water and air dried. The seeds without Zn were washed only with deionized water and dried.

The trial: Randomized blocks are arranged in 3 repeats and 10 plants per repeat according to the trial pattern. The

seeds were sown into seedling trays filled with a mixture of peat: perlite: vermiculite (2:1:1) and placed in a plant growth chamber (DAIHAN WGC-1000), in which temperatures were 26/18 ^{o}C (day / night), with approximately 70% RH and a light intensity of 450 molµm^{-2}s^{-1} (Khadri et al., 2006). The water needs of the plants were started as 20 mL of ultrapure water daily until the first true leaves (FTL) were emerged, and then the salt applications [0 (control), 50, 100 and 150 mM NaCl] were started and treated for two weeks.

Determination of ions: At the end of experiment, the aboveground parts of the plants [FTL, trifolia leaflet (TL), and stem] and root parts were separated, and were washed with tap water and then with deionized water. The plant parts were dried in the drying cabinet at 65 °C until it reached a constant weight. After the weights of the aboveground parts of the dried plant samples were determined, they were milled in the tungsten coated plant-grinding mill. 0.2 g of the milled plant samples were ashed at 550 °C according to the dry ashing method and dissolved in 3.3% HCl (20 mL) and filtered with blue tape filter paper. Potassium, Ca and Na in the strainer were determined in the flame photometer (Thermo Aquamete-2000E) (Richards, 1954).

Tolerance Ratio (TR): Tolerance ratio was used to compare green bean genotypes based on reactions to different NaCl concentrations (Chandler et al., 1986). Tolerance ratio was calculated based on root, stem, FTL, and TL dry weights for each genotype and each salt concentration with and without Zn pre-treatment according to the formula below. Thus, genotypes were not compared to real figures determined in saline environment, but with their proportional development against control in an environment with a certain concentration of salt.

$TR = T_x / T_0$

T_x = dry weight gain on x mM NaCl,

T_0 = dry weight gain on 0 mM NaCl,

The ability of keeping the balance for K/Na and Ca/Na ratios in the aboveground and root parts of the plants were also considered as the other parameters to compare green bean genotypes reaction to salinity.

The results obtained were evaluated using the "IBM SPSS Statistics 20" statistics program. The difference between applications was compared with the Tukey's multiple comparison test at $p < 0.01$ and 0.05 significance levels.

Results and Discussion

Tolerance Ratio (TR): The TR values of aboveground and root parts of Zn priming and Zn non-priming two green bean genotypes under saline conditions are shown in Table 1. According to the average values, Zn priming increased the TR values only in concerning the TR calculated on the basis of FTL dry weight. However, no statistically significant differences were found between genotypes and salt applications in terms of the TR calculated on the basis of FTL dry weight. Besides, the data showed that, 'Şeker Fasulye' (TR=0.91) was more tolerant to salinity than 'Local Genotype' (TR=0.77) exclusively according to TR values calculated on the stem dry weight. Additionally, it is clear from the data, the TR values generally decreased on the basis of the dry weight calculated in all parts of the plants in both genotypes depending on the increases in NaCl concentrations.

High salt concentrations in the irrigation water result in reduced plant growth, limiting leaf expansion and changing the relationship between the aerial and root parts (Acosta-Motos et al., 2017). In this study, although the TR values calculated on the basis of root and TL dry weights were found higher in 'Local Genotype' than those in 'Şeker Fasulye' genotype, 'Şeker Fasulye' was more tolerant than 'Local Genotype' to salinity according to the TR values calculated on the basis of stem dry weight. In our previous work it has been found that

'Şeker Fasulye' genotype has more fresh and dry weight in all sampling organs (except dry weight of FTL) than 'Local Genotype' thus was found to be relatively more salt tolerant according to these parameters (Gulmezoglu et al., 2016). Therefore, it is suggested that the higher TR values calculated on the basis of stem in green bean plants could possibly be one of the factors involved in conferring salt tolerance. There have been no reports demonstrating this subject in green bean plants. However, it was determined that the highest TR values calculated on the basis of leaf and root dry weight in cv. Camarosa which was more salt tolerant strawberry cultivar than cv. Chandler (Turhan & Eris, 2007). Besides, it is a fact that different results can occur between species or even varieties depending on the salt concentrations and the duration of salt exposure (Acosta-Motos et al., 2017).

K/Na ratio: The significant differences in K/Na ratio of green beans'parts on Zn, salt and genotype were shown in Table 2. Seed Zn priming had only a significant effect ($p<0.01$) on K/Na ratio in stem. Therefore, K/Na ratio was not affected by Zn in the other parts of the green beans genotypes. However, salt application was found significant ($p<0.01$) on K/Na ratio of all parts of the green bean. While K/Na ratio in stem was found to be significant on genotypes, there was no difference that of root, FTL and TL. Salt × Zn interaction was significant on K/Na ratio in the all parts of green bean plants. The interactions of Zn × Genotype, Salt × Genotype and Zn × Salt x Genotype were significant ($p<0.05$ or 0.01) only on K/Na ratio of stem and TFL.

The Zn × Genotype interaction among the studied parts of the plants, the least amount of K/Na ratio was seen in the root (Fig. 1). The K/Na ratio of 'Local Genotype' was higher in root, TFL and TL without-Zn priming but K/Na ratio in stem had higher both without-Zn priming and with-Zn priming. The K/Na ratio in stem of 'Şeker Fasulye' genotype had no big difference according to Zn priming. The K/Na ratio at without-Zn priming in TL of 'Şeker Fasulye' was lower than that of 'Local Genotype'; however, Zn priming caused the K/Na ratio of 'Şeker Fasulye' to increase and that of 'Local Genotype' to decrease. When considering all parts of both genotypes, K/Na ratio of 'Şeker Fasulye' was affected positively in plants from Zn priming seeds (Fig. 1). K/Na ratio in FTL and TL of 'Şeker Fasulye' had higher than 'Local Genotype' in plants from Zn primed seeds. The K/Na ratio under salt stress is an important data and K/Na was affected significantly with Na^+ and K^+ concentrations. These elements showed changes genotypically negative and positive directions when Zn applied to seeds (Fig.1). Also, Zn × Genotype interaction was found to be significant and similar findings were found in tomato (Alpaslan et al., 1999), wheat (Singh et al., 2015) and mung bean (Samreen et al., 2017).

The Salt × Genotype variation of K/Na ratio in stem of 'Local Genotype' was higher than that of 'Şeker Fasulye' according to salt ratio increased. However, K/Na ratio in TL was higher in 'Şeker Fasulye' than that of 'Local Genotype'. Trifoliate leaflet of both genotypes was affected positively by 150 mM salt concentration which K/Na ratio was the highest. However, 'Şeker Fasulye' also showed a positive effect on transportation of K/Na from root to young leaves (TL) under salt condition. It is known that the high K/Na ratio in the plant is directly proportional to salt resistance (Wei et al., 2017). There is a positive correlation between the amount of K^+ content in the plant leaves and the increase in plant resistance under saline conditions and high K/Na ratio are directly proportional to salt resistance (Wei et al., 2017). It was pointed out by tolerance rate that 'Şeker Fasulye' were more tolerant than the 'Local Genotype' to salt stress. The increase that occurs in the Na^+ amount usually disturbs the osmotic regulation and nutrition balance, therefore enters into the specific ion toxicity, and starts competing with K^+ ion due to the similarities in their ionic diameters and

electric charges. Consequently, this increase in Na^+ prevents the intake of K^+ ion (Levitt, 1980). Romero et al. (1997) assert that the increasing Na^+ amount in the leaves might cause K^+ deficiency as a result of the antagonistic impact of Na^+ and K^+ ions. As a result, Zn application from seed decreased the Na^+ intake to the cell, and increased K/Na ratio in the plant parts with less Na^+ intake. Similar results were found by some researchers (Saleh et al., 2009; Shabala et al., 2010; Singh et al., 2015) as well.

The Salt × Zn interaction was significant on all parts of green bean. The highest K/Na ratio in all parts of green bean genotypes was obtained without salt applications and this increase decreased with increasing ratio of salt. However, Zn priming of seeds changed increasingly K/Na ratio with increasing salt ratio. The K/Na ratio of TFL was positively affected by Zn priming. The decreases in K/Na ratio in all parts of green beans with increasing salt concentrations were observed in different plants (Eker et al., 2013). Zinc is a very important on strength of cell membranes. When Zn is deficient in plants, membrane permeability increases, especially under stress conditions (Cakmak, 2000). This may increase uptake of Na^+ to a toxic level for plants (Chakraborty et. al., 2018).

The Salt x Genotype x Zn interaction was found to be be statistically significant on K/Na rate of stem and TL ($P<0.01$) (Table 2). Besides K / Na ratio showed differences according to genotypes in organs determined to be statistically significant. In plants from Zn primed seeds of 'Şeker Fasulye' had higher K/Na ratio at 150 mM salt application than that of 'Local Genotype' (Fig. 1).

Ca/Na ratio: The variance analysis results of Ca/Na ratio of belong to root, stem, FTL and TL of two green bean genotypes are shown in Table 2.

Table 1. Mean and analysis of variance of TR of different parts of two green bean genotypes from with or without zinc primed seeds under salinity conditions. Legend: (*) Significant within column at $p<0.05$, (**) Significant within column at $p<0.01$, (ns) - non-significant.

Trait	Tolerance ratio (TR)			
	Root	**Stem**	**FTL**	**TL**
		Zinc (Zn)		
(-) Zn	0.67a	0.89a	0.89b	0.94a
(+) Zn	0.52b	0.79b	1.17a	0.61b
		Genotype (G)		
Local Genotype	0.66a	0.77b	1.07	0.83a
Şeker Fasulye	0.52b	0.91a	0.99	0.72b
		Salinity (S) (mM)		
0	-	-	-	-
50	0.72a	0.97a	1.13	0.94a
100	0.64a	0.85b	0.99	0.72b
150	0.42b	0.70c	0.97	0.67c
		***F* test**		
Zn	**	*	**	**
G	**	**	ns	*
ZnxG	**	**	ns	**
S	**	**	ns	**
ZnxS	**	ns	ns	ns
GxS	ns	*	ns	ns
ZnxGxS	**	*	ns	ns

Table 2. Analysis of variance belongs to K/Na and Ca/Na ratio of different parts of green bean genotypes from with or without zinc primed seeds under salinity conditions. Legend: see Table 1.

	K/Na ratio				Ca/Na ratio			
Trait	**Root**	**Stem**	**FTL**	**TL**	**Root**	**Stem**	**FTL**	**TL**
Zinc (Zn)	ns	**	ns	ns	**	**	**	**
Salt (S)	**	**	**	**	**	**	**	**
Genotype (G)	ns	**	ns	ns	ns	**	**	**
Zn × G	ns	**	ns	*	ns	**	ns	*
S × G	ns	**	ns	**	ns	**	*	**
S × Zn	**	**	**	**	**	**	**	**
S × G× Zn	ns	**	ns	**	ns	**	*	**

A significant variance ($p < 0.01$) was determined for Ca/Na ratio of the root, stem, FTL and TL in terms of Zn and salt. Genotypic difference was not found only Ca/Na ratio of root. The Zn × Salt interaction was significant on Ca/Na ratio of root, stem, FTL and TL. The Zn × Genotype interaction was found significantly on Ca/Na ratio in stem and TL while Genotype × Salt and Zn × Genotype × Salt interactions were not significant only root in terms of Ca/Na ratio.

The Zn × Genotype interaction was examined that Zn priming to seed decreased the Ca/Na ratio in all parts of plant (Fig. 1). The Ca/Na ratio from the highest to the lowest was changed between sampled organs and arranged in order of FTL > stem >TL >root from the highest to the lowest, respectively. The content of Ca decreased in the roots, stems, FTL and TL of both genotypes due to Zn priming. Priming of seeds by Zn in this study may affected negatively on Ca concentrations of green bean plants. The Ca/Na ratio in root and stems of 'Şeker Fasulye' was affected much more by Zn priming than 'Local Genotype' but Ca/Na ratio was found lower in FTL and TL and 'Şeker Fasulye' than that of 'Local Genotype'.

Increasing salt concentrations decreased Ca/Na ratio all parts of green bean genotypes. The Salt × Genotype interaction was not efficient on root. The mean of Ca/Na ratio in 'Şeker Fasulye' was higher than that of 'Local Genotype' in all organs. Thus, Ca/Na ratio in 'Local Genotype' was affected negatively. The Ca/Na ratio in organs decreased in order; FTL > stem> TL> root. Roots were damaged firstly from salt because of being contact organ. Salt tolerance of most plants at high concentrations depends on being impermeable to salt (Chakraborty et al., 2018). However, for the cell to preserve its selective permeability, it depends on the balance of the monovalent (K^+, Na^+) and divalent (Ca^{2+}) cations (Chakraborty et al., 2018).

In this study, it has been shown that the Ca/Na ratio decreased as a result of higher Na ion uptake under salt conditions. It is expected that a plant which is protected from salt by passively keeping the salt away from its body, will have low permeability against Na salts at fairly high salt concentrations. In addition, Ca^{2+} is the main cation in order for the aforementioned balance in permeability to be protected (Tuna et al., 2007).

Depending on Salt x Zn, Ca and Na concentration has dropped as the Ca/Na ratio in the parts of the plant increased slightly with Zn priming (Fig. 1). It has been observed that Na concentration is high in parts of the plant with the salt application, whereas the Ca/Na ratio is low in plants in which Zn priming has not been applied. It was showed that Zn application to green bean seeds restrained to uptake of Ca. The Ca/Na ratio in organs due to Zn priming was lower when compared to without-Zn priming and it was on the decline in order; FTL > stem> TL> root. There were contrary results for Ca/Na ratio of some plants studied Zn applications under salt stress.

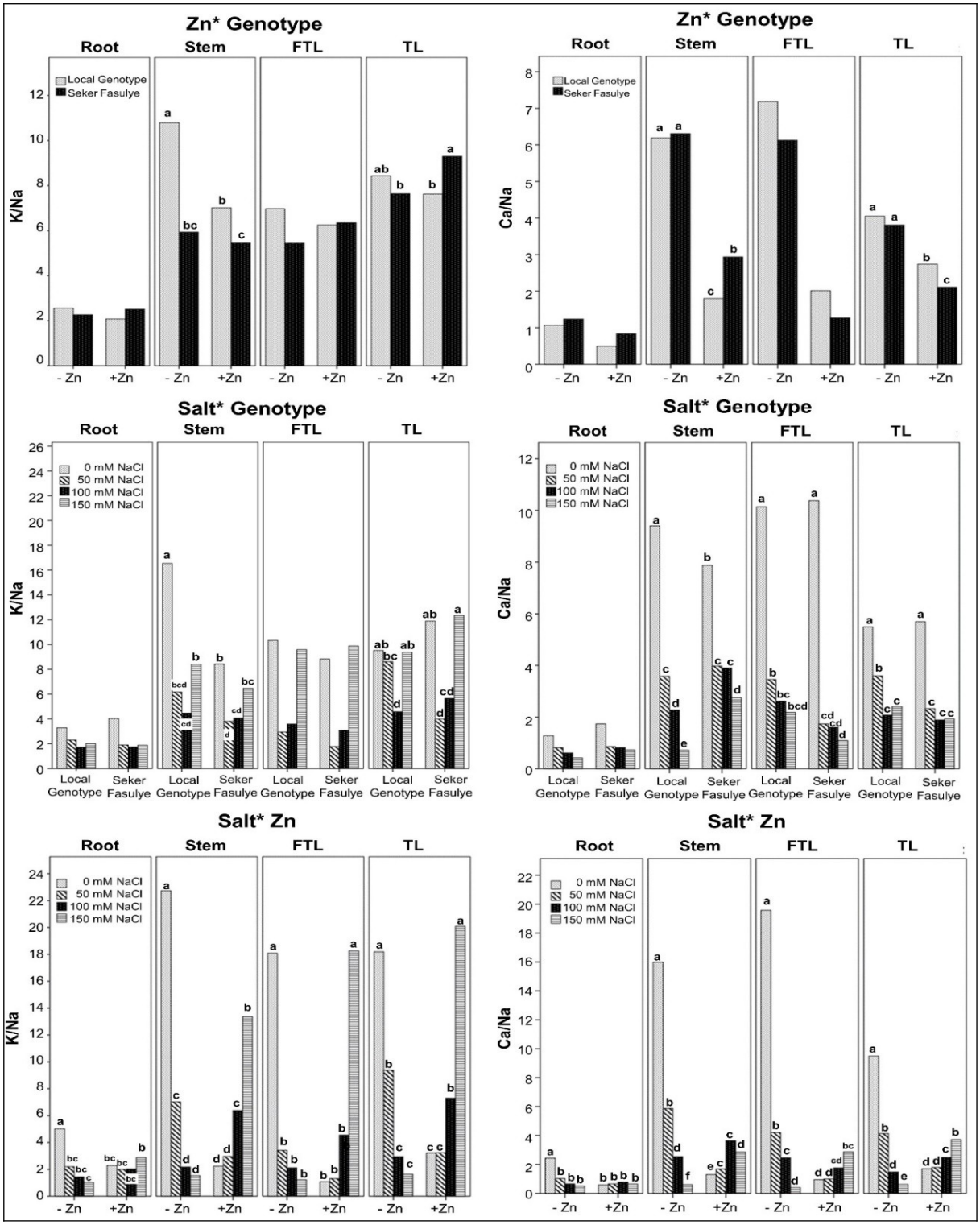

Fig. 1. The interactions (Zn x Genotype, Salt x Genotype and Salt x Zn) of NaCl on K/Na and Ca/Na ratio of different parts of green bean genotypes from with or without zinc primed seeds. Different letter(s) represented on each bar show(s) statistically significant differences at $p < 0.05$.

Researchers attributed that Ca/Na ratio in plant parts increase with-Zn application but it was decreased by salt treatments (Eker et al., 2013; Hejazi-Mehrizi et al., 2011).

The Salt x Genotype x Zn interaction was found to be significant on Ca/Na rate of stem, FTL and TL (Table 2), besides in plants from Zn primed seeds of 'Şeker Fasulye' had higher Ca/Na ratio at 150 mM salt application than that of 'Local Genotype' (Fig. 1). Higher Ca/Na and K/Na ratios in the leaf organs of 'Şeker Fasulye' were found to be effective in salt tolerance.

It has been identified that there is a significant difference ($p<0.01$) between applications and genotypes, and their interactions to the ratio of the stem and Ca/Na concentration. The Ca/Na ratio of stem has reached the highest value after the Ca/Na ratio of FTL and increased in 'Local Genotype' with the application of Zn and 100 mM NaCl. The Ca/Na concentration of FTL has been determined to be higher in without Zn application. The Ca/Na ratio in FTL of both genotypes under Zn condition has decreased as the salt ratio increased.

Conclusion

In conclusion, the higher TR values calculated on the basis of dry weight indicate that tolerance of stems could possibly be one of the factors involved in conferring overall salt tolerance in green bean plants. Also, this research has shown the importance of Zn priming of seeds to alleviate the effects of salt stress on green bean plants by increasing K/Na and Ca/Na ratio. Positive react of genotypes to Zn priming was observed at 'Şeker Fasulye'. It is recommended to examine the effect of Zn priming to seed of green beans on salt tolerance, K/Na and Ca/Na ratios under the field conditions.

Acknowledgements

The authors thank to the Eskisehir Osmangazi University Research Foundation for supporting this study (Project No: 201323D15) financially.

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Received: 03.06.2020
Accepted: 12.09.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 97-110

Microbial Physiological Diversity Assessment in Protected Wetlands: A Case Study in Maritza River Basin, South Bulgaria

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Abstract. This study explored the spatial differences and physiological diversity of soil microbial communities in natural wetland and constructed rice paddies in the Maritsa River Basin, protected under the Birds Directive 2009/147/EC as natural habitats. Community level physiological profiling was used to characterize the metabolic function of the communities. The microbial activity expressed, as average well colour development (AWCD) and substrate richness and diversity was higher in the non-flooded soils with no significant spatial differences. Individual carbon source utilization patterns (CSUPs) segregated the paddy fields profiles from the natural wetland sediments and showed that communities` metabolic functions were strongly influenced by rice cropping and not by the sample location. The CUSPs revealed that easily degradable carbohydrates were the most widely used substrates followed by amino acids, carboxylic acids, and polyols. Natural wetland communities held an isolated position with lower nutrient supply and intensive catabolic activity regarding somewhat biochemically inert substrates polyols, phenolic acids, and amines indicating higher functional diversity. The presented study is evident for the good ecological potential of the Zlato pole wetlands and demonstrates that the extent of carbon uptake is determined by the habitat but also reflects the anthropogenic impact.

Key words: Wetlands, rice paddies, Biolog EcoPlates, AWCD, Community level physiological profile, PCA.

Introduction

Wetlands are found at the interface of aquatic and terrestrial ecosystems. In Bulgaria, they vary in size from large water bodies such as lakes (> 100 ha) to small water complexes (< 0.1ha) (Stoyneva & Michev, 2007). Wetlands play a crucial role in nutrient cycling as they are estimated to contain 20% to 30% of the global soil carbon pool, yet their area worldwide is shrinking as a result of constant urbanization, agricultural pressure and climate change (He et al., 2015). Wetlands located along the Bulgarian part of the Maritsa River basin are part of the Ramsar Convention and represent specific types of ecosystems rare for the state and with international importance. They include natural wetlands and periodically flooded rice paddy fields located in a densely populated area and are therefore very sensitive to human activities related to water management and forestry. The studied wetlands play a crucial role in biodiversity support of many protected animal and plant species. According to Weber (2016), rice paddies can also be

classified as "treatment wetlands" (TW) due to their role of catchment areas for water pollution and water quality improvement.

Monitoring wetland health and functioning has been an ongoing goal of their management. Over the years the studies have been focused on microbial communities` structure and diversity as biological indicators for wetlands ecosystem stability due to the relative ease of identifying and counting them (Urakawa & Bernhard, 2017). Microbial communities are considered as functional units that consist of the sum of the metabolic properties of individual bacteria (Garland, 1997). They play fundamental role in wetland biogeochemistry, and are directly related to the rate of decomposition of organic matter and, in turn, depend on the factors of the environment, such as quantity and quality of organic matter, pH, moisture, etc. (Boivin et al., 2006; Kenarova et al., 2014). In this regard, rice paddy fields are considered unique model ecosystems to study wetland soil microbial ecology (Hussain et al., 2012).

Intensive rice cultivation relates to the usage of organic and chemical fertilizers. This practice could have a negative impact on the soil ecosystem (Luo et al., 2016) affecting the microbial composition and function. The extreme sensitivity of the microorganisms to even small fluxes in the environment renders their metabolic diversity arguably more relevant measures for detection of early signs of degradation in wetland ecosystems (Merkley et al., 2004). The functional diversity and catabolic potential of the communities can be evaluated using culture-based methods such as community level physiological profiling (CLPP) (Garland, 1997; Liu et al., 2013). Biolog EcoPlateTM technique is designed to estimate CLPPs of bacteria by measuring the relative utilization of various ecologically relevant organic substrates. Despite its limitations (Garland, 1999), it has proven to be a useful tool for the detection of differences in CLPPs of bacteria in wetlands (Button et al., 2015; Luo et al., 2016; Zhang et al., 2010).

The objective of the present study was to explore the spatial differences in the metabolic activity of bacterial communities in natural and constructed wetlands in the Maritsa River Basin. The effect of rice cropping as a major factor affecting the soil chemical composition and differentiating bacterial community physiological profiles was tested in rice planted soil versus naturally flooded wetland.

Materials and Methods

Site description and sampling

Two wetlands along the Bulgarian part of Maritsa River were studied – Zlato Pole wetland and Tsalapitsa rice paddies ("Orizishta Tsalapitsa"), both protected under the Birds Directive (79/409/EEC). Protected zone Zlato Pole (42°2.207', 25°42.938') is the largest natural wetland (BG0002103) along the Bulgarian part of the Maritsa River. It is located several kilometers away from the town of Dimitrovgrad, south of the Zlato pole village. Protected zone "Orizishta Tsalapitsa" (BG0002086) is a complex of rice paddies situated between the village of Tsalapitsa (42°13.600', 24° 33.804') and the city of Plovdiv (42°10.307', 24°40.502').

Soil samples from the topsoil 0-10 cm were collected in triplicates in July 2017 and July 2018 during the rice maturity stage (after application of all fertilizers), from flooded and non-flooded rice paddies and from the sediments and the non-flooded area at Zlato Pole. Nine random soil cores within each site were mixed to provide one sample (Luo et al., 2016). Each sample was partitioned into two subsamples: one was partially air-dried and sieved through a 1 mm sieve for chemical analysis, and one was placed in sterile 50 ml containers and stored at 4°C in the dark for microbiological analysis for no longer than 24 h.

Soil parameters

The sieved samples were used to determine soil moisture, pH, $N\text{-}NH_4^+$ (ammonia), $N\text{-}NO_3^-$ (nitrate), ON (organic nitrogen), OM (organic matter), AP

(available phosphorus). Soil moisture was determined by calculating the weight of lost water after drying the sample at 105°C for 24 h. Soil pH was determined in a 1:2.5 soil:deionized water suspension with a pH meter (WTW/SET). Inorganic N-NO_3^- and NH_4^+ were analysed after soil extraction with 0.1 N KCl according to (Motsara & Roy, 2008). Organic nitrogen was determined by the Kjeldahl method after mineralization with selenium in DK-6 (VELP-Scientifica) module for decomposition and a semi-automatic analysis system for distillation UDK-132 (VELP-Scientifica). OM was measured by calculating the loss of weight on ignition at 600°C. AP was determined spectrophotometrically by the Olsen's method (Motsara & Roy, 2008).

Microbial community profiles

The pattern of utilization of individual carbon sources by the soil microbial communities was assessed using BIOLOG-EcoPlates (Biolog Inc., Hayward CA, USA), containing 31 different C sources in three replicates. Tetrazolium redox dye that changes from colourless to purple was used as an indicator of the ability of microorganisms to metabolize the organic carbon. Cell suspensions were prepared from 1 g soil suspended in 99 ml sterile saline solution (0.85% w/v NaCl) on a rotary shaker at 200 rpm for 30 min and then left to settle for 10 min. The supernatant was filtered subsequently through 8.0 and 3.0 µm nucleopore membranes (Whatman). Each well in the plates was inoculated with 150 µl of the cell suspension. The plates were incubated at 22°C in dark for 14 days. Colour development was measured as described by Garland (1997). Optical density (OD) at 590 nm of each well was recorded with MULTISKAN FC microplate reader (Thermo Fisher Scientific, Shanghai, China) every 24 h.

Prior to analysis, the OD value of the control wells was subtracted from the individual substrate absorbances and the negative readings (OD < 0) were excluded. Data were represented by the mean (n = 3). The microbial activity was expressed by average well color development (AWCD) according to Garland and Milles (1991):

$$AWCD = \sum \frac{(n_i - c)}{31}$$

where n_i and c were the average absorptions of the three wells of the substrate and the control wells (without a C source), respectively. AWCD were calculated daily throughout the incubation period to reveal the kinetic profiles. EcoPlate substrata were grouped into six biochemical categories including carbohydrates, carboxylic acids, amino acids, amines, polyols, and phenolic acids following Kenarova et al. (2014).

Data Analysis

Pearson correlation, analysis of variance (ANOVA) and cluster analysis were carried out with Statistica software (StatSoft, version 10). Principal component analysis was conducted with PRIMER 6 (Clarke & Gorley, 2005). The AWCD 0.75 value (between 0.25 and 1) was chosen as a reference point for all further statistical analysis as recommended by Garland et al. (2001) and standardization of the data was performed to reduce any bias due to inoculum density differences. Calculation of precise OD values at the point of $AWCD_{0.75}$ was achieved by linear interpolation based on the assumption that the relevant part of the curve follows a linear function (Salomo et al., 2009). The normality, homoscedasticity and the number of linear correlations of data set were evaluated, followed by principal component analysis (PCA) based on AWCD for reduction of the dimensionality of the n-dimensional data set and extraction of an orthogonal set of principal components (PCs) made up of linear subsets of the original ordinates. Subsequent cluster analysis (CA) based on the squared Euclidian distances was performed to validate the results obtained from the PCA, and to group and visualize

the similar bacterial CLPPs in a dendrogram (Weber & Legge, 2014). Shannon richness, diversity and substrate evenness indices were calculated to assess the functional diversities of the communities. Richness [S] values were calculated as the average number of oxidized C substrates in the three replicates. The substrate diversity (Shannon index [H]) was calculated as follows: $H = -\Sigma pi(\log_2(p_i))$, where p_i is the ratio of the activity on each substrate (OD_i) to the sum of activities on all substrates (ΣOD_i). The evenness [E] was calculated as $E = H/\log_2(S)$ (Luo et al., 2016).

Results

Environmental parameters

The soil profiles of the two types of samples were highly differentiated, with parameter values varying in a relatively wide range between the stations located in different zones as well as between the samples of the non-flooded and flooded areas (Table 1). The sediment samples from Zlato Pole (ZP1) and the samples from the surrounding non-flooded area (ZP2) were characterized as sandy. The samples taken from the sediments at Plovdiv (P1) and Tsalaptsa (C1) were characterized as clay and silty-clay – for the soils from the adjacent non-flooded zones at Plovdiv (P2) and Tsalapitsa (C2) rice paddies. The soil water content (SM) of the sediment was at 25-47%, with significantly higher values in the clay soils ($p < 0.05$). For ZP2, P2, and C2 the moisture was at 7-9% with no significant differences. The soil pH was neutral to slightly alkaline (6.9-7.3) with the exception of C1 and C2 (5.12-5.25) which were defined as acidic. The distribution of nitrogen forms, AP and organic matter was site-specific with higher values in the paddy filed samples, compared to ZP. In general, the sediments were less abundant in ammonium ($N\text{-}NH_4^+$), nitrate ($N\text{-}NO_3^-$), organic nitrogen and organic matter than the non-flooded soils (P2 and C2) and ZP2 ($p = 0.039$), and were more abundant in ammonium nitrogen ($N\text{-}NH_4^+$). A significant positive correlation was established for $N\text{-}NO_3^-$ with organic matter ($r = 0.892$) (Table 2). A high concentration of available phosphorus was found at all stations, explaining the lack of correlation between AP and the substrate utilization rates by the microbial communities.

Comparative analysis of the sampling sites performed by PCA (Fig. 1A) and cluster analysis (Fig. 1B) suggested distinct physicochemical characteristics between the flooded and the non-flooded areas as well as significant spatial differences between stations. The PCA generated two principal components accounting 80.32% of the total data variance, separating the samples in four groups - of acidic sediment C1 (quadrant 1) and non-flooded soil C2 (quadrant 2), ON and OM depleted sediments ZP1 and P1 (quadrant 3), and nutrient-rich dry soils P2 and ZP2 (quadrant 4). With high loadings in PC1 were SM (0.904), $N\text{-}NH_4^+$ (0.737), $N\text{-}NO_3^-$ (-0.873), ON (0.521) and AP (-0.910), while in PC2 variability was caused by pH (0.843) and OM (-0.775). Cluster analysis showed similar grouping, differentiating sites based on their specific environmental characteristics (Fig. 1B).

Table 1. Average values for the environmental parameters from the sampling sites at the studied wetlands.

Station	SM %	pH (H_2O)	$N\text{-}NH_4^+$ $mg.kg^{-1}$	$N\text{-}NO_3^-$ $mg.kg^{-1}$	ON %	OM %	AP $mg.kg^{-1}$
ZP1	25.36	7.55	86.6	5.7	1.18	3.53	83.5
ZP2	9.11	7.21	18.8	14.4	10.1	44.64	72.5
P1	37.51	7.24	68.8	10.3	3.98	12.02	264.0
P2	7.18	7.33	26.9	39.9	12.2	59.90	99.5
C1	44.20	5.12	70.0	7.5	3.76	9.40	175.0
C2	7.60	5.25	48.7	20.8	6.05	86.07	78.0

Table 2. Pearson correlation matrix of the environmental parameters, AWCD and substrate classes utilization by the bacterial communities inhabiting the studied wetlands. Legend: * - Correlation is significant at the 0.05 level (2-tailed). ** - Correlation is significant at the 0.01 level (2-tailed).

	SM	pH	NH_4^+	NO_3^-	ON	OM	AP	AWCD	PM	CH	CA	PA	AA	AM
SM	1													
pH	-.140	1												
NH_4^+	**.855***	-.116	1											
NO_3^-	**-.818***	.128	-.693	1										
ON	-.684	.188	**-.962****	**.835***	1									
OM	-.809	-.280	-.694	**.892***	.651	1								
AP	**.903***	-.028	.425	-.320	-.360	-.533	1							
AWCD	**-.726***	-.282	**-.875***	**.845***	**.827***	**.900***	-.395	1						
PM	.504	.234	**.871***	-.557	-.802	-.717	.097	**-.936****	1					
CH	-.071	-.431	-.607	.433	.603	.440	.278	**.825***	**-.871***	1				
CA	**-.899***	.045	-.524	.348	.393	.782	-.775	.641	-.376	-.092	1			
PA	**-.668***	.668	-.404	.486	.477	.195	-.704	.081	.084	-.403	**.749***	1		
AA	.644	.333	**.858***	-.680	-.808	**-.875***	.291	**-.985****	**.954****	**-.792***	-.516	-.014	1	
AM	.125	.110	.663	-.644	-.767	-.296	.839	-.535	.710	**-.853***	.226	.002	.649	1

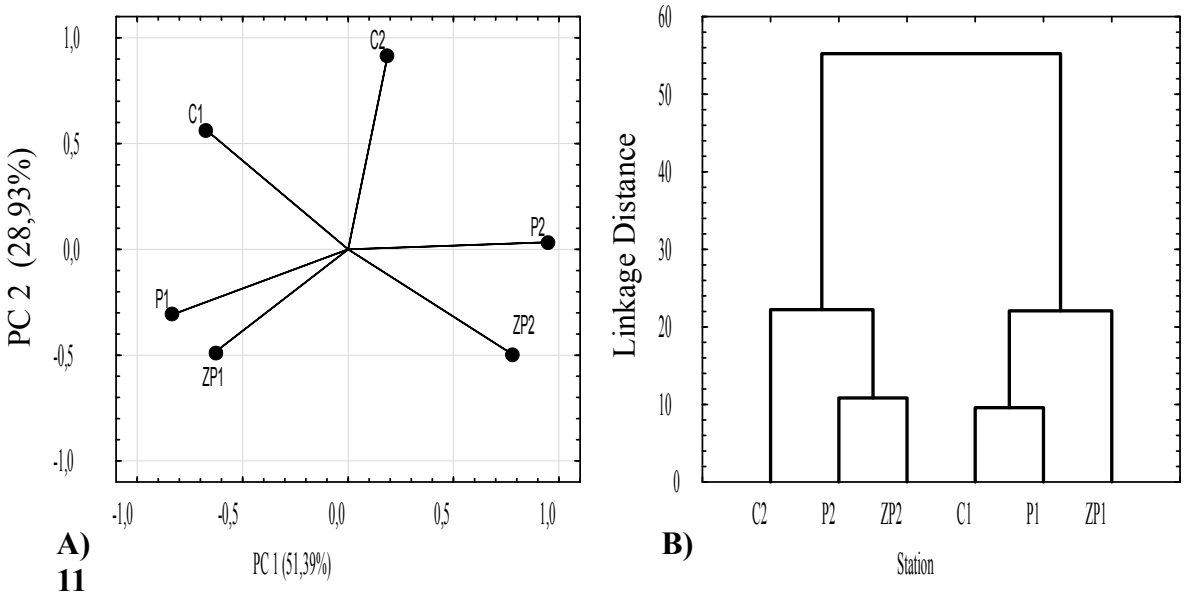

Fig. 1. Soil plots based on the environmental parameters by principal component analysis(**A**) and cluster analysis (**B**).

AWCD and CLPP

The rate of the average well color development (AWCD) over time for all six samples followed a sigmoidal curve, with short lag phase (< 24 h) nearly equal in all soil samples (Fig. 2). The kinetic curves demonstrated differences in the substrate utilization pattern. The non-flooded soil bacteria (ZP2, P2, and C2) were generally more active on the EcoPlates, with higher utilization rates and steeper slopes than the flooded communities indicating microbial activity from fast-growing heterotrophs. The AWCD values in the rice paddies sediments were greater than the ZP1, which suggests that rice cropping also stimulated

the growth of fast-growing heterotrophs. In the ZP1 samples, carbon sources were by trend slowest metabolized, differing significantly from the other samples ($p < 0.05$). The $AWCD_{0.2}$ value for all samples was reached over a relatively large time period 24-40 h, whereas the evaluation point, chosen for further analysis $AWCD_{0.75}$ occurred after 48-96 h showing clear differences based on sample types. Such differentiation is based on the physicochemical soil properties expressed as a strong positive correlation of the AWCD with OM ($r = 0.900$), nitrate ($r = 0.827$) and organic nitrogen ($r = 0.845$).

After 14 days of incubation with the exception of ZP1, the studied microbial communities were able to utilize 26 to 30 of the 31 carbon sources in the EcoPlates. None of the analyzed communities metabolized the γ-hydroxybutyric acid. ZP1 bacteria showed a reduced catabolic diversity, being unable to oxidase 50% of the tested carbohydrates: α-cyclodextrin, glycogen, β-methyl-D-glucoside, α-D-lactose, Glucose-1-phosphate and D, L,-α-glycerol phosphate. The rates of substrates utilization by categories are shown in Fig. 3A. The profiles were biochemically dependent and corresponded to the habitat type. With the exception of ZP1, carbohydrates (CH) were the most widely used substrates by the studied communities followed by amino acids (AA), carboxylic acids (CA), and polyols (PM).

The CLPP profiles based on the PCA showed obvious similarities between rice paddies sediments plots (P1 and C1) as well as between the non-flooded samples at all sites (Fig. 3B). ZP1 was characterized by lower CH and higher AA, PM and AM utilization rate and its CLPP apparently held an isolated position. PC1 accounted for 57% of the total variance. It demonstrated that substrate classes of CH, PM, and AA had the highest factor loadings and are responsible for the similarities in C1 and C2 plots regarding carbon utilization rates. The plotted vectors of CA and PA are orientated parallel to the PC2 axis between the ZP1 and the dry soils plots (ZP2, P2, and C2) indicating that they are utilized equally in these samples.

The substrate level utilization patterns of the microbial communities were further analyzed based on the plots of individual carbon sources utilization profiles (CUSPs) (Fig. 4). The non-flooded soils surrounding the wetlands showed extremely high similarity regarding the utilization of the majority of substrates in the Ecoplate, while C1 and P1 differed at substrate level despite their high resemblance regarding substrate classes. The carbohydrates were the most widely used substrates, affecting significantly the overall AWCD ($r = 0.825$) (Table 2). The PCA plot segregated the communities in three separate quadrants (Fig. 4A). The non-flooded soils communities (ZP2, P2, and C2) metabolized easily degradable monosaccharides with a phosphate group, such as glucose-1-P and α-glycerol-P, while sediment communities preferentially oxidized xylose and cellobiose, both products of plant decomposition. The other carbohydrates were evenly metabolized in the different samples. ZP1 showed individual significantly different utilization pattern, where five of the carbohydrates were not utilized at all. All communities were able to degrade the tested carboxylic acids, expect the biochemically inert γ-hydroxybutyric acid, with an equal rate for the majority of them (Fig. 4B). The utilization of phenolic acids was rather site-specific. They were predominantly oxidized by the flooded soils communities. The degradation pattern also revealed a remarkably high similarity between rice paddies sediment communities. In contrast to carbohydrates, the Zlato Pole sediments were characterized by a broader spectrum and utilization rates compared to the other sites. ZP1 communities showed the best growth on hydrogenated carbon sources (polyols) (Fig. 4C).

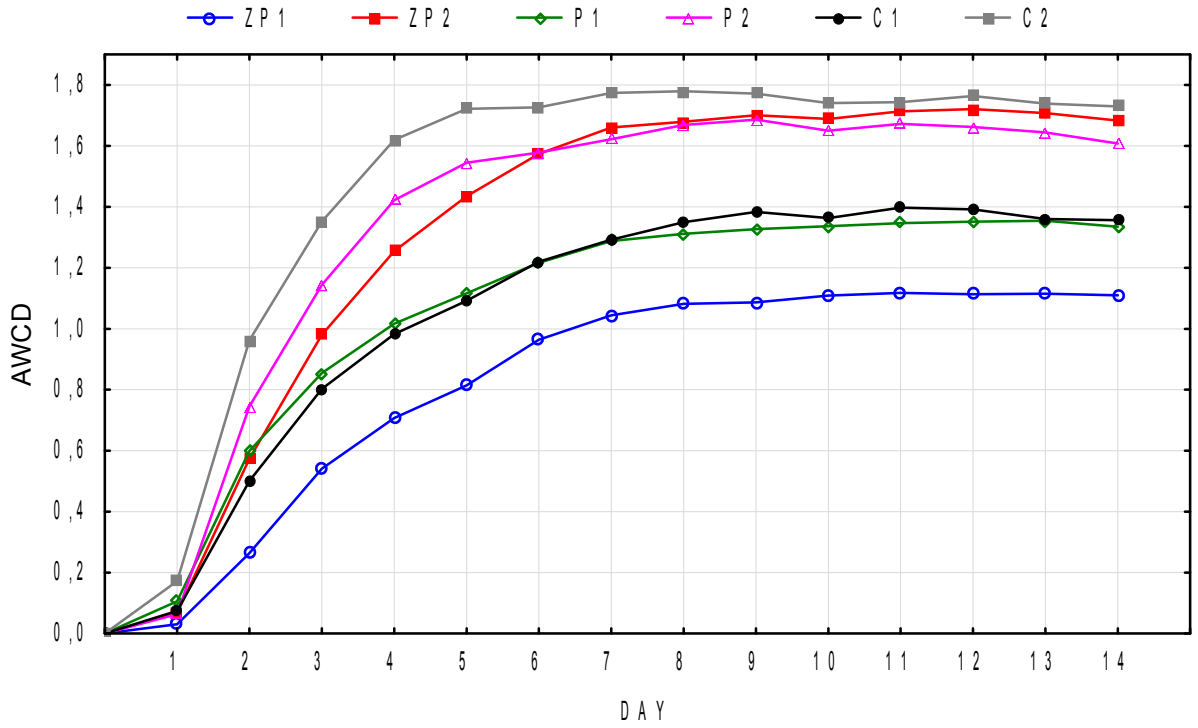

Fig. 2. Kinetic curve of carbon sources utilization by the communities inhabiting wetlands in the Maritza River Basin expressed as AWCD.

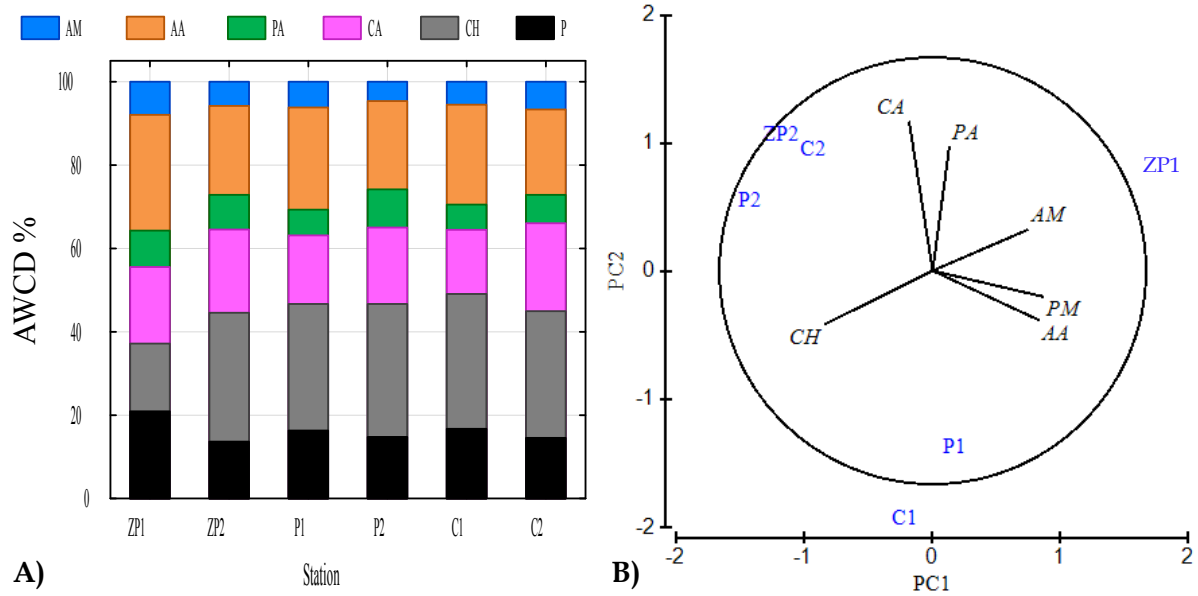

Fig. 3. Bacterial activity (**A**) expressed as a percentage of the total substrate utilization by categories: amines (A); amino acids (AA); phenolic acids (PA); carboxylic acids (CA); carbohydrates (CH); polyols (PM) and PCA (**B**) score plot (PC1 – 57.8%; PC2 – 28.4%) of the community level physiological profiles (CLPPs).

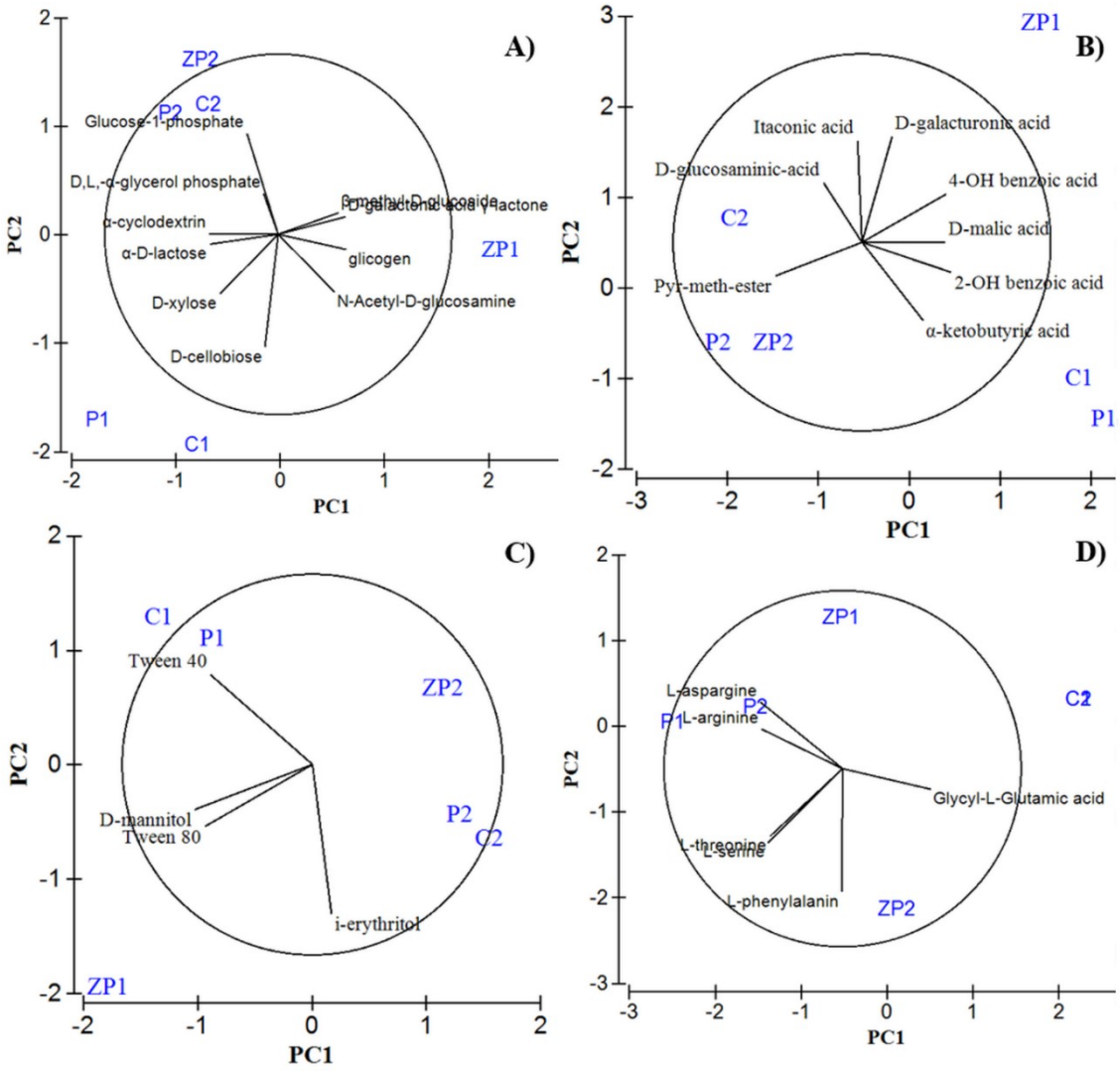

Fig. 4. Substrate level utilization pattern of the microbial communities (PC – part of total data variance (%)): **A** – carbohydrates (PC1 – 65.2%; PC2 – 21.4%); **B** – carboxylic and phenolic acids (PC1 – 45.1%; PC2 – 27.8%); **C** – polyols (PC1 – 55.4%; PC2 – 38.6%); **D** – amino acids (PC1 – 56.6%; PC2 – 27.4%).

Amino acids/amines PCA plot was significantly different and revealed a distinct dissimilarity between the two paddy fields (Fig. 4D). The highest OD was measured for arginine and asparagine for all samples, while only the P1 and P2 communities utilized two of the amino acids: threonine and glycyl-L-glutamic acid. An interesting fact was that ZP1 and ZP2 communities were unable to grow on serine and threonine despite their similar chemical structure with asparagine. The growth on amino acids/ amines was inversely proportional to the carbohydrates oxidation (r = -0.792/ -0.853). The result suggested that the amino acid utilization pattern was site rather than habitat determined. Both polyols and amino acids were negatively correlated with the AWCD (r = -0.936; r = -0.985).

The data on the substrate utilization was used for calculation of the microbial functional diversity indices (Table 3). $AWCD_{0.75}$ was chosen as a reference point, in order to access the whole community activity and not only that of the fast-growing heterotrophs. The samples differed

significantly in their substrate richness and diversity ($p < 0.05$). Shannon index was high in the range of 3.9-4.4; hence, the bacterial activity in the samples must be quite uniformly distributed with few or no dominant species. The *H* values showed higher functional diversity of the non-flooded soils communities than that in the flooded areas. The Pielou`s evenness index was affected by the sample type segregating flooded and non-flooded soils and detected significant differences between the rice paddies sediments plots (C1 and P1) and natural wetland sediments (ZP1). This indicates that microbial diversity and potential for carbon source utilization was slightly higher in natural wetland than in the paddy fields, despite the relatively similar environmental conditions. Hence, the microbial functional activity and diversity are affected by the studied environmental parameters, but they are also significantly influenced by the anthropogenic pressure expressed as flooding with waters with high organic loads, rice cropping, and other agricultural activities.

Table 3. Substrate richness (S), diversity (H), and evenness (E) indices based on the microbial functional soil bacteria inhabiting flooded and non-flooded wetlands.

Station	Richness [S]	Shannon [H]	Evenness [E]
ZP1	24.7±0.67	4.05±0.02	0.88±0.02
ZP2	30.0±0.0	4.22±0.00	0.80±0.00
P1	26.7±1.5	3.85±0.01	0.79±0.00
P2	30.0±0.0	4.47±0.04	0.90±0.00
C1	28.0±1.0	3.83±0.00	0.77±0.00
C2	30.0±0.0	4.39±0.10	0.90±0.01

Cluster analysis of the CLPP revealed different site grouping compared to that based on the physicochemical parameters and separated the plots into three clusters with site-specific physiological profiles (Figure 5), comprising the rice paddies sediments (CA1), non-flooded substrata (CA2), and Zlato Pole sediments (CA3). The CLPPs clustered in CA2 (P2 and C2) were characterized by lower linkage distance than those of the flooded communities (P1 and C1). The distance between rice paddies CLPPs and that of ZP1 (CA3) was much higher.

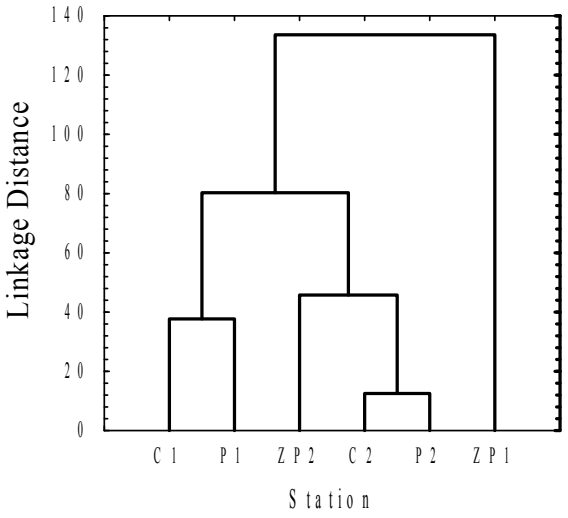

Fig. 5. Dendrogram of the soil plots based on bacterial CLPPs.

Discussion

One of the main purposes of this study was to reveal the metabolic profiles of bacterial communities using CLPP in wetland soil environment and to analyze their activity driven by the environmental factors and agricultural activities. The results indicate that the physiological profiles and the community diversity indices of the flooded and non-flooded areas significantly differed, reflecting the differences in their physicochemical parameters. Certain soil properties were promptly related to the substrate utilization patterns, suggesting their ecological relevance to soil microbial communities.

In particular, soils with high water content, caused by the wetland flooding, were associated with lower AWCD and reduced diversity (*H* values). Our findings

suggest that such soils support lower numbers of microbial species as these zones demonstrate reduced substrate utilization rate. The result confirmed that SM has a negative impact on the soil structure, microbial communities and mineralization rate in general as described by Borowik and Wyszkowska (2016) and Marhova et al. (2018). Water content and water holding capacity of the soils seem to be a major factor influencing the quantitative and qualitative composition of microbial communities in the sediments of wetlands and lake ecosystems (Ma et al., 2018).

Our results indicate a high organic matter and nitrogen concentrations in the non-flooded areas compared to the flooded rice paddies and natural wetland, exceeding several times the values established by other authors for the Plovdiv region (Krasteva-Milanova, 2017, Plovdiv – pers. comm.). The rice paddies were also characterized by a more nutrient-rich environment than the Zlato Pole wetland, due to their regular drainage and flooding leading to the introduction of organic matter with incoming waters (Das & Chakrabarti, 2013), and moreover due to the regular fertilization. However, the significantly lower concentrations of nitrate-nitrogen in rice sediments compared to the adjacent non-flooded areas ($p = 0.039$), is a result of the higher availability of $N\text{-}NO_3^-$ for plants that can absorb a large quantity of nitrates as described by Farrell et al. (2014). The organic inputs affect food-web interactions and are expected to be one of the primary mechanisms that influence microbial activity (Cartwright et al., 2016). So not surprisingly soil OM and ON were strongly positively related to AWCD ($r = 0.900$) and H ($r = 0.916$), suggesting that carbon, nitrogen, and natural vegetation rich non-flooded areas supported more diverse bacterial communities. Marhova at al. (2018) also draws a similar conclusion studying the effect of organic nitrogen content on the quantity and biodiversity of microorganisms in wetland sediments. Our results are consistent with the findings of a significant increase in AWCD, and *H* several months after organic amendment application (Gomez et al., 2006). In the present study, high phosphate levels were found at all stations and the absence of correlation between soil phosphorus and any of the studied microbiological parameters may indicate that AP was not a limiting factor despite its low natural bioavailability and rapid mineralization rates.

The Biolog Eco-plate technique was applied to provide data for the physiological activity of the culturable fraction of the microbial communities (Kirk et al., 2004). The results demonstrated differences in the adaptation period to the artificial environment as well as a significant differentiation regarding the utilization of the carbon sources by the natural wetland and rice paddies communities. The studied groups of energy sources were utilized rather similarly but there were significant differences in the utilization of individual carbon substrates by stations. With the exception of the flooded natural wetland (ZP1), the Biolog data suggests the presence of fast-growing heterotrophs with a relatively higher carbon utilization activity. This shows that soil types and the environmental factors related to and cropping systems have a leading role in the CLPP over the geographical location of the studied areas.

The AWCD highlighted all non-flooded areas as places with the highest number of utilized substrates, confirming the importance of soil water content. The communities showed clear similarities regarding the substrate utilization patterns especially for the group of carbohydrates and carboxylic acids. Easily degradable CH such as glucose-1-P and α-glycerol-P and CA, which are mainly the products of carbohydrate metabolism, were better utilized in comparison with the flooded areas. They represent a preferred energy source for the majority of microorganisms (Button et al., 2015). According to the results,

the communities in these areas have adapted to metabolize accessible carbohydrates, due to their excess in the environment, but also possessed the potential to utilize other diverse energy sources. The observed similarity between non-flooded soils could be explained with the presence of a high variety of plant species transforming the whole land as a rhizosphere. A number of authors (Baudoin et al., 2003; Frac et al., 2012) confirmed that root exudates of in undisturbed natural soils compared to fertilizer-amended soils stimulated bacterial activity and diversity. The catabolic evenness and diversity values are evident for high stress and disturbance resistance of the communities in the non-flooded areas. Such stability is typical for pastures and soils with high vegetation score (Cartwright et al., 2016; Degens et al., 2001). In these ecosystems, OM, cation exchange and microbial biomass are also reported to be greater (Marhova et al., 2018; Sparling et al. 1992). They offer stress resistance to the microbial communities and could enhance the recolonization in the event of environmental disturbances (Degens et al., 2001).

The communities in the flooded Tsalapitsa rice paddies (P1 and C1) were characterized by a preferential growth on α-cyclodextrin, xylose, and cellobiose as well as amino acids and polyols. Such patterns are typical for rice cropping systems where over time the plant residues deposit over the surface layer and cellulose represent a major carbon input, subjected to decomposition by the sediment microbial communities. The microorganisms in the surface sediment layer further metabolize products like xylose and cellobiose (Salomo et al., 2009). The better utilization of α-cyclodextrin and Tween 40 also suggests that the flooded communities receive substances from plant residues and confirms that the communities in such soils are well adapted to such carbon sources (Das & Chakrabarti, 2013), but at the expense of their limited ability to utilize carboxylic and phenolic acids.

The natural flooded wetland was strongly influenced by the lacking availability of organic matter, and nitrogen and always held an isolated position regarding the carbon utilization patterns. The bacterial community of ZP1 area is well adapted to polyols, phenolic acids, amino acids, and amines, suggesting that the bacteria in the natural environment are limited in substrates. Transferring bacteria from such conditions to an artificial medium with high substrate concentrations could lead to "substrate-accelerated death" (Straskrabova´, 1983). In this regard, the polyols seem to be a better substrate than the easily degradable CH, as they pass an initial hydrolysis step and "eliminate sudden exposure" to substrates (Chin et al., 1999). The utilization of a wide variety of substrates is related to higher diversity and catabolic evenness compared to the rice paddies.

Conclusion

Microbial metabolic function in the studied wetlands differed significantly and demonstrated that the substrate utilization profile of the communities is not determined by a single environmental factor. In fact, a broad spectrum of parameters such as soil water content, pH, organic matter, and nitrogen forms in the soils was all dominant. The PCA and cluster analysis clearly revealed that the long-term agricultural exploitation is related to changes in soil properties and bacterial communities, grouping the rice paddies in a separate cluster. The continuous rice cropping in the Tsalapitsa wetland lead to soil acidification and are related to a higher overall metabolic activity but a lower catabolic richness and substrate diversity which makes the microbial communities sensitive to stress and external factors. The higher substrate diversity, along with the sandy river like sediments, the low concentration of organic nitrogen, organic matter, and phosphates, are evident for the good ecological potential of the Zlato Pole wetland. This is also

confirmed by the higher metabolic activity regarding the more difficult for utilization polyols and amino acids due to the lack of easily digestible carbohydrates.

Acknowledgement

The study was funded under Project № FP17-BF-001 by the Scientific research Fund, Plovdiv University Paisii Hilendarski, and supported by the National Scientific Program for Young Scientists and Postdoctoral Fellows, Bulgarian Ministry of Education and Science. Special thanks to Dr. Ivelin Mollov, who provided the soil samples for the analysis!

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Received: 28.07.2020
Accepted: 14.09.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 111-119

Lichenized Fungi (Ascomycota) from the Tisata Reserve (Bulgaria)

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Abstract. Forty species of lichens are reported from the nature reserve area as a result of field and laboratory work in 2014. Thirteen species, *Amandinea punctata*, *Caloplaca subpallida* s.l., *Cladonia coniocraea*, *C. fimbriata*, *Evernia prunastri*, *Lecanora carpinea*, *Lecidea confluens*, *Lecidella elaeochroma*, *Opegrapha atra, Parmelina quercina, Pertusaria albescens, Rinodina confragosa,* and *Sarcogyne privigna,* are reported for the first time from the valley of Struma River. *Opegrapha atra* is less known in Bulgaria. Brief notes and information on their distribution, locations and substrata are included.

Key words: biodiversity, lichen mycota, protected area, Tisata.

Introduction

The Tisata Reserve was founded on 5 December 1949. Afterwards its name became 'Tisova Barchina', and it occupied an area of 19 ha. The nature reserve is situated near the villages of Gorna Breznitsa and Kresna (Southern Struma River Valley). Its constant area and borders were finally adopted in 1991, with a total of 574.5 ha. This nature reserve has been created with the purpose to protect one of the most abundant, biggest and the only natural locality of *Juniperus excelsa* M. Bieb. (the Greek juniper).

Biodiversity of the lichenized fungi in the Tisata Reserve is poorly studied. Prior to the present work only twelve species were known – *Candelariella reflexa*, *Physcia biziana*, *P. dimidiata*, *P. dubia*, *Rinodina oleae*, *R. sophodes*, *Lepra amara*, *Lepraria nylanderiana*, *Melanelixia glabratula*, *M. subargentifera, Physconia enteroxantha,* and *Porpidia macrocarpa* (Atanassova & Mayrhofer, 2012; Mayrhofer et al., 2020).

This study aimed to contribute to the knowledge on lichen mycota of the Tisata Reserve area.

Material and Methods

Field and laboratory studies were carried out by the author on 15 March 2014, at the side of Maleshevska Mt, near Curkvishte locality, and 10 May 2014, on the opposite side of Kresna Village, at the side of the Pirin Mts, at altitudes 230-260 m. Locality data on the collection sites were taken with the help of Garmin Etrex 10 GPS receiver. Field work was performed following the linear transect method. The host plant species and substrata were identified in the field. Semi-permanent microscope slides for studying lichen morphology were prepared in tap-water, and observed under Boeco BM-180/T/SP LM. Chemical spot tests with 10% KOH (K), calcium hypochloride (C), iodine reactions, and UV tests were checked in the laboratory. The studied materials were

documented *ex situ* with the aid of Canon PS A460 digital camera under Boeco B-3500 binocular stereomicroscope. Colour macrophotographs of thalli of 16 species, made under Boeco microscope, are composed in 16 figures. Collected specimens were identified by the keys in Hodgetts (1992), Nimis (1992), Wirth (1995), Giordani et al. (2002), Smith et al. (2009), Dobson (2011), Atanassova & Mayrhofer (2012), Szczepańska & Kossowska (2014). The nomenclature generally follows Mayrhofer et al. (2005), and Smith et al. (2009). Diagnostic characters are given for some taxa. The size of apothecia, asci and ascospores are presented with their minimum-maximum values, or in the form: (min-) mean±standard deviation (-max), *n*, where '*n*' - denotes the number of measurements. Most of the examined materials were housed at the Mycological Collection of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia (SOMF). New records for the valley of Struma River are designated in the text with an asterisk (*).

Results

As a result of the present work 40 lichen species (including 1 subspecies) are recorded in the Tisata Reserve. Thirteen species, *Amandinea punctata*, *Caloplaca subpallida* s.l. (=*Rufoplaca subpallida*), *Cladonia coniocraea*, *C. fimbriata*, *Evernia prunastri*, *Lecanora carpinea*, *Lecidea confluens*, *Lecidella elaeochroma*, *Opegrapha atra, Parmelina quercina, Pertusaria albescens (=Lepra albescens), Rinodina confragosa* and *Sarcogyne privigna*, are reported for the first time from the valley of Struma River. *Opegrapha atra* is less known lichen in Bulgaria.

List of taxa

**Amandinea punctata* (Hoffm.) Coppins & Scheid., s.l. on bark of *Carpinus orientalis* Mill., N41°46′04″, E023°08′58″, alt. ca 234±3 m, 15.03.2014; on bark of *Fraxinus*, Curkvishte locality, N41°45″59″, E023°09′05″, alt. 228±3 m, 15.03.2014, SOMF 30360, spot test C(-); on bark of old tree, N41°46′02″, E023°08′57″, alt. 235±3 m, 15.03.2014; on bark of Greek juniper, N41°46′02″, E023°08′57″, alt. 232±3 m, 15.03.2014 (Fig. 7). - Notes: apothecia superficial, small, black, less than 0.5 mm in diam. Asci (35-)45-70(-80) × (11-)13-15(-17) µm, *n*=15. Ascospores (11-)13-15 × (5-)5.5-7.5 µm, 1-septate, brown; on *Fraxinus*: asci 40-50 × 11-13 µm, *n*=6, ascospores darker brown, 1-septate, slightly constricted at septum, (10-) 11±0.6 (-12) × (4.5-) 5.1±0.4 (-6) µm, *n*=15 (SOMF 30360). Recently reported from West Frontier Mts (Mayrhofer et al., 2020).

Aspicilia cinerea (L.) Körb., s.l., on siliceous rocks, 41°46′03″, E023°08′59″, 234±3 m alt., 15.03.2014, SOMF 30359. - Note: spot test K+(red).

Aspicilia caesiocinerea (Nyl. ex Malbr.) Arnold, s.l. [=*Circinaria caesiocinerea* (Nyl.) A. Nordin, Savić & Tibell], on rock, Curkvishte locality, N41°45′59′, E023°09″05′, alt 225±3 m, 15.03.2014, SOMF 30354, on rock, N41°46′02″, E023°08′59″, alt. 228±3 m, 15.03.2014. - Note: spot test K(-).

**Caloplaca subpallida* H. Magn., s.l. [=*Rufoplaca subpallida* (H. Magn.) Arup, Søchting & Frödén], on siliceous rock in the open sunny place, Curkvishte locality, N41°45′59″, E023°09′05″, alt. 223±3 m, 15.03.2014, SOMF 30358 (Fig. 6).

Apothecial disc orange red, (0.2-) 0.5±0.1 (-0.8) mm in diam, *n*=30, apothecia developed directly on the substrate surface, often in groups, round or angular in shape, spot test: K(+) intense red; disc margin proper, 30-40(-45) µm wide, bright orange red, C(-). Amphitecium with numerous green algal cells. Epihymenium with crystals turning dark red with K. Hymenium hyaline, 90-100(-125) µm. Paraphyses simple, about 3 µm wide, rarely branched near the apex; apical cells not or slightly thickened (up to 4-4.5 µm wide). Asci 8-spored, about 40-50 × 13.5-15 µm, with polarilocular thin-walled, hyaline spores. Ascospores narrowly ellipsoid, 12.5-16(-18) × (4-) 4.5-6.5 µm [12.5-16 × (4.5-)5-6.5 µm, n_1=20; (13-) 14.5±1.2 (-18) × (4-) 5.5±0.7 (-6.5), n_2=30; (12.5-) 14.5±1.3 (-

18) × (4-) 5.3±0.6 (-6.5) µm, n_3=35], septum 2-3(-3.5) µm. Conidia not seen. - Note: according to Smith et al. (2009) *Caloplaca subpallida* differs from *C. arenaria* in having paler apothecia. The group of '*C. ferruginea*' is taxonomically difficult and therefore is in urgent need of a thorough revision worldwide (Szczepańska et al., 2013).

Candelariella vitellina (Hoffm.) Müll. Arg., on large rock, N41°46′03″, E023°08′59″, alt. 231±3 m, 15.03.2014, SOMF 30376; on rock, N41°45′59″, E023°09′05″, alt. 223±3 m, 15.03.2014, SOMF 30365 (Fig. 9) - mixed with *Polysporina simplex*. – Notes: Apothecia 0.3-1 mm. Asci 50-65 × (15-)18-23 µm, *n*=8, 24-32 spored. Ascospores hyaline, (9-) 10.5±0.8 (-12) × (3-) 4.6±0.7 (-5.5) µm, in aquaeous lactophenol, *n*=15. *Candelariella reflexa* (Nyl.) Lettau has squamulose thallus, 8-spored asci and somewhat broader ascospores.

Chrysothrix candelaris (L.) J.R. Laundon, on bark of *Fraxinus*, near N41°45′58″, E23°09′05″, alt. 228 m, 15.03.2014.

**Cladonia coniocraea* (Flörke) Spreng, s.l., on bark of old tree, Curkvishte locality, alt. ca 229 m, 15.03.2014, SOMF 30367. – Note: *C. coniocraea* was reported in the Central Rilski Reserve near the base of old conifer trees (Stoykov, 2020).

**Cladonia fimbriata* (L.) Fr., on soil, among moss, and near the rock base, N41°46′02″, E023°08′59″, alt. 213±3 m, 15.03.2014, SOMF 30366.

Cladonia foliacea (Huds.) Willd., on soil among moss, above Struma River, N41°45′50″, E23°08′58″, alt. 223±3 m, 15.03. 2014, SOMF 30378; on soil, N41°43′51″, E23°10′33″, alt. 250 m, 10.05.2014.

Cladonia rangiformis Hoffm., on soil among moss, N41°46′03″, E023°08′59″, alt. 231±3 m, 15.03.2014, SOMF 30370. - Note: spot test K(+) yellow.

Diploschistes scruposus (Schreb.) Norman, on rocks, N41°46′03″, E023°08′59″, alt. 231±3 m, 15.03.2014, SOMF 30380; on rocks, N41°43′51.3″, E023°10′37″, alt. 273 m, 10.05.2014 (Fig. 8). - Notes: Apothecia 1-1.3 mm, ascospores 21-30 × 11-16(-17.5) µm, n=10; on siliceous rocks, Curkvishte locality, along with *R. geographicum*, 15.03.2014, SOMF 30356, on light gray areolate thallus, exciple K(+) red, asci 8-spored, ascospores 16.5-27(-32.5) × 9.5-15(-17.5) µm, *n*=10, hyaline, rarely brownish, muriform. *Diploschistes scruposus* was reported on mica schists from Slavyanka Mt (Mayrhofer et al., 2020).

**Evernia prunastri* (L.) Ach., on bark of Greek juniper, N41°46′02″, E023°08′58″, alt. 234±3 m, 15.03.2014, SOMF 30375, accompanied by *P. tiliacea*.

**Lecanora carpinea* (L.) Vain., on bark of *Carpinus orientalis* Mill., N41°46′04″, E023°08′58″, alt. ca 229±3 m, 15.03.2014, SOMF 30353. - Notes: thallus C (-), K(+) yellow, asci up to 55-60 × 18-20 µm. Ascospores hyaline, 9.5-13.5 × 6-8 µm, *n*=15 (n - number of spores measured in tap water), hymenium about 55-60 µm, I(+) blue. New record for the valley of Struma river (Fig. 11).

Lecanora gangaleoides Nyl., s.l., on big rocks, N41°46′03″, E023°08′59″, alt. 231±3 m, 15.03.2014, SOMF 30352 (Fig. 10). - Notes: apothecia (0.7-)1-1.4(-1.7) µm, hymenium up to 120 µm, I(+) in iodine. Asci 50-55 × 14-16 µm. Ascospores ellipsoid to ovoid, simple, 9.5-13.5 × 4.5-8 µm, *n*=15. Spot tests C(-), K (+) yellowish, CK(-).

**Lecidea confluens* (Weber) Ach., on siliceous rock, N41°46′03″, E023°08′59″, alt. 231±3 m, 15.03.2014, SOMF 30368. – Notes: spot tests C(-), epithecium K(+) floating, medulla I+(blue), ascospores 7-10 × 4-5.5 µm.

**Lecidella elaeochroma* (Ach.) M. Choisy, s.l., on bark of *Fraxinus*, N41°45′58″, E023°09′05, alt. 228±3 m 15.03.2014 (Fig. 16).

Lepraria incana (L.) Ach., s.l., on moss on the ground, N41°46′03″, E023°08′59″, alt. 231±3 m, 15.03.2014, SOMF 30372, spot tests: K, C (-), UV (-); on bark of old Greek juniper, N41°46′02″, E023°08′57″, alt. 220±3 m, 15.03.2014; on moss on the ground, N41°46′02″, E023°08′59″, alt. ca 231±3 m, spot tests: K, C (-); on soil, near the base of a tree, Curkvishte locality, N41°46′02″, E023°08′57″, alt. ca 220 m; on soil, near the trunk of *Carpinus orientalis* Mill., among moss, N41°43′49″, E023°10′36″, alt. 253±3 m, 10.05.2014.

Leptogium gelatinosum (With.) J.R. Laundon, on rocky slope, on soil, N41°43′49″, E023°10′36″, alt. 253±3 m, 10.05.2014.

Melanelixia fuliginosa (Fr. ex Duby) O. Blanco, A. Crespo, Divakar, Essl., D. Hawksw. & Lumbsch subsp. *glabratula* (Lamy) J.R. Laundon [=*Melanelixia glabratula* (Lamy) Sandler & Arup], on bark of *Fraxinus*, near Curkvishte locality, N41°45′58.5″, E23°09′05″, alt. 228±3 m, 15.03.2014. - Note: spot tests C(+), K(+) reddish. Reported in Tisata Reserve on *Juniperus* (Mayrhofer et al., 2020).

Melanohalea elegantula (Zahlbr.) O. Blanco, A. Crespo, Divakar, Essl., D. Hawksw. & Lumbsch, on *Quercus*, Curkvishte locality, N41°46′03″, E023°08′59″, alt. 231±3, 15.03.2014; on *Fraxinus*, N41°46′02″, E023°08′57″, alt. 229±3 m, 15.03.2014, SOMF 30369 - mixed with *Parmelina tiliacea*. - Note: spot test of thalline sections C(-).

**Opegrapha atra* Pers., on bark of *Fraxinus*, near Curkvishte locality, after N41°45′58.5″, E023°09′05″, alt. 228±3 m, 15.03.2014 (Fig. 4). - Note: Ascospores observed under LM were hyaline, 3-septate, straight, sometimes slightly thinner only near the one end, 13-18 × 4-5.5 µm, *n*=16. The species is rarely recorded in Bulgaria.

**Parmelina quercina* (Willd.) Hale, s.l., on twigs of broadleaf tree, above Curkvishte, N41°46′02″, E023°08′59″, alt. 229±3 m, 15.03.2014 (Fig. 5).

Parmelina tiliacea (Hoffm.) Hale, s.l., on old *Fraxinus*, above Curkvishte, N41°46′02″, E023°08′59″, alt. 229±3 m, 15.03.2014, upper part isidiate, spot reactions: thallus C(+) carmine red, K(-), KC(+) carmine red, idem., N41°46′02″, E023°08′59″, alt. 235±3 m, 15.03.2014; on rocks under *Fraxinus*, idem., 15.03.2014, SOMF 30364, fertile, with small apothecia, isidia coralloid, with brown tips, under surface black, brownish near the margins only, spot tests K(-), C(-), KC(+) reddish, CK(-); on bark of old Greek juniper, along with *Evernia prunastri*, N41°46′02″, E023°08′57″, alt. 231±3 m, 15.03.2014; on bark of old *Fraxinus*, Curkvishte locality, N41°46′02″, E023°08′58″, alt. 234±3 m, 15.03.2014, SOMF 30369; on piece of bark from *Quercus*, idem., N41°45′60″, E023°09′00″ alt. 232±3 m, 15.03.2014, spot tests C(+) red, K(+) red, KC(+) red. - Notes: different hemotypes examined, upper surface gray, thalli up to 2-2.5 cm in diam, lobes about 3 mm (SOMF 30369), isidia short, dark, apothecia not present, spot test reactions of thalline sections: C(+), K(-), CK(+) red, KC(+) red.

**Pertusaria albescens* (Huds.) M. Choisy & Werner [= *Lepra albescens* (Huds.) Hafellner], on bark of old *Fraxinus*, N41°46′02′′, E023°08′57′′, alt. 235±3 m, 15.03.2014, SOMF 30361, UV(-) (Fig. 12).

Pertusaria amara (Ach.) Nyl. [=*Lepra amara* (Ach.) Hafellner], on big rock, N41°46′03″, E023°08′59″, alt. 231±3 m, 15.03.2014, SOMF 30357. - Note: thallus with white soralia, slightly bitter if tasted, UV(-). Reported from the Tisata Reserve on rock (Mayrhofer et al., 2020).

Physcia adscendens H. Olivier, on bark of *Fraxinus*, N41°45′58.5″, E023°09′05", alt. 228±3 m, 15.03.2014, SOMF 30362. – Note: Upper thalline surface whitish, cilia white, long, up to 1-1.7 mm; spot test of the upper cortex K(+) yellow. Also found on bark of old Greek juiniper, above Curkvishte, alt ca. 231±3 m, 15.03.2014, spot test of the upper cortex K(+) yellow.

Physcia biziana (A. Massal.) Zahlbr., on bark of old Greek juniper, N41°46′02′′, E023°08′57′′, alt. 220±3 m, 15.03.2014, SOMF 30373. - Note: spot reaction of the upper cortex K(+) yellow. Published from the Tisata Reserve also on Greek juniper by Atanassova & Mayhofer (2012).

Physcia dubia (Hoffm.) Lettau, on pebble, N41°46′ 02″, E023°08′59″, alt. 231±3 m, 15.03.2014. - Note: published from the Tisata Reserve by Atanassova & Mayrhofer (2012).

Polysporina simplex (Davies) Vežda, on rock, N41°45′59″, E023°09′05″, alt. 223±3 m, 15.03.2014, SOMF 30365 (Fig. 1). – Note: hymenium 90-120 µm in cross section, I(+); paraphyses up to 1.8-2 µm; asci polyspored,

62.5-67.5(-75) × 17.5-22.5(-25) µm, I(-); ascospores hyaline, bacilliform, 3-4(-5) × 1-1.5 µm, *n*=10.

Porpidia crustulata (Ach.) Hertel & Knoph, on siliceous rock, Curkvishte locality, N41°45′49″, E023°08′58″, alt. 229±3 m, 15.03.2014, SOMF 30363. – Note: thallus inconsicuous, pale grey, apothecia in groups, about 1 mm in diam, numerous, black; hymenium ca 80 µm, I(+) blue.

Protoparmeliopsis muralis (Schreb.) M. Choisy, on rock, Curkvishte locality, N41°45′ 59;″, E023°09′05″, alt. 225±3 m, 15.03.2014, SOMF 30371, accompanied by *Xanthoparmelia pulla*, idem., SOMF 30355 - mixed with *Rinodina confragosa*.

Ramalina farinacea (L.) Ach., on bark of trees, above Curkvishte locality, near N41°45″59″, E023°09′05″, alt. 228±3 m 15.03.2014.

Rhizocarpon geographicum (L.) DC., on rocks, N41°45′59′′, 023°09′05′′, alt. 228±3 m, 15.03.2014, SOMF 30356 (mixed with *Diploschistes scruposus*); on big rock, N41°46′02″, E023°08′59″, alt. 231±3 m, 15.03.2014, SOMF 30374. - Note: ascospores in tap water (35-)40-43 × 13-17(-19) µm, *n*=10, muriform, medulla I+ (blue).

**Rinodina confragosa (*Ach.) Körb., on rock, accompanied by *Protoparmeliopsis muralis*, 15.03.2014, SOMF 30355. - Notes: thallus thin, pale grey, apothecia black, less than 0.5 mm in diam, ascospores 16.5-23 × 9-13 µm, *n*=17, brownish, with angular locules. The species was known from the Black Sea coast, Vitosha and Belasitsa Mts (Mayrhofer et al., 2005).

Rinodina sophodes (Ach.) A. Massal., on bark of *Fraxinus*, N41°46′02″, E023°08′57″, alt. 231±3 m, 15.03.2014 (Fig. 2). – Note: reported from the Tisata Reserve on *Paliurus spina-christi* (Mayrhofer et al., 2020).

**Sarcogyne privigna* (Ach.) A. Massal., on siliceous rocks, N41°43′51″, E023°10′33″, alt. 250±3 m, 10.05.2014; N41°44′42″, E023°09′34″, alt. 259 m, 10.05.2014. (Fig. 3). – Notes: ascospores hyaline, 3.5-5 × 1.4-1.8 µm, *n*=10. Known from the Rhodopi Mts (Mayrhofer et al., 2005).

Xanthoparmelia conspersa (Ach.) Hale, on rocks, N41°43′51″, E023°10′33″, alt. 250±3 m 10.05.2014 (Fig. 15).

Xanthoparmelia pulla (Ach.) O. Blanco, A. Crespo, Elix, D. Hawksw. & Lumbsch, on rocks, Curkvishte locality, N41°45′59″, E023°09′05″, alt. 225±3 m, 15.03.2014, spot tests K, C, KC(-), along with *Protoparmeliopsis muralis*; on rock accompanied by *Diploschistes scruposus,* N41°46′03, E023°08′59″, alt. 231±3 m, 15.03.2014, SOMF 30379 (Fig. 14), spot tests C, CK(-), KC(+) floating pinkish red. - Notes: it was recorded from the open pasture, and on big rocks exposed in the sun. Known from the valley of Struma River (Mayrhofer et al., 2020).

Xanthoparmelia stenophylla (Gyeln.) Hale, on rocks, N41°46′02″, E023°08′59″, alt. 229±3 m, 15.03.2014, on rocks, N41°46′02″, E023°08′57″, alt. 231±3 m, SOMF 30381 (Fig. 13). - Notes: spot tests C(-), CK(+) yellow-orange. Known from the West Frontier Mts and valley of Struma River (Mayrhofer et al., 2020). *Xanthoparmelia conspersa* and *X. pulla* have black lower thalline surfaces, while *X. stenophylla* has brownish lower part (Hale, 1990, Giordani et al., 2002; Szczepańska & Kossowska, 2014; Tsurykau et al., 2018). These three species of the genus *Xanthoparmelia* could be distinguished also on the base of ascospore characters, thalline chemical compounds (by spot tests and thin-layer chromatography).

Xanthoria parietina (L.) Th. Fr., on twigs and bark of *Fraxinus*, N41°45′58″, E023°09′05″, alt. 228±3 m, 15.03.2014, SOMF 30377; idem., N41°46′02″, E023°08′59″, alt. 235±3 m, 15.03.2014.

Discussion

During this study, 40 species of lichenized fungi were reported. It is supposed, that due to the different habitats and climate conditions in both parts of the Tisata Reserve (at the side of Maleshevska Mt, and on the opposite side of Kresna Village, at the side of Pirin Mts) the number of lichenized fungi will increase.

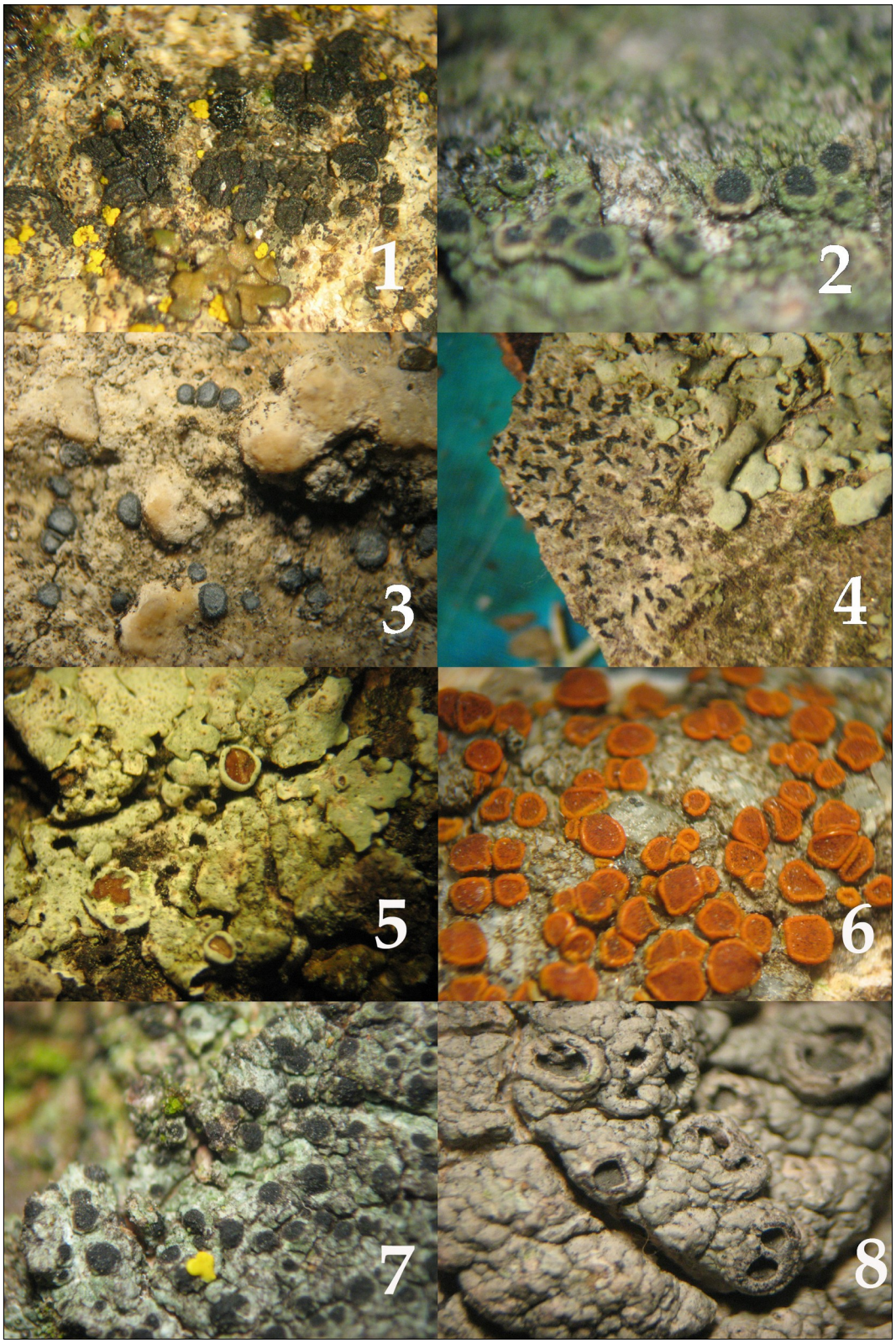

Fig. 1-8. Thalli: 1. *Polysporina simplex*; 2. *Rinodina sophodes*; 3. *Sarcogyne privigna*; 4. *Opegrapha atra*; 5. *Parmelina quercina*; 6. *Caloplaca subpallida*; 7. *Amandinea punctata*; 8. *Diploschistes scruposus*.

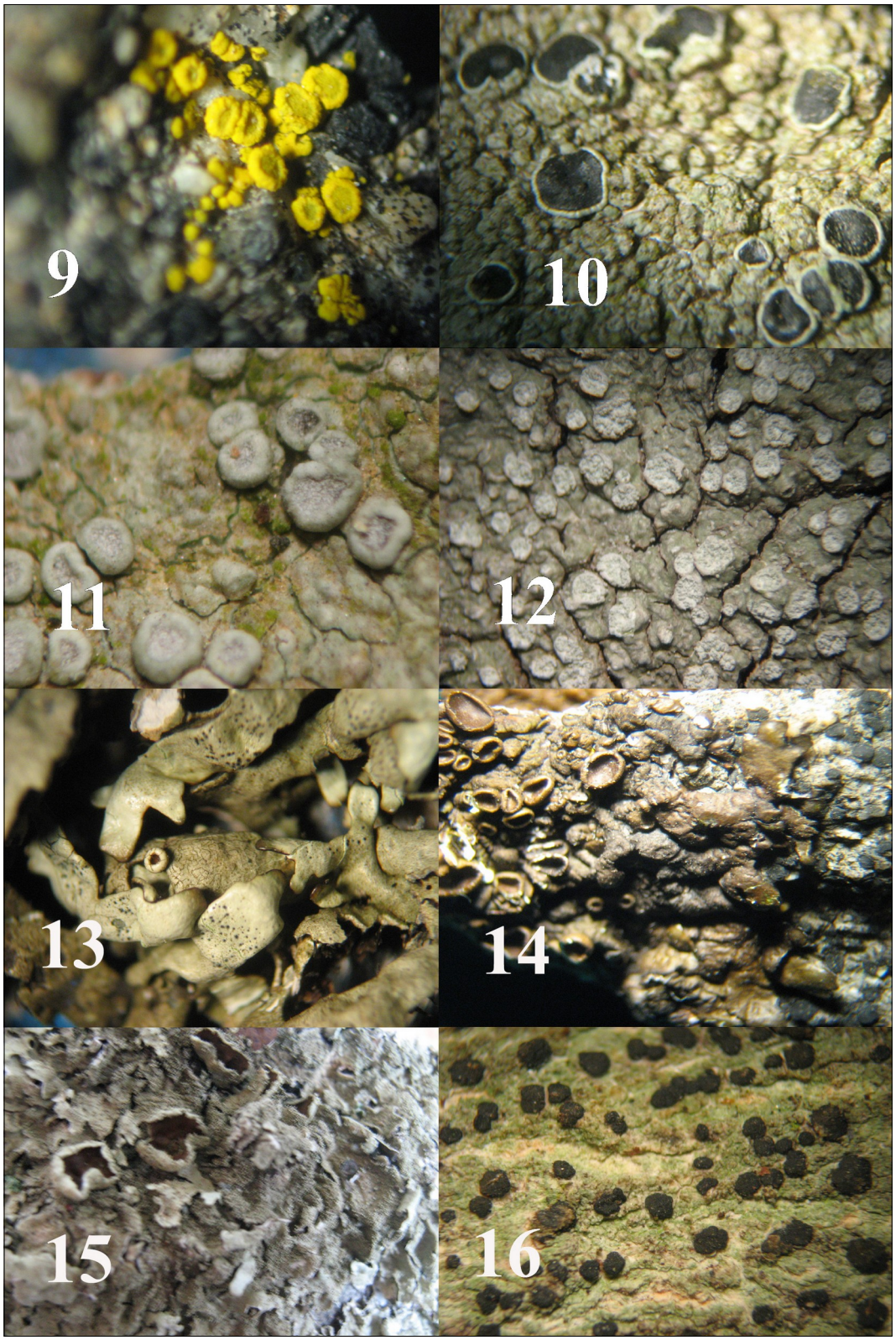

Fig. 9-16. Thalli: 9. *Candelariella vitellina*; 10. *Lecanora gangaleoides*; 11. *L. carpinea*; 12. *Pertusaria albescens*; 13. *Xanthoparmelia stenophylla*; 14. *X. pulla*; 15. *X. conspersa (in situ)*; 16. *Lecidea elaeochroma*.

Amandinea punctata was reported on bark of trees in the Tisata Reserve in the area of Curkvishte. It was known from the Black Sea coast, Northeast Bulgaria, Forebalkan, Stara Planina Mts, Sofia region, Vitosha region, West Frontier Mts and the Rhodopi Mts (Mayrhofer et al., 2005, 2020).

Caloplaca subpallida (=*Rufoplaca subpallida*) was recorded on basalt and trachyte rocks in Bulgaria from the Rhodopi Mts (Mayrhofer et al., 2020), while *C. arenaria* (Pers.) Müll. Arg. was keyed under *Caloplaca ferruginea* (Huds.) Th. Fr., as var. *lamprocheila* (DC.) H. Olivier, by Zhelezova & Popnikolov (1964) on siliceous rocks, with ascospores 13-15 × (5-)6-6.5 µm, and was known from the Black Sea coast, Vitosha and Rhodopi Mts (Mayrhofer et al., 2005; Vondrák & Slavíková-Bayerová, 2006). *Caloplaca subpallida* differs from *C. crenularia* (With.) J.R. Laundon by its smaller orange red apothecia, inconspicuous thallus, thinner ascospores with shorter septum. Nimis (1992), Wirth (1995) and Szczepańska et al. (2013) considered *Caloplaca subpallida* and *C. arenaria* different species.

Lecidea confluens was known from Stara Planina Mts, Vitosha region, Pirin, Rila and Rhodopi Mts, and the Thracian Lowland (Mayrhofer et al., 2005).

Opegrapha atra was known so far from the Black Sea coast only (Mayrhofer et al., 2005). The first report for the country appeared in Szatala (1929). The photograph of *O. atra* and its first description in Bulgarian was included in Popnikolov & Zhelezova (1964).

Physcia adscendens was previously known from the valley of Struma River in Rupite locality on sandstone tomb and oak (Atanassova & Mayrhofer, 2012).

Pertusaria albescens (= *Lepra albescens*) and *Lecidella elaeochroma* s.l., known from the present study on bark of ash trees, during 2008 have been recorded in the Central Balkan National Park on beech, and in the Strandzha Nature Park on oak trees (Spier et al., 2008). *Pertusaria albescens* (=*Lepra albescens*) and *Protoparmeliopsis muralis* have been reported recently in the Central Rilski Reserve, Rila Mts (Stoykov, 2018).

Pertusaria amara (=*Lepra amara*) was known from the Black Sea coast, Stara Planina, the valley of Struma River, Belasitsa, Rila and Strandzha Mts on beech, oak trees and trachyte (Mayrhofer et al., 2005, 2020; Spier et al., 2008; Stoykov, 2020).

Polysporina simplex was known from Stara Planina Mts, the valley of Struma River, Pirin and Rhodopi Mts (Mayrhofer et al., 2005).

Sarcogyne privigna was known so far from the Rhodopi Mts (Mayrhofer et al., 2005). This report from the Tisata Reserve is the second find from Bulgaria.

Acknowledgements

Field work was supported by the 'Management Plan of Tisata Reserve project (2014)'. This study is held within the framework of 'Phylogeny, distribution and sustainable use of fungi' project.

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Received: 02.08.2020
Accepted: 26.09.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 121-129

Characteristics of Alluvial Soils from Aydemir and Vetren Islands of Lower Danube

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Abstract. Soil characteristic is an important factor for conducting sustainable management of the territories. In the present work a general characteristic of the soils (texture, pH, humus%, total N and humus composition) of Aydemir and Vetren Islands from the Lower Danube is done. The results show a distinction between two soil types, characterized by different duration of soil formation processes: those from the periphery of the islands and those from the interior of the islands. The peripheral soils, which are newer formations with shorter soil-forming process, are lighter in texture and have a smaller part of the clay fraction. The soils from the inside have a texture from slightly to heavy sandy-clay and slightly clay. Soil acidity from both islands is slightly alkaline. In Aydemir Island the pH in the range of 7.2 ± 0.9 and in Vetren Island it is respectively 7.3 ± 0.6. The humus content varies greatly from (0.9 - 2%) in the sandy soils from the periphery of the islands, to 2.8 - 4.3% for the interior of the islands. Particularly high humus content has the soils under old *Salix* spp. forest of Vetren Island, where the quantity of total carbon is up to 8%. Humus composition of soils from both islands shows higher share of fulvic acids compared to the more stable humic acids. The soils from the periphery of the islands show fulvic type of humus, while those from the interior humic-fulvic type of humus.

Key words: soils, texture, soil organic matter, humus composition.

Introduction

The islands of the Lower Danube, which spans the course of the river from Turnu Magurele Town to the Danube delta, are very rich in ecosystems and provide unique conditions for the development of flora and fauna. Their favorable climatic and ecological characteristics, as well as the pronounced recreational and sanitary-hygienic functions make them a good basis for the development of eco-tourism, fishing, forestry, etc. The natural functions and significance of the islands for the economy have been a prerequisite for their study and description since the beginning of forestry science in Bulgaria (Minchev, 1936; Stoyanov, 1948). Such studies were made until the 80s of the last century, but there are no newer ones, as well as studies related to the humus formation process, which led us to conduct the current study.

The islands are complex element of the landscape, mobile and non-permanent formations, which are formed over the time and sometime destroyed by the waters of the Danube River. Currently, the Bulgarian territory includes 75 islands with unique biodiversity and on which 14 habitat types,

which are protected. Largely, the natural forest vegetation has been destroyed by man and in many places it has been replaced by successfully growing plantations of Euro-American poplar *Populus euroamericana* (Dode) *Guinier cv. regenerate* (Marinov & Fakirov, 1977). The natural vegetation, where it occurs is represented by well-developed and viable populations of the typical native species of *Salix* spp. and *Populus* spp. where occurs natural vegetation is presented. On the higher elevations of the Danube islands, the native species *Ulmus* spp., *Fraxinus* spp, and *Quercus* spp. are well represented, and in some places almost pure associations are preserved (Green Balkans, WWF - Greece, 2001). These natural phytocenoses have high conservation value and ecological functions. The policies are aimed at identifying activities to protect existing natural floodplain forests and create forests of local species that are more adaptable and sustainable. There is a Strategy and Action Plan (2000), which outlines the measures to no-allowed further replacement of natural forests and restoration of existing ones, as well as to comply with Bulgaria's commitments to establish a green corridor along the Lower Danube. The preservation of the rich biodiversity of vegetation and habitats in the Danube Islands, as well as the sustainable management of natural resources is also related to good knowledge of the components of natural environment, including soils. Soils are this important factor on which depends the good development of vegetation cover (Bogdanov, 2014), also its productivity and normal functioning (Ilinkin et al., 2018).

Soils from Danube river islands are classified as *Fluvisols,* with weak or no profile differentiation (IUSS Working Group WRB, 2014). The main factor of their formation is the river deposits. According the Basic Classification of Soils in Bulgaria (Penkov et al., 1992) the studied soils are classified as *Alluvial,* which are part of Class *Fluvisols*. Thus, these soils are specified as *Alluvial Fluvisols* in the soil description. They are complex, dynamic systems with no genetic connection between different layers (Donov, 1993; Pavlović et al., 2015). Their properties depends on alluvial river materials that form them (West et al., 2017; Ilinkin, 2019). In the Lower Danube, the *Alluvial Fluvisols* are usually deep, fine-grained, surface humified, formed on alluvial soil-forming materials of sand, loess and clays (Naumov, 1959). The soil-forming process takes place on these sediments and these processes are under the influence of moisture-loving woody, shrubby and grassy vegetation (Tsanov, 1992). As the soils are formed from alluvial materials accumulated by river during periodic seasonal floods, the soil formatting process is irregular. These soils do not have a clear morphological differentiation and genetic horizons with exception of top humus horizon (Ah), and they are composed of separate layers - Ah, I, II, III etc. (Donov, 1979; 1993; Galić, 2010; Koynov et al., 1998). The Danube collects waters from 19 countries, and carries different compounds from urban, industrial and agricultural sites (Liška et al., 2008). Thus, elements and nutrient contents, as well as physicochemical properties of these soils are varied. Humus content ranges from 0.1 to 4% and in some sites up to 6% (Biolchev et al., 1963; Donov, 1993). Regarding the humus composition there is no research in the literature. Nitrogen contents is varied too, commonly in the range from 0.1 to 0.4% (Biolchev et al., 1963; Donov, 1979) and its mobile forms from 66 up to 126 mg/100g soil (Donov, 1979). Soil pH values is mainly from moderately acid to alkaline (Donov, 1993; Illinkin, 2019). Soil texture depends on the duration of periodic floods and commonly varies from sandy to loamy, with predomination of sand fraction (Naumov, 1959; Ilinkin et al., 2018; Illinkin, 2019). In the periphery of the island these soils are poor in fertility, sometimes presented only from no fertile sands and gravel (Donov, 1993). However the studied soils show very good and high fertility (Stoyanov, 1948; Tsanov,

1974) and are favorable for fertilization (Denev, 1974; Shishkov & Kolev, 2014).

The aim of this study is to characterize some main properties of *Alluvial Soils* from Lower Danube with a case study from the islands Aydemir and Vetren.

Thus will give a clear view on the features of soil formation processes, especially on humus formation and humus composition from the surface layers of the island soils.

Material and Methods

Aydemir (Chayka) and Vetren (Ciocăneşti) islands are situated in the Danube River nearly the towns of Silistra and Călăraşi. The island of Aydemir is divided between Bulgaria and Romania, and the island of Vetren (Ciocăneşti) is claimed by both countries at the same time, but factually is controlled by Romania. Both islands are included in NATURA 2000 protected areas. The island of Aydemir has an area of 77 ha, while Vetren (Ciocăneşti) Island covers 280 ha. Both islands are located in the Mizian Forest-plant Zone. According to vertical zoning they are in hilly and pre-mountain belt of oak forests and more precisely in its sub-belt of floodplain and riparian forests (Zahariev et al., 1979). Climate is typical continental, with high temperatures in summer and low in winter. The average annual temperature is 11.7 ^{o}C, and the amount of precipitation is the lowest in the Country (518 - 579 mm) (Tsanov, 1992). Climate, along with the nature of alluvial materials, moisture-loving vegetation and periodic flooding are those important factors that determine the formation of these intrazonal alluvial soils.

Five sample plots (Sps) under typical woody vegetation are set on the Aydemir and Vetren Islands. The description of SPs is presented in Table 1.

Three soil samples were taken from each sample plot at depths: 0-20 cm, 20-40 cm. An average sample was formed for each SPs and a total of 20 samples were given in the laboratory for analysis. It was determined: pH – potentiometrically by pH-meter "Placitronic, MV 88" according ISO 10390, 2002; Soil texture: titration method, with HCl treatment; Bulk density: by the method of Kaczynski; Total carbon (C%) and humus (%): by the Thurin method; Total nitrogen (N%): by the method of Kjeldal– all above mantioned methods are included in Donov et al. (1974). We studied also the composition of humus in soils by the method of Kononova-Belchikova (1961): total content of humic and fulvic acids with a mixed solution of 0.1N $Na_4P_2O_7$ and 0.1M NaOH; free acids and bound to the sesquioxides (R_2O_3) with 0.1M NaOH; aggressive fulvic acids with 0.05M H_2SO_4.

Result and Discussion

The characteristics of soil texture which is the relative contents of different in size mineral elements in solid phase of soils is important in relation with consideration of issues about soil fertility. The soils with more clay content are more fertile due to the presence of mineral colloids that retain a significant amount of nutrient elements because of adsorption/desorption processes. Certainly, these soils are more prone to adsorption of contaminants too.

The results of soil texture analysis from the SPs are presented in Table 2.

The studied soils of the islands differ in texture. In practice, the texture is in the range from clayey-sandy to medium-clayey according to Kachinski's scale (Donov, 1993). However, the soils fall mainly in the range from light-sandy-clay to heavy-sandy-clay and light-clay, which corresponds to the studies of Tsanov (1992) and Biolchev et al. (1963) for the Danube River basin and islands.

The *Alluvial Fluvisols* of SP1 has the lightest texture (clay-sandy). Morphologically it is represented by a large amount of sand fraction. The interesting thing about this area is that it is located in the eastern periphery of Aydemir Island, on newly formed sand embankments.

Table 1. Characteristics of the studied sample plots (SPs).

SPs	Coordinates	Altitude m	Description
		Aydemir Island	
SP1	N44°08'033" / E27°09'269"	13.5	*Populus hybrid* forest; canopy closure 35%; flooding up to 1.30m; area 200m^2.
SP2	N44°08'069" / E27°09'339"	10	*Populus* hybrid, *Tamarix ramosisima*, *Amorfa Fruticosa*, canopy closure 60%; flooding up to 1.20m; area 200m^2.
SP3	N44°07'561" / E27°10'368"	27	*Populus Nigra* (P.h.), *Fraxinus pensilvanica*, *Ulmus; Amorfa Fruticosa* mixed forest, natural origin; the populous tree have a poor vegetation status; canopy closure 70%; no flooding; area 500m^2.
SP4	N44°07'691" / E27°10'268"	10	*Salix* forest, natural habitat, hybrid species; *Amorfa Fruticosa*; canopy closure 55%; flooding up to 0.4m; area 500m^2.
SP5	N44°07'922" / E27°09'766"	22	*Salix Alba* hybrid forest. Poor vegetation status; canopy closure 50%; flooding up to 1m; area 500m^2.
		Vetren Island	
SP6	N44°08'270" / E27°03'247"	12	*Salix Alba* hybrid forest; *Amorfa Fruticosa*; canopy closure 60%; flooding up to 1.4m; area 500m^2.
SP7	N44°08'433" / E27°03'286"	13	*Fraxinus*; *Negundo*; *Morus*; *Ulmus* mixed forest, natural origin; canopy closure 65%; flooding up to 0.4m; area 500m^2.
SP8	N44°08'320" / E27°03'925"	17	*Salix*; *Fraxinus*; *Morus*; *Ulmus* mixed old forest, natural origin; canopy closure 55%; flooding up to 0.9m; area 500m^2.
SP9	N44°08'551" / E27°04'551"	12	*Populus* hybrid forest with *Negundo*; *Fraxinus*; *Ulmus*; canopy closure 60%; flooding up to 1.3m; area 500m^2.
SP10	N44°08'708" / E27°03'966"	12	*Salix Alba* forest - many trees are doubled. The stems were cut at the level of water - to be regenerated as sprouts; canopy closure 55%; flooding up to 1.4m; area 500m^2.

Table 2. Characteristics of the soil texture in the studied SPs.

SPs	Depth cm	Particle density	Fractions, %						Mechanical composition, %		Waste from HCl, %
			Sand		Ash			Clay	Physical clay < 0.01	Physical sand 1 – 0.05	
			Coarse and middle size	Small	Coarse	Middle	Small				
			1-0.25	0.25-0.05	0.05-0.01	0.01-0.005	0.005-0.001	<0.001			
						Aydemir Island					
SP1	0-20	2.8	17.19	44.54	17.14	no	4.07	4.06	19.37	80.63	11.00
	20-40	2.7	15.23	46.23	18.12	1.25	5.08	3.02	19.35	80.65	10.00
SP2	0-20	2.7	23.13	30.28	16.34	2.37	6.09	8.47	27.13	72.87	10.20
	20-40	2.7	14.37	33.23	15.37	4.82	9.27	5.38	29.87	64.13	10.40
SP3	0-20	2.7	1.79	9.14	21.06	21.05	14.32	22.00	68.01	31.99	10.64
	20-40	2.6	12.37	15.18	14.38	22.15	8.23	17.47	60.15	43.85	12.30
SP4	0-20	2.3	1.66	8.43	47.67	4.14	12.42	12.43	42.24	57.76	13.25
	20-40	2.4	6.12	18.47	28.29	12.87	13.72	15.38	44.72	54.28	12.75
SP5	0-20	2.5	1.68	24.22	no	11.7	21	29.42	74.10	25.90	11.98
	20-40	2.4	9.36	18.12	7.28	15.3	16.18	18.32	61.98	38.02	12.18
						Vetren Island					
SP6	0-20	2.7	2.53	39.46	36.13	4.36	no	4.05	21.88	78.12	13.47
	20-40	2.6	4.32	32.78	30.18	5.80	6.25	6.38	29.59	68.41	11.16
SP7	0-20	2.8	2.04	10.74	42.23	3.37	12.44	16.58	44.95	55.05	12.56
	20-40	2.8	2.70	20.72	34.28	9.18	10.28	8.45	40.95	59.05	13.04
SP8	0-20	2.0	2.09	10.53	41.75	4.18	16.10	12.53	45.63	54.37	12.22
	20-40	2.3	3.06	8.06	11.71	27.87	13.18	20.04	72.79	27.21	11.70
SP9	0-20	2.4	2.77	13.16	no	25.51	21.25	25.51	84.07	15.93	11.80
	20-40	2.5	4.32	8.18	12.17	17.15	24.07	20.34	73.59	26.41	12.03
SP10	0-20	2.3	3.62	25.55	21.30	4.26	8.52	25.56	49.53	50.47	11.19
	20-40	2.6	2.18	24.50	22.21	8.12	10.45	20.78	50.15	49.85	10.80

It should be noted that SPs, which are located in the peripheral parts of the islands (SP2 and SP6), in greater proximity to the flowing waters of the Danube, also have a lighter texture (lightly sandy-clay) in comparison with other SPs. On the other hand, SPs from the interior of the islands show heavier texture, with content of physical clay fraction from 42.24% (SP4) to 84.07 (SP9). The most clayey (slightly to average clay) is the soil from SP9, which inwards to the central part of Vetren Island (150 m from the shore). This area is under a mixed forest dominated by *Populus* spp. hybrid. The soil from SP8 also has heavier soil texture (heavy sandy-clay to slightly clay). It is located in the interior of the island too (80 m towards the cost) and, at the same time has the highest altitude (17m). This trend is also confirmed by the soils of Aydemir Island. Here, the most clayey (slightly clay) is also the soil from the interior of the island (130 m towards the cost), with the highest altitude (27m) and no traces of flooding - SP3. In this case the alluvial materials are the oldest and the soil formation process took the longest time. In addition, the SP3 is under mixed forest with domination of *Populus Nigra* and *Fraxinus pensilvsnica*. *Salix* spp. Which develops on slightly to heavy sandy-clay soils.

In general, it can be concluded that the soils from the periphery of the islands have lighter texture, and those from the interior of the islands, where the floods are shorter and less and the soil formation process lasts longer, have heavier composition.

In terms of particle density, the whole range of 2.8 to 2 is covered.

The distribution of mineral particles by size in vertical duration is much diverse which confirm the research of Galić (2010) about soils in the islands of the Middle Danube. Ilinkin (2019) finds an increase in silt and cay fraction into depth in *Fluvusols* from the countryside (Sofia district), but here such regularity is not observed. There is no such tendency in the distribution of clay fraction in soil depth, too. Obviously, the texture of the considered soils was formed by different in structure alluvial materials during periodic flooding.

The most important chemical properties and characteristics of studied soils are presented in Table 3.

The chemical characteristics of studied soils vary, which is reported by other authors (Illinkin et al., 2018; Ilinkin, 2019; Galić, 2010), as well.

The acidity is neutral to slightly alkaline, varying in the range of 6.95 ÷ 7.50. This is normal for the soils that are formed from the periodic waters of the Danube River. Similar are the studies of Naumov (1959) for carbonate-free soils from the Danube Islands and of Fakirov & Tsanov (1967) for the islands near Oryahova and Tutrakan. Biolchev et al. (1963) obtained slightly higher pH values of 7.6 ÷ 9.1 for the floodplains of the Belene and Vardim islands, probably due to high Ca content in soils. Illinkin (2019) also observed variations without a trend in pH changes in soil depth.

The organic carbon content in studied soils varies from very low to very high 0.96 ÷ 8.16%. The published data are also contradictory: from poor 0.55 ÷ 1.90% (Naumov, 1959; Fakirov & Tsanov, 1967) to medium-humus (≤ 3.83%) (Biolchev et al., 1963; Tsanov, 1992) and very high to the river soils named "talog", which are extremely rich in organic matter, and where turbulent vegetation develops (Styanov, 1948). Largely, the content of organic matter is determined by the composition of sediments deposited on the river (Pavlović et al., 2015), as well as by periodic floods, which can either enrich the soil with organic matter (Stoyanov, 1948) or wash away organic forest litter (Mikelsen & Vesho, 2000).

The analysis of the results shows two tendencies: reduction of the carbon content in soil depth and lower content in the soils from the periphery of the islands (SP1; SP2 and SP6) compared to the other soils from the

Table 3. Chemical characteristics of the soils in the studied SPs.

SP	Depth, cm	pH	C %	N %	C / N
		Aydemir Island			
SP1	0-20	6.96	0.96	0.15	6.4
	20-40	7.20	0.95	0.14	6.7
SP2	0-20	6.90	2.5	0.17	14.7
	20-40	7.10	1.17	0.14	8.3
SP3	0-20	7.26	4.56	0.20	22.8
	20-40	7.30	3.12	0.19	16.4
SP4	0-20	7.37	2.88	0.17	16.9
	20-40	7.50	2.75	0.16	17.2
SP5	0-20	7.24	3.48	0.20	17.4
	20-40	7.38	3.20	0.18	17.8
		Vetren Island			
SP6	0-20	7.36	1.96	0.05	39.2
	20-40	7.45	0.98	0.02	49.0
SP7	0-20	7.27	3.36	0.18	18.3
	20-40	7.50	2.23	0.17	13.1
SP8	0-20	7.29	4.32	0.21	20.6
	20-40	7.05	3.45	0.21	16.4
SP9	0-20	7.31	4.50	0.30	15.0
	20-40	7.28	3.76	0.29	12.9
SP10	0-20	7.32	8.16	0.41	19.9
	20-40	7.48	7.23	0.33	21.9

Table 4. Composition of soil organic matter in the studied SPs. Columns 2, 3, 4, 5, 7, 9, 10. are given as a percentage to the weight of soil sample.

SPs	Depth, cm	Organic C% Extracted with 0.1 M $Na_2P_4O_7$ + 0.1MNaOH			Cx / Cf	Organic C% Fractions of humic acids		Non extracted organic C % (humin)	"Aggressive" fulvic acids
		Extracted totally	Humic acids Cx	Fulvic acids Cf		Free or bounded with R_2O_3	Bounded with Ca		
1	2	3	4	5	6	7	8	9	10
				Aydemir Island					
SP1	0-20	0.05	0.01	0.03	0.30	100	0	1.56	0.01
	20-40	0.04	0.00	0.01	0.00	100	0	1.48	0.03
SP2	0-20	0.97	0.23	0.65	0.35	100	0	1.45	0.06
	20-40	0.68	0.17	0.43	0.40	100	0	1.63	0.07
SP3	0-20	1.10	0.40	0.52	0.77	100	0	1.56	0.08
	20-40	0.98	0.47	0.53	0.88	100	0	1.20	0.07
SP4	0-20	0.82	0.38	0.40	0.95	100	0	0.46	0.06
	20-40	0.75	0.25	0.42	0.60	100	0	1.15	0.08
SP5	0-20	0.90	0.40	0.42	0.95	100	0	2.06	0.08
	20-40	1.03	0.34	0.63	0.54	100	0	1.23	0.06
				Vetren Island					
SP6	0-20	0.42	0.03	0.35	0.08	100	0	2.58	0.02
	20-40	0.74	0.15	0.54	0.28	100	0	1.17	0.04
SP7	0-20	1.28	0.48	0.72	0.67	100	0	0.54	0.08
	20-40	1.12	0.52	0.54	0.96	100	0	0.95	0.06
SP8	0-20	1.58	0.58	0.97	0.59	100	0	2.08	0.09
	20-40	1.35	0.54	0.73	0.74	100	0	1.27	0.09
SP9	0-20	1.82	0.68	0.81	0.83	100	0	2.74	0.13
	20-40	1.53	0.73	0.85	0.85	100	0	1.35	0.10
SP10	0-20	2.54	1.02	1.32	0.77	100	0	2.68	0.19
	20-40	2.04	0.98	1.18	0.83	100	0	1.75	0.20

interior. Soils from peripheral sites SP1; SP2 and SP6 are poor of humus (≤ 2% C). The soil of SP4 on Aydemir Island has the highest content (C% in the top layer 4.56). This soil has the highest altitude (27m) and is not flooded by the river. High content of organic carbon have the soil of SP10 on Vetren Island as well as SP8 and SP9, which are internal to the island and have high content of clay soil colloids. The stock of soils with total N also varies. Slightly (N% 0.1 ÷ 0.15) are supplied with nitrogen sandy soils of SP1 and SP6. Sandy fraction is vary inert material and does not hold nutrients (Ilinkin, 2018). Well-stocked (N% 0.2 ÷ 0.25) are soils rich in organic matter from SP3 and SP8, and very well-stoked (N% ≤ 0.25) are those of SP9 and SP10.

Important for he processes of mineralization and humification of organic residues in the soil is the C/N ratio. The smaller value ratio leads to faster mineralization processes of soil organic matter, and more mature is the humus, and vise versa. At C/N ˂ 14 the humus system is defined as Mull, between 14 ÷ 25 as Moder, and > 25 as More. For most of the soils in the area the ratio is low (C/N 17 ÷ 24), which indicates relatively fast mineralization process and formation of Mull and Moder type of humus. The mineralization processes in the soil of SP1, SP2 and SP9 (C/N <16) are very fast, and very slow in the soils of SP6.

An analysis of the humus composition in studied soils from both islands was made. It represents the content of humic and fulvic acids, as well as their ratio in the soils. The results are presented in Table 4.

Humic acids are the stable part of organic matter, insoluble in strong acids, which gives stability and "maturity" to the humus in the soil. On the contrary, fulvic acids are more mobile and short-chain and their participation in the composition of humus gives it higher lability and instability. The results show that in all cases, the amount of fulvic acids is higher than that of humic acids in the soils of both islands. The quantity of "aggressive" fulvic acids, which are the most unstable part of the soil humus is also high. This is expected result, due to the short-term soil-formation process, interrupted by periodic flooding from the Danube. The participation of fulvic acids in SP10 is the highest under old natural forest from *Salix* spp., but here is also the high participation of humic acids, due to the highest percentage of organic C% extracted with 0.1 M $Na_2P_4O_7$ + 0.1 M NaOH (2.54%). Another trend is observed - in the sample areas from the peripheral parts of the islands (SP1; SP2; SP6), which have the lowest percentage of organic C% extracted with 0.1 M $Na_2P_4O_7$ + 0.1 M NaOH, and also the lowest amount of both humic and fulvic acids. This tendency for differences in the composition of humus for peripheral soils, compared to those of the internal parts of the islands is confirmed by considering the Ch/Cf ratio which shows soil humus type (Zhyianski et al., 2012). For the soils from the peripheral parts (SP1; SP2; SP6) the type of humus is fulvate (Ch/Cf <0.5), as soils which are the latest formations of the islands, with the lowest degree of humus-formation processes. For all other areas falling within the islands, the Ch/Cf ratio is > 0.5p, which defines the humus as humic-fulvic. Kirillov et al. (2015) also find a fulvic-humic and humic type of humus system in soils from the Danube lowlands. Another feature showing the "immaturity" of humus in the soils of the islands is that the humic acids in all Sps are 100% free or bound to R_2O_3 sesquioxides and do not form stable complexes with Ca ions. This shows the mobility of organic matter in soil profile depth and its low degree of retention in the surface soil layers.

Conclusions

The characteristic of *Alluvial Fluvisols* from Aydemir and Vetren Islands of the Danube River distinguish two groups of soils. The newly formed soils from the periphery of the islands, which are closest to the river waters, are characterized by a

lighter texture (clay sandy) and a lower participation of the clay fraction in the soil texture, as well as a lower content. of organic carbon, with fulvic type of humus, and the largest predominance of fulvic acids over the humic acids. The soils from the interior of the islands, where the soil formation processes are more advanced, have heavier texture, between heavy sandy-clay to slightly clay, with a larger amount of humus, which is of humic-fulvic type. Fulvic acids predominate over humic acids in the composition of humus. This shows the instability of organic matter in soils and its greater lability in the soil profile depth and potential to release larger amounts of carbon gases into the atmosphere. In this respect, forest vegetation would contribute for stabiliztion of carbon in these soils.

Acknowledgments. The author expresses her gratitude to the Bulgarian Academy of Sciences and Romania Academy of Sciences which financed the project "Invasive alien plant species in protected areas on the Danube islands in Bulgaria and Romania", leader Assos. Prof. Georgi Hinkov, part of which is this work.

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Received: 22.08.2020
Accepted: 08.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 131-139

Heavy Metals and Metabolite Profiling - A Case Study of Achillea millefolium L.

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Abstract. Mining is one of the industries that has had the greatest impact on natural resources. Ore extraction is a mining process that has a significant impact on the environment. Heavy metals at concentrations higher than the normal levels inhibit plant growth. Studies by different authors show that various plants can be used for phytoremediation. The aim of this study was to investigate the relations between heavy metal content in soils and plants and metabolite content in *Achillea millefolium* L. in a heavy metal contaminated environment due to ore mining. The results of our investigation lead to the conclusion that heavy metal contamination does not have a negative influence on the normal growth and development of *Achillea millefolium* L., and the species is suitable for phytoremediation use because it is able to produce sustainable communities.

Key words: heavy metals, metabolites, *Achillea millefolium*.

Introduction

The sustainable management of natural resources is a particularly relevant issue both in Bulgaria and worldwide (LeDuc & Terry, 2005; Bogdanov, 2014; Teoharov & Hristov, 2016; Glogov & Pavlova, 2016). Mining is one of the industries that has had the greatest impact on the natural resources. Its effects can be either direct or indirect and people's health can be adversely affected (Samecka-Cymerman & Kempers 2004; Alexander et al., 2006; Bogdanova et al., 2016; Fiket et al., 2019). The mining activities can result in the loss of topsoil, habitat destruction, landscape changes, etc. (Donov et al., 1978; Nenova et al., 2018). The territories around mined areas or areas, where technological processes related to mining, are carried out are often subjected to very strong anthropogenic pressure (Kumpiene et al., 2007; Tsolova et al., 2014; Nenova et al., 2015). Ore extraction is a mining process that has a significant impact on the environment (Samecka-Cymerman & Kempers 2004; Kumpiene et al., 2007).

Heavy metals (some of which at certain concentrations can be nutrients) adversely

affect the normal growth of plants at higher concentrations (Gorbanov et al., 2005). The toxicity of heavy metals varies with different species and different concentrations and can inhibit the function of certain organs or of the whole plant, can cause changes in pigment content and ratios, can inhibit photosynthesis and respiration or cause other changes in plant growth and development (Kuboi et al., 1986; Gorbanov et al., 2005; Alexander et al., 2006; Monterroso et al., 2014).

Studies by different authors show that various plants can be used for phytoremediation (Chaney, 1997; Salt et al., 1998; Van der Ent, 2012; Monterroso et al., 2014). Most studies focus on phytoaccumulation and phytostabilization (Sekara et al., 2005, Yoon et al., 2006; Singh, 2012).

The aim of this study was to investigate the relations between heavy metal content in soils and plants and metabolite content in *Achillea millefolium* L. in a heavy metal contaminated environment due to ore mining.

Materials and Methods

The objects of study are:

- a population of *Achillea millefolium* L. and soils of the *Luvisols – Chromic Luvisols* groups (WRB, 2014) located to the north of the village of Petarch (Sofia Region, Bulgaria) – control group (SP1);
- a population of *Achillea millefolium* L. and soils of the *Technosols* group (WRB, 2014) located in the vicinity of the village of Lokorsko (SP2), Sofia Region, Bulgaria. The soils have been formed as a result of the ore mining activities of Kremikovtsi Metallurgical Plant and are characterized by high concentrations of heavy metals.

According to Bulgarian forest vegetation zoning (Zahariev et al., 1979), the studied sites are located in the Moesian forest vegetation area, Lower forest vegetation zone.

Methods of study. Five soil and five plant samples were collected from each site, accounting to a total of ten soil and ten plant samples. The samples were taken using the systematic sampling technique according to Petersen & Calvin (1996). The taxonomy of the plant species is presented according to Delipavlov & Cheshmedjiev (2003). Plant samples were collected during their flowering period. The above-ground part of the plants was used for analysis. Each sample was formed by combining of parts of five different plants. The samples were air-dried, ground to a fine powder (in an agate mortar to prevent their contamination with metals when using grinding machines) and dry matter content was determined (Sparks et al., 1996; ISO 638:2008). All results were recalculated based on the absolutely dry weight.

The soil samples were taken at depths of 0-20 cm. The analysis of the soil characteristics listed below was made by utilizing the following methods:

- Preparation of samples by Sparks et al. (1996);
- Soil Organic Matter (SOM, %), oxidation with a solution $K_2Cr_2O_7/H_2SO_4$ according to Donov et al. (1974);
- Total Kjeldahl Nitrogen (TKN, %) according to ISO 11261:2002;
- P_2O_5 ($mg.100g^{-1}$) and K_2O ($mg.100g^{-1}$) according to Ivanov (1984);
- Plant available metals (Fe, Pb, Cu, Cd, Mg; $mg.kg^{-1}$) using a 1 $mol.L^{-1}$ NH_4NO_3 (ISO 19730:2008).

Plant samples. The metals were determined by atomic absorption analysis (ISO 5961:1994). The Kjeldahl method was used to determined total nitrogen (Brashnarova & Stanchev, 1981). Phosphorous contents was determined by Ammonium molybdate spectrometric method (ISO 6878:2004).

Extraction procedure. The dry, ground plant material (100 mg) and internal standards of 50 µg of 3,4 dichloro-4-hidroxy benzoic acid were extracted with 1 mL methanol by classical maceration for 24 h. An aliquot of 300 µL from the extract was placed in glass vial and evaporated. The dry

extract was silylated with 50 µL of N,O-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) in 50 µL of pyridine for 2 h at 50°C.

GC/MS analysis. The GC/MS spectra were recorded on a Termo Scientific Focus GC coupled with Termo Scientific DSQ mass detector as described by Nikolova et al. (2018).

Spectrophotometric analysis. Total phenolic content of the studied samples was determined by Folin-Ciocalteu reagent and gallic acid as standard (Nikolova et al., 2013) Total flavonoid content was determined according to Miliauskasa et al. (2004), using rutin as a reference compound.

Data analysis. The relationships among the soil characteristics, heavy metal content in the aboveground portion of the plants and the metabolites under investigation were analysed using Pearson's product-moment correlation. SPSS for MacOS was used to generate pairwise correlation coefficients. A significance level of α=0.05 was chosen. The statistical significance of the differences in the soil characteristics between SP1 and SP2 was tested at α=0.05 c t-Test (Excel for MacOS).

Results and Discussion

The sample plots are laid out in two grass communities. A sample plot (control SP1) was set up in a grassland composed of 27 species belonging to 14 families and 27 genera, with the most representative families being Poaceae and Asteraceae, co-dominant being *Poa pratensis* L. and *Festuca valesiaca* Schleich, ex Gaud., with greater abundance were the species: *Agrimonia eupatoria* L. and *Fragaria vesca* L., with the single participation were the species such as: *Bromus mollis* L., *Sambucus ebulus* L., *Eryngium campestre* L., etc. Perennial herbaceous plants predominated - 85%, annual herbaceous were 7%, biennial herbaceous were absent, representatives of the shrubs - *Rosa canina* L. and *Crataegus monogyna* Jacq. were about 7%.

The second sample plot of heavy metal contamination (SP2) was set in a grass community consisting of 18 species belonging to 10 families and 18 genera. The families Asteraceae and Fabaceae had the most representatives. Dominant was *Poa pratensis* L., with a greater share was the species *Achillea millefolium* L., single participation had species such as: *Plantago media* L., *Potentila argentea* L., *Euphorbia cyparissias* L. and others. Perennial herbaceous plants predominated - 78%, biennial herbaceous plants were 11%, annual herbaceous plants were 6% and there was a single share of shrubs - *Rosa canina* L.

Under natural conditions (SP1) correlations were found among the chemical elements studied and SOM in the soil on the one hand and the metabolites studied in the aboveground portion of the plants on the other hand, where the group of the phenolic acids (4 correlations) showed the highest number of correlations, followed by the group of the saccharides and saccharide derivatives (2 correlations) and the total phenols (1 correlation). The group of the organic acids didn't show any correlations.

Under the influence of heavy metal contamination, the highest number of correlations among the chemical elements studied and SOM in the soil on the one hand and the metabolites studied in the aboveground portion of the plants on the other hand were found in the group of the saccharides and saccharide derivatives (8 correlations) (where these data are consistent with the results from other studies (Fryzova et al., 2017), followed by the group of the phenolic acids (2 correlations), organic acids (1 correlation) and total phenols (1 correlation).

Under the influence of heavy metal contamination, Inositol 1 was the metabolite with the highest number of correlations (4 correlations), where only the correlation with available phosphorous was positive, and the others were negative. Sucrose had three correlations with the soil characteristics studied, where only the correlation with potassium was positive. Salicylic acid had two negative correlations (with Cu and Cd).

Table 1. Table of the Pearson correlation coefficients among SOM and the chemical elements studied in the soil and the metabolites.

	Metabolites	Sample Plot	N SSD, %	P, mg.kg^{-1}	K SSD, mg.kg^{-1}	SOM %	Fe SSD, mg.kg^{-1}	Pb SSD, mg.kg^{-1}	Cu SSD, mg.kg^{-1}	Cd SSD, mg.kg^{-1}	Mg SSD, mg.kg^{-1}
Phenolic acids	Salicylic Acid	SP1	0.444	0.986**	0.638	-0.826	0.232	-0.399	-0.418	0.560	0.504
		SP2	-0.250	0.795	0.252	-0.813	-0.684	0.073	-0.940*	-0.883*	-0.714
	Protocatechuic acid	SP1	-0.130	0.689	0.802	-0.884*	0.189	0.045	-0.136	-0.251	0.116
		SP2	0.130	0.087	-0.340	0.055	-0.031	-0.128	-0.219	-0.060	0.063
	Quinic Acid	SP1	0.354	0.635	0.281	-0.615	-0.282	-0.818	-0.722	0.811	0.057
		SP2	0.170	0.668	0.735	-0.684	-0.561	0.034	-0.579	-0.497	-0.684
	Caffeic Acid	SP1	-0.329	-0.017	-0.283	-0.312	-0.460	-0.566	-0.403	0.424	-0.676
		SP2	-0.333	0.020	-0.192	-0.152	0.024	0.522	-0.220	-0.289	-0.029
	Chlorgenic acid cis	SP1	-0.243	-0.608	-0.263	0.271	-0.904*	-0.530	-0.487	-0.004	-0.735
		SP2	0.497	-0.346	0.431	0.358	0.302	-0.110	0.639	0.645	0.223
	Chlorgenic acid trans	SP1	0.469	0.182	-0.357	0.082	-0.136	-0.617	-0.384	0.948*	0.143
		SP2	0.597	-0.118	0.556	0.131	0.164	0.109	0.343	0.435	0.031
Organic acids	Phosphoric Acid	SP1	-0.289	-0.566	-0.311	0.211	-0.865	-0.540	-0.471	0.055	-0.776
		SP2	0.294	0.210	0.225	-0.240	-0.041	0.597	-0.322	-0.166	-0.180
	Succinic Acid	SP1	-0.608	0.290	-0.099	-0.681	0.090	-0.074	0.025	0.109	-0.570
		SP2	-0.842	0.195	-0.386	-0.270	-0.315	-0.302	-0.327	-0.553	-0.180
	Malic Acid	SP1	-0.583	0.402	0.095	-0.800	0.123	-0.041	0.002	0.018	-0.488
		SP2	-0.385	0.709	0.690	-0.707	-0.843	-0.948*	-0.418	-0.593	-0.785
	Pyroglutamic Acid	SP1	-0.171	0.287	-0.044	-0.549	-0.364	-0.645	-0.508	0.544	-0.471
		SP2	0.073	-0.010	0.696	-0.038	-0.127	-0.514	0.408	0.244	-0.176
Saccharides and saccharide derivatives	Fructose 1	SP1	-0.851	-0.391	-0.264	-0.196	-0.385	-0.012	0.017	-0.365	-0.972**
		SP2	-0.294	0.202	0.485	-0.352	-0.266	-0.037	-0.057	-0.247	-0.348
	Fructose2	SP1	-0.355	0.014	-0.644	0.077	0.800	0.606	0.790	0.075	-0.010
		SP2	-0.186	0.147	0.725	-0.252	-0.298	-0.499	0.216	-0.022	-0.349
	Monosaccharide 1	SP1	-0.474	-0.778	-0.622	0.434	-0.648	-0.232	-0.115	-0.079	-0.847
		SP2	0.159	-0.333	-0.144	0.427	0.238	-0.429	0.471	0.462	0.328
	Glucose	SP1	-0.214	-0.353	0.392	-0.021	-0.774	-0.272	-0.457	-0.504	-0.462
		SP2	0.443	0.144	0.538	-0.024	-0.174	-0.563	0.154	0.230	-0.175
	Inositol 1	SP1	-0.086	0.152	-0.507	-0.178	0.006	-0.383	-0.123	0.717	-0.285
		SP2	0.026	0.939*	0.664	-0.909*	-0.824	-0.163	-0.911*	-0.805	-0.888*
	Monosaccharide 2	SP1	-0.327	-0.329	-0.340	-0.032	-0.713	-0.593	-0.463	0.264	-0.780
		SP2	0.235	0.676	0.974**	-0.657	-0.644	-0.383	-0.398	-0.362	-0.741
	Inositol 2	SP1	-0.173	-0.121	-0.151	-0.196	-0.736	-0.742	-0.630	0.396	-0.649
		SP2	-0.600	-0.182	0.149	-0.008	-0.014	-0.219	0.351	-0.005	-0.015
	Disaccharide	SP1	-0.577	-0.119	0.441	-0.370	-0.294	0.157	-0.049	-0.754	-0.502
		SP2	0.021	0.310	0.870	-0.372	-0.396	-0.455	0.051	-0.086	-0.476
	Sucrose	SP1	-0.123	-0.721	-0.670	0.599	-0.646	-0.388	-0.227	0.218	-0.576
		SP2	-0.291	0.814	0.919*	-0.860	-0.891*	-0.695	-0.522	-0.672	-0.918*
	Trisaccharide	SP1	0.498	0.965**	0.824	-0.774	0.266	-0.255	-0.363	0.328	0.642
		SP2	0.062	0.064	0.747	-0.120	-0.187	-0.478	0.324	0.165	-0.249
	Total flavonoids	SP1	-0.243	-0.404	-0.809	0.584	0.626	0.678	0.840	-0.086	0.020
		SP2	-0.811	0.220	-0.287	-0.361	-0.284	0.054	-0.403	-0.618	-0.229
	Total phenols	SP1	0.940*	0.328	0.088	0.209	-0.029	-0.503	-0.424	0.790	0.734
		SP2	-0.271	-0.727	-0.896*	0.641	0.702	0.590	0.448	0.371	0.745

Legend: SSD indicate that there is a statistically significant difference between the content of the chemical elements/chemical compounds in SP1 and that of their corresponding counterparts in SP2 ($p<0.05$); * indicate statistically significant correlation at $p\leq0,05$; ** indicate statistically significant correlation at $p\leq0,01$.
indicate statistically signifint correlation at SP1; indicate statistically significant correlation at SP2

There were number of correlations among the content of the chemical elements studied (in the aboveground portion of the plants) and the metabolites in the control group plants and in the plants exposed to heavy metal contamination (Table 2).

The groups of the saccharides and saccharide derivatives and of the phenolic acids showed the highest number of correlations with the elements studied (6 each with the control group and 8 and 4 respectively with the plants exposed to heavy metal contamination). The group of the organic acid had 4 correlations with the control group plants and one correlation with the plants exposed to heavy metal contamination. The group of the total phenols and flavonoids had only one correlation with the plants exposed to heavy metal contamination.

Under natural conditions without anthropogenic pressure, the elements studied had a positive influence on the synthesis of metabolites, where only the salicylic acid and Mg showed the negative correlations. Copper was the element that had a positive effect on the largest number of metabolites in 3 out of 4 metabolic groups (which confirmed the results of studies conducted by other authors (Kumar et al., 2004), whereas iron did not show any statistically significant correlations.

Under the influence of heavy metal contamination, the main nutrients (N, P, K) showed positive correlations with the metabolites. All other elements showed negative correlations, with the exception of iron and cadmium, which showed both positive and negative correlations. The plants exposed to heavy metal contamination had the highest number of correlations (negative) among Mg and the metabolites.

The correlations found among the soil characteristics and the metabolites in this study confirmed data found by other authors (Akula & Ravishankar, 2011; Fahimirad & Hatami, 2017) on the influence of the environment on the synthesis of metabolites. The different heavy metals both in the soil and in the aboveground portion of the plants had a different effect (positive and/or negative) on the different metabolites, which was consistent with studies carried out by other authors (Misra, 1992; Macnair, 1993; Tumova & Blazkova, 2002; Tumova et al., 2001).

Low concentrations of some heavy metals could be used (as nutrients) to increase the synthesis of a certain metabolite or a group of metabolites. Such data have also been presented by other authors (Kumar et al., 2004).

Conclusion

The heavy metals studied (both in the soil and in the aboveground portion of the plants) have the strongest influence on the group of the saccharides and saccharide derivatives, whereas the group of the organic acid has remained relatively stable under the influence of the soil characteristics.

The heavy metal contamination does not have an adverse effect on the successful growth and development of *Achillea millefolium* L, and the species is able to create sustainable communities, which makes it suitable for the purposes of phytoremediation.

Acknowledgements

This work was supported by the University of Forestry, Sofia, Bulgaria under project "Metabolic profile of plants species of phytocoenosis located on Technogenic soils" approved by SRS – B – 1011/27.03.2019.

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Table 2. Table of the Pearson correlation coefficients among the chemical elements in the plants and the metabolites.

	Metabolites	Sample Plot	N, %	P, mg.kg^{-1}	K, mg.kg^{-1}	Fe, mg.kg^{-1}	Pb, mg.kg^{-1}	Cu, mg.kg^{-1}	Cd, mg.kg^{-1}	Mg, mg.kg^{-1}
Phenolic acids	Salicylic Acid	SP1	0,932*	0,249	0,493	-0,659	-0,063	0,123	-0,063	-0,933*
		SP2	0,662	0,445	-0,207	-0,016	0,689	0,127	-0,898*	0,087
	Protocatechuic acid	SP1	0,656	0,039	0,427	-0,252	-0,322	-0,027	-0,204	-0,331
		SP2	-0,309	-0,216	0,900*	0,261	0,237	-0,497	0,444	0,789
	Quinic Acid	SP1	0,584	0,739	0,601	-0,326	0,534	0,685	0,386	-0,831
		SP2	0,959**	0,871	-0,515	-0,654	0,159	-0,069	-0,866	-0,673
	Caffeic Acid	SP1	-0,163	0,991**	0,73	0,472	0,961**	0,984**	0,880*	-0,229
		SP2	0,223	-0,064	-0,857	0,357	0,556	0,865	-0,719	-0,105
	Chlorgenic acid cis	SP1	-0,517	0,546	-0,005	0,521	0,638	0,714	0,414	0,410
		SP2	0,093	0,302	-0,189	-0,658	-0,771	-0,292	0,333	-0,779
	Chlorgenic acid trans	SP1	0,122	0,509	0,230	-0,209	0,596	0,46	0,439	-0,604
		SP2	0,471	0,59	-0,456	-0,767	-0,476	-0,178	-0,093	-0,906*
Organic acids	Phosphoric Acid	SP1	-0,514	0,642	0,113	0,563	0,731	0,789	0,525	0,347
		SP2	0,723	0,545	-0,811	-0,257	0,468	0,410	-0,824	-0,482
	Succinic Acid	SP1	0,019	0,802	0,981**	0,481	0,662	0,688	0,809	-0,291
		SP2	-0,442	-0,579	0,298	0,708	0,302	0,262	-0,004	0,708
	Malic Acid	SP1	0,156	0,711	0,961**	0,380	0,506	0,596	0,666	-0,314
		SP2	0,227	0,343	0,524	-0,45	-0,451	-0,677	-0,023	-0,145
	Pyroglutamic Acid	SP1	0,141	0,983**	0,817	0,230	0,853	0,941*	0,777	-0,487
		SP2	0,177	0,355	-0,171	-0,709	-0,894*	-0,332	0,157	-0,858
Saccharides and saccharide derivatives	Fructose 1	SP1	-0,523	0,654	0,548	0,863	0,647	0,696	0,702	0,413
		SP2	0,453	0,328	-0,834	-0,300	-0,161	0,421	-0,625	-0,755
	Fructose 2	SP1	-0,277	0,016	0,373	0,314	0,179	-0,165	0,457	-0,092
		SP2	0,286	0,361	-0,401	-0,607	-0,733	-0,094	-0,141	-0,884*
	Monosaccharide 1	SP1	-0,792	0,526	0,068	0,773	0,733	0,647	0,616	0,542
		SP2	-0,591	-0,332	0,818	-0,011	-0,553	-0,599	0,924*	0,301
	Glucose	SP1	-0,15	0,068	-0,224	0,223	-0,033	0,257	-0,194	0,513
		SP2	0,110	0,404	0,633	-0,702	-0,677	-0,932*	0,487	-0,29
	Inositol 1	SP1	-0,072	0,803	0,692	0,245	0,861	0,699	0,854	-0,496
		SP2	0,901*	0,814	-0,068	-0,507	0,352	-0,304	-0,823	-0,252
	Monosaccharide 2	SP1	-0,38	0,874	0,441	0,56	0,915*	0,950*	0,758	0,070
		SP2	0,855	0,929*	-0,151	-0,932*	-0,313	-0,515	-0,499	-0,791
	Inositol 2	SP1	-0,151	0,913*	0,492	0,372	0,878*	0,980**	0,691	-0,129
		SP2	0,806	0,709	0,251	0,909	0,477	0,353	0,835	0,399
	Disaccharide	SP1	-0,074	0,044	0,131	0,366	-0,139	0,125	-0,082	0,460
		SP2	0,523	0,614	-0,357	-0,801	-0,65	-0,263	-0,262	-0,949*
	Sucrose	SP1	-0,689	0,442	-0,109	0,514	0,698	0,562	0,505	0,365
		SP2	0,646	0,668	-0,039	-0,667	-0,333	-0,412	-0,5	-0,578
	Trisaccharide	SP1	0,985**	-0,049	0,252	-0,776	-0,384	-0,146	-0,376	-0,790
		SP2	0,281	0,431	-0,264	-0,739	-0,829	-0,285	0,026	-0,911*
	Total flavonoids	SP1	-0,571	-0,299	-0,129	0,340	0,012	-0,39	0,210	0,282
		SP2	-0,095	-0,366	-0,353	0,634	0,513	0,687	-0,505	0,344
	Total phenols	SP1	0,456	-0,152	-0,352	-0,767	-0,118	-0,160	-0,321	-0,587
		SP2	-0,674	-0,834	-0,359	0,883*	0,357	0,859	0,195	0,470

Legend: * indicate statistically significant correlation at p≤0,05; ** indicate statistically significant correlation at p≤0,01 . indicate statistically significant correlation at SP1; indicate statistically significant correlation at SP2.

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Received: 04.09.2020
Accepted: 08.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 141-151

CP43 and CP47 Proteins of Photosystem II (PSII) as Molecular Markers for Resolving Relationships between Closely Related Cyanobacteria

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Abstract. Cyanobacteria are the most primitive photosynthetic organisms on the Earth. Their classification is traditionally based on morphological characters in both botanical and bacteriological systems. Due to the enormous diversity and the lack of clear diacritic morphological features between the closely related species, for resolving the evolutionary relationships and classification of Cyanobacteria during the last years is used a polyphasic approach including sequencing data. Although many molecular markers are improved, new suitable markers for resolving relationships within cyanobacteria at species and generic level are still needed. Our objective was to examine whether the sequences of the photosystem II proteins CP43 and CP47 are suitable markers for such purposes. Phylogenetic analyses based on the CP43 and CP47 amino acid sequences showed that most of the cyanobacterial species/strains belonging to different genera are clustered in separate clades supported by high bootstrap values. The comparison between the CP43 and CP47 trees, and the 16S phylogenetic trees showed that the CP43 and CP47 proteins are more suitable markers in resolving phylogenetic relationships within Cyanobacteria at generic and species level than the conserved 16S rRNA gene sequence. The correct taxonomic classification and identification of the cyanobacterial strains is very important for all studies related to the biological activity of cyanobacteria, their biotechnological application or in the management and monitoring of water.

Key words: Cyanobacteria, molecular marker CP43, CP47, phylogeny.

Introduction

Cyanobacteria are the oldest microorganisms performing oxygenic photosynthesis. They can be found in almost all ecosystems on Earth including freshwater lakes, rivers, ponds, oceans, hot springs and deserts (Scanlan, 2001). Many cyanobacterial strains produce intracellular and extracellular metabolites with various biological activities (antibacterial, antifungal, antiviral, immunostimulation (Baldev et al., 2015), but they are also capable of extensive growth, resulting in bloom events and toxin production that can cause a significant threat to human and animal health (Carmichael, 1992).

Within prokaryotic groups, Cyanobacteria is one of the most morphologically diverse groups (Castenholz et al., 2001 & Shih et al., 2013). Their evolutionary relationships and classification are, at present, poorly understood (Castenholz et al., 2001 & Komarek et al., 2002). The taxonomy of Cyanobacteria has been debated vigorously and revised many times. One way to improve cyanobacterial taxonomy is a polyphasic approach including molecular, cytological, ecological, morphological, and physiological data (Rajaniemi et al., 2005). The morphological and cytological features are the basis for the conventional identification and taxonomy of cyanobacteria. But it is found that most of the cyanobacterial species change their morphology when grow at different ecological conditions. Therefore, the conventional methods are useless for identification of cyanobacteria at the species level. As a much more reliable technique for such identification is recommended the usage of molecular markers (Baldev et al., 2015).

Molecular phylogenies of cyanobacteria were mainly inferred with the 16S rDNA sequences. This molecular marker is generally conserved and provide many thousands of well-aligned informative sites. Furthermore, it is the best represented in the GenBank database, and phylogenies based on their sequences have so far been considered quite efficient for cyanobacterial phylogeny at the genus level (Hoffmann et al., 2005; Miller & Castenholz, 2001; Rajaniemi et al., 2005). The phylogenetic resolution of 16S rDNA, however, is limited, and often not sufficient to resolve relationships neither among very closely nor very distinctly related organisms (Johansen, 2005). Various protein coding sequences have also been used for inferring phylogenies within cyanobacteria (*rpoC1*, *rpoB*, *gyrB*, *rbcLX*, *cpcBA-IGS* and 16S-23S-*ITS)*(Sciuto et al., 2012; Seo & Yokota, 2003; Boyer et al., 2001; Nubel et al., 1997; Premanandh et al., 2006). The 5S ribosomal RNA, the outer membrane efflux protein (OMER), the Light-Repressed Protein (LRP) and the Psb27 protein have been recently proposed as new molecular markers (Teneva, 2019). The revolution in molecular phylogenetic approaches has had a profound effect on the description and classification of taxa (Garcia-Pichel et al., 2020).

Photosystem II (PSII) is the first component of the photosynthetic electron transfer chain located in the thylakoid membranes of cyanobacteria, algae and plants. Active cyanobacterial PSII consists of 17 transmembrane protein subunits, three peripheral proteins and about 80 cofactors such as chlorophylls, carotenoids and lipids (Guskov et al., 2009). The membrane-embedded core complex of PSII consists of the D1 and D2 reaction center (RC) subunits, the inner chlorophyll (Chl)-binding antenna proteins, CP43 and CP47, and a number of smaller polypeptides (Sánchez-Baracaldo & Cardona, 2020). CP47 and CP43 are encoded by the *psb*C and *psb*B genes in the genomic DNA of cyanobacteria. The main purpose of CP43 and CP47 is to deliver energy to the RC for driving electron transfer and, in the case of CP43, to help ligate the $CaMn_4$ cluster (Barber, 2006; Sánchez-Baracaldo & Cardona, 2020). CP43 and CP47 have 473 and 510 amino residues, respectively, and both of them have six transmembrane α-helices, which are separated by five extrinsic loop domains (Bricker & Frankel, 2002).

Taxonomy and classification have always been a challenge in the cyanobacteria. It is important to identify other reliable molecular markers for more precise cyanobacterial classification at the generic and subgeneric level. We decided to focus on the CP43 and CP47 proteins of Photosystem II, since so far, it is not exploited for phylogenetic analyses of Cyanobacteria. In this study, we investigated the phylogenetic relationships of cyanobacterial strains based on the CP43 and CP47 protein sequences, and compared the phylogenetic position of cyanobacterial strains with phylogenetic trees based on 16S rDNA.

Material and Methods

The CP43 and CP47 protein sequences used in this study were obtained from NCBI database. We chose to use amino acid sequences rather than nucleotide sequences because the latter are more strongly affected by saturation over long time scales (Li et al., 2014). The sequences were aligned to observe the phylogenetic relationship. Multiple sequence alignments of the proteins were created using ClustalW program of the phylogenetic software MEGA-7 (Kumar et al., 2016). Two data sets were constructed. The first one included 129 taxa (with *Escherichia coli str. K-12* as outgroup) and was based on CP47 sequence alignment. A second data set included 133 taxa (with *Arabidopsis thaliana* as outgroup) and was based on CP43 sequence alignment. The size of the analyzed CP47 amino acid sequences varied between 456 aa and 538 aa, and between 393 aa and 490 aa for CP43. The minimum evolution (ME), maximum parsimony (MP), maximum-likelihood (ML), and neighbor-joining (NJ) algorithms were used to construct phylogenetic trees, and the reliability of each branch was tested by 1,000 bootstrap replications. All positions containing gaps and missing data were removed from the dataset using the "complete deletion" option. For ME and NJ, the evolution distances were calculated using the Maximum Composite Likelihood method. For ML trees, the General Time Reversible (GTR) model with Corrected Invariable Sites (I), Gamma Distribution Shape Parameters (G), and Nearest-Neighbor-Interchange algorithm was used. In order to compare the topology of the taxa, the phylogenetic reconstruction was conducted also with 16S rDNA nucleotide sequences of the same strains used in the CP47 and CP43-phylogenetic trees. 16S rRNA gene sequences were aligned using the ClustalW multiple sequence alignment tools in Version 7.0 of MEGA phylogenetic software (Kumar et al., 2016). Trees based on the 16S rRNA gene were constructed by the same manner as described above. 16S rDNA nucleotide sequence from *Escherichia coli* str. K-12 was used to root the trees.

Results and Discussion

Our objective was to examine whether or not phylogenetic analysis based on CP47 and CP43 amino acid sequences supports the division Cyanobacteria. The phylogenetic trees based on different methods (Minimum Evolution (ME), Maximum Parsimony (MP), Maximum-Likelihood (ML), and Neighbor-Joining (NJ)) obtained in this study exhibited a high degree of similarity with minor topological differences. Here we represented only the ML trees.

CP43

In the present study, we have performed phylogenetic analyses based on the CP43 amino acid sequences. As can be seen in Fig. 1, cyanobacterial strains are grouped in eight larger clades (*Synechococcus* clade 1; *Candidatus Synechococcus* clade; *Planktothrix* clade; *Fischerela* clade; *Leptolyngbya* clade 1; *Nostoc* clade 1; *Nostoc* clade 2; *Pseudoanabaena* clade). Most members of the genus *Synechococcus* (23 strains) were grouped in two separate clades (Fig. 1, *Synechococcus* clade 1 (20 strains, 86% bootstrap supports), *Synechococcus* clade 2 (3 strains, 99% bootstrap supports)). The *Nostoc* strains also were clustered in two separate clades: *Nostoc* clade 1 (6 strains, 72% bootstrap support) and *Nostoc* clade 2 (4 strains, 65% bootstrap support, Fig. 1). All representatives of the other main clades were clustered in separate monophyletic clades: *Candidatus Synechococcus* (6 strains, 94% bootstrap support), *Planktothrix* (5 strains, 98% bootstrap support), *Pseudanabaena* (4 strains, 87% bootstrap support), and *Fischerella* (4 strains, 88% bootstrap support). The *Leptolyngbya* strains were clustered in three separate clades: *Leptolyngbya* clade 1 (4 strains, 69% bootstrap support, Fig. 1), *Leptolyngbya* clade 2 (3 strains, 54% bootstrap support, Fig. 1) and *Leptolyngbya* clade 3 (2 strains, 79% bootstrap support, Fig. 1). According to the phylogenetic analysis, the polyphyletic nature

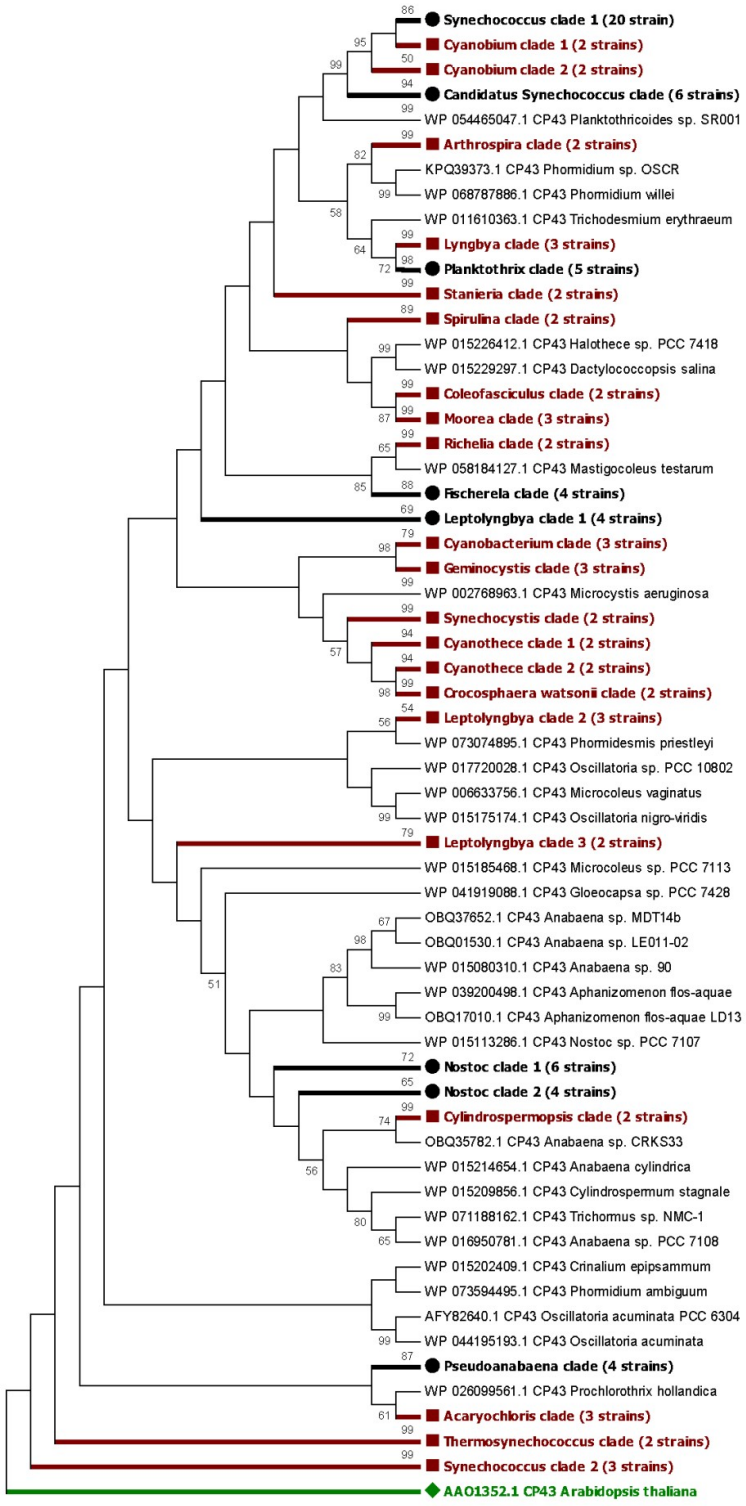

Fig. 1. Phylogenetic tree based on CP43 amino acid sequences from 133 cyanobacterial strains. The reconstruction has been performed by using ML analysis applying the GTR+I+G evolutionary model. The numbers above branches indicate the bootstrap support (>50%) from 1,000 replicates. *Arabidopsis thaliana* was used as an outgroup. ● - big monophyletic clades; ■ - small monophyletic clades.

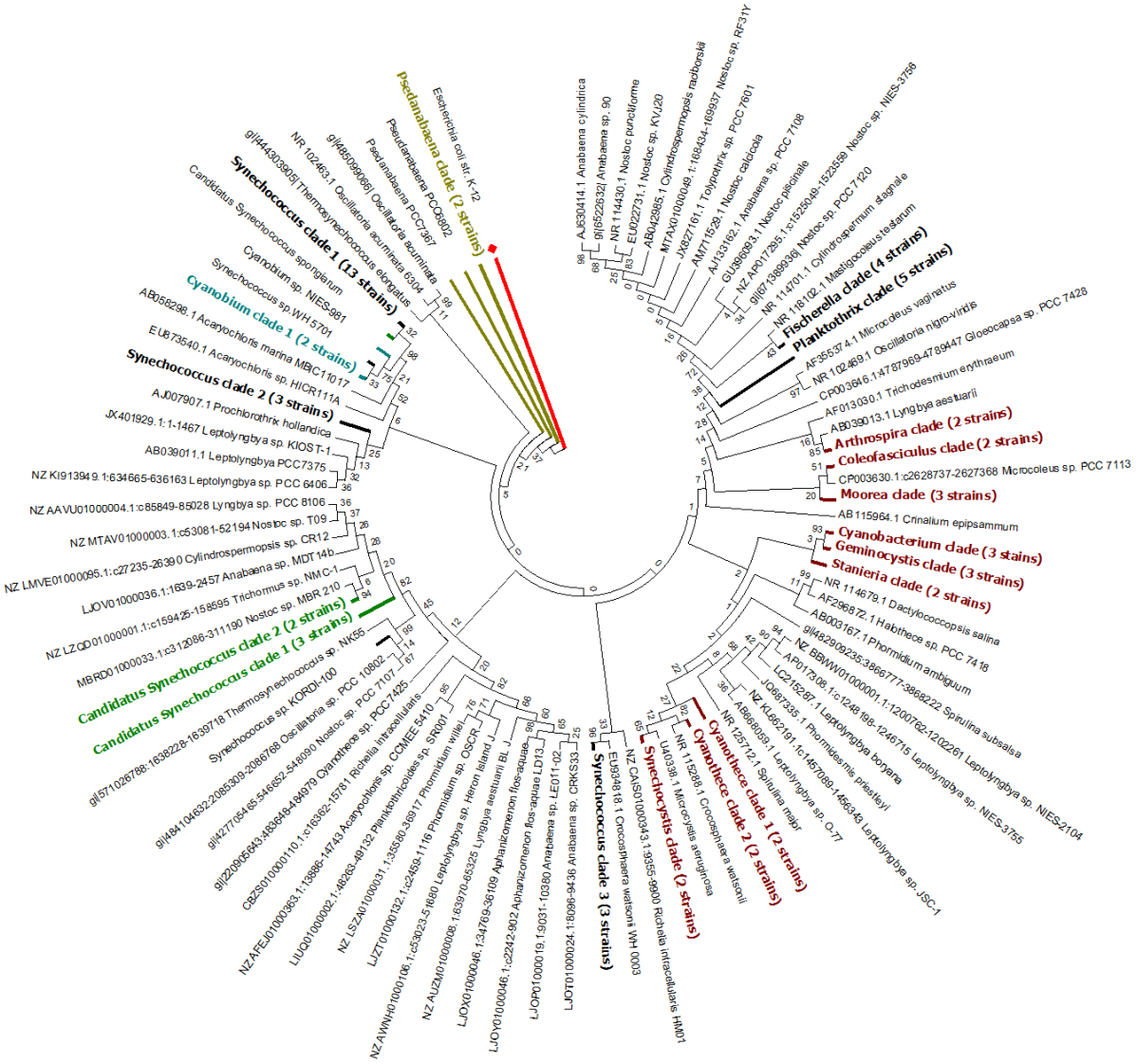

Fig. 2. Phylogenetic tree based on 16S rDNA sequences of 133 cyanobacterial strains. The tree was reconstructed by using ML analysis and the GTR+I+G evolutionary model. 16S rDNA sequence from *Escherichia coli* str. K-12 was used as an outgroup. The numbers near branches indicate bootstrap support from 1,000 replicates.

of order Oscillatoriales including genus *Oscillatoria, Microcoleus* and *Phormidium* was confirmed. Previous studies have also shown the polyphyletic nature of order Oscillatoriales based on the 16S rRNA gene sequences (Ishida et al., 1997; Marquardt & Palinska, 2007).

A number of smaller clades of cyanobacterial strains belonging to one genus, which are comprised of between 2 and 3 species/strains, are also observed in the tree shown in Fig. 1: *Cylindrospermopsis* clade (2 strains, 99% bootstrap support); *Moorea* clade (3 strains, 99% bootstrap support); *Arthrospira* clade (2 strains, 99% bootstrap support); *Spirulina* clade (2 strains, 89% bootstrap support); *Lyngbya* clade (3 strains, 99% bootstrap support); *Geminocystis* clade (3 strains, 99% bootstrap support); *Cyanobacterium* clade (3 strains, 79% bootstrap support); *Stanieria* clade (2 strains, 99% bootstrap support); *Richelia* clade (2

strains, 99% bootstrap support); *Synechocystis* clade (2 strains, 99% bootstrap support); *Cyanothece* clade 1 (2 strains, 94% bootstrap support); *Cyanothece* clade 2 (2 strains, 94% bootstrap support); *Thermosynechococcus* clade (2 strains, 99% bootstrap support); *Coleofasciculus clade* clade (2 strains, 99% bootstrap support); *Synechococcus* clade 2 (3 strains, 99% bootstrap support); *Cyanobium* clade 1 (2 strains, 50% bootstrap support); *Cyanobium* clade 2 (2 strains, 94% bootstrap support); *Acaryochloris* clade (3 strains, 99% bootstrap support); *Crocosphaera watsonii* clade (2 strains, 99% bootstrap support); *Leptolyngbya* clade 2 (3 strains, 54% bootstrap support); *Leptolyngbya* clade 3 (2 strains, 79% bootstrap support) (Fig. 1).

The phylogenetic trees based on the markers CP43 (Fig. 1) and 16S rDNA (Fig. 2) showed similar topologies, but differences in the positions of some strains were observed. Some of the species that showed monophyly with high bootstrap supports in the CP43 tree did not group together in the 16S tree. Intermixing of the members of genera *Synechococcus*, *Candidatus Synechococcus*, *Pseudanabaena*, *Nostoc*, *Thermosynechococcus, Cylindrospermopsis*, *Crocosphaera watsonii*, *Richelia* and *Spirulina* was observed in the phylogenetic tree based upon 16S rRNA, while in the phylogenetic tree constructed on CP43 they were grouped in separate monophyletic clades. The representatives of the genera *Fischerella, Planktothrix, Arthrospira, Coleofasciculus, Moorea, Cyanobacterium, Geminocystis, Stanieria,* and *Cyanothece* were grouped in separate monophyletic clades as in the phylogenetic tree based on CP43, but with low bootstrap supports (Fig. 2). The evolutionary relationships among different cyanobacterial taxa seen in this work are similar to those observed by Shih and colleagues (Shih et al., 2013). These results confirmed the usefulness of 16S rRNA gene as a valuable tool for identification of cyanobacteria up to order or genus level.

The phylogenetic trees based on the protein sequences and the 16S rRNA gene sequences created in this study showed that the CP43 is a suitable marker in resolving phylogenetic relationships within Cyanobacteria at generic and species level. The search for more stable molecular markers has become essential for understanding the phylogeny and taxonomy of cyanobacteria (Gribaldo & Brochier, 2009 & Makarova et al., 1999).

CP47

The phylogenetic tree based on CP47 amino acid sequences showed that most cyanobacterial species/strains belonging to one genus were clustered together and they were supported by high bootstrap values (Fig. 3). Eight distinct large monophyletic clades could be distinguished in the phylogenetic reconstruction, here named *Nostoc* clade 1, *Anabaena* clade 1, *Calothrix* clade, *Planktothrix* clade, *Pseudanabaena* clade, *Candidatus Synechococcus* clade, *Cyanobium* clade and *Synechococcus* clade 1. Representatives of the *Nostoc* (11 strains) were located in two separate clades (Fig. 3, *Nostoc* clade 1 (89% bootstrap supports), *Nostoc* clade 2 (53% bootstrap supports)). The *Anabaena* strains also were grouped in two separate clades: *Anabaena* clade 1 (5 strains, 91% bootstrap support) and *Anabaena* clade 2 (2 strains, 87% bootstrap support, Fig. 3). All representatives of the other main clades were clustered in separate monophyletic clades: *Calothrix* (4 strains, 97% bootstrap support), *Planktothrix* (6 strains, 100% bootstrap support), *Pseudanabaena* (4 strains, 67% bootstrap support), *Candidatus Synechococcus* (4 strains, 100% bootstrap support) and *Cyanobium* (4 strains, 78% bootstrap support). Most members of the genus *Synechococcus* were clustered in two separate clades: *Synechococcus* clade 1 (16 strains, 99% bootstrap support, Fig. 3) and *Synechococcus* clade 2 (3 strains, 98% bootstrap support, Fig. 3).

The other cyanobacterial species belonging to one genus were grouped in smaller separate clades: *Nodularia* clade (2 strains, 100% bootstrap support); *Cylindrospermopsis*

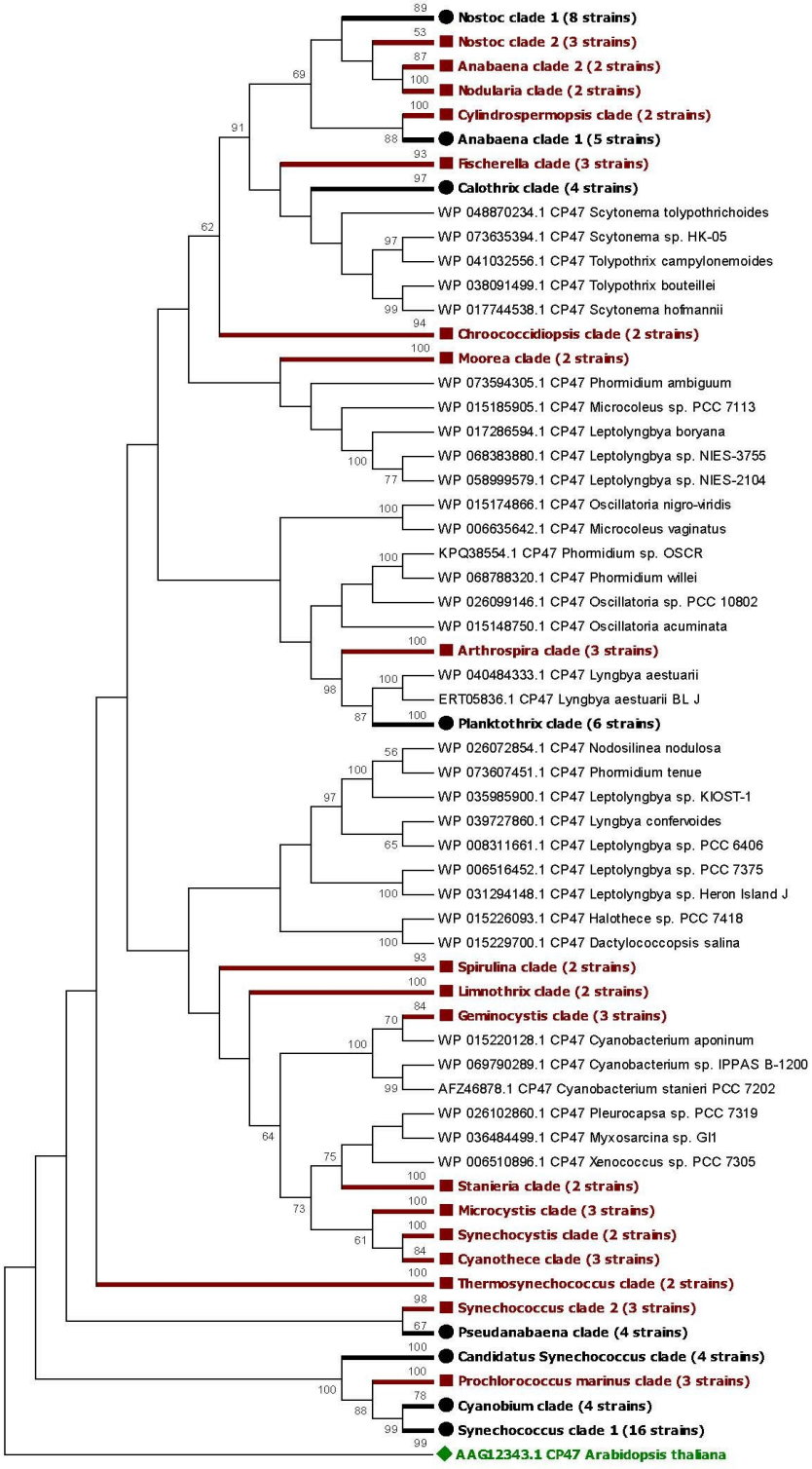

Fig. 3. Phylogenetic tree based on CP47 amino acid sequences from 129 cyanobacterial strains. The reconstruction has been performed by using ML analysis applying the GTR+I+G evolutionary model. The numbers above branches indicate the bootstrap support (>50%) from 1,000 replicates. *Arabidopsis thaliana* was used as an outgroup. ● - big monophyletic clades; ■ - small monophyletic clades.

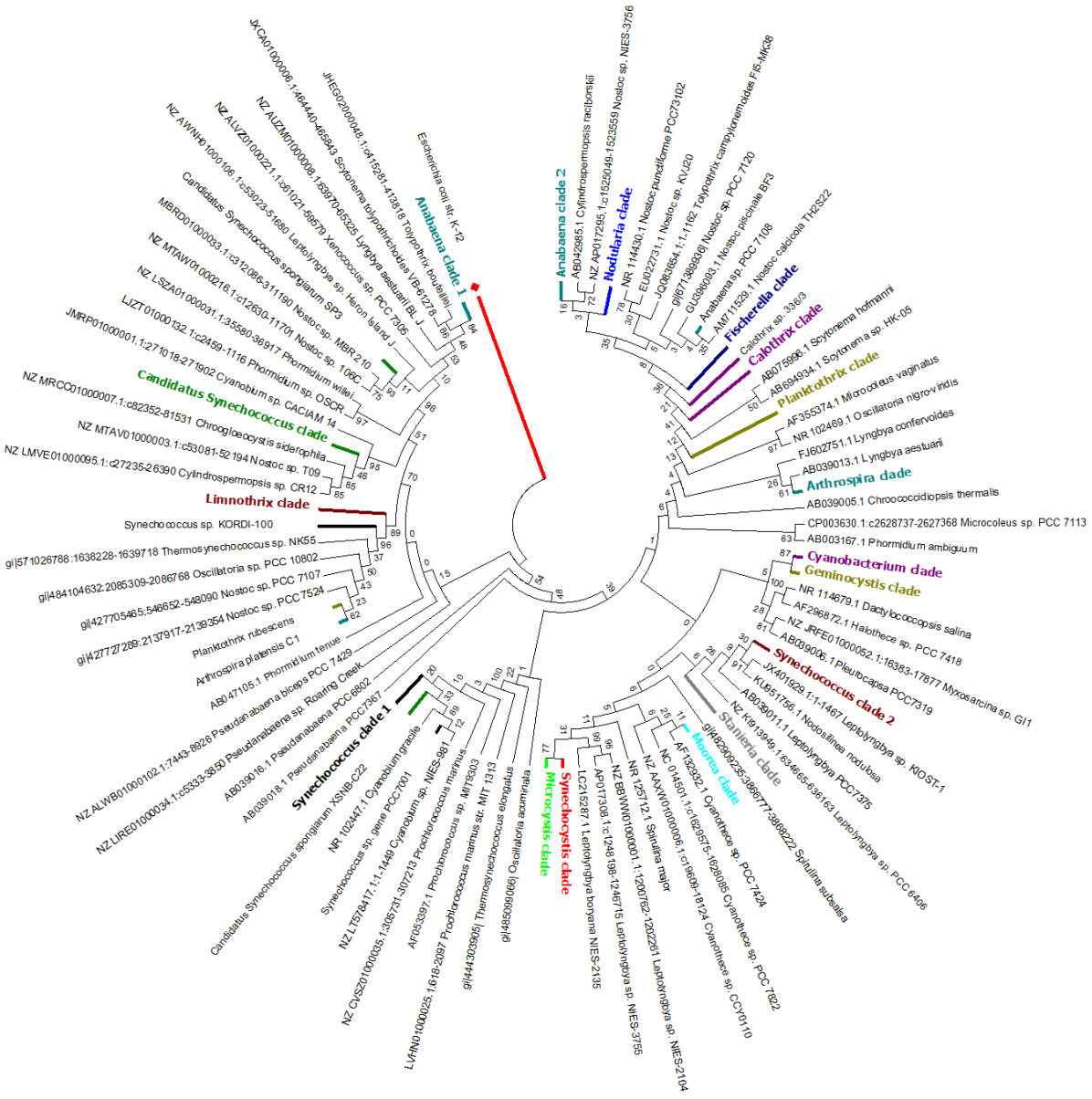

Fig. 4. Phylogenetic tree based on 16S rDNA sequences of 129 cyanobacterial strains. The tree was reconstructed by using ML analysis and the GTR+I+G evolutionary model. 16S rDNA sequence from *Escherichia coli* str. K-12 was used as an outgroup. The numbers near branches indicate bootstrap support from 1,000 replicates.

clade (2 strains, 100% bootstrap support); *Fischerella* clade (3 strains, 93% bootstrap support); *Chroococcidiopsis* clade (2 strains, 94% bootstrap support); *Moorea* clade (2 strains, 100% bootstrap support); *Arthrospira* clade (3 strains, 100% bootstrap support); *Spirulina* clade (2 strains, 93% bootstrap support); *Limnothrix* clade (2 strains, 100% bootstrap support); *Geminocystis* clade (3 strains, 84% bootstrap support); *Stanieria* clade (2 strains, 100% bootstrap support); *Microcystis* clade (3 strains, 100% bootstrap support); *Synechocystis* clade (2 strains, 100% bootstrap support); *Cyanothece* clade (3 strains, 84% bootstrap support); *Thermosynechococcus* clade (2 strains, 100% bootstrap support); *Prochlorococcus marinus* clade (3 strains, 100% bootstrap support);

Synechococcus clade 2 (3 strains, 98% bootstrap support); *Nostoc* clade 2 (3 strains, 53% bootstrap support); *Anabaena* clade 2 (3 strains, 87% bootstrap support); (Fig. 3). The examined cyanobacterial species formed a number of strongly supported clades in this tree.

For comparison, the 16S phylogenetic tree (Fig. 4) was constructed with 129 nucleotide sequences for the same cyanobacterial strains as in Fig. 3 using Mega 7 (Kumar et al., 2016). Comparing the topology of CP47 and 16S trees, it can be seen that the clades within the CP47 tree are clustered much better than within the 16S tree. Most of the species that showed monophyly with high bootstrap supports in the CP47 tree did not group together in the 16S tree. For example, *Nostoc, Pseudanabaena, Cyanobium, Anabaena, Calothrix, Synechococcus, Arthrospira, Candidatus Synechococcus* and *Planktothrix* (Fig. 4). Some of the other cyanobacterial species are also grouped in smaller separate clades as in the phylogenetic tree based on CP47, but with low bootstrap supports: *Nodularia* clade (2 strains, 85% bootstrap support); *Fischerella* clade (3 strains, 34% bootstrap support); *Geminocystis* clade (3 strains, 77% bootstrap support) (Fig. 4). Representatives of the *Cyanobacterium* (3 strains) were located in a separate clade within the 16S tree. This topology was supported by a bootstrap value of 64%.

The results presented herein strongly support CP43 and CP47 as markers of choice for cyanobacterial phylogenetic studies and emphasize the importance of using multiple molecular markers to prevent erroneous conclusions.

Conclusions

The taxonomic resolution offered by 16S rRNA genes is insufficient for discrimination of closely related organisms. The results obtained from this study have contributed greatly to the knowledge of cyanobacterial diversity. However, more phylogenetic studies are needed with other molecular markers to confirm the phylogenetic position of previously unidentified cyanobacterial isolates.

Acknowledgements

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

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Received: 11.09.2020
Accepted: 14.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 153-164

Phytoplankton, Macrophytes and Macroinvertebrates in Reservoirs: Response to Eutrophication

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Abstract. Eutrophication impact on key aquatic communities (phytoplankton, macrophytes and macrozoobenthos) was studied in three standing water bodies in Bulgaria (Kardzhali, Koprinka and Batak). The reservoirs had a high summer nutrient level and were between eu- and hypertrophic category. In the meso-eutrophic condition the highest number of phytoplankton species and functional diversity (Kardzhali Reservoir) and domination of functional groups (FGs) B, F, X3 and T were registered. The most intensive cyanobacterial growth was recorded in eu- to hypertrophic conditions (Koprinka Reservoir), as well as lower functional and species diversity, and domination of FGs M and J. Hydrological regime in hydroelectric reservoirs was a serious pressure for aquatic macrophytes and macrozoobenthos. Along the nutrient gradient, the Batak Reservoir had highest phytoplankton biovolume and macroinvertebrate taxa richness, well developed macrophyte community, dominated by eutrophication-tolerant species. The results suggested that measures for mitigating ecological impacts from pressures should be implemented without significant adverse effect on water use.

Key words: eutrophication, trophic state, biotic metrics, reservoirs.

Introduction

Eutrophication is one of the major environmental problems in reservoirs leading to water quality deterioration and restriction of their use (Kelly et al., 2016; Padedda et al., 2017). Among the symptoms of *eutrophication* is often the substantial loss of submerged plants and their replacement by dense *phytoplankton* communities (algal blooms). As key factors that reflect macroinvertebrate communities were found chlorophyll *a* and biomass of submerged macrophytes (Pan et al., 2015). The same research reported a loss of macroinvertebrate taxa richness and alteration of species composition along the eutrophication gradient. As far as eutrophication has a widespread impact, often its effects on invertebrate fauna and the remaining water biota is the result of combinations of other pollutants, hydromorphological changes and alien species (Donohue et al., 2009).

The trophic state of the Koprinka Reservoir for the period 2009-2011 was determined as mesotrophic, with good trophic integrity and with benthic community composed mostly of deposit feeders (Kenderov et al., 2014). The algal communities of the same reservoir were reported to be dominated by chlorophytes, cyanoprokaryotes and diatoms (Dochin et al., 2017a). The trophic state of the Kurdzhali Reservoir was assessed as meso- to eutrophic (Traykov, 2005). The last published data on phytoplankton of the Kurdzhali Reservoir reported 137 taxa from 6 divisions (Dochin & Stoyneva, 2014). Transparency, conductivity, pH, dissolved oxygen, total nitrogen were among the variables with the highest impact on the phytoplankton in the reservoir (Dochin et al., 2017b).

The phytoplankton community in Batak Reservoir included a total of 106 phytoplankton taxa and was dominated by diatoms, green algae and blue-green algae (Dochin et al., 2018).

The literature data on phytoplankton communities of Koprinka, Kurdzhali and Batak reservoirs focused mainly on phytoplankton taxonomic structure (species composition, dominant complexes, number of species), spatial and seasonal dynamics of the abundance and provided no data on macrophyte communities. Current research made an attempt to test new phytoplankton-based metrics sensitive to eutrophication and additionally to study how dominant functional groups, growth of cyanobacteria, species and functional diversity react along eutrophication gradient. The aim of this paper was to answer the question how phytoplankton, macrophytes and macroinvertebrates were affected by eutrophication in three large reservoirs.

Material and Methods

Study on three reservoirs Batak, Kardzhali and Koprinka (Table 1) was conducted in three sampling periods during 2011-2015. Water temperature (T, °C), pH, electrical conductivity (C, μS cm^{-1}) and dissolved oxygen (DO, mg L^{-1}) were measured *in-situ* using WTW pH/Conductivity/Oxygen meters. Water sampling followed EN ISO 5667-6. Total nitrogen (TN) and phosphorous (TP) and chemical oxygen demand (COD) were analyzed following the standards EN ISO 11905-1, EN ISO 11885, ISO 15705: 2002.

The nomenclature followed Lee (2008) for phytoplankton, Delipavlov et al. (2003) for vascular plants. Macroinvertebrate taxonomy followed Fauna Europaea (2013).

Phytoplankton was sampled three times during the vegetation season (June-October), close to the reservoir walls. Phytoplankton sampling and laboratory determination, including chlorophyll *a*, followed international standards: ISO 5667-3:2012, EN 15204:2006, ISO 10260:2002. Functional groups (FGs) of the phytoplankton species were determined by their codons following Reynolds et al. (2002) and Padisák et al. (2006, 2009). The descriptor species were selected based on their relative biovolume > 5% of the total biovolume. Percent cyanobacteria (% Cyano) was calculated as relative share of eutrophic species towards total biovolume. Trophic State Index (TSI) of Carlson (1977) was applied: TSI_{TP}, TSI_{SD} and TSI_{CHL} were calculated, and additionally TSI_{TN} (Kratzer & Brezonik, 1981). The classification of trophic categories was after Carlson & Simpson (1996): TSI < 40 - oligotrophy; 40 < TSI < 50 - mesotrophy; 50 < TSI < 70 - eutrophy; TSI > 70 -hypertrophy. Shannon index (Shannon, 1948) and functional diversity according to Borics et al. (2012) were calculated additionally for the phytoplankton communities.

The macrophyte surveys were carried out during the main vegetation period (end of June until September) in belt transects in correlation to the lake size. Species, their abundance and additional relevant parameters were recorded for the defined depth zones (0–1; 1–2; 2–4; and > 4 m). The abundance of each species was noted on a five-degree scale after Kohler (1978).

The macrozoobenthos sampling was made in compliance to the multi-habitat

sampling method (Cheshmedjiev et al., 2011) and in accordance with the standards BDS EN ISO 5667-1:2007 and BDS EN ISO 5667-3:2012. After primary processing and taxonomic determination of the macrozoobenthos, a checklist was published (Vidinova et al., 2016). Feeding groups' affiliation according Cheshmedjiev & Varadinova (2013) was made.

Ten metrics were applied: functional phytoplankton diversity (HFGsDiv) and species diversity (HSpD) of the phytoplankton, total phytoplankton biovolume, chlorophyll *a* (Chl *a*), % cyanobacteria (% Cyano), transparency according to Secchi (Transpar), depth zones of macrophyte colonization (DepthZ-MPH), number of macrophyte species (N-MPH), number of macrozoobenthos taxa (N-MZB) and PETI trophic index (Schweder, 1990).

We examined relationship between TN, TP and COD, and the ten metrics based on phytoplankton, macrophytes and macroinvertebrates with Principal component analysis (PCA). The data were transformed (x' = log (x+1)), automatically centered and standardized with Canoco v.5 program (Smilauer & Budejovice, 2014).

Results

Trophic state

All three reservoirs had high summer nutrient levels at the border between eu- and hypertrophic category (Fig. 1, Table 2). Kardzhali Reservoir was between mesotrophic (TSI_{CHL}, TSI_{SD}) and eutrophic (TSI_{TP}, TSI_{TN}) category, while Koprinka Reservoir was in eutrophic condition with TSI_{TP} values in hypertrophy (Fig. 1). The strongest variation was observed for TSI in Batak Reservoir: from mesotrophic (TSI_{CHL}, TSI_{SD}), through eutrophic (TSI_{TN}) to hypertrophic (TSI_{TP}) state.

Phytoplankton

Phytoplankton species belonged to 7 taxonomic groups in the three reservoirs: Cyanoprokaryota, Chlorophyta, Chrysophyta, Bacillariophyta, Cryptophyta, Euglenophyta, Dinophyta (Table 2). Nine descriptor species were recorded in Batak Reservoir belonging to 8 FGs: **B**, **C**, **N**, **P**, **E**, $\mathbf{L_0}$, $\mathbf{X_3}$ and **Y**. *Tabellaria fenestrata* var. *asterionelloides* **(N)** and *Fragilaria crotonensis* **(P)** had highest relative biovolume during the three sampling events (Table 2). The cryptophyte *Cryptomonas marssonii* from FG **Y** was recorded at the end of the vegetation season.

Table 1. Morphometric and typology characteristics of the selected reservoirs. Legend: L3 - Mountain lakes in the Eastern Balkans; L11 - Large deep reservoirs (Cheshmedjiev et al., 2010); HMWB - Heavily Modified Water Body.

Features	Batak	Kardzhali	Koprinka
Latitude/longitude	41.95786; 24.15654	41.63333; 25.31666	42.62183; 25.27348
Altitude, m a.s.l.	1106	330	380
Surface area, km^2	22.08	16.7	11.2
Maximal depth, m	30	93	43
Mictic type	dimictic	dimictic	dimictic
Volume, hm^3	310	533	140
Lake Type	L3	L11	L11
Water Body Category	HMWB	HMWB	HMWB
Use	Hydroelectric Power Plant, Water supply, Fishfarming	Hydroelectric Power Plant, Irrigation, Fishfarming	*Hydroelectric Power* Plant, Fishfarming

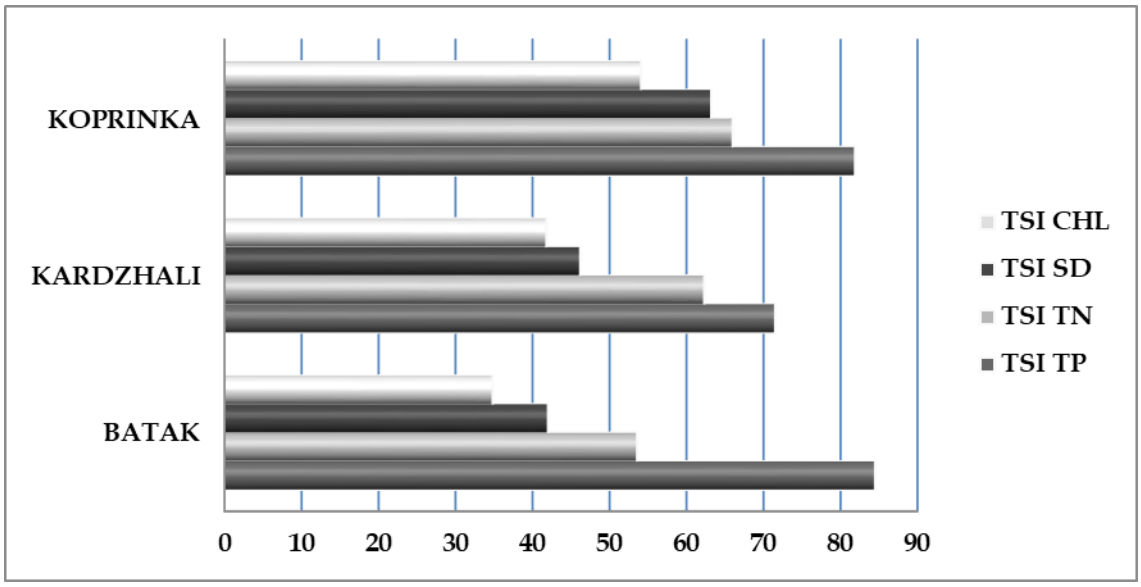

Fig. 1. Trophic State Index (TSI) for Chlorophyll *a* (TSI_{CHL}), Transparency (TSI_{SD}), total phosphorus (TP) and total nitrogen (TN). Classification of trophic categories: TSI < 40 - oligotrophy; 40 < TSI < 50 - mesotrophy; 50 < TSI < 70 - eutrophy; TSI > 70 -hypertrophy.

Table 2. Selected physico-chemical parameters and biota taxa at studied reservoirs. Legend: *average seasonal values for the main physicochemical and biological parameters (surface measurements) in the reservoirs; **descriptor species (> 5% of the total biomass), established in at least one sample and their FGs in parenthesis; ***relative taxa share (%) in the total biovolume during July/September/October.

	Batak	Kardzhali	Koprinka
T,°C*	16.0	23.8	21.7
Secchi depth, m*	3.5	2.6	0.8
pH*	7.45	7.97	8.56
C, µS cm^{-1}*	131.37	317	375.3
DO, mg L^{-1}*	7.32	6.32	8.05
TN, mg L^{-1}*	1.443	1.716	2.221
TP, mg L^{-1}*	0.263	0.107	0.22
COD, mg L^{-1}*	4.53	4.05	5.34
Phytoplankton-Descriptor species**	**Cyanoprokaryota (=Cyanobacteria)** *Woronichinia naegeliana*-***0/0/6.1 **(L_0)** **Chlorophyta** *Pseudosphaerocystis lacustris*-0/0/8.0 **(X_3)** **Chrysophyta** *Mallomonas caudata*-	**Chlorophyta** *Coelastrum microporum*-***9.6/0/0 **(J)** *Eutetramorus planctonicus*-0/4.7/0 **(F)** *Gloeotila monospora*-14.9/0/0 **(T)** *Oocystis lacustris*-7.8/0/0 **(F)**	**Cyanoprokaryota (=Cyanobacteria)** *Microcystis aeruginosa*-***0/18.5/0 **(M)** *Microcystis wesenbergii*-0/41.8/40.7 **(M)** **Chlorophyta** *Coelastrum reticulatum*-38.7/0/0 **(J)**

	0/0/9.1 **(E)** **Bacillariophyta** *Asterionella formosa*-0/0/5.1 **(C)** *Cyclotella meneghiniana*-0/7.4/0 **(C)** *Cyclotella ocellata*-0/12.9/0 (**B**) *Fragilaria crotonensis*-26.9/38.8/0 **(P)** *Tabellaria fenestrata* var. *asterionelloides*-70.1/36.0/45.3 **(N)** **Cryptophyta** *Cryptomonas marssonii*-0/0/19.0 **(Y)**	*Oocystis marssonii*-8.2/0/0 **(F)** *Closterium aciculare*-0/4.7/0 **(P)** *Cosmarium* sp.-0/12.1/0 **(N)** **Chrysophyta** *Chrysococcus minutus*-17.1/0/0 **(X_3)** *Chrysococcus rufescens*-18.6/0/0 **(X_3)** **Bacillariophyta** *Aulacoseira granulata*-0/14.6/0 **(P)** *Cyclotella radiosa*-0/0/15.3 **(B)** *Fragilaria crotonensis*-10.0/32.2/71.5 **(P)** *Fragilaria ulna* var. *angustissima*- 0/11.0/0 **(D)** **Dinophyta** *Ceratium furcoides*-0/15.1/0 (**L_0**)	*Eudorina elegans*-0/4.8/0 **(G)** *Oocystis marssonii*-0/8.5/0 **(F)** *Pediastrum simplex*-24.4/0/0 **(J)** *Staurastrum gracile*-11.4/0/0 **(P)** **Chrysophyta** *Chrysococcus rufescens*-12,7/0/0 **(X_3)** **Bacillariophyta** *Asterionella formosa*-0/0/6.1 **(C)** *Cyclostephanos invisitatus*-0/0/5.0 **(A)** *Cyclotella meneghiniana*-0/12.6/0 **(C)** *Cyclotella pseudostelligera*-0/0/7.5 **(B)** **Euglenophyta** *Trachelomonas volvocinopsis*-0/0/9.1 **(W_2)** **Dinophyta** *Ceratium furcoides*-0/0/15.3 **(L_0)**
Macrophytes	*Ceratophyllum demersum* *Elodea canadensis* *Elodea nuttallii* *Myriophyllum spicatum* *Potamogeton nodosus*	Macrophyte depopulation	*Myriophyllum spicatum*

Fourteen descriptor species were registered in Kardzhali Reservoir which belonged to 9 FGs: **B**, **F**, **N**, **X_3**, **P**, **D**, **J**, **T** and **L_0** (Table 2). The following FGs **F**, (*Oocystis lacustris*, *O. marssonii*), **X_3** (*Chrysococcus rufescens*, *Ch. minutus*) and **T** (*Gloeotila monostroma*) had highest relative abundance in midsummer. They were replaced by FGs **P** (*Fragilaria crotonensis, Aulacoseira granulata*) – 46.8% and **L_0** (*Ceratium furcoides*) – 15.1% during the seasonal succession. The same species from FGs **P** (71.5%) dominated at the end of the season.

Fourteen descriptor species from 11FGs: **A**, **B**, **C**, **F**, **J**, **P**, **M**, **G**, **X_3**, **W_2** and **L_0** (Table 2) were recorded in Koprinka Reservoir. The seasonal succession began with domination of FGs **J** (*Coelastrum reticulatum*, *Pediastrum simplex*), **X_3** (*Chrysococcus rufescens*) and **P** (*Staurastrum gracile*), replaced by FGs **M** (*Microcystis aeruginosa, M. wesenbergii*) and **L_0** (*Ceratium furcoides*) at the end of the season.

Seasonal succession in the Batak Reservoir was characterized by domination of *Tabellaria fenestrata* var. *asterionelloides* from FG **N** during the whole season: **N→N,P→N,Y**.

In the meso-eutrophic Kardzhali Reservoir the seasonal succession of the dominant FGs had the following order: **X_3,F→P,L_0→P,** while for eutrophic state of the same lake type (Koprinka Reservoir) the order was different: **J→M→M,L_0**. Specific FGs for meso-eutrophic state of lake type L11 were **B**, **F**, **X_3** and **T,** for eutrophic were **M** (*Microcystis* species) and **J** (*Coelastrum*, *Pediastrum*) (Table 3).

Table 3. Dominant phytoplankton FGs (with average season abundance > 5%) in lake type L11. Legend: grey: common FGs for both reservoirs; black: specific FGs.

Reservoir	B	F	P	X3	M	J	Lo	T
Kardzhali	5.11	8.22	49.36	11.93			5.02	4.96
Koprinka			5.53		33.89	21.84	6.79	

The highest biovolume was registered in Batak Reservoir (Fig. 2). The biovolumes at Kardzhali and Koprinka Reservoirs were similar, but the percent of eutrophic cyanobacteria was very high in Koprinka Reservoir (36.6%). The parameters for species and functional diversity (H SpD, H FGs Div) had higher values in Kardzhali Reservoir (Fig. 2), while in eutrophic Koprinka Reservoir, species and functional diversity were lower.

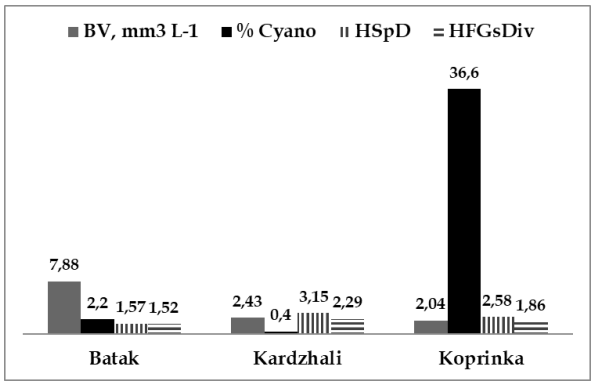

Fig. 2. Midseason values of phytoplankton metrics. Legend: BV – Total phytoplankton biovolume; % Cyano - % cyanobacteria; HSpD - Species diversity; HFGsDiv - Functional phytoplankton diversity.

Macrophytes

Five macrophyte species were registered in Batak Reservoir at 3 depth zones (Table 2). *Elodea nuttallii* was the species recorded with highest abundance and at maximum depth (2-4 m). Two of the registered species (*Ceratophyllum demersum* and *Elodea nuttallii*) were reported as tolerant to eutrophication (Penning et al., 2008).

Only one macrophyte species with low abundance was registered in Koprinka Reservoir, while Kardzhali was in macrophyte depopulation. Water abstraction in such large deep reservoirs results in water level fluctuations and specific conditions suppressing macrophyte communities' development.

Macroinvertebrates

Benthic invertebrates were under the negative influence of fluctuations of the water level caused by permanent water use in the result of negative for aquatic ecosystem anthropogenic activities such as water supply, hydroelectric power plant, fishfarming (Table 1). In the three studied reservoirs taxonomic composition of the macroinvertebrates was dominated by tolerant chironomids and aquatic oligochaetes (Fig.3).

The Sørensen coefficients demonstrated 21% similarity between the Batak and Kardzhali Reservoirs, 19% between Batak and Koprinka. The greatest resemblance (26%) was observed between the Kardzhali and Koprinka Reservoirs. It should be noted that benthic samples were collected close to the reservoir wall, as well both standing water bodies belong to national type L11 (large deep reservoirs) and had a similar hydromorphological and hydrogeological characteristics. Batak was distinguished by the richest taxonomic composition, the prerequisite of which was smallest depth, well-formed sampling littoral zone, where more diverse environmental conditions and changes in the trophic status were observed. During the studied period Batak Reservoir was determined in maximum ecological potential according metric H-MZB, Kardzhali and Koprinka were defined in good ecological potential. (Varadinova, 2013; Varadinova et al., 2019).

Incomplete trophic structure of the bottom communities was formed in the studied modified water bodies. Permanent presence of deposit feeders, tolerant taxa of scrapers and predators were observed. The group of shredders was significantly reduced or completely missing. The deposit feeders (fam. Tubificidae, subfam.

Chironominae) predominated in the trophic structure of the macrozoobenthos in the three reservoirs. This functional group coposed 70% of the benthic community in the Koprinka and Kardzhali and more than half of the invertebrate taxa in Batak (Fig.3).

Relationship between metrics and pressure

Among the tested 10 metrics, chlorophyll *a* levels and percent share of cyanobacteria positively correlated with COD and total nitrogen, while PETI was in negative correlation with COD (Fig. 4). The eigenvalues of the first two PCA axes were 0.662 and 0.338. The second axis revealed the importance of total phosphorous. Aquatic macrophyte community in Batak Reservoir was species richer and occupied deeper zones, as well as macrozoobenthos was represented by highest number of species.

Discussion

The studied communities were highly influenced by site-specific conditions formed by the hydrological regime and environmental variables. They often are correlated to each other, so it is difficult to separate the influence of single factors in determining the assemblage composition (Larocque et al., 2001).

Common result for the three reservoirs was that nutrients indicated higher trophic status then chlorophyll *a* and transparency. The obtained result $TSI_{TP} > TSI_{Chl} = TSI_{SD}$ could be linked to zooplankton grazing or nitrogen limitation (Brown & Simpson, 1998). The type-specific for L11 functional groups, linked with meso-eutrophic state were FGs **B**, **F**, $\mathbf{X_3}$ and **T** (Kardzhali Reservoir), replaced by **M** and **J** in eutrophic state (Koprinka Reservoir). Habitat templates of codons **M** and **J** are highly enriched systems, eutrophic to hypertrophic (Padisák et al., 2009). It was confirmed that blooming cyanobacteria FG **M** (*Microcystis*) domination results in lower phytoplankton functional diversity (Borics et al., 2012). The intense cyanobacterial growth in Koprinka Reservoir could be linked to total nitrogen concentration (2.22 mg L^{-1}) due to the fact that in high phosphorus levels, cyanobacterial development has linear link with nitrogen (Dolman et al., 2012).

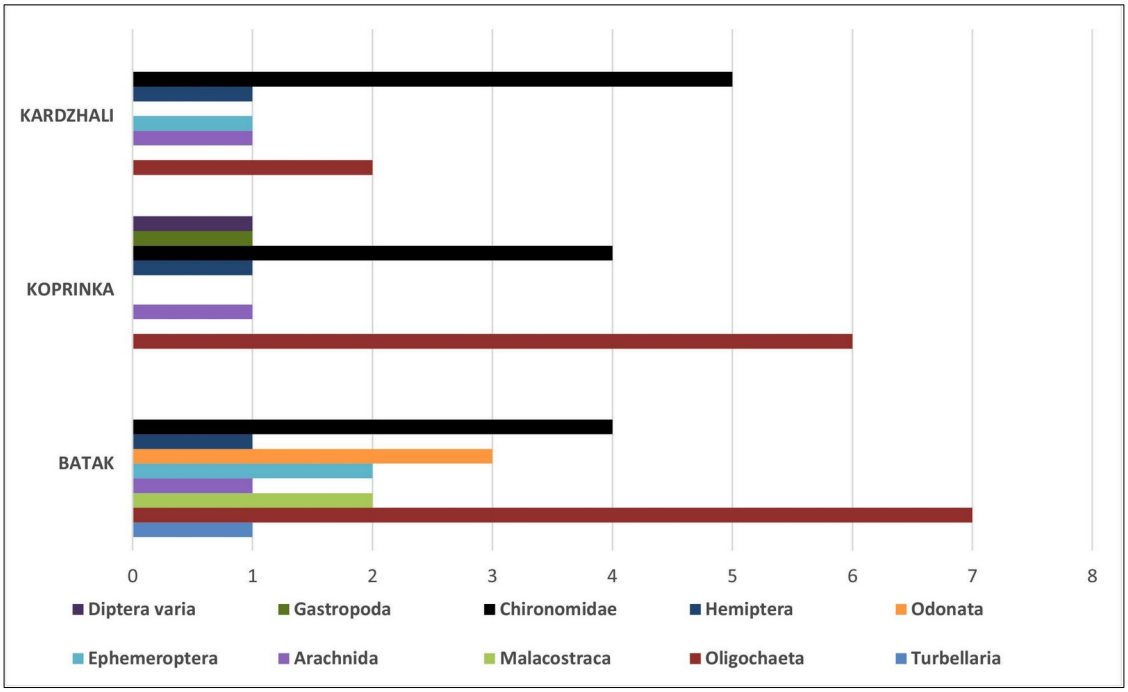

Fig. 3. Distribution of main taxonomic groups of the macrozoobenthos in the studied reservoirs.

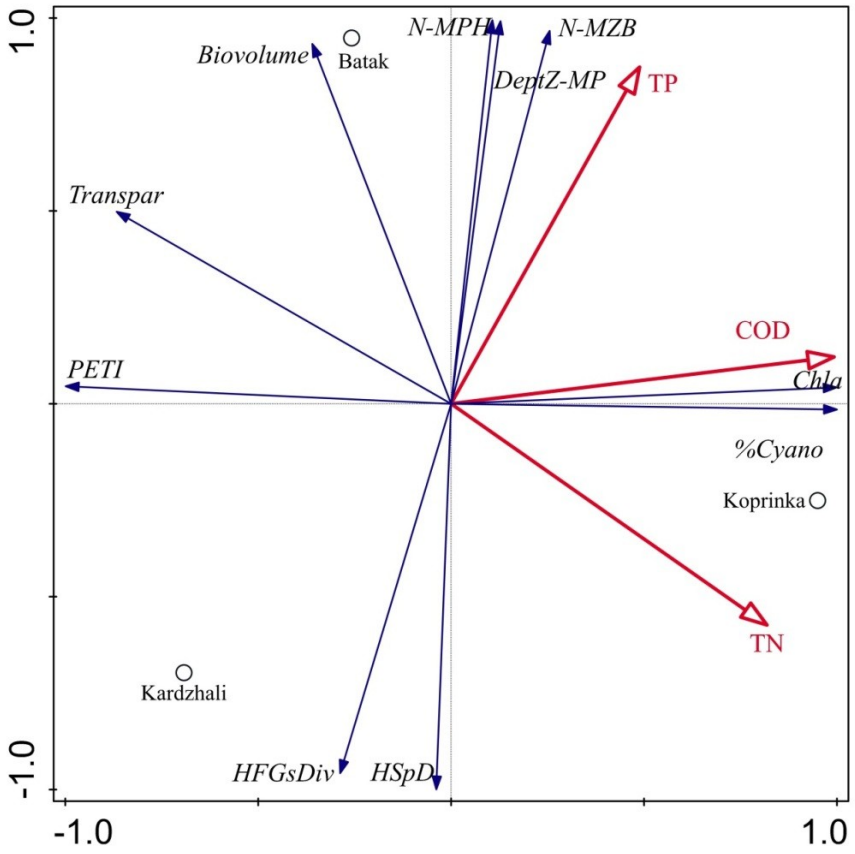

Fig. 4. PCA scatterplot of the studied metrics and selected physico-chemical variables (Chla - Chlorophyll *a*; COD - chemical oxygen demand; DepthZ-MPH – depth zones of macrophyte colonization; HFGsDiv - Functional phytoplankton diversity; HSpD - Species diversity; N-MPH - number of macrophyte species; N-MZB - number of macrozoobenthos taxa; PETI - trophic index PETI; TN - total nitrogen; TP - total phosphorous; Transpar - transparency; % Cyano - % of cyanobacteria).

Water level fluctuations have great influence on aquatic plant communities (Coops et al., 2003). Aquatic macrophytes both in Kardzhali and Koprinka Reservoirs were not represented mainly due to the rapid water level change (> 10 m increase in spring/about 10 m decrease in summer). These results suggested that in such cases, some mitigation measures to reduce impact of maintenance could be recommended. Nevertheless, if such measures will impact the specified water uses significantly, then macrophytes can be applied only as supplementary indicators to eutrophication pressure.

On the contrary, in Batak Reservoir mass development of submerged disturbance indicators was recorded.

Benthic taxa composition in aquatic environments depends mainly on factors such as substratum type, water trophic status, and hydro-period (Sanseverino et al., 1998; Kownacki et al., 2000). Specific invertebrates' communities were formed in the littoral zone of the reservoirs not only as a result of the environmental factors, but also under the conditions of permanent fluctuation of the water level. Important factor for the macrozoobenthos species distribution was and inflow of nutrients and

the trophic resources availability. However, when organic pollution is more intense, it is oxygen concentration rather than food that limits the species survival and determines the community composition (Larocque et al., 2001). Although the majority of benthic organisms do not have a strict differentiation with regard to the trophic status, the predominant part of the found species were pollution-tolerant, adapted to nutrient heavily loaded aquatic environment. Some oligochetes like a *Limnodrilus hoffmeistery* and chironomids are probably the most useful profundal indicator of trophic status (Solimini, 2006). They occur over the whole spectrum of nutrient conditions but individual species water worms and non-biting midges have specific ecological preferences. The predominant part of these species belongs to the group of deposit feeders. However, in this study no close connection was found between the trophic status and the percentage of the deposit feeders. Thus, Kardzhali (meso-eu-trophic) and Koprinka (eutrophic) are characterized by different trophic conditions, but deposit feeders in the both reservoirs formed the same share in the trophic structure of the macrozoobenthos. Batak Resevoir, which trophic status varied (meso-ey-hyper-trophic), had the lower percentage of the deposit feeders and maximum ecological potential.

Positive correlation between chlorophyll *a* and % cyanobacteria with COD and TN confirmed previous results of Phillips et al. (2008) and Borics et al. (2013) that the link Chl a = f (TP) is linear only in conditions of low phosphorous levels (TP < 5-100 μg L^{-1}) and that when nitrogen levels are high (TN ≤ 1700 μg L^{-1}) a linear connection was established with nitrogen (Chl a = f (TN)). The number of macrophyte and macroinvertebrate species, as well as the colonized depth zones were positively correlated with the total phosphorous gradient. This could be a result of interactions between *macrophytes* and *phytoplankton* that had led to indirect facilitation among plants and the maintenance of higher *macrophyte* diversity in *eutrophic* conditions.

Conclusion

Eutrophication changes the aquatic environment conditions and has a structurally significant impact on aquatic communities. Phytoplankton was characterized by (i) replacement of FGs: **B**, **F**, **X3** and **T** were replaced by **M** and **J**; (ii) growth of cyanobacteria from FG **M** (*Microcystis* species) and thus a loss of species and functional diversity along eutrophication gradient. Macrophytes are growing at the littoral zone and even small changes in water level affect their distribution and lead to macrophyte depopulation registered in Kardzhali Reservoir and similar limited development in Koprinka. Reduction in macrophytes had a negative impact on the macrozoobenthos because plants provide refuges and impede predation. Anthropogenically induced water level fluctuation and changes in the trophic status alter the food base, which reflects on the taxonomic composition and the trophic structure of the macrozoobenthos. Thus, biota communities' reaction towards eutrophication in highly modified water bodies should be first differentiated from hydromorphological pressure. The determination of HMWBs' types based on the use (e.g. for electric power, fish farming, etc.) is therefore crucial. Given the received preliminary results, phytoplankton-based metrics sensitive to eutrophication could be suggested as key metrics in assessing eutrophication in HMWBs.

Acknowledgements

The publication of the current article is financed by Dundee Precious Metals Inc.

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Received: 11.05.2020
Accepted: 14.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 165-174

Antimicrobial Potential of Methanolic Extracts from Betonica bulgarica Degen et Neič. (Lamiaceae)

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Abstract. *Betonica bulgarica* Degen et Neič. (syn. *Stachys bulgarica* Hayek) is a Bulgarian endemic plant included in Red Data Book of Bulgaria under the category "endangered". The aim of the present study is to provide data about the antimicrobial activity of *B. bulgarica* leaf, flower, seed, stem and root methanolic extracts against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus cereus, Aspergillus ochraceus* 2002 IM-BAS, *Fusarium moniliforme* 394 FN-9, *Fusarium graminearum* 2294 IMI 155426 and *Penicillium verrucosum* 2003 NRRL F-143. Antimicrobial activity of the extracts was evaluated by agar well diffusion method. Root extracts of *B. bulgarica* exhibited the highest antibacterial activity against *S. aureus* and *B. cereus* with large zones of inhibition. All extracts demonstrated either low and statistically insignificant activity against *E. coli* or a lack thereof. As a whole, extracts of Ablanovo area (in Sinite kamani National Park) exerted the highest activity against *S. aureus, B. cereus* and *E. coli*. Leaf, flower, stem and root extracts of *B. bulgarica* showed either a lack of antifungal activity or low and statistically insignificant one.

Key words: antimicrobial activity, methanol extracts, *Betonica bulgarica*, *Stachys bulgarica*.

Introduction

Betonica bulgarica Degen et Neič. (syn. *Stachys bulgarica* Hayek) is a Bulgarian endemic plant included in Red Data Book of the Republic of Bulgaria under the category "endangered" (Genova, 2011). The known locations of this species are Balkan Range (Central and Eastern), Tracian Plain and

Sinite Kamani Natural Park near to Sliven (Grozeva et al., 2014, 2016; Gerdzhikova et al., 2015).

Betonica (*Stachys*) species are widely used in folk medicine and recently in official medicine (Bankova et al., 1999). They have anti-inflammatory, immunomodulatory, antimicrobial, anti-cancer and antioxidant properties (Khavani et al., 2005; Amirghofran et al., 2007; Salehi et al., 2007; Saeedi et al., 2008; Morteza-Semnani & Saeedi, 2009; Hajdari et al., 2010; Serbetci et al., 2010; Šliumpaitė et al., 2013; Jassbi et al., 2014; Tzanova et al., 2018). Nowadays, the more frequent use of plant extracts as natural food preservatives and safer alternative of antimicrobial agents brings about the necessity to study their antimicrobial activity (Mostafa et al., 2018). *B. bulgarica* is known to have a high content of polyphenols and flavonoids, which not only have antioxidant, but also antimicrobial activity (Bankova et al., 1999; Tzanova et al., 2018; Yakoub et al., 2018). Moreover, it is found that many species from *Betonica* (*Stachys*) genus are rich in essential oils, which also have antimicrobial activity (Skaltsa et al., 2003; Grujic-Jovanovic et al., 2004; Vundac et al., 2006; Salehi et al., 2007; Morteza-Semnani & Saeedi, 2009; Rusenova & Parvanov, 2009; Ebrahimabadi et al., 2010; Hajdari et al., 2011; Dimitrova-Dyulgerova et al., 2015). Because of that, even though there are not data about the antimicrobial activity of *B. bulgarica*, it could be assumed that it exists.

Because the chemical polymorphism of medicinal plants largely depends on various factors such as geographic conditions, collection time, vegetation phase, etc., the survey of medicinal flora present in various growing sites and countries is important part of plant studies (Dimitrova-Dyulgerova et al., 2015; Igwaran et al., 2017). The differences of the chemical constituent's content could lead to variations of antimicrobial activity of the cultivated plants, because many chemical constituents exert antimicrobial activity (Das et al., 2009).

The available literature is lacking data about the antimicrobial potential of extracts from different organs of *B. bulgarica*. This motivated the present study which aims to provide such findings from *B. bulgarica* leaf, flower, seed, stem and root methanolic extracts and to compare the antimicrobial activities of different populations of *B. bulgarica*.

Material and Methods

Plant material and extract preparation. Aerial parts of *Betonica bulgarica* were harvested from July to September in four locations from naturally growing populations in Bulgaria (Table 1; Figure 1). The roots were collected at the end of the vegetative period. The voucher specimens from studied populations are kept in the herbarium of the Agricultural University in Plovdiv (SOA). Plant material was air-dried in shade at room temperature and grounded in a mechanical grinder (final powder size less than 400 μm). The samples were stored in the dark and cool rooms at 16 – 18 °C prior to the analysis.

Table 1. Basic characteristics of the populations whence the plant materials of *Betonica bulgarica* were collected (by Tzanova et al., 2018).

Population No	Location, voucher number	North	East	Elev. m a.s.l.	Ecological conditions
1	Balkan Foothill Region, Lovnidol village, Pashova Livada area (SOA 062252)	42°59.079′	25°15.846′	368	Soil type – Cambisols (WRBSR, 2006). Herbaceous community dominated by *Festuca pratensis*. The terrain is slightly sloped (4° – 5°), non-eroded, facing south-west.

2	Balkan Foothill Region, Lovnidol village, Above Avdjiiski trap area (SOA 062253)	43°01.327′	25°15.154′	503	Soil type – Cambisols (WRBSR, 2006). Herbaceous community dominated by *Trifolium pratense* L. The terrain is very slightly sloped (2° – 3°), non-eroded, facing north-east.
3	Eastern Balkan Range, Sinite kamani Natural Park, Karandilska poliana area (SOA 062254)	42°42.688′	26°22.872′	972	Open meadow of the clifs northwest. Herbaceous community dominated by *B. bulgarica.* The terrain is slightly sloped (4° – 5°), non-eroded, facing north-east.
4	Eastern Balkan Range, Sinite kamani Natural Park, Ablanovo area (SOA 062255)	42°42.638′	26°17.262′	540	Soil type – Chromic Luvisols (WRBSR, 2006); Open meadow on the edge of a mixed deciduous forest comprising *Carpinus betulus L., Quercus robur L., Ulmus minor Mill., Fraxinus ornus L. and Crataegus monogyna Jacq.* The herbaceous community is dominated by *B. bulgarica*. The terrain is very slightly sloped (3° – 4°), non-eroded, facing south-east.

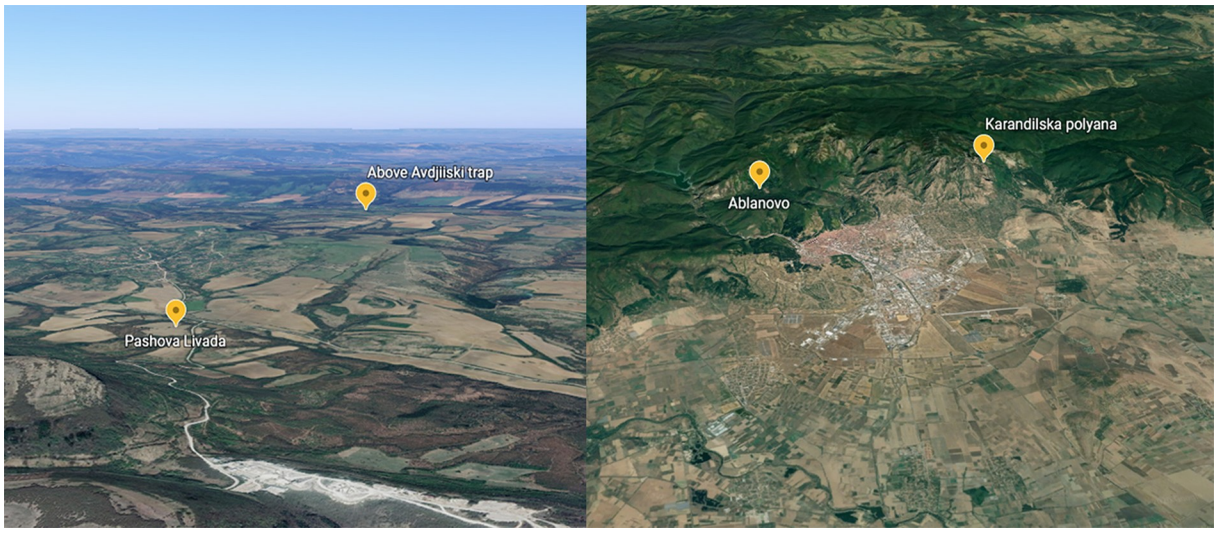

Fig. 1. Map of the four locations of *Betonica bulgarica* populations.

The target compounds were extracted by Soxhlet method, for 8h. As solvent was used methanol in the ratio of plant material: solvent 1:10. After filtration through 0.45 µm membrane, the extracts were concentrated by rotary vacuum evaporator at 30°C (Hossain & Rahman, 2015).

Tested microorganisms. In this study were included reference bacterial strains (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922) and a clinical bacterial isolate (*Bacillus cereus*). The fungi

strains were *Aspergillus ochraceus* 2002 IMBAS, *Fusarium moniliforme* 394 FN-9, *Fusarium graminearum* 2294 IMI 155426 and *Penicillium verrucosum* 2003 NRRL F-143. The bacterial strains were stored at -20 °C. Prior to use they were restored by trypticase soy blood agar (Himedia, India). The fungal strains were grown on Potato-glucose agar (glucose 20.0 g, potatoes 200.0 g, yeast extract 2.0 g, agar 20.0 g., pH 5.6).

Antimicrobial activity. Antimicrobial activity of the extracts was evaluated by agar well diffusion method described by Velichkova et al. (2018). In brief, for measuring of antibacterial activity, inoculums were prepared in saline corresponding to 0.5 of the McFarland standard (1.5x 10^8 CFU/mL) from 24 h bacterial colonies incubated on trypticase soy blood agar. 20 mL of Mueller Hinton agar (Himedia, India) was poured in every *Petri* dish. The wells were formed with a sterile 6 mm cork borer after pre-application of the inoculum with a sterile cotton swab. The wells were filled with 100 µL of the extracts. Positive control with gentamicin at a concentration of 12.5 µg/mL and negative with methanol was performed. The plates were incubated at 37 °C for 24 h under aerobic conditions.

For measuring of antifungal activity, 72 h old fungal cultures were grown on Potato glucose agar. 20 mL of Potato glucose agar was poured in every *Petri* dish. After solidification, 0.1 mL inoculum of the fungal strains (1-2 x 10^4 CFU/mL) was introduced on the agar plate surface and the wells were made by sterile cork borer of size 6.0 mm. The wells were filled with 0.1 mL of the methanol extract. An incubation period of 3-7 days at 23°C was maintained.

Antimicrobial activity was evaluated by measuring zones of inhibition of microbial growth surrounding the plant extracts in the wells. The zones of inhibition were measured in millimeters. Antibacterial activity was assumed in the presence of a growth inhibition zone ≥ 8.0 mm. The tests were performed in triplicate to determine the reproducibility of the results. The complete experiment was carried out under strict aseptic conditions.

Statistical analysis. All analytical assays were carried out in triplicate and expressed as mean values ± standard deviation (SD). Statistical analysis was performed with Statistica 10, StatSoft Inc. (2007).

Results and Discussion

According to the experimental data (Table 2) only leaf, flower and root methanolic extracts of *Betonica bulgarica* exhibited activity against *S. aureus*. The root extracts have the highest antibacterial activity. The zones of inhibition (ZI) at concentration of 16 mg/mL were very large (15.5 – 18.17 mm). Moreover, further dilutions of the root extracts showed concentration-dependent effect against *S. aureus* and some activity even at concentration of 0.25 – 1 mg/mL. Root extracts from the area of Ablanovo exhibited the highest activity against *S. aureus* (ZI 18.17 mm) and were active at concentration of 0.25 mg/mL (at 0.5 mg/mL there was statistically significant difference with the negative control). The leaf and flower extracts demonstrated low and statistically insignificant activity against *S. aureus*. Leaf and flower extracts from the area of Ablanovo again have the highest inhibitory activity against *S. aureus* with ZI of 9.0 mm and 9.33 mm, respectively.

All methanolic extracts of *B. bulgarica* exhibited either low and statistically insignificant activity against *E. coli* or a lack thereof. Ablanovo root extracts showed the highest activity (ZI 9.17 mm) followed by leaf extracts from Karandilska polyana (ZI 9.00 mm).

Only root methanolic extracts showed activity against *B. cereus*, with large ZI (11.17 – 12.5 mm) and statistically significant difference with methanol control. Ablanovo root extracts again demonstrated the highest antimicrobial activity (ZI 12.5 mm).

As a whole, methanolic extracts from *B. bulgarica* of Ablanovo area exhibited the

highest antibacterial activity. This is probably due to the type of soil and climatic conditions of Ablanovo area, which are presumably favourable for the growth of plants rich in antimicrobial constituents.

ZI of the positive control (gentamicin at concentration of 12.5 mg/mL) for *S. aureus*, *E. coli* and *B. cereus* were 21 mm, 17 mm and 23 mm, respectively, which means that all microorganisms were sensitive to positive control.

Our results regarding the antibacterial activity of *B. bulgarica* methanolic extracts are corresponding to the findings of Leblebici et al. (2016) who found that ethanolic extracts of 6 *Stachys* species (*S. annua* ssp. *cilicia*, *S. setifera* ssp. *lycia*, *S. sosnowskyi*, *S. tmolea*, *S. cretica* ssp. *anatolica* and *S. iberica* ssp. *iberica* var. *densipilosa*) were more inhibitory against *S. aureus* and *B. cereus* than *E coli*. Lotfipour et al. (2008) reported higher activity of methanolic extracts of *S. fruticulosa* and *S. schtschegleevii* against *S. aureus* than *B. cereus* and lack of any inhibitory activity against *E. coli.* Saeedi et al. (2008) found that methanolic extracts of four *Stachys* species (*S. byzantina*, *S. inflata*, *S. lavandulifolia* and *S. laxa*) demonstrated higher activity against *S. aureus* than *E. coli* as a whole. Moreover, methanolic extracts of *S. laxa* were not inhibitory against *E. coli*. Mahboubi et al. (2012) reported much higher activity of ethanolic extracts of *S. byzantina* against *S. aureus* than *E. coli*.

On the other hand, our findings are somewhat dissimilar to the data of Jassbi et al. (2014), who evaluated antibacterial activity of methanolic extracts from 9 *Stachys* species (*S. acerosa*, *S. benthamiana*, *S. byzantina*, *S. obtusicrena*, *S. lavandulifolia S. pilifera*, *S. pubescens*, *S. spectabilis* and *S. persica*) through nutrient broth microdilution assay. These authors found that the extracts from *Stachys* species showed similar activity against *S. aureus*, *E. coli* and *B. cereus* as a whole, with small differences. The extracts were the most active against *B. cereus*, followed by *E. coli* and *S. aureus*. Dulger & Aki (2009) reported much higher activity of ethanolic extract of *S. pseudopinardii* against *B. cereus* (ZI 25 mm) than *S. aureus* (ZI 13 mm). The extract was not inhibitory against *E. coli*. However, Abichandani et al. (2010) found that methanolic extract of *S. schtschegleevii* exhibited similar activity against *S. aureus* and *E. coli* (ZI 10 mm), but did not inhibit *B. cereus*. According to Yavuz et al. (2017) methanolic extract of *S. annua* inhibited *E. coli* (ZI 17 mm) but not *S. aureus* (ZI 7 mm).

According to the experimental data leaf, flower, stem and root extracts of *B. bulgarica* showed either a lack of antifungal activity or low and statistically insignificant one (Table 3).

These results are similar to the data of Saeedi et al. (2008), who found that methanolic extracts of four *Stachys* species (*S. byzantina*, *S. inflata*, *S. lavandulifolia* and *S. laxa*) did not show any antifungal activity against *Aspergillus niger*. Skaltsa et al. (2003) studied antimicrobial activity of essential oils from eight *Stachys* species (*S. alopecuros, S. scardica, S. cretica* ssp. *cretica*, *S. germanica* ssp. *heldreichii, S. recta, S. spinulosa, S. euborica, S. menthifolia*) and reported better activity against bacterial species (*P. aeruginosa, E. coli, B. subtilis, B. cereus, Micrococcus flavus* and *Staphylococcus epidermidis*) than fungi (*Aspergillus niger*, *Penicillium ochrochloron*, *Epidermophyton floccosum*, *Candida albicans* and *Trichophyton menthagrophytes*), which is similar to our findings. On the other hand, the essential oils of *Stachys pubescens* exhibited noticeable antifungal activity against *Fusarium oxysporum*, *Aspergillus flavus* and *Alternaria alternata* (Mohammadi et al., 2014). According to Lazarević et al. (2010) the antifungal activity of *Stachys* spp. extracts was very dependent on the type of solvent. Diethyl ether extracts of *S. germanica*, *S. plumosa* and *S. scardica* exhibited very high activity against *Aspergillus niger* (with ZI at least 24 mm), but ethyl acetate extracts were not inhibitory against this mould. As a whole, there is a lack of enough literature data about the antifungal activity of *Betonica* (*Stachys*) species which hinders the comparison of our data with similar findings.

Table 2. Diameter of inhibition zones (mm) of methanolic extracts from *B. bulgarica* (n=3, mean± SD). Legend: * – no activity. Different letters in the table denote significant differences between zones of inhibition of plant extracts and negative control (methanol) values according to LSD test ($p \leq 0.05$).

***B. bulgarica* Population/Organs**	**Methanol extract (mg/mL)**	***S. aureus***	***E. coli***	***B. cereus***
Pashova livada				
Leaves	16	8.33 ± 0.58^{a}	-	-
Flowers	16	8.0 ± 0.0^{a}	-	-
Seeds	16	-	8.17 ± 0.29^{a}	-
Stems	16	-	8.83 ± 0.29^{a}	-
Roots	16	16.0 ± 0.5^{b}	8.5 ± 0.5^{a}	11.33 ± 0.29^{ab}
	8	14.83 ± 0.86^{ab}		
	4	13.83 ± 1.04^{ab}		
	2	11.33 ± 0.58^{ab}		
	1	8.5 ± 0.5^{a}		
	0.5	-		
Above Avdjiiski trap				
Leaves	16	7.67 ± 0.58^{a}	-	-
Flowers	16	7.33 ± 0.58^{a}	-	-
Seeds	16	-	-	-
Stems	16	-	-	-
Roots	16	15.83 ± 0.29^{ab}	8.0 ± 0.5^{a}	11.5 ± 0.5^{ab}
	8	14.33 ± 0.58^{ab}		
	4	13.33 ± 0.29^{ab}		
	2	11.33 ± 0.58^{ab}		
	1	8.5 ± 0.5^{a}		
	0.5	-		
Karandilska polyana				
Leaves	16	-	9.0 ± 0.0^{a}	-
Flowers	16	8.67 ± 0.58^{a}	-	-
Seeds	16	-	7.17 ± 0.29^{a}	-
Stems	16	-	-	-
Roots	16	15.5 ± 0.5^{ab}	8.17 ± 0.29^{a}	11.17 ± 0.29^{ab}
	8	14.33 ± 0.58^{ab}		
	4	13.5 ± 0.5^{ab}		
	2	11.0 ± 0.0^{ab}		
	1	8.33 ± 0.29^{a}		
	0.5	-		
Ablanovo				
Leaves	16	9.0 ± 0.0^{a}	-	-
Flowers	16	9.33 ± 0.58^{a}	-	-
Seeds	16	-	7.5 ± 0.87^{a}	-
Stems	16	-	8.17 ± 0.29^{a}	-
Roots	16	18.17 ± 0.29^{ab}	9.17 ± 0.76^{a}	12.5 ± 0.5^{ab}
	8	16.17 ± 0.29^{ab}		

	4	14.17 ± 0.29 [ab]		
	2	13.67 ± 0.58 [ab]		
	1	13.0 ± 0.0 [ab]		
	0.5	10.83 ± 0.29 [ab]		
	0.25	8.0 ± 0.0 [a]		
	0.12	-		
Methanol	0	7.0 ± 0.0 [a]	7.0 ± 0.0 [a]	6.0 ± 0.0 [a]
Gentamicin	12.5	21 ± 0.0	17 ± 0.0	23 ± 0.0

Table 3. Diameter of inhibition zones in mm of methanolic extracts (32 mg/mL) from *B. bulgarica* (mean± SD). Legend: * – no activity. According to LSD test ($p \leq 0.05$) the results denote no significant differences between zones of inhibition of plant extracts and negative control (methanol) values.

Betonica bulgarica **Population/Organs**	***A. ochraceus***	***F. moniliforme***	***F. graminearum***	***P. verrucosum***
Pashova livada				
Leaves	7.17 ± 0.29 [a]	7.0 ± 0.0 [a]	-	-
Flowers	-	7.17 ± 0.29 [a]	-	-
Stems	-	-	-	-
Roots	-	7.17 ± 0.29 [a]	7.0 ± 0.0 [a]	-
Above Avdjiiski trap				
Leaves	-	-	-	-
Flowers	8.67 ± 0.58 [a]	-	-	-
Stems	-	-	-	-
Roots	-	7.17 ± 0.29 [a]	7.0 ± 0.0 [a]	--
Karandilska polyana				
Leaves	-	-	-	-
Flowers	-	7.0 ± 0.0 [a]	-	7.0 ± 0.0 [a]
Stems	-	-	-	6.83 ± 0.29 [a]
Roots	-	7.0 ± 0.0 [a]	7.0 ± 0.5 [a]	-
Ablanovo				
Leaves	-	-	-	8.17 ± 0.76 [a]
Flowers	-	-	-	-
Stems	-	-	7.0 ± 0.0 [a]	8.0 ± 0.0 [a]
Roots	-	8.0 ± 0.0 [a]	8.17 ± 0.29 [a]	7.83 ± 0.29 [a]
Methanol	6.0 ± 0.0 [a]	6.0 ± 0.0 [a]	6.0 ± 0.0 [a]	6.0 ± 0.0 [a]

We see some experimental data concerning the antibacterial and antifungal activity of *Betonica* (*Stachys*) species that sometimes differ greatly from our results. The distinctions could be primarily attributed to factors such as plant species, bacterial strains and method of extract preparation, but also to geographic conditions, collection time and vegetation phase of the plants, etc. (Dimitrova-Dyulgerova et al., 2015; Igwaran et al., 2017). Climatic differences and geographical area may change the amount and types of secondary metabolites of plant species. Moreover, the plants of the same genus differ in chemical composition and content of antimicrobial substances such as monoterpenes, sesquiterpenes, diterpenes, triterpenes, flavonoids, biflavonoids, glycosides, phenolic acids, etc. (Leblebici et al., 2016). These factors could strongly influence antimicrobial activity of the plants

examined which could explain the differences found.

Conclusions

In the present study root extracts of *Betonica bulgarica* exhibited the highest antibacterial activity against *S. aureus* and *B. cereus* with large zones of inhibition. All extracts demonstrated either low and statistically insignificant activity against *E. coli* or a lack thereof. Seed and stem extracts did not have any activity against *S. aureus*. Leaf, flower, seed and stem extracts were not inhibitory against *B. cereus*. As a whole, methanolic extracts from *B. bulgarica* of Ablanovo area showed the highest activity against *S. aureus, B. cereus* and *E. coli*. Plant extracts of *B. bulgarica* exhibited either a lack of antifungal activity or low and statistically insignificant one.

Acknowledgements

This work was supported by the Bulgarian Ministry of Education and Science under the National Research Programme "Healthy Foods for a Strong Bio-Economy and Quality of Life" approved by DCM #577/17.08.2018.

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Received: 16.10.2020
Accepted: 14.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 175-185

The Hatching Period of Winter and Summer Populations of Thaumetopoea pityocampa (Lepidoptera: Notodontidae) in Bulgaria

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Abstract. The dynamics of the larval emergence of the "summer form" and "winter form" of *Thaumetopoea pityocampa* was studied in the period July-October 2020 in laboratory conditions. The biological material was collected from two sampling sites – Sandanski in the western foothills of Prirn Mts. and Dobrostan on the northern slope of the Rhodopes Mts. The region of Sandanski is occupied by the winter form and the region of Dobrostan – by the summer form. The phenological calendars of the two populations of *T. pityocampa* were found to be in agreement with the known temperature thresholds of the species. The culmination of the larvae hatching of the winter form was in early October – well after midsummer heats, while the culmination of the summer forms was in early August and coincided with the hottest period. In both populations, however, the culmination of hatching occurred at average monthly maximum air temperatures of about 28°C.

Key words: larvae hatching, phenological forms, pine processionary moth, temperature threshold.

Introduction

The pine processory moth (PPM) *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) is a forest insect, which extends its geographical range as a result of climate warming (IPCC, 2007; Raev et al., 2011). The climate change affects the vital activity of insects through various processes, which may increase their presence in some areas and decrease it in others. For example, rising winter temperatures are expected to favor the more northern outbreaks of southern pine beetles, but to reduce them in more southern ones (Ungerer et al., 1999). A similar shift of the attacks of the pine processionary moth was observed in Bulgaria. Zaemdzhikova et al. (2020a) reported an increase in the frequency of *T. pityocampa* attacks at the northern border of its range and a reduction in the south. In any case, the global warming, at least initially, will benefit thermophilic insects, increasing the frequency of pest outbreaks. The expansion of the PPM in Europe is due to rising winter temperatures, which favor the winter feeding and survival of caterpillars in new habitats located to the north and higher altitudes (Battisti et al., 2005, 2006; Buffo et al., 2007; Robinet et al., 2007, 2015).

Expansion of PPM has also been established in Bulgaria (Mirchev et al., 2017; Zaemdzhikova et al., 2018).

Phenology is the time schedule of seasonal activities of plants and animals such as flowering or reproduction (Moore & Allard, 2008). As the phenology of organisms depends on temperature, it can be expected to be affected by climate change. Expected reactions to rising temperatures are faster life cycles of insects, earlier emergence of their larvae and adults and increasing duration of the flight period (Menéndez, 2007). Members of the order Lepidoptera again provide the best examples of such phenological changes (Roy & Sparks, 2000; Stefanescu et al., 2003).

There are two phenological forms of PPM – "summer form" (SF) and "winter formr" (WF), as they are often termed in the entomological literature (Mirchev et al., 2019). It is not yet known whether there are genetic differences between them or they are behavioral strategies of the same species in response to different environments. For this reason, many authors prefer the terms summer population and winter population. Whatever the truth, two types of phenological calendars of the species are observed. The summer form flies early (in May-June, before the summer solstice) (Zaemdzhikova, 2020), its larvae feed in summer, pupate in the soil in autumn and overwinter there (Georgieva et al., 2019; Mirchev et al., 2019; Santos et al., 2011, 2013). The winter form flies late (in July-August, after the solstice), its larvae develop in winter, pupate in the soil in the spring and remain there in the obligatory diapuse until July (Mirchev et al., 2017; Santos et al., 2007, 2011, 2013; Tsankov & Mirchev, 2000; Zaemdzhikova, 2020).

The air temperature is a major limiting factor for the vital activity of insects. Especially the temperature extremes can be fatal for the survival of the PPM. However, the temperature thresholds are different for the different instars so the temperature should affect the two forms differently. For example, for the survival of WF caterpillars in the period October-February, temperatures at -13°C (L2) and -16°C (L3/L4) are lethal (Buffo et al., 2007), as well as some diurnal temperature fluctuations - the insufficient number of the so-called "favorable days for feeding" (the days with an activation temperature >9°C during the day and air temperatures >0°C at night) is also deadly (Battisti et al., 2015). These temperature thresholds are essential for the survival of WF, but do not affect SF. Conversely, summer extremes (air temperatures above 32°C and 40°C) affect rarely the development of the WF, but are crucial for the survival of young caterpillars of SF, which develope during the hot months (Georgieva et al., 2019; Robinet et al., 2013; Santos et al., 2011).

The winter form is worldwide much more widespread and long known (Drenovski, 1923; Mirchev et al., 2000, 2019; Zankov, 1960). However, Bulgaria is perhaps unique, at least for now, in that the summer form is also widespread (about the half of the studied sites) and long known. In 1926, for the first time, Chorbadzhiev reported it – he reported caterpillars collected from the Rhodope Mts., which completed their development in the autumn. Later, in the 1950s, Zankov (1960) also reported habitats in the Sub-Balkan Valleys and the Central Rhodopes, where some of the caterpillars completed their development before the cold winter months. These are perhaps the first scientific reports of atypical PPM populations, which were re-discovered in Portugal in 1997 much later (Pimentel et al., 2006).

The aim of the present work is to compare the periods of larval emergence of the phenologically different populations of the PPM and to look for a correlation with the temperature thresholds of the species.

Material and Methods

The biological material (egg-batches) of PPM was collected from two sampling sites – the lands of Dobrostan vill. and the town of Sandanski, for which according to literature data the phenological form of the species is known: SF in Dobrostan and WF – in Sandanski (Mirchev et al., 2019; Tsankov & Mirchev, 2000). Dobrostan is located in a mountainous region on the northern periphery of the Western Rhodopes Mts., in South-central Bulgaria (41°54′05.57′′N, 24°55′22.84′′E, 1110 m a.s.l.). Sandanski is located in the Sandanski-Petrich valley, at the foot of Pirin Mts., in the Southwestern Bulgaria (41°34′07.53′′N, 23°17′33.84′′E, 310 m a.s.l.). Both sites are within "Southern-Frontier Forest Vegetation Province", which has a transitional-Mediterranean climate (Sabev & Stanev, 1959).

The egg-batches of *T. pityocampa* were collected in Austrian black pine plantations (*Pinus nigra* Arn.) in July-September 2020, i. e. before the caterpillars start hatching in both sites. The eggs were stored in laboratory at room temperature (daytime 22°C, nighttime 17°C). The number of hatching egg-batches was counted daily.

To characterize the climate data, we used the average monthly maximum air temperatures in Sandanski and Dobrostan provided by the National Institute of Meteorology and Hydrology – NIMH (Bulgaria).

Results

The dynamics of the hatching by sites is shown in Table 1. The hatching of the summer form began at the end of July, that of winter form – in mid-September, i.e. a month and a half later. The average duration of the hatching period of the both populations was about 3 weeks, slightly more in winter populations than in summer ones.

Figures 1 and 2 show the timing and dynamics of larval hatching in both sites. To describe the daily dynamics of hatching, both the curve of the empirical distribution and the cumulative distribution are given. From empirical distributions it is seen that in both forms the distribution was multimodal, i. e. there was more than one culmination and more precisely – three of them. In both figures two weaker culminations precede the peak of caterpillar hatching.

Another criterion of the culmination of hatching was the 50th percentile read from the cumulative distribution. The peak of summer form's hatching was observed in the first ten days of August (Fig. 1) and that of the winter form – in early October (Fig. 2). This result shows, that the culmination of hatching in the winter populations in Sandanski occurred a month and a half later than that of the early developing summer population in Dobrostan.

Table 1. Periods of hatching of collected egg-batches of the PPM's summer and winter form in laboratory.

Observations	Dobrostan (SF)	Sandanski (WF)
Date of collection of egg batches	18.08.2019	14.08.2019
Egg batches, number	46	25
Beginning of hatching	26.07.2020	16.09.2020
50% of hatching	07.08.2020	02.10.2020
End of hatching	14.08.2020	11.10.2020
Duration (days)	19	25

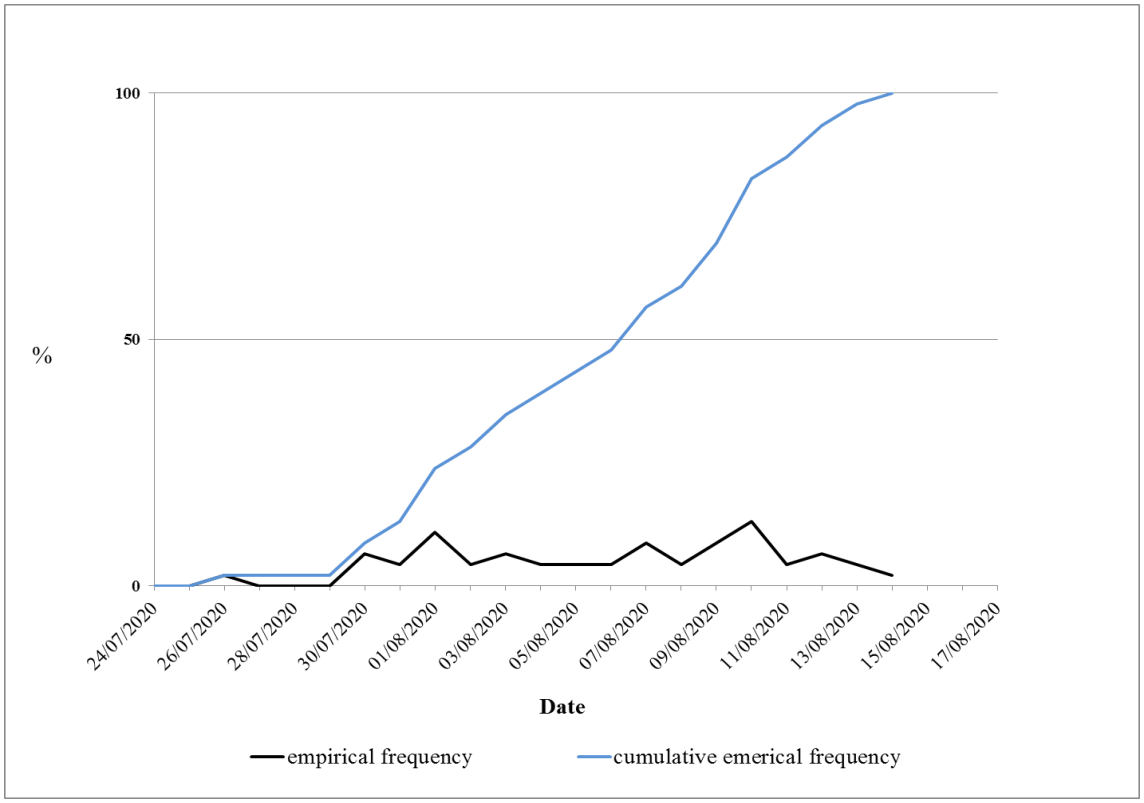

Fig. 1. Dynamics of the hatching of egg batches from Dobrostan (summer form of PPM).

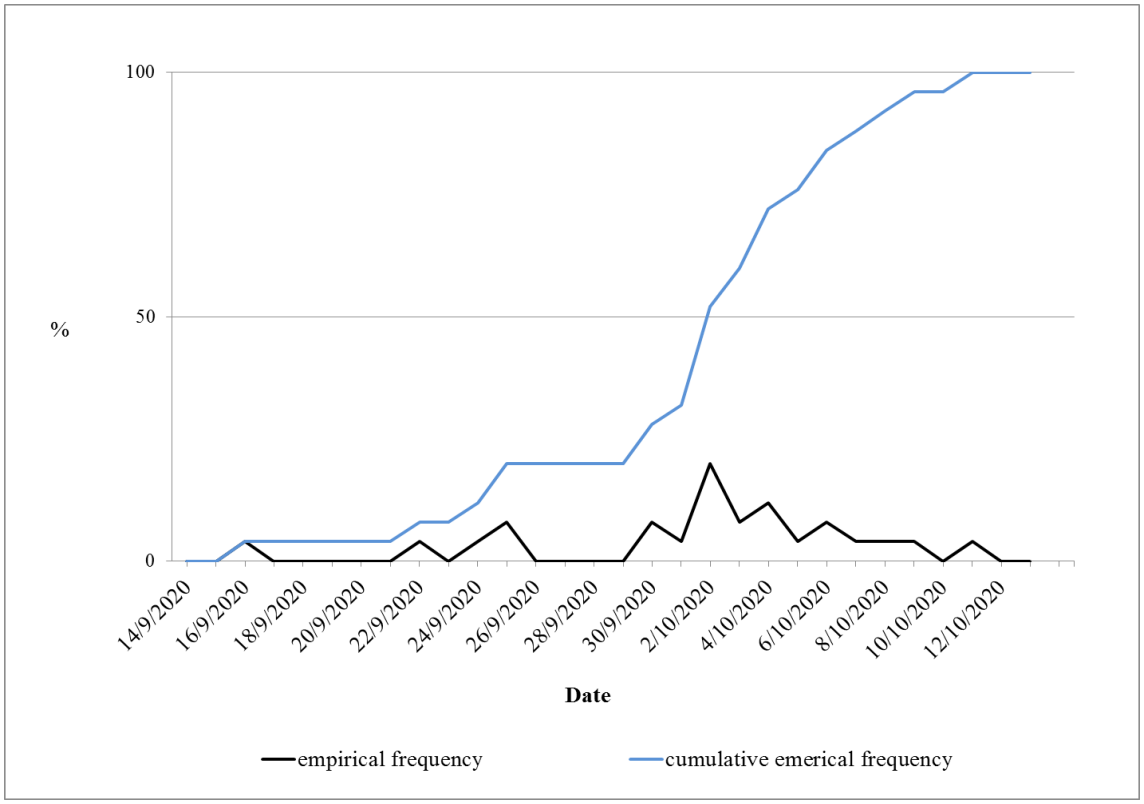

Fig. 2. Dynamics of the hatching of egg batches from Sandanski (winter form of PPM).

In Figures 3 and 4 the hatching period of egg batches from Dobrostan and Sandanski has been plotted together with the average monthly maximum air temperatures of the region of origin. The hatching period is given by the hatching of the first and the last egg-batches for the season (absolute hatching period), as well as by its peak determined the 50-th percentile. In the hottest region of the country – Sandanski, the PPM hatching took place well after the hottest time of the year and its peak came in a moment, when the temperatures did not exceed 28°C (Fig. 3), which is well below the threshold of 32°C known as lethal (Battisti et al, 2015). On the opposite, the hatching of larvae of the summer population in Dobrostan occurred during the hottest period (July and August), but in this region the average monthly maximum air temperatures did not exceed 28°C nevertheless (Fig. 4).

Discussion

Thaumetopoea pityocampa is one of the most common defoliator insects found in pine plantations in Bulgaria. Its heavily attacks for the period 2003-2018 reach 2700 ha per year (Zaemdzhikova et al., 2019). Significant money is spent each year to combat its attacks, thus preventing losses of growing stock and increment. In Bulgaria, so far no monetary valuation of the damage from PPM has been made, but other countries' studies show serious monetary value of annual increment and growing stock losses caused by strong attacks of pine processionary moths (CABI, 2020). Regardless of their relatie merits, both criteria – the price of control measures and the amount of losses, calculate significant moneys. In this regard, it is important to take into account that the phenological calendar of the pest is of great importance for the effectiveness of control measures: In the case of PPM, in order to maximize the effect of control measures, they should be applied against the caterpillars of the first and second larval stage, when the young larvae have not yet created dense nests.

The phenological cycle of *T. pityocampa* is strongly influenced by the temperature regime of the habitats. Temperature thresholds are crucial for the emergence of adults and larvae, and are the primary regulator of the duration of the pupal diapause (Robinet et al., 2013; Salman et al., 2016). It is known that in the south and at low altitudes, the adults of the PPM fly in autumn avoiding high summer temperatures, while in the north and at high altitudes they fly in summer (Robinet et al., 2013). French publications usually refer to the winter form of PPM, while in Bulgaria things are complicated by the very widespread distribution of summer form. In Bulgaria, however, regardless of the phenological forms, the flight of moths is during the hottest months of the year, culminating before the peak of the heat waves. In the most observed cases, the flight of PPM is quite long – two or three months and in some places even five or six months. There is a great local variety of the flight period, but in the places inhabited by the summer form, the flight begins and ends a month and a half earlier (Zaemdzhikova, 2020; Zaemdzhikova et al., 2020b). Given the mountainous relief of the habitats, characterized by strong variations in altitudes, as well as the flying abilities of males up to 50 km (Mirchev et al., 2013), it is quite possible that individuals from distant populations are present in the catch of one pheromone trap. Distant populations higher of lower in the mountain may have quite different calendars than the local population and may even belong to a different phonological form. The participation of distant populations may explain the extended period of flight in most Bularian sites regardless of the established local phonological form usually established y using pheromone traps. For this reason, for a country like Bulgaria, the use of pheromone traps as a means of monitoring the flight is quite uncertain and can not be used to identify the phonological form. This calls into question and its suitability, as a monitoring tool, in predicting the emergence of young caterpillars.

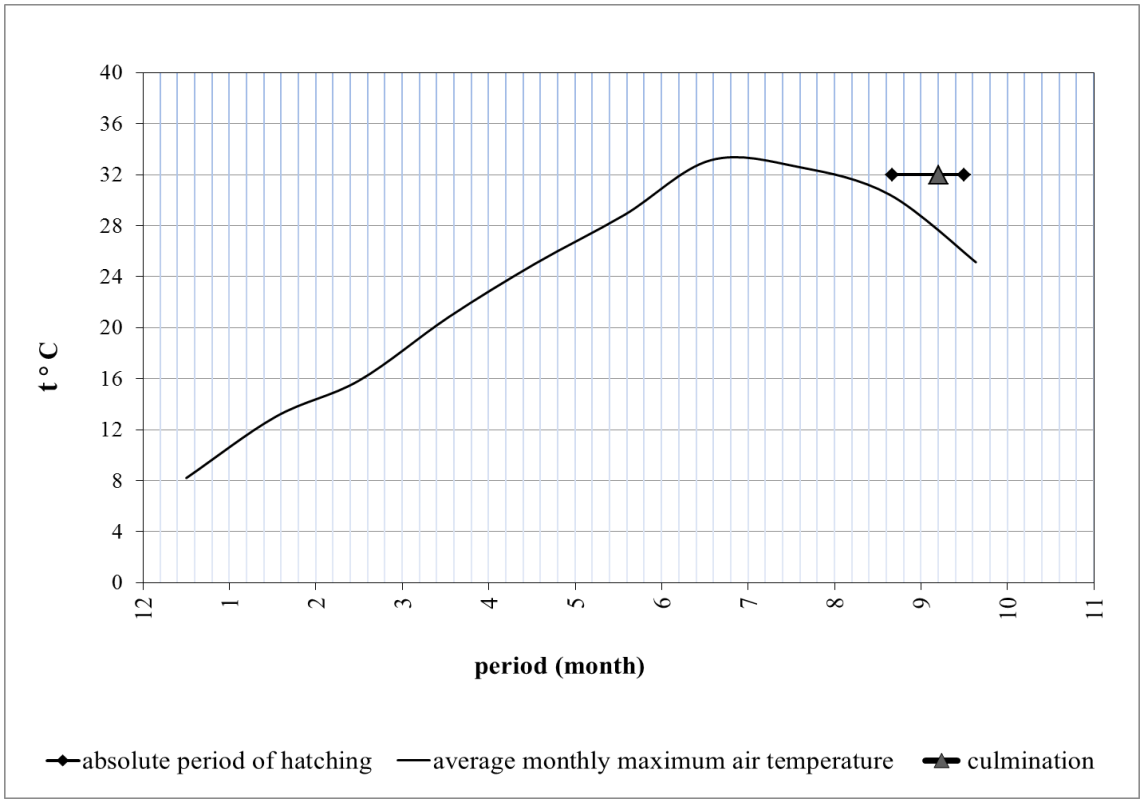

Fig. 3. Comparison of the hatching period and its peak with the temperature profile in Sandanski.

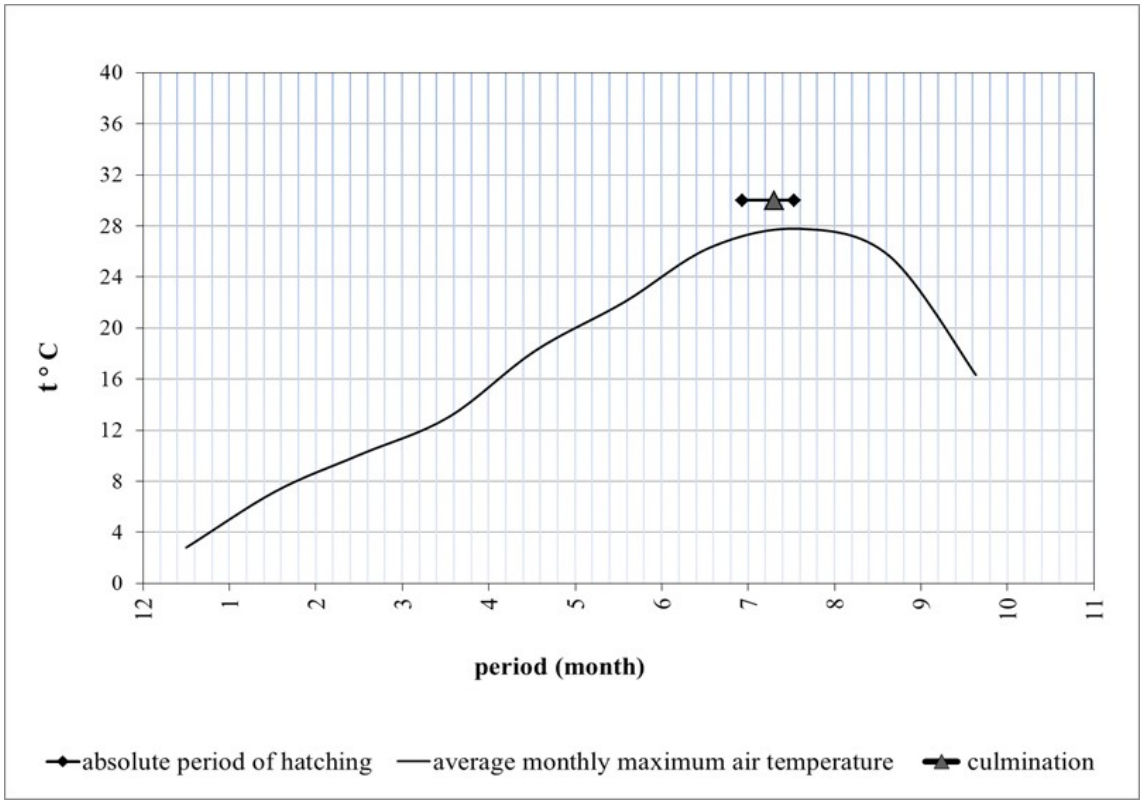

Fig. 4. Comparison of the hatching period and its peak with the temperature profile in Dobrostan.

The result of the present study shows, that the established hatching dates of the caterpillars correspond to the phenological calendar of the two populations (winter type or summer type calendar), as well as to the temperature thresholds limiting their survival. In Sandanski, the development of the larvae is within the known temperature optimum, with the culmination of hatching after the summer extremes. Of course, delaing the hatching after the hot waves there in July and August does not save the WF from all effects of heat since hot wawes have a negative effect on the embryonic stage of PPM. In the literature, the temperature maxima above 42°C which are reported periodically in Sandanski (Zaemdzhikova et al., 2020b) are considered lethal for development of the embryos of PPM (Robinet et al., 2013; Rocha et al., 2017). A higher percentage of unhatched dead caterpillars in the winter form – 7.3% against 3.2% in the summer form was proved for the Bularia in a study by Mirchev et al. (2020). In the region of Dobrostan, due to the mountainous terrain and the high altitude (over 1000 m a.s.l.) no heat waves are registered. In our records, the hatching of caterpillars from Dobrostan egg batches began in late July, culminating in early August, similar to the already known hatching period in the region of Kirkovo (Georgieva et al., 2019).

Considering the potential effects of the future climate warming scenarios, scientific considerations suggest that the southern regions will become less favorable for winter populations of *T. pityocampa*, as a result of the expected increase in summer extremes, which will increase the risk of the embryo survival. This may be the reason for the reduction of PPM attacks in Southern Bulgaria, found by (Zaemdzhikova et al., 2020a). On the other hand, global warming may be causing a shift in the phenological calendar of the winter form to the winter months, which is observed compared to the data from the 1950s. In more recent times For the region of Sandanski, Tsankov & Mirchev (2000) reported the beginning of the larvae hatching of winter populations in 2000 on 8th September with a culmination on 15th September, which is two weeks earlier than the established deadlines set by us in 2020. A quite probable explanation of this difference is that during a period of twenty years a shift in the phenology calendar of winter populations in their southern border has occurred, which is an expected result of global warming. The survival of the larvae of the WF seems to require a shift of the flight period towards the winter months.

The influence of temperature thresholds in Bulgaria is complicated by the orography and the presence of phenologically different populations, In addition to the climatic region and altitude, temperature extremes are strongly influenced by other environmental factors that enhance or mitigate the local impact of the temperatures. Such are: the relief forms, the exposure, the proximity of water area, etc. For instance, the towns Kirkovo and Sandanski are located at equal altitudes (average 300 m a.s.l.). Nevertheless in Kirkovo the SF is present, while in Sandanski – it is the WF (Mirchev et al., 2019). This difference is obviously due to the terrain forms that model the microclimate in these habitats. Kirkovo is located in a hollow field that keeps the cold air masses. For this reason in Kirkovo winters and summers are cooler. The minimum temperatures in Kirkovo are often from -10 to -12°C, and in some cases -24 ÷ -26°C while in the Petrich-Sandanski climatic region the minimum temperatures fall to -8 ÷ -9°C and in extremely cold weather can drop to –18÷ -19°C (Georgieva et al., 2018). The cold winters in Kirkovo are probably an explanation for the presence of the summer populations there.

Another example deliver the villages Dobrostan and Muldava also inhabited by phenologically different populations. The distance between them is 10-12 km as the crow flies. Both villages are assigned to the

continental-Mediterranean climate area (Sabev & Stanev, 1959). In the land of Muldava the winter form is found, and in Dobrostan – the summer form. Due to the serious difference in altitude of about 800 m a.s.l. (Mirchev et al., 2019), the temperature thresholds in them should be quite different. In the higher site Dobrostan (1110 m a.s.l.) it is assumed that the winters will be colder than in Muldava (300 m a.s.l.) and conversely the summers in Muldava will be hotter than Dobrostan. In the first case (Sandanski – Kirkovo) the determining factor for the phenological form is the oreography, and in the second (Dobrostan – Muldava) the determining factor is the altitude.

Conclusions

While the flight of PPM in Bulgaria lasts several months, usually two or three, sometimes even five or six, the hatching of the larvae takes a much shorter period – in our case it took about 3 weeks. This supports the assumption that pheromone traps catch both specimens from the local population and from remote ones, which makes it difficult to establish the phenological calendar of the local population.

Our case study in Sandanski and Dobrostan confirms with new data the observation that in Bulgaria the development of the winter form follows with a delay of a month and a half the development of the summer form.

The larvae hatching of the winter forms of PPM culminates in the beginning of October and occurs after the summer heats. On the other hand, the culmination of the summer form's hatching ocurs in early August and coincides with the summer heats. In both cases, however, the culmination of hatching occurs at average monthly maximum air temperatures of about 28°C.

In the phenological calendar of the winter form there is a change in the hatching period as compared to 2000, which is a supposed effect of global warming.

Acknowledgements

This work has been carried out in the framework of the National Science Program "Environmental Protection and Reduction of Risks of Adverse Events and Natural Disasters", approved by the Resolution of the Council of Ministers № 577/17.08.2018 and supported by the Ministry of Education and Science (MES) of Bulgaria (Agreement № D01-322/18.12.2019).

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Received: 08.11.2020
Accepted: 14.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 187-196

Study on the Distribution of Climbing Plants (Climbers) on the Territory of the Danube Island Aydemir, Bulgaria

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Abstract. The purpose of the present study is to describe the diversity, distribution and participation of the climbing plants (woody and herbaceous climbers) in the different plant communities in natural habitats of the Danube island Aydemir which is part of NATURA 2000 and to investigate their participation in the succesional processes. The methodology includes setting up Permanent Sample Plots (PSP) in different parts of the island with natural and semi-natural vegetation. The composition, cover abundance and occurrence of all the plant species in the PSP and their affiliation to a specific habitat type of NATURA 2000 were determined, as well as some soil properties as a major part of natural conditions giving specificity of plant composition. During the study, 36 species of vascular plants were identified belonging to 33 genera and 26 families from *Magnoliophyta* division - 41,4% of all the species and 66,7% of the vines are diagnostic for habitat 91EO Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (Alno-Padion, Alnion incanae, Salicion albae). The study shows that the distribution and diversity of climbers on the territory of Aydemir island, are mainly determined by vertical structure and age of forest communities and the high species richness in them. Among the climbers found on the island, the invasive alien species *Sicyos angulatus* has the strongest influence on the condition of natural habitats, and represents a real threat to their diversity.

Key words: Danube island, invasive alien species, lianas, climbers, NATURA 2000.

Introduction

Climbing plants (woody lianas and vines like *Periploca graeca* and *Vitis sylvestris*) and annual and perennial climbers (herbaceous climbers like *Humulus lupulus* and *Sicyos angulatus*) play an increasingly important role in forest regeneration, species diversity and ecosystem level processes such as transpiration and carbon sequestration (Schnitzer & Bongers, 2002). In world's scale and especially in the tropics, lianas influence forest dynamics by increasing the size of treefall gaps, and thereby increasing tree turnover rate (Phillips & Gentry, 1994). According to Tang et al. (2012) lianas are considered structural parasites, having a negative impact on the forests such as the reduction of aboveground biomass of trees (Laurance et al., 2014). The low occurrence of lianas in temperate regions has been ascribed to frequent low winter temperatures that may

cause freezing-induced embolisms in lianas' long vessel systems and lead to unrecoverable damage (Tang et al., 2012). The regular and active forest management (Addo-Fordjour et al., 2012), and indirect role of fishing and trampling also affects the climbers' abundance.

Climbers are principal physiognomic components of some specific European habitats such as riparian and longoze forests (91EO, 91FO). In Bulgaria, part of the places where these habitats and the natural processes in them can be observed are the Danube Islands, most of which are currently difficult to access and relatively poorly visited due to natural conditions (periodic flooding) and prohibition regimes related to border control and biodiversity conservation measures.

The flora and vegetation of the Danube islands has been subject of numerous studies (Yordanov & Kochev, 1981; Hinkov et al., 2006; Tzonev, 2005; 2007; 2008; Pedashenko et al., 2012) some of which date from the first half of the last century (Petkov, 1940; Stoyanov, 1947, 1948). Many of these publications are focused on climbers as part of the local floristic composition of the study areas, but the inter-storey vegetation on the Danube Islands has not been the subject of an independent study to date.

The purpose of the present study is to describe the diversity, distribution and participation of climbers in the different plant communities of the Aydemir island which is a part of NATURA 2000 and to investigate their role in the succession processes. Some major characteristics of soils also have been done.

Material and Methods

The research activities took place on the territory of the Danube island Aydemir, in natural habitats of riparian vegetation, where investigations were made on the presence of vines (including invasive alien and local species) in the phytocoenoses. Aydemir Island (also named Chayka) the largest island in the Chayka archipelago, has been designated as a Community Important Area (SCI), code BG0000534 by Commission Decision (CD, 2009) and a protected area under Directive 92/43/EEC for the conservation of natural habitats and wild fauna and flora with the Council of Ministers' decision No122 of March 2, 2007 (State Gazette, 2007).

Fig. 1. PSP locations on Aydemir (Chayka) island.

The island of Aydemir has an area of 77 ha on department 5 "a" (SFE Silistra, 2011). The main habitat classes are Bogs, Marshes, Water fringed vegetation, Fens and Broad-leaved deciduous woodland. Other characteristic habitat type are floodplain broad-leaved forests of *Salix alba* L., *S. triandra* L., with *Populus nigra* L., *P. alba* L., *P. canescens* Sm., *Populus x canadensis, Ulmus laevis* Pall., *Morus nigra* L., *Fraxinus pennsylvanica* Marschal, *Fr. americana* L., and *Acer negundo* L. The following habitats listed in Annex 1 to Directive 92/43/EEC are identified on the territory of the island: Habitat 3130 – Oligotrophic to mesotrophic standing waters with vegetation of the *Littorelletea uniflorae* and/or *Isoëto-Nanojuncetea* with an area of 2,52 ha; 3150 - Natural eutrophic lakes with *Magnopotamion* or *Hydrocharition*-type vegetation (0,1 ha); 3270 - Rivers with muddy banks with *Chenopodion rubri* p.p. and *Bidention* p.p. vegetation (5,82 ha) and 91EO - Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-Padion*, *Alnion incanae*, *Salicion albae*) (86,35 ha) (NATURA 2000 - Standard data form, 2020).

The study was conducted in October 2019. It is part of the project "Invasive alien plant species in protected areas of the Danube Islands on the territory of Bulgaria and Romania" funded under the bilateral science cooperation program between Romania and Bulgaria.

The methodology includes setting up Permanent Sample Plots (PSP) with a size for one plot of 500 sq. m in different parts of the island with homogenous vegetation (Hinkov et. al., 2020). The geographical coordinates of the plot centre were registered using GPS devices (Garmin GPSMAP64s) and marked with paint on the nearest tree. All trees, shrubs and herbaceous plants present within the PSP boundary were inventoried. The cover abundance/abundance of species was established at PSP level in % using the Braun-Blanquet (1964) scale (Table 1).

The composition and the cover abundance of all plant species in the PSP and their diagnostic role to a specific habitat type of NATURA 2000 were presented. The species occurrence was calculated as a ratio between the number of PSP in which one species was found and the total PSP number (Pavlov, 1998). The constancy (in scores from I to V) of each species was derived from the occurrence (Table 2).

Table 1. Braun-Blanquet scale of cover abundance.

Braun-Blanquet scale	Range of cover
R	< 5%; very few individuals
+	< 5%; few individuals
1	< 5%; numerous individuals
2	6-25%
3	26-50%
4	51-75%
5	76-100%

Table 2. Calculation of species constancy by Zielonka (2004).

Constancy	Occurrence (%)
I	0-20
II	21-40
III	41-60
IV	61-80
V	81-100

The cover abundance and constancy of each species were presented individually in five phytocenotic layers (Pavlov, 1998): A1, A2, B, C and D. For the purposes of this study the first two layers of trees A1 and A2 are united in one common layer - A, and the tall grass layer (C) and short grass and mosses layer (D) are united in the layer C.

The characteristic species for the habitats of Annex 1 to Directive 92/43/EEC are according to Kavrakova et al. (2005). The taxonomic nomenclature and the determination of plant taxa is by Kozhuharov (1992) and Delipavlov & Cheshmedzhiev (2003). Invasive alien species (IAS) have been identified according to Petrova et al. (2013), Drescher & Bohdan (2016), Invasive Species Compendium (CABI, 2020), Global invasive species

database (ISGG, 2020) and their nomenclature is given according to The Plant List (2013) database. The soil analyses were made according Donov et al. (1974).

Some main soil characteristics were determined such as pH – potentiometrically by pH-meter "Placitronic, MV 88" according ISO 10390, 2002; mechanical composition (texture) by titration method, with HCl treatment, and total carbon (C%) and humus (%) by the Thurin method and total nitrogen (N%) by the Keldal method – all of methods described by Donov et al. (1974).

Results

During the study 10 Permanent Sample Plots were set up and 36 species of vascular plants belonging to 33 genera and 26 families from one division - *Magnoliophyta* (Table 3) were identified.

The ratio between the number of species of the classes *Liliopsida* and *Magnoliopsia* is respectively 6 and 30. The species richest families are *Asteraceae* (4 species), and *Poaceae* (6 species). Trees and shrubs sum up 13 species, and herbs a total of 23 species. The climbers are represented with 6 species - 4 herbaceous climbers (*Calystegia sepium*, *Humulus lupulus, Sicyos angulatus*, *Tamus communis*) *and 2 woody climbers* (*Periploca graeca*, *Vitis sylvestris*).

Two of the PSPs are set up in monodominant plantations of hybrid poplars (*Populus x canadensis* Moench) and 4 PSPs are in plantations of hybrid willows (*Salix* × *rubens*). The rest of the PSPs are situated in mixed plantations of *Populus x canadensis*, *Salix* × *rubens*, *Ulmus laevis* and *Fraxinus pennsylvanica*. The total cover of the tree layer in the PSP's is between 35 and 80%, on shrub layer - between 0 and 95% and on the herb layer - between 0 and 70%.

The highest number of species (26 species or 52% of the total floristic composition of the all PSPs) was found in PSP 5, and the lowest - 14 species (28%) - in PSP 10. The richest diversity (over 50%) of all vines was found in 2 sample plots (or 20% of all PSPs) - PSP 5 (4 species, 66,4%) and PSP 7 (3 species, 49,8%). Climbers were not registered in PSPs 1, 2 and 9 (30% of all PSPs). Their participation in the rest of the PSPs is between 16,6 and 33,2% (1 or 2 species). Species cover abundance and constancy in the sample plots is highlighted in Table 3.

Of the all species (41,4%) and 66,7% of the climbers are diagnostic for habitat 91EO Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-Padion*, *Alnion incanae*, *Salicion albae*).

The variety of vines is higher in the PSPs set up in the primary forests dominated by *Populus nigra, Salix* × *rubens and Ulmus leavis* (3-5 species) than in the secondary communities of *Fraxinus pennsylvanica* (1-2 species).

Eleven species or 30,6% of the PSPs floristic composition are IAS. The largest number of IAS is found in PSPs 1 and 2 (5 species), and the least is in PSP 8 (1 species). One of them (S*icyos angulatus*) is climber (Fig. 2). This species has the highest constancy and cover abundance in the PSPs.

Table 3. Species cover abundance and constancy in the sample plots.

Species, Layer (A, B,C)	Family	No of PSP and species cover abundance (%)										Con-stan-cy	Species diagnos-tic for Natura 2000 Habitats	IAS
		1	2	3	4	5	6	7	8	9	10			
Total cover abundance (%) of Layer A		35	45	70	65	55	60	70	60	80	55			
Total cover abundance (%) of Layer B		18	20	75	15	55	10	15	95	30	5			
Total cover abundance (%) of Layer C		60	70	35	25	70	45	10	20	5	20			
Acer negundo L. *(A)*	Aceraceae										3	I		V

Acer negundo L. *(B)*	Aceraceae								1		I		V
Acer negundo L. *(C)*	Aceraceae				1				1		II		V
Agrostis capillaris L. *(C)*	Poaceae	2	1								I		
Amorpha fruticosa L. *(B)*	Fabaceae			2		4	2	2			III	91EO	V
Amorpha fruticosa L. *(C)*	Fabaceae			2	2	3	2	2			III	91EO	V
Aristolochia clematitis L.	Aristolochiaceae								+		I		
Bidens frondosa L.	Asteraceae					2	2				II	3270	V
Calystegia sepium (L.) R.Br.	Convolvulaceae							+			I	91EO	
Chenopodium glaucum L.	Chenopodiaceae		1								I	3270	
Cirsium arvense Scop.	Asteraceae					r					I	91EO	
Digitaria sanguinalis (L.) Scop.	Poaceae	2	1								II		V
Echinochloa crus-galli (L.) Beauv	Poaceae	1	2								II	3270	
Erigeron annuus (L.) Pers.	Asteraceae	2	1	1		+	+			1	IV	91EO	V
Fraxinus pennsylvanica Marshall *(A)*	Oleaceae				4						I		V
Fraxinus pennsylvanica Marshall *(B)*	Oleaceae			2	2					1	II		V
Fraxinus pennsylvanica Marshall *(C)*	Oleaceae		1	2	2			1		3	II		V
Galium album Mill.	Rubiaceae						+				I		
Humulus lupulus L. *(B)*	Cannabaceae							+			I	91EO	
Lycopus europaeus L.	Lamiaceae					r	r				II	91EO	
Morus nigra L. *(A)*	Moraceae								3		I		
Morus nigra L. *(B)*	Moraceae			2		1		2	1	1	III	91EO	
Morus nigra L. *(C)*	Moraceae				+				1	+	II		
Myosotis palustris L.	Boraginaceae						r				I		
Panicum capillare L.	Poaceae	1	2								II		V
Periploca graeca L. *(C)*	Apocynaceae				+	1	+	1		1	III	91EO	
Persicaria maculosa Gray	Polygonaceae					+					I		
Phalaris arundinacea L.	Poaceae	2									I		
Polygonum persicaria L.	Polygonaceae					+					I		
Polypogon viridis (Gouan) Breistr.	Poaceae	2	1								II		
Populus x canadensis Moench *(A)*	Salicaceae	3	2	4	2						III	91EO	
Populus x canadensis Moench *(B)*	Salicaceae	2	2								II	91EO	
Populus x canadensis Moench *(C)*	Salicaceae	1	1	1							II	91EO	
Populus nigra L. *(A)*	Salicaceae							3		4	II	91EO	

Portulaca oleracea L.	Portulaca-ceae	+	1								II	3130		
Rubus caesius var. *agrestis* Weihe & Nees	Rosaceae			2				1			II	91EO		
Salix × *rubens* Schrank *A)*	Salicaceae		3		2	4	4		4		3	III	91EO	
Salix × *rubens* Schrank *(B)*	Salicaceae		1									II	91EO	
Sicyos angulatus L. *(B)*	Cucurbita-ceae					2	3		5			II		V
Sicyos angulatus L. *(C)*	Cucurbita-ceae								3			I		V
Solanum nigrum L.	Solanaceae					2					1	II		
Stellaria nemorum L.	Cario-phyllaceae						+		+			II	91EO	
Tamarix ramosissima Ledeb. *(B)*	Tamari-caceae	2	2									II		V
Tamus communis L.	Diosco-reaeceae			1		1						II		
Urtica dioica L.	Urticaceae								+			I		
Ulmus laevis Pall. *(A)*	Ulmaceae							3		1	1	II		
Ulmus laevis Pall.*(B)*	Ulmaceae			3				2		1		II		
Ulmus laevis Pall.*(C)*	Ulmaceae							1	+	1		I		
Vitis sylvestris C.C.Gmel. *(B)*	Vitaceae			1		1					1	II	91EO	
Vitis sylvestris C.C.Gmel. *(C)*	Vitaceae			1		1						II	91EO	
Xanthium italicum Moretti	Asteraceae	2	3									II	3270	V

Fig. 2. Invasive potential of the alien species *Sicyos angulatus* (Photo: G. Hinkov).

Another point of investigation is characteristic of natural habitat conditions. Among them the soils conditions are this characteristic that has a major role in determined specificity of plant diversity and abundance of plant species. All soils of Aydemir are *Alluvial Fluvisols* and have similar characteristics. They have neutral to slightly alkali reaction (pH 6,96 – 7,37). The organic carbon in PSP 1 is with lowest value (0,9%), but in the rest plots the content of organic carbon is normal (~ 2%). The value of nitrogen follows the trends of organic carbon values, and varied among average values 0,15-0,20%. Detailed characteristics of soils of Aydemir island is given by Kachova (2020).

Discussions

The highest variety of climbers is in PSP 5, where the cover abundance of the tree and shrub layers is around the average (55%), and the herbaceous layer is high (70%). The soils of this plot have not special characteristics - normal content of carbon (2,88%), and slightly alkali reaction (7,37). The highest species diversity was also found in the same PSP. This corresponds to the finding by Nabe-Nielsen (2001) that the density of climbers increased with the density of small trees and the areas with high tree-saplings had a high diversity of lianas.

The lack of understory, poor species composition and low grass cover abundance is associated with increased participation of the invasive alien species *Sicyos angulatus* (Star cucumber). This species is the most competitive not only compared with the rest of the herbaceous climbers and lianas but for all other plant species in the PSPs. Its occurrence in Danube island Belene was recorded 15 years ago by Tzonev (2005) who predicted the expansion of its distribution in Bulgaria. The Star-cucumber populations form dense monodominant sinusia on all layers, being favoured by seasonal flooding and rapid growth thanks to the species strong environmental adaptability.

The relationship between the diversity of climbers and primary vegetation, confirms at this stage the findings of Roeder et al. (2010) that species richness and the proportion of climbers decreased with the increase in distance from the primary forest. According to Campbell et al. (2015) lianas preferentially impact certain ecological "guilds" of tree species such as late – successional / climax species.

The absence of climbers in some of the sample plots could also be explained by the presence in the herbaceous layer of a large number of cereals (including invasive alien species) such as *Agrostis capillaris, Phalaris arundinacea, Digitaria sanguinalis, P. capillare* etc.

The species diversity and abundance of climbers on the territory of Aydemir island are close to those of other Danube islands such are Belene (Stoyanov, 1947), Cama and Barzina (Mică) island (Schneider-Binder, 2009). The results confirm important role of climbers for the biodiversity of the forests (Schnitzer & Bongers, 2002). The intercrown connections provided by them are of great importance to animals that can't fly or glide long distances and also increase the likelihood of trees pulling down their neighbours when they fall (Putz, 2012). All the climbers contain active substances as follows: *Calystegia sepium* - glycosides, tannins, resins (Konstandi, 2020 – pers. com.), *Humulus lupulus* - alfa-acids, terpenes, flavonoides (Killeen et al., 2017), *Sicyos angulatus* - flavonoid glycosides and sterols (An et al., 2019)., *Tamus communis* - polyphenols and flavonoids (Shaheen et al., 2009) „*Vitis sylvestris* - (tannins and *Periploca graeca* - steroids, terpenoids, phenylpropanoids, flavonoids, quinines (Mingjin at al., 2019). Some of them are edible such as *Vitis syvestris*, other are toxic (*Periploca graeca).*

Conclusions

The study shows that the distribution and diversity of climbers on the territory of Aydemir island depend to some extent on the well-defined and balanced structure of

forest communities and their rich biodiversity.

Among the climbers found on the island, the invasive alien species *Sicyos angulatus* has the strongest influence on the condition of natural habitats and represent a real threat to their diversity.

The remaining IAS registered on the island pose an indirect threat to the climbers, as well as to the entire habitat 91EO, causing appearance of secondary communities and reducing its natural floristic diversity.

Acknowledgment

The publication was made with the financial assistance of a project for bilateral cooperation between Bulgarian Academy of Sciences and the Romanian Academy of Sciences.

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Received: 22.07.2020
Accepted: 15.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 197-201

Short note

Length–Weight Relationships and Condition Factors of Three Sturgeon Species (Acipenseridae) from the Danube River

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Abstract. Length–weight relationships (LWRs), Fulton (*K*) and relative (K_{rel}) condition factors for *Acipenser ruthenus* Linnaeus, 1758, *Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833 and *Acipenser stellatus* Pallas, 1771 from the Danube River were estimated. Fish were captured by bottom drifting trammel nets in the period June – August, 2015–2019. A total of 790 specimens were measured and analyzed. More than 80% of the specimens were young of the year (YOY) fish with total length (TL) up to 35 cm. The values of the parameter *b* of the weight – length relationship for fish with lengths up to 35 cm ranged from 2.433 (*A. ruthenus*) to 2.859 (*A. gueldenstaedtii*). For specimens with TL > 35 cm the values of parameter *b* ranged from 3.227 (*A. stellatus*) to 3.668 (*A. ruthenus*). Relative condition factor (K_{rel}) ranged from 1.003 ± 0.020 (*A. gueldenstaedtii*) to 1.144 ± 0.018 for *A. ruthenus*. Fulton's condition factor (*K*) ranged from 0.300 ± 0.003 for *A. stellatus* to 0.514 ± 0.011 for *A. gueldenstaedtii*.

Key words: Sterlet, Stellate sturgeon, Russian sturgeon, Fulton factor, relative condition factor, allometric growth, Danube River.

Introduction

The fish family Acipenseridae includes some of the most endangered fish species worldwide (IUCN, 2010). Historically, six members of the family were widely distributed throughout the Danube River and Black Sea: Beluga (*Huso huso* Linnaeus, 1758), Russian sturgeon (*Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833), Stellate sturgeon (*Acipenser stellatus* Pallas, 1771), Sterlet (*Acipenser ruthenus* Linnaeus, 1758), Atlantic sturgeon (*Acipenser sturio* Linnaeus, 1758) and Ship sturgeon (*Acipenser nudiventris* Lovetzky, 1828), yet the last two are now considered extinct from the region. In the past these species constituted the main part of the traditional fishing in the Lower Danube River (Kynard et al., 2002). However, in the last 50 years, their populations have been drastically reduced due to habitat degradation, overexploitation, disturbance of the river longitudinal connectivity, loss of spawning sites, and water pollution (Bristein et al., 1997; Raischi et al., 2017). Danube sturgeons are anadromous, except the Sterlet that spends its entire life in the river (Bacalbasa-Dobrovici, 1997). All sturgeon species have been listed in the IUCN Red list as threatened or endangered (IUCN 2009), and in Annex I and II of the CITES (Bristein et al., 1997). Since 1999, support for Danube sturgeons has been initiated

through restocking and catch-limiting bans (Hubenova et al., 2009, WSCS & WWF, 2018). The maintenance and restoration of self-sustaining sturgeon populations will require knowledge of their biology and life history (Bristein et al., 1997). The length–weight relationships (LWRs) help to determine biomass from length observations, and the conversion of growth-in-length equations to growth-in-weight, it is also applied when assessing the well-being of individuals and comparing life histories of separated populations of the same species in different water bodies and regions (Craig et al., 2005; Froese, 2006). Body condition indices are commonly used in fisheries biology because they provide information on the stock condition. These indices can be very useful to fisheries management, especially as an alternative non-lethal method of providing information on rare and protected species without the need to sacrifice specimens (Le Cren, 1951, Froese et al., 2011). Currently, the knowledge on the length–weight relations of the sturgeon fishes in the Danube River is limited.

The aim of this work was to determine the length – weight relationships (LWRs) and condition indices for three representatives of wild sturgeon species inhabiting the Lower Danube River.

Material and Methods

The study was carried out in the Lower Danube River section, near Vetren Village (44.141538° N, 27.029863° E), Bulgaria. Sturgeons were caught as bycatch during the annual fish monitoring conducted by WWF-Bulgaria; they were captured in the period from June until August during five-year monitoring carried out from 2015 to 2019. YOY specimens of Stellate sturgeon (up to 35 cm TL) were captured in the Danube, while the specimens with TL > 35 cm were caught in the Danube and the Black Sea. The weight and length data of 18 specimens of the Stellate sturgeon species caught along to the Black Sea coast, near to Krapets Village (43.38493° N, 28.34325° E) were provided by professional fishermen. The sampling in Danube River were conducted using bottom drifting trammel nets. The nets were 100 m long × 2 m wide, with three different finer-meshed central layers, with mesh size of 20, 50 and 70 mm. Five to nine nets per day were used with an average 30 min. exposition. All individuals were identified *in situ* according Kottelat & Freyhof (2007). The fish were subsequently photographed, weighed, and immediately released back into the river. Total weight (W) was measured to the nearest 0.1 g using a digital portable balance Kern EMS 6K0.1 and the total length (TL) was measured to the nearest 0.1 mm. The Ministry of Food, Agriculture and Forests and the Executive Agency of Fishery and Aquaculture of Bulgaria authorized field samplings performed in this study.

The LWR parameters were estimated according to the formula: $W = aL^b$ (Le Cren 1951) by the least squares method through the transformed equation: $\log W = \log a + b \log TL$. The statistical significance level of the coefficient of determination (r^2) and 95% confidence limits of a and b were computed for all equations. Obvious outliers were identified and removed, according to the plot of the log W over log TL (Froese 2006). The differences between the value of b of the LWR and the value of isometric growth (b = 3) were compared by t-test (Froese et al. 2011). Fulton (K) and relative (K_{rel}) condition factors (Le Cren 1951, Sutton et al. 2000), were computed as $K=100*W/TL^3$ and $K_{rel} = W/aTL^b$ respectively, where a and b are the parameters from the LWR equations. Length and weight data were pooled together for each species without sex differentiation. All analyses were performed using Statistical Package SPSS version 22 (SPSS Inc. Ltd.) and Excel software (Microsoft Office, 2016).

Results

A total of 772 sturgeon specimens were caught on the Danube River and 18 specimens in the Black sea. Descriptive statistics and estimated parameters of the LWR are shown in Table 1. All length–weight relations were statistically significant ($P < 0.001$).

For *A. ruthenus* and *A. stellatus* with length up to 35 cm, the estimated growth type was negative allometric, while at *A. gueldenstaedtii* was isometric. Larger specimens of *A. ruthenus* (> 35 cm TL) (b=3.668) and *A. stellatus* (b=3.227) exhibited positive allometric growth.

Table 1. Descriptive statistics and length–weight relationships (LWRs) for three sturgeon species from the Danube River. Abbreviations: a - intercept, b - slope, CI - confidence interval, n - sample size, r^2 - coefficient of determination; SD - standard deviation. * All length–weight relations were statistically significant (P < 0.001).

Species name	n	Total length [cm]		Body weight [g]		LWR Parameters					
		Range	Mean±SD	Range	Mean±SD	*a*	*b*	SE *b*	95% CI of *b*		r^2
Acipenser ruthenus (< 35 cm TL)	546	14.8-29.0	21.9±2.7	12.0-98.0	43.8±13.6	0.023	2.433	0.037	2.36	2.51	0.886
Acipenser ruthenus (> 35 cm TL)	67	35.2-68.0	45.5±6.3	180.0-2015.0	457.5±286.2	0.0003	3.668	0.119	3.43	3.91	0.936
Acipenser stellatus (< 35 cm TL)	130	16.7-30.0	21.9±2.7	14.0-74.0	32.3±11.4	0.010	2.609	0.070	2.47	2.75	0.916
Acipenser stellatus (> 35 cm TL)	25	36.0-139.0	62.0±21.5	140.0-10200.0	1047.1±2136.8	0.001	3.277	0.186	2.89	3.66	0.931
Acipenser gueldenstaedtii	22	12.6-33.0	24.4±6.2	13.0-168.0	86.1±53.8	0.008	2.859	0.074	2.70	3.01	0.987

Relative condition factor (K_{rel}) and Fulton's condition factor (K) for four sturgeon species are shown in Table 2. In this study, the K_{rel} ranged from 1.003 ± 0.020 (*A. gueldenstaedtii*) to 1.144 ± 0.018 (*A. ruthenus*). The minimum and maximum of Fulton's condition factors (K) were 0.300 and 0.514 ± 0.011 for *A. stellatus* and *A. gueldenstaedtii* respectively.

Table 2. Fulton's condition factor (K) and relative condition factor (K_{rel}) for three sturgeon species from the Danube River. Legend: max - maximum; min - minimum; n - sample size; SE - standard error.

Species name	n	Fulton's condition factor (K)			Relative condition factor (K_{rel})		
		mean	SE	min - max	mean	SE	min - max
Acipenser ruthenus (< 35 cm TL)	546	0.404	0.0025	0.263-0.682	1.006	0.0048	0.669-1.438
Acipenser ruthenus (> 35 cm TL)	67	0.439	0.0085	0.303-0.641	1.144	0.0178	0.869-1.527
Acipenser stellatus (< 35 cm TL)	130	0.300	0.0028	0.237-0.418	1.009	0.0084	0.800-1.350
Acipenser gueldenstaedtii	22	0.514	0.0113	0.434-0.650	1.003	0.0201	0.880-1.244

Discussion

In our study we examined the parameters of the LWRs of juveniles (immature) and adult Sterlet and Stellate sturgeon specimens separately, to determine more rigorously the slope *b*. Sterlet mature at 35 cm TL for male specimens (Muus & Dahlström 1968). All captured Russian sturgeon were YOY specimens up to 35 cm TL. We suspect that the origin of this specimens were most likely from a restocking carried out in 2019 in the Lower Danube.

The parameters of LWRs for *A. ruthenus* (b = 2.94, n = 285) obtained in the present study was similar to those that have been observed by Lenhardt et al. (2004) under morphological analysis of Sterlet population from the Serbian part of the Danube River. Froese (2006) observed ontogenetic changes in body shape related with size.

In the present study a positive allometric growth (b= 3.227) was observed in the case of *A. stellatus* (> 35 cm TL). The results obtained for the same species from Caspian Sea (Fazli & Moghim 2014, Mousavi & Ghafor 2014) show isometric growth, while Ceapa et al. (2002) found out that the Stellate sturgeon brood fish collected during reproductive migration in the Romanian part of Danube River had a negative allometric growth (b = 2.284 for males, n = 128). The observed differences might be due to the different sample size (number of specimens used), sex of specimens or sampling periods. Therefore, extrapolation of LWR parameters of *A. stellatus* to different length ranges should be handled with caution. Isometric growth (b=2.859) was observed for *A. gueldenstaedtii*, which corresponds to the results reported by Mousavi & Ghafor (2014) for the same species from the Caspian Sea.

For the Stellate sturgeon, the Fulton's condition factor (K) has the lowest value (0.300 ± 0.003), while the Russian sturgeon has a higher value (0.514 ± 0.011), which may be due to the fact that the Russian sturgeon specimens were probably hatched on a farm for restocking purposes where they were fed *ad libitum* in their first three months.

Conclusion

This study provided the basic information on the length–weight relation parameters and condition indices for the YOY and juvenile specimens of *A. ruthenus, A. stellatus* and *A. gueldenstaedtii* from the Lower Danube. Such data is valuable for establishing a monitoring program and enforcing management solutions for the protection of the natural stocks of these endangered species.

Acknowledgements

The funding for this study was provided by WWF-Bulgaria and the Sofia University Scientific Fund, Grant 80-10-136/2020. The authors would like to thank Stoyan Mihov and Veselin Koev for the technical support and their valuable and constructive advice during the whole study.

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Received: 14.07.2020
Accepted: 15.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 203-205

Short note

Frogs at the Sea – Unusual Breeding Site of Pelophylax ridibundus (Pallas, 1771) (Amphibia: Anura) at the Black Sea Coast (Bulgaria)

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Abstract. Unusual breeding site of the Marsh frog (*Pelophylax ridibundus*) is reported from Ropotamo Reserve in the south Black Sea Coast. Few adult and subadult individuals, producing mating calls, as well as eggs and newly hatched larvae were observed in brackish standing water pools on a rocky shore.

Key words: Marsh frog, water salinity, breeding, Black Sea, Ropotamo Reserve, Bulgaria.

Anurans have limited ability to handle increased salinity of the water basins they inhabit, but sometimes aquatic frogs of the *Pelophylax* genus can be registered in brackish water bodies (Litvinchuk et al., 2015). Such observations are recorded from Bulgarian Black Sea coast for *P. esculentus* complex by Natchev et al. (2011) at Shablenska Tuzla Lagoon; Natchev et al. (2016) at the regions of Shabla and Ezeretz Lakes; the sand beach in the region of Durankulak Lake; The Bolata Bay and the sand beach in the region of Shablenska Tuzla Lagoon. Covaciu-Marcov et al. (2006) report occurrence of *P. ridibundus* in brackish waters in Romania. Litvinchuk et al. (2015) report several observations *P. esculentus* complex in brackish water basins from Kaliningrad Region in Russia.

Aquatic frogs of the *P. esculentus* complex are adapted to handle increased sodium concentrations and to withstand the osmotic gradient when inhabiting brackish sea waters (Kuzmin, 1999). According to the same author *P. ridibundus* is resistant to high water salinity from 0.9 to 8.3‰.

It is relatively rare occasion to observe breeding of aquatic frogs in brackish waters. On 25.06.2020 unusual breeding site of *Pelophylax ridibundus* was discovered. The frogs were found in three standing water basins on the rocks, just few meters away from the sea in Ropotamo Reserve (Bulgaria), at approximate coordinates N42°20.114 E027°47.052 (WGS 84 datum), 1-2 m a.s.l. (Fig. 1 & 3). Few subadult and adult individuals, producing mating calls, were observed in the pools (Fig. 2 & 4), as well as eggs and newly hatched larvae in one of them. Unfortunately, the salinity or the conductivity of the water could not be measured, but the presence of *Enteromorpha (Ulva) intestinalis* and *Cladophora sp.* algae and the marine isopod *Idotea baltica* was det-

Fig. 1. Small stagnant water basin with brackish water, few meters away from the sea, where eggs, larvae and adult individuals of *P. ridibundus* were recorded. Photo: I. Mollov.

Fig. 2. Subadult *P. ridibundus*, surrounded with *Entheromorpha (Ulva) intestinalis* algae. Photo: I. Mollov.

Fig. 3. Another standing water basin, 2 meters away from the first one, where adult specimens of *P. ridibundus* where observed. Photo: I. Mollov.

Fig. 4. Close-up of and adult *P. ridibundus* from one of the pools. Photo: I. Mollov.

ected in the pools. Based on that, we assume the waters were brackish. They are probably supplied with water by the sea waves crushing into the rocky shore (this could probably also explain the presence of *I. baltica*) and of course rain water.

Kuzmin (1999) reported a reproduction of *P. ridibundus* in marine water with low salinity, at 0.5 to 1 m from the shore for a population inhabiting the Apsheron Peninsula of the Caspian Sea. On the base of the results from the five year monitoring study Natchev et al. (2016) report that in four studied localities in the NE Black Sea coast line in Bulgaria, the green frogs from the *P. esculents* complex have developed adaptation to live and breed in mixoligohaline waters.

Acknowledgements

The author would like to express his sincerest gratitude to Dr. Anelia Dakova

(University of Plovdiv "Paisij Hilendarski", Faculty of Physics and Technology, Department of Physics) for her valuable help. The publication of the current short note is financed by Dundee Precious Metals Inc.

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Received: 24.11.2020
Accepted: 15.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 207-211

Short note

New Distribution Records of an Endemic Terrestrial Isopod Species (Trachelipus trilobatus) in the Romanian Southern Carpathians

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Abstract. *Trachelipus trilobatus* is an endemic terrestrial isopod species, present only in the Herculane Spa area situated in the Cerna River valley, southwestern Romania. Here we present two new records of this endemic species, which increases its known distribution. Both locations are situated in the Domogled-Valea Cernei National Park. One distribution record is situated at approximately 10 km upstream from its closest previously known location, extending the species` distribution area with approximately a third. Both distribution points present the typical habitats of the species, represented by steep and moist limestone slopes, with caves, and gorge-like areas covered by beech forests.

Key words: Cerna valley, distribution, range extension, endemic species, habitats.

The number of terrestrial isopod species in Romania is higher than in the neighboring countries (Sfenthourakis & Hornung, 2018). A high amount of these species are endemic (Sfenthourakis & Hornung, 2018), many of them being recorded in caves (Tabacaru & Giurginca, 2013). A region in Romania with numerous endemic terrestrial isopods at different geographic levels is the Herculane Spa area from southwestern Romania (e.g. Pop et al., 2019). The terrestrial isopod fauna has a particular relation with the small town of Herculane Spa, especially with its older part (Pop et al., 2019), which has a two-millennium history (e.g. Spânu, 2012; Boda et al., 2017). Among the isopods recorded in the Herculane Spa, *Trachelipus trilobatus* has the smallest distribution range (Pop et al., 2019). Furthermore, within the species of this genus, *T. trilobatus* has one of the smallest distribution ranges in the country (Tomescu et al., 2015), as it is present only in Romania (Schmalfuss, 2003). Records of this species are known only from the Herculane Spa area and its neighboring region (e.g. Tomescu et al., 2015). Although *T. trilobatus* is considered to have a similar appearance with some isopod species from the Mediterranean region (Radu, 1958), its exclusive link to natural areas has recently reconfirmed its status as a native species (Pop et al., 2019). Until now, this species was recorded only in nine locations, all from Herculane Spa and its neighboring region (see in Tomescu et al., 2015; Pop et al., 2019), including some caves (e.g. Tabacaru & Giurginca, 2013). Only one of these nine records is situated outside the Cerna River

valley, on the other slope of the Mehedinți Mountains, but it is considered an uncertain record by the authors (Ilie et al., 2002). Because of its small distribution range and its peculiar zoogeographic significance, any new distribution record of *T. trilobatus* is of high importance, as for other endemic isopod species (e.g. Ferenți et al., 2016; Ferenți & Covaciu-Marcov, 2017; Recuero & Rodríguez-Flores, 2019). The present note reports two new distribution records of *T. trilobatus*.

The two new distribution points were recorded in July 2020. Our initial objectives were not the terrestrial isopods of the area, but as we sighted suitable habitats for *T. trilobatus*, we started to search for the species on wet limestone walls, cracks or under stones. Because of the unique features of this species, the specimens were identified in situ, none of them was collected. Both new distribution records are situated in the Cerna River valley upstream the Herculane Spa. One location is situated near the Vânturătoarea Waterfall. The other one is located further upstream in the Cerna River valley, with some hundreds of meters upstream from the intersection between the road to Baia de Aramă and the one to Cerna village (Fig. 1). Although both new distribution records are situated in the Cerna River valley, the second one is situated at approximately 10 km upstream from the closest previously known distribution record, the Prisăcina Gorge (Tomescu et al., 2015). This new record extends the distribution area of *T. trilobatus* in the Cerna River valley by almost a third. Even if 10 km do not seem too much, in proportion to this species distribution range it represents an important extension. Moreover, the areas between the new and the old distribution records contain numerous habitats potentially used by this species (Tomescu et al., 2015). Thus, although rare and strictly geographically located, *T. trilobatus* is probably continuously distributed in the Cerna Valley, at least between its lower limit (Pecinisca Gorge) and the new distribution record.

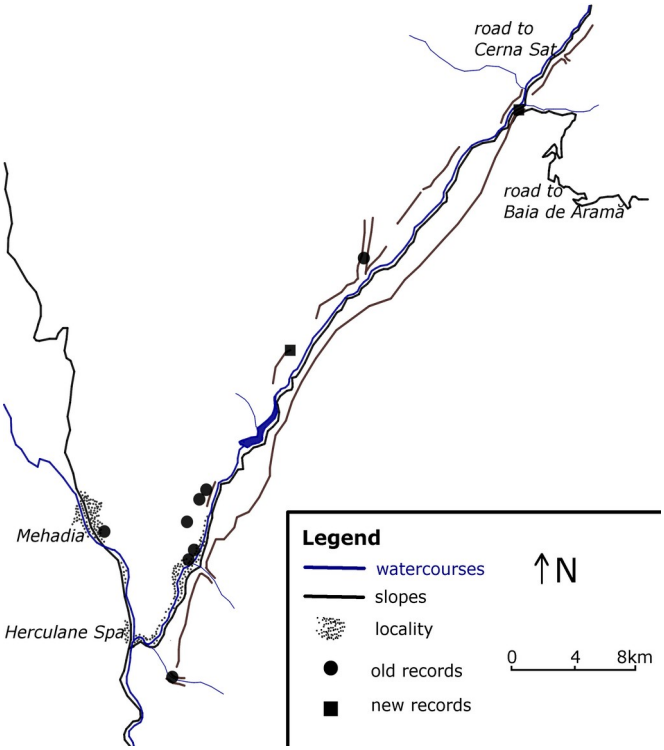

Fig. 1. The old (after Tomescu et al., 2015 and Pop et al., 2019) and new records of *Trachelipus trilobatus* in the Cerna River valley.

Nevertheless, despite the new distribution records and range extension, *T. trilobatus* remains strictly related to restrictive ecological conditions. The new habitats are similar to the previous ones described in the literature (Tomescu et al., 2015). In both cases, *T. trilobatus* was identified on steep (almost vertical) limestone walls, with water infiltrations, present in areas covered with beech forests (Fig. 2a, b). These limestone walls contain numerous cavities and small caves, as *T. trilobatus* have populations both outside and inside the caves (Tomescu et al., 2015), as it is a troglophilous species (Tabacaru & Giurginca, 2013). *T. trilobatus* individuals (Fig. 2c) were identified either under rocks or directly on limestone walls. In the upstream location on a single square meter of limestone wall in a couple of minutes we observed two individuals under two rocks, two individuals directly on the wall, two shed skins, and fragments from one dead individual.

Fig. 2. The habitat (a) and microhabitat (b) of *Trachelipus trilobatus* (c) in the upstream new distribution point.

The extension of the distribution range of other endemic terrestrial isopod species was also reported in other recent studies (Recuero & Rodríguez-Flores, 2019; Hughes, 2020), including Romania in the case of another endemic *Trachelipus* species (Ferenți & Covaciu-Marcov, 2017). Certainly, new studies on the Cerna River valley will lead to further extension of this species distribution range, but only in its restrictive habitats. Our data confirms the fact that this stenobiont species have probably been evolving for a long time in the peculiar conditions of this region (Tomescu et al., 2015). Its survival seems to be ensured by the fact that the species uses restrictive and hard-to-reach

habitats, in a region where even old-growth forests are affected by wood harvesting (Vijulie et al., 2017). Even if law (O.U.G. 57/2007) does not protect *T. trilobatus*, the fact that it is an endemic and relict species makes it worthy for Domogled-Valea Cernei National Park, since this protected area seems to shelter all its known populations. The importance of species that are not protected but have zoogeographic value for protected areas was already mentioned in Romania, for example in the case of an endemic species with a larger distribution range, namely the Carpathian scorpion (Covaciu-Marcov & Ferenți, 2019). Thus, we consider that *T. trilobatus* should be protected in the future and deserves to be included at least in a national red list.

Acknowledgments

Our study was made in collaboration with the administration of Domogled-Valea Cernei National Park.

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Received: 16.10.2020
Accepted: 15.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 213-217

Short note

A Second Record of the Species Clathrus ruber P. Micheli ex Pers. in Romania, and Notes on its Distribution in Southeastern Europe

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Abstract. In this short note, the fungal species *Clathrus ruber* is reported for the second time in Romania, 60 years after the first record. It was identified in the northeastern part of the country, in Iaşi region, in a *Lolium* sward from a private garden. Twenty fruitbodies were counted between June and October 2020. It is not known how the species was brought into the garden.

Key words: stinkhorns, rare species, distribution, new occurrence.

Introduction

Clathrus ruber P. Micheli ex Pers. (syn. *C. cancellatus* Tourn. ex Fr.) is one of the two species of the genus *Clathrus* (*Phallaceae*, *Phallales*) that have been identified in Romania (besides *C. archeri* (Berk.) Dring (Bereş, 1996)). The species was first described by the Italian botanist Pier Antonio Micheli in 1729 (Micheli, 1729). Species in the genus *Clathrus* are characterized by fruitbodies with bright colors and bizarre shapes. Etymologically, the name comes from the Greek "*kleithron*" = grid, box, cage and the Latin "*ruber*" = red (Bresson, 1996). The popular French name of "witch heart" comes from the Spanish and Serbian vernacular names (Courtecuisse & Duhem, 2013). According to some authors, *C. ruber* is quite common in the Mediterranean region where it can be found on sandy and calcareous soils, in coniferous forests (Mallorca, Sardinia, Corsica), but also on the French Atlantic coast (Breitenbach & Kränzlin, 1986; Gerhardt, 1999). It is a species with a Mediterranean origin, occurring throughout the year in southern France. Sporadic occurrences have been reported in Central Europe. The species is absent in Northern Europe.

In Romania, *Clathrus ruber* (Fig. 1) was considered a rare macromycete species, with only one reported occurrence in the Black Sea coastal area (Toma et al., 1962). It was regarded as vulnerable and included in Romanian Red List of Macromycetes (Tănase & Pop, 2005). Also, it was included in the Red Lists of other countries, such as Ukraine, Bulgaria, Macedonia, and Slovakia (Didukh et al., 2009; Gyosheva et al., 2006; Karadelev & Rusevska, 2013; Lizoň, 2001).

Fig. 1. Fully developed fruitbody of *Clathrus ruber* in Valea Adânca, Northeastern Romania (Photo: Birsan C.)

In Bulgaria it was identified from the coastal region of the Black Sea and sporadically found in the city of Sofia, and the Vitosha and Rodopi Mountains, which was an extension of its habitat compared to the first discovery in 1936 in the Botanical Garden of Sofia (Hinkova, 1961). It was also identified from the northern slopes of Mount Sakar, in plantations of *Pinus nigra* and mixed forests of *Fraxinus* sp., *Quercus* sp., and *Pinus* sp., on sandy soils (Lacheva, 2015). Recently, in 2016, the species was discovered in three new localities in the southwestern part of Bulgaria on decaying plant material in *Platanus orientalis* and *Castanea sativa* forests. The fungus produced fruitbodies between May and November and was found at altitudes ranging between 10-600 m a.s.l. (Uzunov et al., 2016). The distribution of the species in Bulgaria is related to milder climates and high humidity in regions adjacent to the Black Sea (Assyov et al., 2010).

In the Ukraine, most reports of this species are from the Crimean region, especially from the Nikita Botanical Garden and neighboring areas. The first published report of *Clathrus ruber* from the Ukraine dates from June 12, 1961 (Zerova, 1962). The fungus was identified in deciduous (*Quercus* sp.) and mixed (*Pinus* sp. and *Quercus* sp.) forests, and frequently occurred in old parks and areas with strong anthropogenic influence (e.g. terrains in the Magarach Institute for Grapes and Wines, Yalta cargo port area). The increase in the records of this species on the southern coast of Crimea agrees with the opinion of mycologists regarding favorable habitat conditions for *Clathrus ruber* in regions around the Black Sea. In September 2017, fruitbodies were identified in the Ivano-Frankivsk region (Heluta & Zykova, 2019) from parks, plantations and natural forests, on clay and mostly basic soils with poorly developed or absent herbaceous vegetation. They were found mainly under the canopy of trees that maintained soil moisture, thus favoring their development. The period for producing fruitbodies in this region was from April to October, with the largest number found in August (Dudka, 2015).

In Serbia, *Clathrus ruber* was discovered in October 1983 during a botanical expedition. It is interesting that the substrate of the habitat in which the species occurred was ultra-alkaline; this was the only specimen found in such habitat in the former Yugoslavia regions (Ivancevic & Tatic, 2004).

In Turkey, the species was found in winter (December), in the province of Izmir, in olive tree plantations and during the summer (June-August) in the Sinop region of the Black Sea, in hardwood forests (Afyon et al., 2004; Yilmaz & Solak, 2004).

In Macedonia it is a rare species identified from five localities in Jakupica, Vodno, Pelister Mountains. In the village of Katlanovo at the St. Bogorodica Monastery the species has been identified in a *Querco-Carpinetum orientalis* forest at 200 m a.s.l., at the end of October (Karadelev et al., 2008).

For the present Note, we identified the species using macroscopic and microscopic characters described in the following literature: Breitenbach & Kränzlin (1986), Courtecuisse & Duhem (2013), Eyssartier & Roux (2013), Gerhardt (1999) and Tănase et al. (2009). The scientific name follows Index Fungorum database. One voucher specimen

from the examined material was deposited in the Herbarium [I] of the "Alexandru Ioan Cuza" University from Iaşi, Faculty of Biology [voucher I 185665].

The distinctive cage like fruitbody develops rapidly (in a matter of days) and cannot be confused with other stinkhorn species as *Clathrus archeri* (star-shaped fruitbody) or *Aseroe rubra* (presenting a stipe with a radiating ring with 6-10 bifid arms). In the first phase it is globular, white or yellow, in the shape of an egg ca. 2–3 cm long. With maturity it becomes cage-like, in the form of a red-orange network, fragile and spongy, with dimensions ranging from 6–10 (length) × 4–7 cm (diam.) (Fig. 1). At the base it has a soft spongy receptacle with a porous appearance. The fungus emits a cadaveric smell at maturity arising from the olivaceous–black gleba, arranged on the inside of the net. The spore cloud is white. The spores measure 5-6 × 2 µm, are more or less cylindrical to elliptical, without ornamentation (Breitenbach & Kränzlin, 1986; Tănase et al., 2009).

All species in the family *Phallaceae* have a gleba (spore-bearing inner mass) with a foetid odour in order to attract insects to facilitate the dispersal of spores. Spores stick to the body of the insects and others are consumed thus ensuring dissemination the species over possibly quite long distances (Fig. 1). Spores are dispersed by many insect species (e.g. near a specimen of *Clathrus ruber* in the Martinazo forest (Spain) an abundance of dipteran species and three beetles (Román, 2008) was noted).

The new Romanian record was registered from the *Lolium* sward of a private property in Valea Adâncă, a locality close to the city of Iaşi (geographical coordinates: 47.134286 N and 27.548369 E), at an altitude of 109 m a.s.l. The manner of introduction is not known. A total of 20 fruitbodies was identified, the first on 24th June 2020 while the last hatched on 17th October 2020. Taking into consideration that the owner has built a new house, it could be assumed that the species was brought in with construction material. It is the second report from Romania, after the first discovery in July–September 1961, when the species was identified in a private garden in the town of Eforie (Toma et al., 1962). In that report it is interesting that the fruitbodies were recorded from April to July, consisting of 10 specimens. The area where the mushroom was found in 1961 is influenced by the Black Sea, presenting a milder climate, typical for the coast, at an altitude of 20 m a.s.l., in a soil with permanent humidity (Fig. 2).

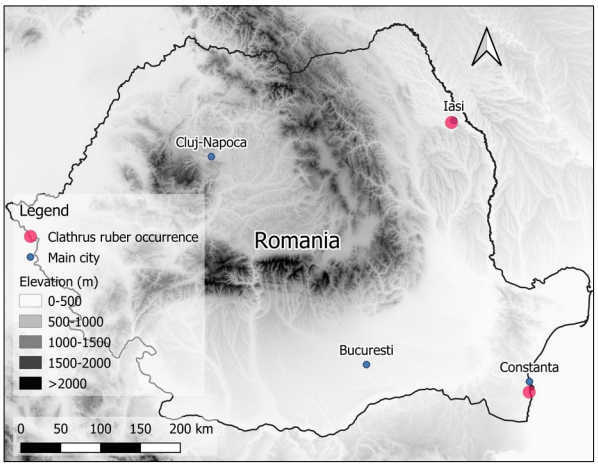

Fig. 2. *Clathrus ruber* occurrences in Romania (map created using the Free and Open Source QGIS, after SRTM digital elevation model).

From a climatic point of view, the new area where the fungus was identified in 2020, near the city of Iaşi, is characterized by an average annual temperature of 9.5 °C, an average annual rainfall of 585.8 mm and prevailing winds from North – Northwest (National Meteorological Agency, 2008). The occurrence of the *Clathrus ruber* species in the Iaşi area could be related to climate change, as both temperature and precipitations-related indices have shown significant modifications in the past decades, such as a decreasing number of days with low and moderate precipitation (Croitoru et al., 2015), and an increasing number of hot and humid days and nights in the area (Croitoru et al., 2012). Also, in the past years, an increase of mean annual temperature has been observed in comparison with the 1961-2009 period,

indicating a temperature positive anomaly (Sfîcă et al., 2017).

The altitudinal range of the localities where *Clathrus ruber* has been found in Romania is from 20 m to 109 m above sea level. *Clathrus ruber* is a thermophilic species, which could potentially be found on the sandy soils of southern Romania (Tănase et al., 2009). In other areas of Europe, it has been identified in deciduous and coniferous forests especially in southern regions of the Continent. It also sporadically occurs in parks, cemeteries, and gardens (Breitenbach & Kränzlin, 1986; Eyssartier & Roux, 2013), especially during the summer.

The free movement of population and merchandise in the European Union can advantage the spread of *Clathrus ruber*. This saprophytic species found mainly in parks and gardens also occurs in forest habitats on decaying plant matter. In the context of global warming, an expansion of the distribution of this species in new territories with favorable climatic and anthropogenic conditions is not unexpected. The intensification of anthropogenic pressure and the changes in land use have led to the decline of macromycete diversity in Europe, especially in developed countries of southern and western Europe (Senn-Irlet et al 2007). Parks and gardens are suitable habitats for introduced fungal species especially for those belonging to the *Phallaceae* that grow on mulch and rotting vegetal matter (Heluta & Zykova, 2019).

Acknowledgements

The authors are grateful to Mr. Răzvan Mocanu for bringing the species to their attention and for the permission to study this species on his property.

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Received: 14.07.2020
Accepted: 15.12.2020

ECOLOGIA BALKANICA
2020, Vol. 12, Issue 2 December 2020 pp. 219-233

Synopsis

The Current Environmental Law on Medical Waste and its Impact as Hazardous Pollutant – A Short Review

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Abstract. In order to guarantee the society a safe living environment, each state regulates rules and normative procedures for their implementation, in accordance with the established international ecological standards. In essence, their subject content covers a wide range of human activities, the secondary result of which is the generation of household, medical and industrial waste. In view of this review, we will examine the applicable national and European acts governing the storage, transport, disposal and incineration of medical waste. Such a regulatory analysis would make a positive contribution to the modernization of the national legal framework, in line with existing good environmental practices within the European Union.

Key words: environmental law, medical waste, environmental pollution.

Introduction

In order for a specific normative act to be fully applicable, it should be approved or transposed in accordance with the procedure provided by law and should regulate as much as possible an aspect of the procedural activity for which it has been approved. When we talk about medical waste, the starting point for regulating the activity should be the content of hazardous substances that pose a serious threat to the environment and human health. In this sense, the pandemic of Coronavirus disease 2019 (COVID-19) also contributed to the discussion and raised a number of issues concerning the direct and immediate consequences for the environment, as a result of the introduced requirements for the use of personal protective equipment worldwide. Disposable masks and gloves used by the public should be treated in accordance with international standards, as medical waste. This raises the question of the extent to which the member states of the European Union, including the Republic of Bulgaria, have managed to create conditions for limiting environmental pollution from the increased amounts of medical waste generated daily by medical and healthcare institutions and society. In order to adequately assess the risk to the environment and biodiversity, in the event of unregulated/unauthorized disposal and storage of medical and biomedical waste as a residual product of medical activity, attention should be paid to the basic European and national legal framework of developed countries, for comparison and formulation of relevant proposals for optimization of the national regulation. Such

an overview would have its practical usefulness in terms of optimizing our national legislation, given the reported gaps and weaknesses in the regulations of these countries.

Common regulatory framework for the management and legal regulation of medical waste within the European Union

The European regulatory framework is a key foundation for the harmonization of procedures and rules in the Member States on medical waste. From a legal point of view, there are a number of council regulations and directives that change with the development of science and technology to meet the needs of modern societies. In the light of this review and timeliness, the most relevant acts transposed into the legislation of most of the Member States will be considered.

- *Council DIRECTIVE 90/385/EEC on Active Implantable Medical Devices (AIMDD) (1990).* The directive regulates all issues concerning implantable medical devices. The basic concepts have been defined, procedures have been outlined regarding their use, storage and destruction. The texts are also in line with the technological progress of the member states in the field of this type of medical devices. Basic concepts are defined in the context of the diversity of this type of medical devices.

- *Council DIRECTIVE 93/42/EEC on Medical Devices (MDD) (1993).* It regulates the use of medical devices and in particular *in vitro* diagnostic medical devices, including reagent, reactive product, calibrator, control material, kit, instrument, apparatus, equipment or system, whether used alone or in combination, intended by the manufacturer to be used *in vitro* for the examination of samples, including donations of blood and tissues obtained from the human body, solely or principally for the purpose of providing information:

- regarding a physiological or pathological condition;

- regarding a congenital anomaly;

- to determine safety and compatibility with potential recipients, or to monitor therapeutic measures.

- *DIRECTIVE 98/79/EC of the European Parliament and of the Council on in vitro Diagnostic Medical Devices (IVDMD) (1998).* It complements the content of Directive 93/42/EEC on Medical Devices concerning the part for self - testing devices. Requirements have been introduced regarding the obligation of Member States to monitor the safety and quality of these devices, as well as to specify the method of disposing of used disposable tests. This Regulation sets high standards of quality and safety for *in vitro* diagnostic medical devices in order to meet the general safety expectations for such products.

- *DIRECTIVE 2000/532 / EC: Commission Decision of 3 May 2000 replacing Decision 94/3 / EC establishing a list of wastes pursuant to Article 1 (a) of Council Directive 75/442 / EEC on waste and Council Decision 94/904 / EC establishing a list of hazardous waste pursuant to Article 1 (4) of Council Directive 91/689 / EEC on hazardous waste (notified under document number C (2000) 1147).* The directive regulates the laws, regulations and administrative provisions related to the classification, packaging and labeling of dangerous substancesA list of wastes in accordance with Article 1 (a) of DIRECTIVE 75/442 / EEC on waste and Article 1 (4) of DIRECTIVE 91/689 / EEC on hazardous waste is also annexed to the Directive. The list is harmonized according to the national laws of the member states and is applied in full by each of them.

- *Regulation (EU) 2017/745 of the European Parliament and of the Council of 5 April 2017 on medical devices, amending Directive 2001/83/EC, Regulation (EC) No 178/2002 and Regulation (EC) No 1223/2009 and repealing Council Directives 90/385/EEC and 93/42/EEC.* This regulation aims to ensure the smooth functioning of the internal market for medical devices, based on a high level of protection of the health of patients and consumers. It sets high standards for the

quality and safety of medical devices in order to meet the general safety requirements for such products. This Regulation harmonizes the rules for the placing on the market and commissioning into service of medical devices and their accessories on the Union market, thus enabling them to benefit from the principle of free movement of goods. As regards Article 168 (4) (c) of The Treaty on the functioning of The European Union(TFEU), this Regulation lays down high standards of quality and safety for medical devices, as well as ways to safely recycle or dispose of them, in order to ensure a safe and healthy environment.

- *Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017 on in vitro diagnostic medical devices and repealing Directive 98/79/EC and Commission Decision 2010/227/EU.* This Regulation establishes high standards of quality and safety for *in vitro* diagnostic medical devices in order to meet the general safety expectations for such products. The Regulation provides guidelines developed for *in vitro* diagnostic medical devices at international level, in particular in the context of the Global Working Group on Harmonization and the Follow-up Initiative, the International Forum of Medical Device Regulators, to promote global regulatory convergence, which contributes to for a high level of global safety protection and for trade facilitation, in particular in the provisions on the unique identification of the device, general safety and performance requirements, technical documentation, classification rules, conformity assessment procedures and clinical evidence.

- *DIRECTIVE (EU) 2018/851 of the European Parliament and of the Council of 30 May 2018 amending Directive 2008/98/EC on waste.* Improving resource efficiency and ensuring that waste is valued as a resource can help reduce the Union's dependence on imports of raw materials and facilitate the transition to more sustainable materials management and a circular economy model. This transition must contribute to the goals of smart, sustainable and inclusive growth set out in the Europe 2020 strategy and create important opportunities for local economies and stakeholders, while helping to increase the synergies between the circular economy and policies in the field. energy, climate, agriculture, industry and research, as well as bring environmental benefits in terms of greenhouse gas savings and the economy. Consistency is envisaged between Directive 2008/98/EC and related Union legislation such as Directive 2009/28/EC of the European Parliament and of the Council (5) and Regulation (EC) № 1907/2006 of the European Parliament and of the Council. The directive also states that many Member States have not yet fully developed the necessary waste management infrastructure. It is therefore essential to set clear long-term policy objectives in order to target measures and investments, in particular by preventing the creation of structural overcapacity for residual waste treatment and blockages of recyclable materials at lower levels of the hierarchy of waste. The directive also states that waste management requires a very complex system, including an efficient collection scheme, an effective system for sorting and properly tracing waste streams, active participation of citizens and businesses, infrastructure adapted to the specific composition of waste, and a complex financing system.

- *DIRECTIVE 2000/532/EC: Commission Decision of 3 May 2000 replacing Decision 94/3/EC establishing a list of wastes pursuant to Article 1 (a) of Council Directive 75/442/EEC on waste and Council Decision 94/904/EC establishing a list of hazardous waste pursuant to Article 1 (4) of Council Directive 91/689/EEC on hazardous waste (notified under document number C (2000) 1147).* The directive stipulates that all waste included in the list of hazardous waste must be included in the European waste catalog defined in Commission Decision 94/3/EC. In order to increase the transparency of the enumeration

system and to simplify the existing provisions, a Unified European Community list should be established, including the list of waste set out in Decision 94/3/EC and that of hazardous waste set out in Decision 94/904/EC requires full harmonization of the general categorization of hazardous waste within the EU. Article 2 of the Directive states that Member States may decide, in exceptional cases, on the basis of documentary evidence duly provided by the holder, that specific wastes listed as hazardous do not show any of the properties listed in Annex III to Directive 91/689/EEC. Without prejudice to the second indent of Article 1 (4) of Directive 91/689/EEC, Member States may decide, in exceptional cases, that the wastes listed as non-hazardous show one or more of the properties listed in Annex III to Directive 91/689/EEC. All such decisions taken by the Member States shall be announced annually at sessions of the Commission(EC). It shall compare these decisions and examine whether the Community list of wastes and hazardous wastes should be amended or supplemented.

- *DIRECTIVE 2000/76/EC of the European Parliament and of the Council of 4 December 2000 on the incineration of waste (OJ L 332, 28.12.2000, p. 91*). Points out that the Protocol on Persistent Organic Pollutants, signed by the Community in the framework of the United Nations Economic Commission for Europe (UN/ECE) Convention on Long-range Transboundary Air Pollution, sets legally binding limit values for dioxins and furans emissions of 0,1 ng/m^2; TE (toxic equivalents) for installations incinerating more than 3 tonnes per hour of municipal solid waste, 0.5 ng/m; TE for installations burning more than 1 tonne per hour of medical waste and 0.2 ng/m; TE for installations burning more than 1 tonne per hour of hazardous waste. The Protocol on Heavy Metals, signed by the Community in the framework of the UNECE Convention on Long-range Transboundary Air Pollution, sets legally binding limit values for particulate emissions of 10 mg/m^3 for the incineration of hazardous and medical wastes and for emissions of 0.05 mg/m^3 mercury for hazardous waste incineration and 0.08 mg/m^3 mercury for municipal waste incineration. The International Agency for Research on Cancer and the World Health Organization indicate that some polycyclic aromatic hydrocarbons (PAHs) are carcinogenic. Therefore, Member States may set emission limit values for PAHs among other pollutants. Furthermore, Article 174 of this Directive provides that Community policy on the environment is to contribute to protecting human health. Paragraph 16 of the Directive clarifies that „The distinction between hazardous and non-hazardous waste is based mainly on the characteristics of the waste before incineration or co-incineration and not on the differences between emissions. The incineration or co-incineration of hazardous and safe waste should be subject to the same emission limit values, but it is appropriate to maintain different incineration techniques or conditions or different co-incineration and different measures to verify the acceptance of different wastes".

Comparative analysis of the legal acts regulating the procedures for medical waste management in the Republic of Germany, the Hellenic Republic, the Republic of Hungary and the Republic of Bulgaria

Germany. In Germany, the management of medical waste is regulated by a number of regulations that aim to cover relatively in detail the procedures for the storage and disposal of medical waste**.** In essence, these acts represent a fundamental plane on the basis of which the management activity is regulated, as well as the subsequent administrative control is exercised in view of regulatory requirements. The Basic Law is the Closed Cycle Waste Management Act, which regulates the prevention, recycling, reuse and disposal of waste (Kreislaufwirtschaftsgesetz vom 24. Februar 2012 (BGBl. I S. 212), das durch § 44 Absatz 4

des Gesetzes vom 22. Mai 2013 (BGBl. I S. 1324) geändert worden ist.). There are also a number of bylaws - regulations and instructions related to the regulation of control over the spread of infections and safety in medical and health care facilities. (Hansen et al., 2014). Procedures are also provided, in the German Dangerous Goods Regulation and the Federal Pollution Control Act, for the transport of medical waste from hospitals to landfills for storage and disposal and destruction. (BImSchG 2013: Bundes – Immissionsschutzgesetz in der Fassung der Bekanntmachung vom 17. Mai 2013 (BGBl. I S. 1274), das durch Artikel 1 des Gesetzes vom 2. Juli 2013 (BGBl. I S. 1943) geändert worden ist, 2013). Germany, as well as a number of other European countries, including Bulgaria, have adopted the standards of division according to the European Waste Catalog, in view of which the lists of hazardous and non-hazardous medical waste are separated in bylaws according to the domestic legislation of each country. (2000/532/EC: Commission Decision of 3 May 2000 replacing Decision 94/3/EC establishing a list of wastes pursuant to Article 1(a) of Council Directive 75/442/EEC on waste and Council Decision 94/904/EC establishing a list of hazardous waste pursuant to Article 1(4) of Council Directive 91/689/EEC on hazardous waste (notified under document number C (2000) 1147). Based on the brief regulatory review of the applicable legislation in the field of medical waste management in the Republic of Germany, it can be concluded that the frequency of audits of waste storage areas should be increased, as well as strict compliance with the adopted rules in the by-laws, according to the safety requirements of the persons working with medical waste (Hansen et al., 2014).

Greece. Unlike a number of European countries, significant progress has been made in the legislation of the Hellenic Republic on medical waste management. With the issuance of the first ministerial decree on medical waste management in 2003, the modernization of the legislation in this field began. The European Directive 2008/2009 „Crimes in the field of the environment" has also been transposed, which marks the beginning of the criminalization of certain types of crimes committed by officials responsible for the management of medical waste in the units entrusted to them. n 2012 Law N4042/2012 – „Framework for waste from production and management" was adopted. The Law contains the Annex with the European Wastes Catalogue (EWC) which isa hierarchical list of waste description established by Commission Decision 2000/532/EC. With its adoption, the Ordinance on Hospital Waste Management (KYA 146163/2012 (ΦΕΚ1537/B/2012)) was approved, in which the following classification for effective management was adopted:

1.Prevention of creation;
2.Preparation for reuse;
3.Recycle;
4.Recovery of raw materials or energy;
5.Disposal.

Explicit prohibitions have also been introduced with the ordinance for storage or disposal of medical waste without supervision (Halazonitis, 2015). At present and in view of the brief overview of the applicable regulations in the Hellenic Republic on medical waste, the following conclusions can be drawn:

- Despite the adapted domestic legislation of Greece in accordance with the European management requirements, the country encounters difficulties in auditing and controlling the management of medical waste generated in the medical establishments located on Its islands.

- The territorial remoteness of these medical and health units increases the cost of transporting medical waste and creates preconditions for the formation of illegal practices that would lead in certain cases to environmental disasters. In this sense, it would be reasonable to strengthen the administrative and control function of local

authorities with regard to the audit of medical waste storage and management procedures.

Hungary. In Hungary, the main piece of legislation governing hazardous waste management is Act CLXXXV of 2012 on waste. The Act covers all wastes, all preventive activities, waste management and waste management facilities. A basic decree in the field of hazardous waste management, and in particular medical waste Decree No. 225 of 2015 (VIII.7)- regulates all activities related to the carriage, distribution, storage and classification of HW. Government decree No. 439 of 2012 (XII.29.) (on the registration and authorization of waste management activities) points out, that waste can only be handed over to authorized intermediates and traders. Formal requirements on the request for permission on related activities can be found in this Decree as well. The competent authority for general permissions is the National Inspectorate for Environment and Nature. Decree No. 1 of 2002 (I. 11.) of the Ministry of Health on waste management in medical institutes regulates the storage of such waste and the quality requirements of the appropriate packings. Infectious medical waste can be stored no longer than 48 hours without cooling, no longer than 30 days in case of storage in dedicated refrigerator, between 0-5 °C. Annex 4 rules the quality requirements on tools used for related activities. Strict regulations regarding the transport of radioactive materials as a residual product have also been adopted. The regime for carrying out activity is mandatory licensing. The main transport mode is road. In most of the cases, the industrial companies arrange their own deliveries, medical institutes mainly contract with a professional forwarder.

Compared to the legal regulation of Greece and Germany in the field of medical waste management, Hungary is significantly less adapted in view of the adopted European standards supplementing by transposing the procedures of domestic legislation. Impressive is the limited range of normative documents that regulate the main issues of the activity of medical waste management. An update in this sense was made in the field of pharmaceutical waste in 2017.

From the review of the regulations relevant to the issues under consideration, it can be concluded that for the most part the procedures related to the regulation of medical waste in Hungary overlap with the adopted general rules for handling hazardous waste. From the current regulatory review, it is to be noted that, unlike Bulgaria, Hungary has not fully adopted the transposition of the European Waste Catalog, which to some extent creates differences regarding the components and categorization of medical waste.

When we talk about state environmental policy, we should keep in mind that in essence these are a set of goals, principles and practical approaches to solving environmental problems of modern society, which are regulated in regulations and programming documents adopted by the state (Penchev, 2017).

Bulgaria. In view of the subject of this review, we will consider in detail the regulatory framework of the Republic of Bulgaria adopted in the field of medical waste. Among the main normative acts is the Environmental Protection Act (EPA). Article 5 specifies the factors that pollute or damage the environment, and can be: natural and anthropogenic substances and processes; different types of waste and their locations; risky energy sources - noise, vibration, radiation, as well as some genetically modified organisms. Chapter Three - „Protection and Use of Environmental Components and Waste Management" of EPA and in particular Article 44 set out requirements regarding the owners and operators of landfills, including tailings, ash dumps and others, as well as of facilities for storage of waste and / or hazardous chemicals, preparations and products and how to organize and operate them in a way

that excludes pollution and damage to soil and other components of the environment. In section seven - „Waste management" of the said law are set out in three provisions the basic requirements that should be met in waste management processes. In essence, the Environmental Protection Act sets a general regulatory framework covering the scope of waste as a residual product of human activity, without being formalized in a specific area of waste.

Next comes The Waste Management Act (WMA) - according to Article 1, paragraph 3 „waste management is carried out in order to prevent, reduce or limit their harmful effects on human health and the environment. "Chapter three of the law regulates the procedures for treatment and transportation of waste. Permits for carrying out activities for recovery and/or disposal of waste, including pre-treatment before recovery or disposal, shall be issued by the director of the regional inspectorate for environment and water on whose territory the activities are carried out or by the Minister of Environment and Water, when the activities are carried out on the territory of more than one regional inspectorate for environment and water. Section five provides that shipments of waste within the European Community with or without transit through third countries, imports into the Community from third countries, exports from the Community to third countries and transits through the Community to and from third countries of waste shall be carried out under the conditions and by the order of Regulation 1013/2006. Regulation 1013/2006 regulates the requirements for notifications for transportation of waste from, to and through the territory of the Republic of Bulgaria, for import or export from or to countries that are not members of the European Union. Chapter Six, Section Two also provides for the forms of administrative violations, as well as the penalties provided for them.

Last but not least in the field of waste management, and in particular medical waste, is the Law on Ratification of the Convention on the Control of Transboundary Movements of Hazardous Wastes and Their Disposal (the Basel Convention) - Article 2, paragraph 1, of the Basel Convention states that „Waste" means substances or objects which are disposed of or are intended to be disposed of, or must be disposed of in accordance with the provisions of national law. Definitions of „environmentally sound management of hazardous or other wastes" have also been introduced, which includes taking all practical action to ensure that management of hazardous or other wastes is such as to protect human health and the environment against the harmful effects of such wastes. The Convention introduces a six-month period for each of the ratifying parties and parties to it to harmonize their categories and definitions in domestic legislation with those provided for in Annex 1 and Annex 2, which are an integral part of the Basel Convention. In this sense, the Republic of Bulgaria has unified all required definitions according to the cited annexes, upon ratification of the Convention.

By-laws, the content of which deals in detail with all relevant regulations related to medical waste management.

Ordinance 1/2015 on the requirements for the activities for collection and treatment of waste on the territory of medical and health establishments, determines in general the circle of the subjects generating medical waste - medical and health establishments. Of course, the division is not exhaustive and in a more detailed analysis can be distinguished subcategories of these two main groups, namely - doctors' offices, dental offices, veterinary facilities, medical laboratories, research centers, etc. According to their nature of work, these entities generate in their daily activities different types of medical waste, which according to the regulations can be classified as hazardous and non-hazardous waste. Experience has shown that if the legal requirements for the management of

hazardous medical waste are met, they do not pose a greater danger to health than non-hazardous waste, and vice versa - their improper management can lead to a significant increase in health risk for the people and the environment (Chartier et al., 2014).

Medical waste from medical and/or healthcare facilities that does not have hazardous properties can be classified into subgroups in general as follows:

- ''Household"/ urban waste;
- Paper, cardboard, plastic, including wrappings;
- Glass and plastic cans of saline, not used in patient transfusion or other infusion systems;
- Ampoules and vials other than those of used vaccines and cytotoxic and cytostatic medicinal products;
- Metal packagings, other than those containing residues of dangerous substances/mixtures or contaminated with dangerous substances/mixtures;
- Bandages, plaster casts, bed linen, clothing and disposable linen, not contaminated with biological fluids, and diapers, excluding waste generated by the operation of isolators in infectious wards/clinics and laboratories for particularly dangerous infections;
- Food waste, excluding food waste from infectious hospitals and clinics/wards.

It should be noted here that the non-hazardous waste, separated from the medical and health establishments, as a waste product of their activity, is managed as household waste and is deposited onto the landfills for non-hazardous waste, after separation of the materials suitable for reuse and recycling – Waste processing is in fact a process in which waste is recycled to produce a raw material that can be used to produce new products. It is an indisputable fact that waste has been a major environmental problem for decades or more precisely since the beginning of the industrial revolution. he recycling process contributes to saving raw materials, on the one hand, and reducing waste and environmental pollution, on the other hand, and therefore recycling technologies are a priority in the policies of public and private companies in environmental terms.

Hazardous waste from medical and/or healthcare facilities are:

- Biomedical:

• Biological medical waste - body parts and organs and other anatomical waste, including blood, biological fluids and pathological waste, which may be distinguished as such by citizens or medical staff and for which, for ethical reasons, a specially treated.

• Laboratory waste - crops and strains containing viable biological agents formed in health establishments operating in the field of hygiene, microbiology and virology, as well as in medical establishments where the multiplication of pathogenic microorganisms may occur, as well as vessels and utensils used for the transport, inoculation and mixing of cultures of infectious agents and infected animals from laboratories.

• Waste contaminated with blood and biological fluids - medical devices and equipment contaminated with blood, blood products, secretions and excreta, whether or not previously tested and categorized as infectious waste that are reasonably believed to carry a potential risk of transmission of infectious agents (bandages, tampons, syringes without needle, infusion sets without needle, bandages, contaminated sheets, underwear, gloves and disposable aprons, etc.).

- Sharp waste - all medical waste with sharp or pointed and/or cutting parts that can cause injury, trauma or cutting/breaking the integrity of the skin of the human body (such as used needles, drainage tubes, syringes with a needle, broken glassware, ampoules, pipettes, scalpel blades, lancets, etc.).

- Waste containing dangerous chemical substances/mixtures - waste from diagnostics, experimental work, cleaning and disinfection activities:

- residues of chemicals/mixtures, fixing solutions, solvents, biocides (disinfectants) and cleaning agents, organic and inorganic chemicals/mixtures;
- unusable batteries containing heavy metals (such as mercury, cadmium, etc.);
- diagnostic instruments and consumables containing heavy metals (such as mercury, cadmium, etc.);
- wastes from dental amalgam, including amalgam residues, particles and fillings, including those contained in used water, as well as teeth or parts thereof contaminated with dental amalgam.

With regard to hazardous medical waste, according to „Ordinance No. 2/2014 on waste classification", potentially infectious waste is classified with code 18 01 03 * - waste, the collection and disposal of which is subject to special requirements in order to prevent infections. Waste Management Act formulates the property H9 - infectivity the property H9 - infectivity. Wastes with this property contain „vital micro-organisms or their toxins which are known or reasonably believed to cause disease in humans or other living organisms". Undoubtedly, such data are of potential interest for periodic monitoring by the competent state authorities, as well as by the World Health Organization (WHO). A safe and healthy environment is one of the fundamental factors for the stability of any society and any ecosystem worldwide. Medical waste is classified as hazardous on the basis of one or a combination of several of the following properties: content of infectious microorganisms; content of toxic chemicals having oxidizing, irritating, flammable properties, etc.; radioactivity, content of sharp objects, etc. The need for safe management of biomedical and medical waste arises from the presumed or actual risk of potential transmission of infectious diseases through accidental injury or contact with infected biological fluids. Disposal of sharp objects (needles, scalpels, etc.) attracts special interest due to the small number of cases of infection of medical workers with hepatitis and human immunodeficiency virus (HIV) due to injuries with sharp objects. Therefore, reducing the risk of injury is a „good practice" in waste management.

For the difference between hazardous and non-hazardous medical waste and the consequences for human health in their improper management and their impact as a pollutant on the environment.

Based on officially released data from the WHO, Safe Management of Wastes from Health-care Activities - Second Edition, by 2019, the most common infectious diseases that occur as a result of improper management of medical waste are presented in Table 1 (Practical guide for safe waste management from medical and healthcare facilities, et, al. 2017). The infections described in Table 1 illustrate the possible negative consequences that can occur directly on human health, if stored improperly, in accordance with the requirements of the regulations on body fluids that can infect waste.

Different concepts and approaches are used in the definition and classification of biomedical and medical waste in the world, especially with regard to infectious waste. The World Health Organization (WHO) follows the concept of „universal precautions", which describes a set of measures formulated to prevent the transmission of contagious diseases. Definitions and criteria for the identification of infectious substances are an area in which international harmonization is important and cooperation with the WHO and the UN Commission of Experts on the Transport of Dangerous Goods plays an important role. (Peiry, 2000). In this regard, the WHO proposed in 2002. the so-called „Universal precautions to prevent the transmission of HIV and other blood-borne infections". These measures eliminated the need for the isolation category - „Precautions in case of contact with blood and biological fluids„. However, the application of universal precautions does not eliminate the need for other isolation precautions, such as measures to protect against airborne infections in the case of influenza, air isolation in cases of pulmonary tuberculosis or contact isolation in methicillin-resistant strains of *Staphylococcus aureus*.

Table 1. Consequences for human health, due to improper management of medical waste.

Type of infection	Examples of causes	Body fluid infecting the waste
1. Respiratory	*Mycobacterium tuberculosis*, *Streptococcus pneumoniae* and other *bacteria*; viruses causing Severe acute respiratory syndrome (SARS).	Secretions from the respiratory tract, saliva.
2. Meningitis	*Neisseria meningitidis*	Cerebrospinal fluid (CSF)
3. Acquired Immunodeficiency Syndrome (AIDS)	Human immunodeficiency virus (HIV)	Blood and other body fluids
4. Viral hepatitis B and C	Hepatitis B and C viruses	Blood and other body fluids
5. Genitourinary	*Neisseria-gonorrhoeae, Herpesvirus*	Urinary secretions and urine

Table 2 presents the pollutants that are released in the form of chemical elements in the air, water and soil during the decomposition and incineration of medical waste as a residual product. The basic data are taken from the special report of the European Court of Auditors „Air pollution - our health is still not sufficiently protected". The report has been drawn up in conjunction with the second subparagraph of Article 287 (4) TFEU. Based on the synthesized analysis presented in the table below, it can be concluded that even in compliance with the normatively defined rules for disposal and destruction there are risks of environmental pollution. Undoubtedly, they are minimized and evaluated by experts as relatively safe for human health. But in the event of unauthorized disposal, the consequences would lead to a serious environmental catastrophe and permanent damage to human health. Regarding the purity and quality of the air in the Republic of Bulgaria, of interest is Case C-488/1 of the European Court in Luxembourg (2017), by whose decision our country was sentenced to pay the financial sanctions related to the systematic and constant non-compliance from 2007 to 2014. including both annual and daily limit values applicable to PM10 concentrations in the following zones and agglomerations: BG0001 Sofia agglomeration, BG0002 Plovdiv agglomeration, BG0004 North, BG0005 Southwest and BG0006 South East.

Methodology for collection, deposit and dissolution of medical waste

The wide variety of types of medical waste classified in these groups, in essence, imply a different methodology for collection, storage, deposit and dissolution. Such a requirement has its logical character, in view of the content and the final residue of the product after its dissolution, incineration or recycling. A basic principle in medical waste management is to preventing generation and searching for opportunities to reduce the amount of waste generated. This principle is accepted for all types of waste in the member states of the European Union. Prior to the final disposal of the waste, the possibilities for reuse and recycling must be used by pre-separating the non-hazardous (household) from the hazardous medical waste (Rushbrook, 2000; Rushbrook & Zghondi, 2004).

Separate waste collection and in-hospital transport - the safe separation of medical waste is the responsibility of the staff of the medical institution. Regarding the color coding of waste containers, Ordinance 1/2015 on the requirements for the activities for collection and treatment of waste on the territory of medical and health establishments regulates the following requirements:

Table 2. Environmental consequences in case of regulated destruction of medical waste and residual elements that are released during the disposal and incineration of waste.

	Atmospheric pollution from incineration of medical waste	**Soil contamination due to improper storage of medical waste**	**Water pollution from improper storage of medical waste**
1.	The pollution is anthropogenic. According to the origin of the pollution: Primary - disposed of directly from identifiable sources. This group includes CO, SO_2, H_2S, NO, NH_3, a small amount of NO_2, hydro-carbons, dust, smoke, soot and others.	Contamination is most often caused by improper disposal and burial of waste. The sites are divided into three categories: landfills for hazardous waste, landfills for safe waste and landfills for inert materials.	The pollution can be physical, chemical or biological.
2.	The main pollutants are carbon monoxide (CO), sulfur compounds (SO_2, H_2S, CS_2), nitrogen compounds (NO_x, NH_3), hydrocarbons (CH), ozone (O_3), photochemical smog and finely divided substances (dust).	The main soil pollutants are metals, a number of organic molecules, pathogens, biologically active mate-rials.	Contaminants are mainly microorganisms, including highly pathogenic microorganisms such as *E. coli, Salmonella sp*. and others. In industrial wastewater, harmful impu-rities in them are very diverse - from abiotic to microbial pollution.
3.	The consequences for the atmosphere during the incineration of waste are expressed in dust and gaseous pollutants. Gaseous pollutants can be divided into organic and inorganic (Guide for pre-treatment before waste disposal in the Republic of Bulgaria, 2014)	The consequences for the soil and groundwater would be irreparable in certain cases of contamination with radioactive medical waste.	The main sources of surface water pollution can be summarized as follows: by physical state are divided into: insoluble, colloidal and dissolved impurities. By their nature they are divided into: mineral, organic, bacterial and biological (Ivanov, 2015).

- non-hazardous waste is collected in transparent polyethylene bags/sacks, placed in containers with lids and pedals, marked with the inscription „Non-hazardous waste".

- hazardous waste is collected separately.

- biomedical waste, with the exception of biological waste, is collected in yellow plastic bags/sacks placed in containers with lids and pedals and marked with the international hazardous waste symbol.

- biological medical waste is collected separately in red plastic bags/sacks/bags.

All used needles are collected together, whether contaminated with biological fluids or not. They are classified as hazardous waste. Containers for collection of used needles must be yellow with the international symbol for hazardous waste, be impenetrable (usually made of high-density plastic) and covered with a lid. These containers must be tight and impermeable so

as to retain not only the needles but also the liquids left in the syringes. In cases where disposable syringes are used, the uncontaminated packaging should be disposed of in the non-hazardous waste container and the used syringe itself in the sharps container (Practical guide for safe waste management in medical and healthcare facilities).

Regarding the methodology for safe storage, collection and disposal of biomedical waste, the legislation of the Republic of Bulgaria provides for the following requirements:

- non-hazardous waste, are collected in collected in transparent plastic bags/sacks, placed in containers with lids and pedals, marked with the inscription «Non-hazardous waste»;

- *hazardous waste* is always collected separately. Biomedical waste, with the exception of biological waste, is collected in yellow plastic bags/sacks placed in containers with lids and pedals and marked with the international symbol for hazardous waste. Biological medical waste is collected separately in red plastic bags/sacks/bags.

- small amounts of chemical or pharmaceutical waste may be collected together with infectious waste using yellow containers. Waste with a high content of heavy metals (eg cadmium or mercury) must be collected separately.

- pharmaceutical products that will not be used or have expired should be collected by the medical establishment until they are returned to the supplier, importer or manufacturer for further disposal.

Containers and bags for waste should be located in each structural unit of the medical institution, and they should be accompanied by written instructions for separation and identification of medical waste in the two groups „hazardous“ and „non-hazardous“.

In order to limit the possibility of environmental pollution, normative rules have been introduced for the transportation of hazardous medical waste on the public road network. This type of waste should be transported in closed secondary packaging in accordance with the method of waste treatment. Secondary packaging (stationary and/or transportable vessels/containers) should be made of rigid impermeable material that does not allow puncture/breakage and meet the requirements of "Ordinance № 40 of 2004 on the terms and conditions for road transport of hazardous goods" and should be marked with the international hazardous waste symbol (Rushbrook, 2000; Rushbrook & Zghondi, 2004).

Treatment and disposal of hazardous medical waste

In view of the entities involved in the waste management process - waste generators; transport and disposal operators; control authorities, good collaboration should be in place to minimize the potential for unregulated pollution or environmental catastrophe. The quantities of non-hazardous medical waste represent 80-85% of the total quantities of all medical waste. Hazardous fractions occupy between 10-15% of the medical waste. The treatment of non-hazardous waste is done by landfilling the designated landfills and they are recycled or incinerated in the facilities for non-hazardous waste. In the case of hazardous waste, the treatment and disposal aims at (WHO, 2018):

- destruction of pathogenic microorganisms;

- destruction/transformation of residual pharmaceuticals and drugs into safe components;

- destruction of sharp and cutting tools and other technical means that can cause physical injuries;

- final disposal/destruction of biological medical waste and other organic materials.

The most common practices in the destruction of hazardous fractions are landfilling, incineration, autoclaving, microwave irradiation, chemical disinfection.

Conclusions

Based on the analysis made in relation to the impact of pollution of medical waste on the environment and the regulations governing the procedures, the following conclusions were drawn:

1. Hazardous medical waste, due to its content of vital microorganisms or their toxins, which can be considered to cause diseases in humans and other living organisms, is the most serious threat to the environment and biodiversity. With the development of European practices in this direction, the national rules and requirements that regulate and control its destruction should be updated. At present, they do not fully comply with established European practices, which creates preconditions for unregulated practices in order to save financial resources.

2. It would also be relevant to review the control powers of the bodies exercising supervision and control over the operators exercising the activity of disposal and destruction of medical and biomedical waste.

3. Checks carried out by the competent authorities should increase their frequency and not be carried out solely on a signal from the institutions or the citizens concerned. Such a practice would impose better control to the behavior and actions of operators treating and disposing of waste, medical institutions and all entities involved in the process.

4. It would be relevant to reconsider the size of the sanctions when we talk about imposing an administrative penalty on violators. The minimum thresholds of restriction create a precondition for neglect of the rules and good practices by the violators.

5. The commissions for ecology and communal activity on the ground, as well as the National Center for Public Health should exercise more actively their powers by regions, in order to prevent preconditions for the formation of pollutants by waste agents. It would be reasonable to establish a register of operators engaged in the disposal of medical waste, in order to exercise more effective control over their activities.

Acknowledgements

This scientific publication is realized under the national program „Young scientists and postdocs" by the Ministry of Education and Science of the Republic of Bulgaria.

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Received: 26.09.2020
Accepted: 12.10.2020

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. 12, iss. 2, 2020 of Ecologia Balkanica.

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

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The standard order of sections should be: Abstract, Key words, Introduction, Material and Methods, Results, Discussion (or Results and Discussion), Conclusions (optional), Acknowledgements (optional) and References.

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

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In-text references must be included following the use of a quote or paraphrase taken from another piece of work. **Direct copy-paste from another source is not acceptable!** Submitted manuscripts will be pre-checked for plagiarism and auto-plagiarism. In-text citations are citations within the main body of the text and refer to a direct quote or paraphrase. They correspond to a reference in the main reference list. These citations include the surname of the author and date of publication only. For example: Smith (2017) states… Or …(Smith, 2017). In case of two authors: the surname of both authors is stated with an ampersand between. For example: Smith & Smith (2017) state… Or …(Smith & Smith, 2017). In case of three or more authors add „et al." after the first author's surname (*et alii*, from Latin means „and others"): Smith et al. (2017) state… Or …(Smith et al., 2017).

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