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ECOLOGIA BALKANICA – Volume 12, Issue 1 / 2020

CONTENTS

Research Articles

Short notes

Synopses

ECOLOGIA BALKANICA

2020, Vol. 12, Issue 1 June 2020 pp. 1-10

Relationships Between Soil Characteristics and Concentrations of Lead and Zinc in Soils and in Oriental Tobacco

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Abstract. Increasing efforts to curb tobacco use worldwide have been made in recent years. Harmful substances in tobacco raw material and cigarettes have been the primary focus of attention. Obtaining information regarding the relationship between soil characteristics and the absorption of heavy metals by tobacco plants could improve the estimations for metal uptake by the plants and the associated health risks with exposure to tobacco smoke. This study was conducted in the Eastern, Central, and parts of the Western Rhodope Mountains using Oriental tobacco plants. The determined soil characteristics were: pH, humus content, and texture. Inductively coupled plasma atomic emission spectroscopy was used to measure the concentration of Pb and Zn in mature tobacco leaves, and the total content and mobile forms of the same elements in the soil. The correlation/regression analyses showed statistically significant linear relationships between the total content and mobile forms of Pb and Zn in soils. The concentration of Zn in tobacco leaves was linearly proportional to the Zn content in soils. The power model adequately reflected the relationship between the Pb total content and mobile forms in soils. The relationships between clay content and the concentrations of Pb and Zn in tobacco leaves were statistically significant.

Key words: zinc, lead, heavy metals, oriental tobacco, soil.

Introduction

Bulgaria is a traditional producer of Oriental tobacco and despite problems in recent years, the tobacco sector continues to be of strategic importance to the country's economy. Oriental tobacco originated from tobacco plants imported through Persia directly from America. They have been planted in the poorest regions in arid climate conditions. *Nicotiana tabacum* L. is a mesophyte that has adapted to dry environments. Thus, two opposing adaptations were combined - those of mesophytic and xerophytic plants. The manifestation of one or the other depends on the water regime during growth and development (Donev et al., 1981).

There has been a lot of pressure in recent years to curb tobacco smoking worldwide. Much attention has been paid to the harmful substances in tobacco and cigarettes. Specialists have directed their research efforts to the alternative uses of tobacco. It has been ascertained that Oriental tobacco contains a high amount of rutin, of greater quantities than in thyme, mint, lemon balm, and chamomile. The content of chlorogenic acid, a powerful antioxidant, is also high. This makes tobacco especially useful in the pharmaceutical industry (Docheva et al., 2012; Popova et al., 2018). Tobacco leaves and stems can be utilized for the production of citric and malic acids, used in the food industry. Additionally, tobacco seeds can also be utilized as a biofuel source, and the stems are suitable both for feed and for the production of pellets (Mijailovic et al., 2014). Another possible application of tobacco could be in phytoremediation due to the ability of these plants to extract heavy metals from the soil (Stojanovic et al., 2012).

The use of tobacco in the pharmaceutical and food industries, as well as for the cigarette industry, requires a strict control over the content of potentially toxic elements in the tobacco products. In the cases where tobacco is grown in regions contaminated with heavy metals, it is crucial to verify the safety of the produce, and, if an increased risk to human health is identified, to offer possible alternative applications.

The content of heavy metals in such tobacco-growing areas is of interest, especially in the regions studied in this paper, due to the high demographic, economic and social importance of this culture (Yancheva et al., 2007).

The main objective of this study was to identify possible contamination with lead and zinc of soils from tobacco production areas, and of *Nicotiana tabacum* L., varietal group Basma, ecotype Krumovgrad grown on them. The statistical relationships between soil characteristics, total content and mobile forms of the heavy metals in soils, and their content in leaves of tobacco plants collected at maturity were investigated.

Material and Methods

The study area covers the eastern and middle parts of the Rhodope Mountains in Bulgaria, as well as parts of the Western Rhodopes. The terrain is mountainous and hilly. The eastern parts of the Rhodope Mountains have the lowest altitude, which increases gradually to the west. There is a shortage of arable land and most of the soils are eroded to varying degrees. According to the FAO soil classification, Fluvisols, Chromic Luvisols, Rendzic Leptosols, Planosols, and Cambisols soils (occurring at elevation of over 800-900 m) are widespread in the studied regions, and Vertisols are found in numerous small areas (Bozhinova & Zheleva, 2007; Yancheva & Stanislavova, 2006; Yancheva et al., 2007).

In three consecutive years (2016, 2017, 2018), Oriental tobacco samples from *Nicotiana tabacum* L., varietal group Basma, ecotype Krumovgrad were collected according to CORESTA Guide N° 13 (2012). As a modification to the protocol, aiming to improve the credibility of the data, 5 mature upper stalk leaves from 10 random plants were collected in the fields. For plant analysis, a mean sample was prepared to represent each sampling point. Subsequently, the data from the three years was averaged, as well.

Collocated soil samples (38 in total) were collected from the surface layer (0-30 cm) in accordance with ISO 10381, during the period 2016-2018, in the following municipalities: Kardzhali, Krumovgrad, Ivaylovgrad, Harmanli, Zlatograd, Ardino, and Kirkovo. Sample preparation was done according to ISO 11464.

The following soil and plant characteristics were determined:

1. pH in aqueous extract - potentiometric, ISO 10390;
2. Humus content - according to Tyurin – a titrimetric method, modified by Ponomareva and Plotnikova (Angelova et al., 2014);
3. Soil texture - pipette method; (Đamić et al., 1996);
4. Total lead and zinc content by *aqua regia* digestion (microwave mineralization), ISO 12914;
5. Concentration of mobile forms of lead and zinc - extraction with 0.005M DTPA + 0.1M TEA, pH 7.3, ISO 14870;

6. Content of Pb and Zn in Oriental tobacco leaves, ISO 14082.

Inductively coupled plasma atomic emission spectroscopy was used as an analytical technique for the determination of lead and zinc content in the soil and the plant samples.

It was determined that the measured values for the concentrations of Pb and Cd in the whole dataset followed a log-normal distribution, verified using a Sapiro-Wilk test ($p = 0.05$). Descriptive statistics and correlation/ regression analyses were performed in IBM SPSS Statistics for Windows, Vers. 19.0 (IBM Corp., 2010) to investigate the relationships between the soil characteristics, total and mobile forms of lead and zinc in soils, and the content of these elements in Oriental tobacco leaves.

Three standard reference materials were employed for accuracy and precision: *CRM045 Silt Clay Soil* was used in measurements of the total content of Pb and Zn in the soils, *NIM-GBW07412A Soil (Brown soil)* was used in measurements of their mobile forms, and *INGT-OBTL-5* (Samczyński et al., 2012) Polish reference material (Oriental Basma Tobacco leaves) was used for the plant analysis. The measured values were found to be in very good agreement with the certified data.

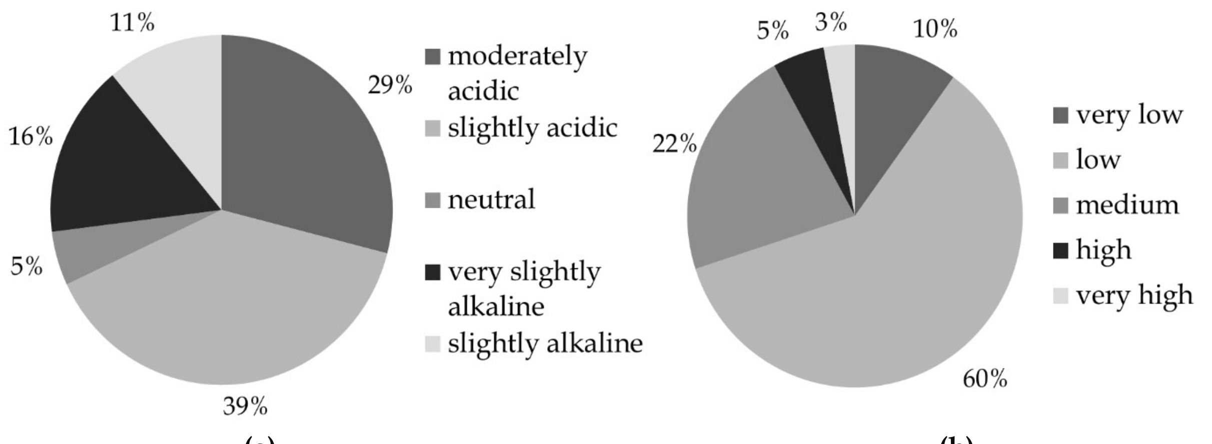
Results and Discussion

Soil

The results of the determined soil characteristics are shown in Table 1. In the studied areas, Oriental tobacco is grown on a wide variety of soils in terms of their texture and degree of erosion. Soils with clay and silt faction content between 10% and 50% (i.e. sandy and clayey-sandy soils) and humus content from 0.5% to 2.8% are considered suitable for Oriental tobacco production. Higher humus content (between 2.5% and 3.8%) could be used in the absence of better soil conditions (Tanov et al., 1978).

The content of the clay and silt fraction (<0.02 mm) varied between 6.3% and 82%. Predominant were the soils with a light texture, favorable for the cultivation of Oriental tobacco; and 27% of the soils were heavy.

Most soil samples were characterized by a slightly acidic to moderately acidic reaction (Fig. 1a). According to the used classification, soil reactions in the range of 5.1 to 6.0 are moderately acidic; between 6.1 and 6.9 – slightly acidic, and a pH of 7.0 is neutral. Very slightly alkaline soil reactions have values between 7.1 and 7.5, and pH levels of 7.6 to 8.0 are slightly alkaline. In some regions, the soils varied greatly in terms of their pH but all the samples had a soil reaction suitable for the normal growth and development of tobacco plants. Humus content of less than 1.0% is considered very low; values from 1.0 to 2.0% define low content; medium content is in range of 2.1 to 3.0%; and high content ranges from 3.1 to 5.0%. Values above 5% define very high humus content and a small proportion of soils, 3%, were characterized by an unsuitably high humus content (Fig. 1b).



(a) **(b)**

Fig. 1. Soil reaction (a) and humus content (b).

Table 1. Descriptive statistics of the soil characteristics determined in the studied regions.

Statistical Parameters	Soil texture fraction content, %					pH	Humus content %
	Coarse sand (2 – 0.2 mm)	Sand (0.2 – 0.02 mm)	Silt (0.02 - 0.002 mm)	Clay (< 0.002 mm)	Silt +Clay (< 0.02 mm)		
Mean	23.49	39.93	13.53	23.03	36.58	6.50	1.85
Standard deviation	17.79	20.09	9.69	16.20	23.48	0.72	1.02
Range	56.68	77.73	33.73	56.66	73.40	2.72	5.27
Minimum	3.35	4.67	3.27	3.68	8.54	5.15	0.86
Maximum	60.03	82.40	37.00	60.34	81.94	7.87	6.13
CV, %	75.73	50.31	62.40	70.34	64.19	11.08	55.14

It was ascertained that 26% of the soil samples were characterized by the optimal for Oriental tobacco-growing sandy and clayey-sandy texture and humus content between 0.8% and 2%. They were collected in the vicinity of the villages: Gluhar, Gradinka, Dryanova Glava, Opalchentsi, Panichkovo, Kondovo, Oreshino, Lyaskovo, Draganovo, and Konush.

Table 2 shows the summarized results for the total lead and zinc content in the soils. The soil status was determined in accordance with Ordinance #3 (2008), which indicates the permissible content of harmful substances in soils and specifies the maximum permissible concentrations (MPCs) and intervention concentrations (ICs) for several heavy metals and metalloids. MPC is defined as the content of a harmful substance in the soil in mg/kg, which, if exceeded, under certain conditions could inhibit soil functions and pose danger to the environment and human health. IC is the content of a harmful substance in the soil in mg/kg, which, if exceeded, leads to inhibition of the soil functions and endangerment of the environment and human health. The concentrations defined in Ordinance #3 (2008) are different depending on the soil reaction, location and land use (i.e. industrial/production sites, permanent grasslands, pastures, in settlements, arable land).

No instances of exceedance of the ICs were determined. Exceedance of the maximum permissible concentration for Pb, set at 60 mg/kg, 100 mg/kg or 120 mg/kg depending on the soil reaction, was observed in samples collected near Visegrad, Kirkovo, and Panichkovo. The MPC for Zn (200 mg/kg, 320 mg/kg or 400 mg/kg based on the soil reaction) was exceeded in one of the soil samples collected in a small tobacco-growing field near the Kardzhali municipal landfill site, however, the IC (900 mg/kg) was not exceeded. Contamination with both Pb and Zn was ascertained in soil samples collected near the Studen Kladenets Reservoir – in the village of Ostrovitsa, located opposite to the non-operational Kardzhali lead-zinc plant, beyond the reservoir (Jeleva et al., 2012).

Table 2. Descriptive statistics of the total content of Pb and Zn in soil (mg/kg).

Statistical Parameters	Pb	Zn
Mean	43.48	82.85
Standard deviation	31.36	72.55
Range	110.00	435.00
Minimum	6.60	17.90
Maximum	116.60	452.90
CV, %	72.12	87.58

Background and precautionary concentrations of heavy metals and metalloids are also established in the referenced Ordinance #3 (2008). By definition, the exceedance of the precautionary concentrations would not lead to inhibition of the soil functions, nor to endangerment to the environment and human health. The values vary according to the soil texture. The percentage of collected samples characterized by very low concentrations of Pb was 37% (lower than the background values) and 74% for Zn. Concentrations of Pb lower than the precautionary values were determined in 63% of the samples, and for Zn – in 92% of the samples. It should be noted that soils (and soil-forming rocks) on the Balkan Peninsula are characterized by the relatively high mineral content (Milev et al., 2007) and this is reflected in the concentration classification system in Ordinance #3 (2008).

Table 3 contains the summarized results for the DTPA-extractable forms of Pb and Zn. According to the classification for concentrations of mobile forms of elements in soils (Horneck et al., 2011; Jones Jr., 2001), 53% of the soil samples had medium Zn concentration, 18% were characterized by low Zn concentration, and 3% had a very low concentration. High concentrations were determined in 8% of the samples, and a very high concentration – in 18%. The highest concentrations of mobile Zn forms were determined in samples from the villages of Ostrovitsa (in the vicinity of the Kardzhali Pb-Zn plant), Vishegrad (near the municipal landfill), Gluhar, Zheltusha, Lyaskovo.

A classification system for the concentrations of mobile forms of Pb was not found but the results could be compared to data from neighboring countries. According to Golia et al. (2008), the concentration of mobile Pb ranges between 2.5 and 4.8 mg/kg in agricultural land, and increases to 7.9 mg/kg in industrial areas. Similar concentrations of mobile Pb were determined in the industrial area of Heraklion, Crete, Greece, by Papafilippaki et al. (2008). According to Jordanoska et al. (2018), the DTPA-extractable Pb content in several Oriental tobacco growing areas of North Macedonia ranged between 0.5 and 4.4 mg/kg.

Table 3. Descriptive statistics of DTPA-extractable forms of trace elements in soils (mg/kg).

Statistical Parameters	Pb	Zn
Mean	2.83	5.18
Standard deviation	3.03	9.87
Range	17.07	50.10
Minimum	0.31	0.39
Maximum	17.37	50.50
CV, %	107.09	190.72

In the present study, the Pb content ranged between 0.31 and 17.37 mg/kg. The highest values were determined in samples from Ostrovica (located opposite to the Pb-Zn smelter in Kardzhali) and from Vishegrad (near the Kardzhali municipal landfill). With the exception of these samples, the determined values for DTPA-extractable concentrations of Pb were similar to those reported in the previously referenced sources (Golia et al., 2008; Jordanoska et al., 2018; Papafilippaki et al., 2008).

Oriental tobacco leaves

Summarized results for the determined Pb and Zn concentrations in tobacco leaves collected at maturity are shown in Table 4.

Plants grown in unpolluted areas have a Pb content in the range of 0.1 to 10 mg/kg, and concentrations in the range of 30 to 300 mg/kg are considered critical (Kabata-Pendias & Pendias, 1992). Bozhinova (2016) ascertained that phosphorus fertilizers do not have a pronounced effect on the concentration of Pb in tobacco leaves, nor in soils (both mobile forms and total content). In this study, high values (maximum concentrations) were ascertained in small crop fields located 10 m away from the Kardzhali municipal landfill, and in the village of Ostrovitsa. However, no visible symptoms of toxicity of the plants were observed.

The determined values for Zn were lower than the critical concentrations for plants (100-400 mg/kg) and consistent with literature data (Kabata-Pendias & Pendias, 1992). According to Stamatov et al. (2015), the concentration of trace elements in oriental tobacco leaves depends on the variety of the plant.

Table 4. Descriptive statistics of Pb and Zn concentrations (mg/kg) in Oriental tobacco leaves. Summarized results for samples collected in 2016, 2017, and 2018.

Statistical Parameters	Pb	Zn
Mean	11.46	89.98
Standard deviation	46.79	66.14
Range	272.83	315.80
Minimum	0.27	22.90
Maximum	273.10	338.70
CV, %	408.29	73.51

Statistical relationships between the data for soils and Oriental tobacco leaves

Table 5 contains the Pearson correlation coefficients for the determined Pb and Zn concentrations in Oriental tobacco leaves and in soils. The significant correlation between the total content of Pb and Zn in the soil (R = 0.475) is explained by the geological features of the region and the presence of polymetallic ores. A significant correlation between the mobile forms of the elements in the soil was observed as well (R = 0.697). Additionally, the total content of the elements and their mobile forms correlated significantly. The concentrations of Pb and Zn determined in Oriental tobacco leaves correlated significantly as well (R = 0.508). The relationships between the mobile forms of the elements and their concentrations in tobacco leaves are of interest. Significant and strong correlations were expected due to the accumulating properties of the studied crop and the availability of the mobile metal forms to plants. The Zn concentration determined in the tobacco leaves and the mobile forms of both Pb and Zn in the soils showed stronger correlations than those of the Pb concentration in tobacco and the mobile forms of Pb and Zn in the soils, however, all were significant.

Regression analyses were performed to investigate possible linear and non-linear statistical relationships between the data. Table 6 contains the regression equations for the whole set of determined soil characteristics, the total content and mobile forms of Pb and Zn in the soils, and their content in Oriental tobacco leaves.

A strong and significant linear relationship between the DTPA-extractable forms of Zn and the Zn concentration in leaves was observed. A similar relationship was ascertained in a study conducted in Greece (Golia et al., 2009). For Pb, the association between the mobile forms and the concentration in the leaves was described by the quadratic regression model. Despite the known effects of soil pH on the mobility and uptake of metals in plants (Kabata-Pendias & Pendias, 1992), no statistically significant relationships were ascertained between the soil reaction and the mobile forms of Pb and Zn in this study. The DTPA-extractable forms of the elements had significant relations to the humus content, described by the power regression model for both. The strong and significant relationships with the total content for the two metals were best described by non-linear regression equations. The power regression model suited the relationship between the mobile forms and the total content of Pb, whereas the cubic model described the dependencies between the total content of Zn and the Zn mobile forms.

The relationships between the soil characteristics and the determined concentrations for the two metals both in the leaves and in the soils were rather similar to those determined in Virginia tobacco grown in Bulgaria (Zaprjanova et al., 2010). The lack of a statistical relationship between the humus content and the Pb concentration in tobacco leaves was an exception. Additionally, the same authors found a linear relationship between the Pb concentration in the leaves and the total content of the element in the soil, whereas, in this study, the relationship was described by the logarithmic regression model.

In a similar study conducted in North Macedonia, it was shown that the composition of the tobacco leaves rarely correlates significantly with the soil characteristics, with the exception of the clay fraction (Jordanoska et al., 2018). The results from this study revealed weak yet significant statistical relationships between the Pb content in the tobacco leaves and both the clay and clay and silt fractions of the soil, both described by the logarithmic regression model.

Table 5. Correlation (Pearson correlation coefficients) between the determined Pb and Zn concentrations in Oriental tobacco leaves and in soils (total *C* – total concentration, mf – mobile forms). Legend: ** Statistical significance at $p \leq 0.01$; * Statistical significance at $p \leq 0.05$.

Statistical parameters	Pb tobacco	Zn tobacco	Pb total *C*, soil	Zn total *C*, soil	Pb mf, soil	Zn mf, soil
Pb tobacco	1					
Zn tobacco	0.508**	1				
Pb total *C*, soil	0.490**	0.532**	1			
Zn total *C*, soil	0.227	0.755**	0.475**	1		
Pb mf, soil	0.409*	0.742**	0.658**	0.511**	1	
Zn mf, soil	0.369*	0.863**	0.567**	0.925**	0.697**	1

Table 6. Regression equations and significance level the determined soil characteristics, Pb and Zn concentrations in Oriental tobacco leaves and in soils. Legend: ** Statistical significance at $p \leq 0.01$; * Statistical significance at $p \leq 0.05$.

Variable X	Variable C – concentrations of the mobile forms of Pb in soil	R^2	*p*
Clay fraction	$C = 18.222 + 7.505 . \ln X$	0.187**	0.007
Clay + silt	no significant relationships	-	-
pH	no significant relationships	-	-
Humus	$C = 1.431 . X^{0.224}$	0.203**	0.004
Pb total	$C = 22.928 . X^{0.586}$	0.506**	0.000
Variable X	Variable C – averaged concentrations of Pb in tobacco leaves	R^2	*p*
Clay fraction	$C = 21.185 + 5.113. \ln X$	0.194**	0.006
silt + clay	$C = 33.510 + 6.503. \ln X$	0.144*	0.019
pH	no significant relationships	-	-
Humus	no significant relationships	-	-
Pb total	$C = 38.836 + 12.874 . \ln X$	0.329**	0.000
Pb mobile	$C = 1.992 + 0.237. X - 0.001 . X^2$	0.709**	0.000
Variable X	Variable C – concentration of the mobile forms of Zn in soil	R^2	*p*
Clay fraction	no significant relationships	-	-
clay + silt	no significant relationships	-	-
pH	no significant relationships	-	-
Humus	$C = 1.052 . X^{1.515}$	0.378**	0.000
Zn total	$C = 4.021 – 0.141 . X + 0.002 . X^2 - 2.91.10^{-6} . X^3$	0.917**	0.000
Variable X	Variable C – averaged concentrations of Zn in tobacco leaves	R^2	*p*
Clay fraction	$C = 32.684. X^{0.292}$	0.173**	0.009
clay + silt	no significant relationships	-	-
pH	$C = 737.205 . 0.704^X$	0.190**	0.006
Humus	$C = 155.339 - 154.826 . X + 79.717 . X^2 - 9.386 . X^3$	0.255*	0.017
Zn total	$C = 32.931 + 0.689 . X$	0.571**	0.000
Zn mobile	$C = 60.062 + 5.778 . X$	0.744**	0.000

A significant statistical relationship was determined between the Zn concentration in tobacco leaves and the clay fraction of the soil described adequately by the power regression model. The compound regression model described the relationship between the reaction of the soil and the Zn concentration in tobacco leaves.

Conclusions

The content of lead and zinc was determined both in soils and in tobacco leaves collected at maturity. In terms of the total content of the metals in soil, no instances of exceedance of the intervention concentrations were observed. The majority of the soil samples can be considered clean, as the determined content of the elements was lower than the precautionary and background concentrations according to the Bulgarian legislation.

The maximum permissible concentration for lead was exceeded in samples collected in the area of Vishegrad, Kirkovo, and Panichkovo. Soil contamination with zinc was ascertained near the village of Ostrovitsa, in the vicinity of the non-operational Kardzhali Pb-Zn plant. Remediation of the affected soils is recommended.

The tobacco samples collected in crop fields located at a distance of 10 m to 600 m from the municipal landfill in Vishegrad and in the village of Ostrovitsa were characterized by high concentrations of both lead and zinc. At the same sites, the concentrations of the heavy metals in the soils were also high (both total content and mobile forms). No symptoms of toxicity of the plants were observed. It was confirmed that Oriental tobacco exhibits accumulating properties, therefore planting in close proximity to known point sources of heavy metal contamination should be prohibited. The observed strong and significant relationships between the DTPA-extractable forms of Pb and Zn in soils with their concentrations in the tobacco leaves show that the mobile forms could be used as a predictor of the metal concentration in the tobacco plants.

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Effect of Cycocel 750 SL on Germination and Initial Development of Some Sorghum Species

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Abstract. During the period 2018-2019, a laboratory test was carried out to determine the effect of Cycocel 750 SL (750 g/l chlormequat chloride) on germination and initial development of selected samples of *Sorghum* species: *Sorghum bicolor* L., *Sorghum sudanense* (Piper.) Stapf. and *Sorghum vulgare* var. *technicum* Körn. Cycocel 750 SL is usually applied as plant growth regulator with retardant activity on wheat (*Triticum* sp.) but some authors have observed a stimulatory effect on germination and root elongation of many crops when it was applied for pre-sowing treatment of seeds. We found, that the combined application of Cycocel 750 SL (as pre-sowing treatment and foliar application) in concentrations 0.75-3.8% w/v leads to the enhanced germination of seeds as well as to the significant decrease of shoot-to-root ratio (expressed by the Coefficient of allometry, CA) in all experimental variants in comparison with the control one. Shoot development was significantly inhibited in all cases while the root growth was depressed only at lower concentration applied (0.75% w/v) and stimulated by higher doses of Cycocel (1.5-3.8% w/v). Seedling vigor index (SVI) revealed 24-48% higher values after the Cycocel 750 SL application on *S. vulgare* var. *technicum* (2.6% w/v), *S. bicolor* (2.6% w/v) and *S. sudanense* (3.0% w/v).

Key words: hormo-priming, germination rate, plant growth regulators, crops.

Introduction

Sorghum is the fifth most produced grain globally (Mundia et al., 2019). High ecological plasticity and increased resistance to various abiotic stress factors (atmospheric and soil drought) determine *Sorghum* spp. as promising crops for inclusion in crop rotation under conditions of global warming and drought (House, 1985; Moyer et al., 2003; Berenji & Dahlberg, 2004; Angelova et al., 2011; Stefaniak et al., 2012). At present, *Sorghum sudanense* (Piper.) Stapf. hybrids are preferred as a fodder due to higher yields and sibling ratios and also their thin stems and higher leaf ratios (Uzun et al., 2009). *Sorghum bicolor* (L.) Moench find various applications as human food, animal feed, industry raw material, biofuel production, etc. (Bibi & Ali, 2012; Serna-Saldívar et al., 2012; Cifuentes et al., 2014). *Sorghum vulgare* var. *technicum* Körn. is mainly utilized for producing brooms, washing brushes, knitting, paper, wallboard, fences, biodegradable materials for packaging due to their peculiar resistance (Popescu & Condei, 2014).

According to the studies of Jamshidi et al. (2011), Fromme et al. (2012), Silva et al. (2014), the exploration of the biological potential of *Sorghum* species, including *S. bicolor*, *S. sudanense* and *S. vulgare* var. *technicum*, is closely linked to overcoming biologically delayed seed emergence and the slow growth rate in the initial stages of their development. This leads to their high sensibility against weed infestation in the first 30-40 days after sawing (Marinov-Serafimov & Golubinova, 2015).

In recent years, research has focused on increasing the viability and germination of seeds in a variety of crops using various chemical and physical methods (Afzal et al., 2006; Lutts et al., 2016; Zheng et al., 2016). The pre-sowing treatment of the seeds aims to increase and synchronize the germination as well as to increase the resistance of the sprouts to abiotic stress. Some authors (Pourmohammad et al., 2013; Singh et al., 2017) find that pre-sowing priming of seeds with growth regulators (with retardant activity) has a stimulating effect on seed germination in a number of crops.

Cycocel is among the most reliable and widely used plant growth regulators with retardant activity on the market today. Cycocel may be used on any crop in the greenhouse or nursery including but not limited to, poinsettias, hibiscus, azaleas, and geraniums to reduce stem elongation, induce early flowering, improve flowering, and to produce compact plants with multiple buds per shoots. In wheat and oats Cycocel 750 stimulates root development, reduces stem length and causes the stem wall to thicken. Primary tiller development is slowed down and secondary tiller development is stimulated (BASF Fact Sheet, 2018).

In their experimental work some authors (Afria et al., 1998; Pirasteh-Anosheh et al., 2016; Singh et al., 2017) found that Cycocel 750 SL could be applied not only on the seedlings but also for priming of seeds. They revealed that the pre-sowing treatment of *Brassica napus* L., *Cyamopsis tetragonoloba* L., *Lens culinaris* Mediec., *Triticum aestivum* L. and *Zea mays* L. had a stimulatory effect on both the germination process and the initial development of plant roots. Similar results have been reported by Ismaeil et al. (1993) which proved that the priming of Sudan grass seeds (*S. sudanense*) with chlormequat chloride decreased the stress effects in plants (inhibition of germination and growing rate) when sowing on saline soils.

Limited studies on the influence of pre-sowing treatment of seeds of some important *Sorghum* crops determines the necessity of such studies in order to establish the effect of priming the seeds as a means of reducing abiotic stress in the germination and initial development of the plants. Aim of the present study was to assess the effect of Cycocel 750 SL (750 g/l chlormequat chloride) on germination and initial development of selected samples of *Sorghum* species: *S. bicolor* (L.) Moench, *S. sudanense* (Piper.) Stapf. and *S. vulgare* var. *technicum* Körn.

Material and methods

Experimental design. Experiment was conducted at laboratory conditions during the period 2018-2019 in the Plovdiv University. Seeds of *S. bicolor* (L.) Moench (1641 hybrid), *S. sudanense* (Piper.) Stapf. (300/43 mutant form) and *S. vulgare* var. *technicum* Körn. (MI 16 N local population from NW Bulgaria) were provided from the Selection Collection of the Institute of Forage Crops in Pleven, Bulgaria.

Number of 108 seeds per species was put into 2.0% NaClO solution for 10 minutes for surface sterilization and then three times thoroughly rinsed with distilled water. After that, the seeds were primed for 24 hours with Cycocel 750 SL (750 g/l chlormequat chloride) at concentrations 0.0 (control), 0.75, 1.5, 2.6, 3.0 and 3.8% w/v following the adapted method of Pourmohammad et al. (2013).

Ex-situ experiment was carried in 0.3 l pots containing a potting mixture of soil, sand and perlite (1:1:1) (Lau et al., 2008). Three seeds per each species and per each test concentration were placed in a pot and left at 22 ± 2°C with 12:12 hours light and dark phase period. All experimental variants were conducted in six replications.

Second application of Cycocel 750 SL was made seven days after seed germination in the phase of the first leaf of the cultures. The treatment with tested growth regulator was carried out by pulverization on the aboveground biomass in all experimental variants at same concentrations as used for seed priming (0.0 (control), 0.75, 1.5, 2.6, 3.0 and 3.8% w/v). Then seedlings were left for another period of seven days at 22 ± 2°C with 12:12 hours light and dark phase. The adapted method of Pourmohammad et al. (2013) was used to assess the effect of Cycocel 750 SL on seed germination and initial development of *Sorghum* crops.

Effect assessment. For evaluation of the experimental results obtained, the following parameters were used: 1. Quantitative parameters - percentage of germination in each treatment (%); 2. Biometric parameters: shoot, root and seedling length (cm) and fresh biomass per plant (g). Length was measured using graph paper and weight - on an analytical balance: 3. Qualitative parameters (calculated by formulas) and statistical processing as described below.

Germination of seeds (GS) was determined by equation, proposed by ISTA (1985), as follows:

$$GS\% = \frac{Number\ of\ seeds\ germinated}{Total\ number\ of\ seeds\ placed} \times 100, \%$$

Inhibition rate (IR) of Cycocel 750 SL on shoot and root growth in experimental variants in comparison to control was calculated according to the adapted formula of Surendra & Pota (1978):

$$IR = \frac{C-T}{C} \times 100, \%,$$

where: IR is the percentage of inhibition, C – control value (germination, number; length, cm; biomass, g); T – treatment value. Values indicate the inhibitory (+) or stimulatory (-) effects in comparison to control treatments.

Seedling vigor index (SVI) was determined using the equation proposed by Islam et al. (2009):

$$SVI = \frac{S \times G}{100},$$

where: S – seedling length in the treatments and control variant, cm; G – germinated seeds in the treatments and control variant, %.

Coefficient of allometry (CA) was calculated by the formula of Nasr & Mansour (2005):

$$CA = \frac{L_s}{L_r},$$

where: L_s is shoot length and L_r is root length, cm.

The seed germination was calculated after preliminary arcsin-transformation following the formula, Y=arcsin√(x%/100), forwarded by Hinkelmann & Kempthorne (1994). All collected data were analyzed using analysis of variance with means separation based on Fisher's least significant difference test at p<0.05 with the software Statgraphics Plus for Windows Ver. 2.1 (1994) and STATISTICA Ver. 10 (StatSoft Inc., 2010).

Results and Discussion

The pre-sowing treatment of seeds of the tested samples of *Sorghum* species with Cycocel 750 SL has a stimulating effect on seed germination in all tested concentration in comparison with the control variants (Table 1) and the differences were statistically significant at p<0.05. The percentage of germination of primed seeds increased from 22.3% up to 44.4% in comparison with the untreated ones and was most pronounced for *S. sudanense* genotype. As a whole, maximum stimulation exerted concentrations in the range 0.75-2.6% w/v while higher doses of Cycocel 750 SL (3.0-3.8% w/v) had lower effect (10.5-14.4% raising). Similar results have been reported by Pirasteh-Anosheh et al. (2016), according to which priming of seeds with Cycocel 750 SL induces a stimulating effect on seed germination in wheat, barley, maize and rapeseed, whereas in sunflower, no primary stimulating effect is detected.

According to the genotype response towards the stimulating effect of Cycocel 750 SL on seed germination, the tested *Sorghum* sp. could be arranged as follows: *S. sudanense* ($IR_{average}$ = -31.02%) > *S. vulgare* var. *technicum*, ($IR_{average}$ = -24.5%) > *S. bicolor* ($IR_{average}$ = -19.12%). Therefore, the priming of *Sorghum* seeds could be used as an effective practice to enhance the germination and to reduce the biologically delayed seed emergence which will increase their competitiveness against weed infestation.

Data from biometric measurements on seedling length make it possible to objectively evaluate differences in the initial stages of plant development depending on the Cycocel 750 SL concentration used (Table 2). Treatment with Cycocel 750 SL exhibit a stimulatory effect on the root elongation mainly in *S. sudanense* seedlings where the Inhibition rate varied in the range from -34.3% to -276.5% ($IR_{average}$ = -99.5%), followed by *S. bicolor* seedlings with an IR values between -16.7% and -126.5% ($IR_{average}$= -76.4%), whereas *S. vulgare* var. *technicum*, seedlings were less influenced – IR was in the range from -5.8% up to -67.7% ($IR_{average}$=-27.7%).

On the other hand, regardless of the applied concentration, Cycocel 750 SL significantly inhibited shoot growth in all studied *Sorghum* species (p<0.05). Maximum inhibition of shoot development (from 33.3% to 59.1% in comparison with the control) was reported after the combined treatment with Cycocel 750 SL at a dose of 1.5% w/v in all studied genotypes (Table 2). The reduction of shoot length in the experimental variants may be due to a decrease in both cell division and cell number. Similar results were reported by Child (1984) immediately after the application of Cycocel 750 SL on oil rapeseed. Our data well correspond with the findings obtained through the experimental work of Pirasteh-Anosheh et al. (2014) according to which the seed priming with Cycocel 750 SL is an effective means of increasing the root growth rate by redirecting much of the assimilate to the root, since the shoot-to-ratio in barley increases after application of the growth regulator.

When regarding the seedling development as a whole, it was less reduced in *S. bicolor* L. genotype ($IR_{average}$ = 6.6%), followed by *S. vulgare* var. *technicum* Körn. ($IR_{average}$ = 11.4%) and strongest reduction of seedling length was observed in *S. sudanense*. Some exceptions were found at higher concentrations of Cycocel 750 SL (2.6% and 3.0%) where a stimulatory effect occurred with IR values in the range from -1.2% to -9.8% (*S. bicolor* L.) and -17.7% (*S. sudanense* (Piper.) Stapf.).

Cycocel 750 SL treatment of *Sorghum* seeds and seedlings significantly influenced the dynamics of fresh biomass synthesis in all studied genotypes (Table 3). The accumulation of fresh biomass in shoots, roots and seedlings is quite lower after the combined treatment with lower doses of Cycocel 750 SL when compared to the untreated control plants. Treatment with higher doses leads to significant increment only of the root biomass quantity of experimental plants, more expressed at *S. bicolor* and *S. sudanense* (p<0.05). Species specific differences in barley's tolerance towards the same growth regulators were reported also by Bahrami et al. (2014). An exception was found in relation to the variant with 2.6% w/v of growth regulator where the significant stimulatory effect occurred in all

Sorghum seedlings. According to the genotype response towards the inhibitory effect of combined treatment with Cycocel 750 SL on the process of biomass synthesis, the tested *Sorghum* sp. could be arranged as follows: *S. bicolor* ($IR_{average}$ = 4.42%) > *S. vulgare* var. *technicum*, ($IR_{average}$ = 2.98%) > *S. sudanense* ($IR_{average}$ = 2.28%).

Table 1. Effect of growth regulator Cycocel 750 SL on laboratory germination of seeds (GS) from *S. vulgare* var. *technicum, S. bicolor* L. and *S. sudanense* (Piper.) Stapf. *Legend:* Statistically significantly differences a, b, c, d, e - LSD at p<0.05.

Concentration, % w/v	*Sorghum vulgare* var. *technicum* Körn.		*Sorghum bicolor* L.		*Sorghum sudanense* (Piper.) Stapf.	
	GS%	*IR*	GS%	*IR*	GS%	*IR*
0.0	69.7^{ab}	0	73.6^{a}	0	63.4^{a}	0
0.75	92.1^{d}	-32.1	94.5^{c}	-28.4	88.0^{c}	-38.8
1.5	92.1^{d}	-32.1	90.0^{c}	-22.3	87.7^{c}	-38.3
2.6	90.0^{d}	-29.1	90.0^{c}	-22.3	91.5^{d}	-44.4
3.0	81.3^{c}	-16.6	82.5^{b}	-12.1	74.4^{b}	-17.4
3.8	78.5^{a}	-12.6	81.3^{b}	-10.5	73.7^{b}	-16.2

Table 2. Effect of growth regulator Cycocel 750 SL on root, shoot and seedling length of *S. vulgare* var. *technicum* Körn., *S. bicolor* L. and *S. sudanense* (Piper.) Stapf. *Legend:* Statistically significantly differences a, b, c, d, e - LSD at p<0.05.

Species	Parameter	Concentration, % w/v					
		0.0	0.75	1.5	2.6	3.0	3.8
Sorghum vulgare var. *technicum* Körn.	Shoot, cm	21.33^{d}	15.6^{b}	14.2^{a}	16.0^{b}	17.6^{c}	17.8^{c}
	IR		26.9	33.3	25.1	17.5	16.7
	Root, cm	6.4^{b}	5.5^{a}	7.9^{bc}	10.7^{d}	6.8^{b}	7.3^{bc}
	IR		14.5	-23.0	-67.7	-5.8	-14.5
	Seedling, cm	27.7^{d}	21.1^{a}	22.1^{ab}	26.7^{cd}	24.4^{c}	25.1^{c}
	IR		24.0	20.3	3.7	12.1	9.5
Sorghum bicolor L.	Shoot, cm	16.73^{c}	10.5^{b}	9.0^{a}	13.9	10.2^{b}	11.5^{b}
	IR		37.1	46.0	16.7	39.2	31.4
	Root, cm	5.4^{ab}	4.9^{a}	9.2^{c}	10.4^{d}	12.2^{e}	6.3^{ab}
	IR		8.7	-70.4	-92.0	-126.5	-16.7
	Seedling, cm	22.1^{c}	15.5^{a}	18.2^{b}	24.3^{d}	22.4^{c}	17.8^{ab}
	IR		30.1	17.6	-9.8	-1.2	19.7
Sorghum sudanense (Piper.) Stapf.	Shoot, cm	16.8^{e}	10.4^{d}	6.9^{a}	9.0^{b}	10.2^{c}	8.1^{b}
	IR		37.9^{a}	59.1^{d}	46.4^{b}	39.3^{a}	51.8^{c}
	Root, cm	3.7^{a}	3.3^{a}	5.0^{b}	5.6^{c}	13.9^{d}	5.0^{b}
	IR		11.6	-34.3	-52.2	-276.5	-35.1
	Seedling, cm	20.5^{cd}	13.7^{b}	11.8^{a}	14.6^{c}	24.1^{d}	13.1^{b}
	IR		33.2	42.2	28.6	-17.7	36.1

Generally, the values of the Seedling vigor index (SVI) in all experimental variants showed a tendency to increase when increasing the concentration of the applied dose of Cycocel 750 SL up to 3.0% w/v (Table 4). The lowest SVI values were observed in seedlings treated with the lowest (0.75% w/v) and the highest (3.8% w/v) doses of studied growth regulator. Maximum values were achieved under the combined application of Cycocel 750 SL (seed priming and shoot pulverization) with concentration of 2.6% w/v at *S. vulgare* var.

technicum and *S. bicolor* and 3.0% w/v at *S. sudanense*. Stimulatory effect on SVI in these seedlings revealed 24.3%, 34.3% and 48.4% respectively when compared to the control variants ($p<0.05$). This result could be explained by the positive influence of Cycocel 750 SL on seed germination and root growth. Our data correlate with the findings of other authors (Pourmohammad et al., 2013; Pirasteh-Anosheh et al., 2014) that seeds priming with an optimal concentrations of Cycocel 750 SL (species specific doses) reduces the adverse effect of the complex influence of abiotic factors through the germination and initial development of barley, maize and rapeseed. According to the genotype response towards the stimulating effect of Cycocel 750 SL on Seedling vigor index, the tested *Sorghum* sp. could be arranged as follows: *S. vulgare* var. *technicum*, ($IR_{average}$ = -5.74%) > *S. bicolor* ($IR_{average}$ = -4.48%) > *S. sudanense* ($SVI_{average}$ = 2%).

The combined treatment with Cycocel 750 SL led to the significant decrement of the values of Coefficient of allometry (CA) in all experimental seedlings in comparison with the control (Table 4). Because the allometric relationships reveal the existence of underlying biophysical constraints to seedling growth, they are an important means to investigate seed and plants after treatment with chemical and/or biological products. Deviations from the expected relationships may also help to explain how plants partition their resources between competing requirements (Daws et al., 2007). Largest variations have been observed in *S. sudanense* (Piper.) Stapf. genotype (CA=1.4-6.2 times lower values), followed by *S. bicolor* L. genotype (CA=0.8-3.2 times lower values) and less affected was *S. vulgare* var. *technicum* Körn. genotype (CA=1.49-2.85 times lower values). Priming of seeds and shoots pulverization with Cycocel 750 SL at doses between 1.5 and 3.0% w/v had the strongest effect on the CA reduction which was due both to the enhanced root elongation and suppressed shoot development (Table 2). Similar results in the experiments of other authors have been explained by a probable blocking of gibberellin biosynthesis (North et al., 2010) or by an increase in ethylene synthesis (Gianfagna, 1995; Shahrokhi et al., 2011).

Table 3. Effect of growth regulator Cycocel 750 SL on fresh biomass accumulation of *S. vulgare* var. *technicum* Körn., *S. bicolor* L. and *S. sudanense* (Piper.) Stapf. *Legend:* Statistically significantly differences a, b, c, d, e - LSD at $p<0.05$.

Species	Parameter	Concentration, % w/v					
		0.0	0.75	1.5	2.6	3.0	3.8
Sorghum vulgare var. *technicum* Körn.	Shoot, g	0.093^c	0.071^a	0.073^a	0.108^d	0.087b	0.101^d
	IR		23.7	21.5	-16.1	6.5	-8.6
	Root, g	0.029^c	0.012^a	0.024^b	0.057^e	0.028^c	0.031^d
	IR		58.6	17.2	-96.6	3.4	-6.9
	Seedling, g	0.122^b	0.083^a	0.097^a	0.165^d	0.115^b	0.132^c
	IR		32	20.5	-35.2	5.7	-8.2
Sorghum bicolor L.	Shoot, g	0.141^d	0.088^a	0.123^c	0.156^e	0.091^a	0.103^b
	IR		37.6	12.8	-10.6	35.5	27
	Root, g	0.049^b	0.018^a	0.122^e	0.075^d	0.061^c	0.071^d
	IR		63.3	-149	-53.1	-24.5	-44.9
	Seedling, g	0.190^d	0.106^a	0.245^e	0.231^e	0.152^b	0.174^c
	IR		44.2	-28.9	-21.6	20	8.4
Sorghum sudanense (Piper.) Stapf.	Shoot, g	0.050^b	0.013^a	0.030^b	0.060^c	0.061^c	0.034^b
	IR		74.0	40.0	-20.0	-22.0	32.0
	Root, g	0.012^a	0.012^a	0.025^d	0.021^c	0.031^d	0.016^b
	IR		0.0	-108.3	-75	-158.3	-33.3
	Seedling, g	0.10^b	0.025^a	0.10^b	0.10^b	0.10^b	0.10^b
	IR		59.7	11.3	-30.6	-48.4	19.4

Table 4. Effect of growth regulator Cycocel 750 SL on initial development of *S. vulgare* var. *technicum* Körn., *S. bicolor* L. and *S. sudanense* (Piper.) Stapf. *Legend: SVI* - Seedling vigor index; *CA* - Coefficient of allometry; Statistically significantly differences *a, b, c, d, e* - *LSD* at p<0.05.

Concentration, % w/v	*Sorghum vulgare* var. *technicum* Körn.		*Sorghum bicolor* L.		*Sorghum sudanense* (Piper.) Stapf.	
	SVI	CA	SVI	CA	SVI	CA
0.0	19.33[a]	3.33[c]	16.29[b]	3.10[c]	13.0[c]	4.54[d]
0.75	19.41[a]	2.85[bc]	14.61[a]	2.14[bc]	12.1[b]	3.19[c]
1.5	20.35[ab]	1.81[a]	16.41[b]	0.98[a]	10.38[a]	1.38[ab]
2.6	24.03[b]	1.49[a]	21.87[d]	1.34[ab]	13.39[c]	1.60[b]
3.0	19.81[ab]	2.60[b]	18.48[c]	0.83[a]	17.95[d]	0.73[a]
3.8	19.70[ab]	2.42[b]	14.45[a]	1.82[b]	9.60[a]	1.62[b]

Conclusions

The seed priming with Cycocel 750 SL in 0.75-3.8% w/v concentrations had a stimulating effect on seed germination of studied *Sorghum* species in comparison with the control (p<0.05). The enhancement of germination process varied from 22.3% up to 44.4% and was most pronounced for *S. sudanense* genotype.

The combined application of Cycocel 750 SL (as pre-sowing treatment and foliar pulverization) in concentrations 0.75-3.8% w/v significantly inhibited shoot growth in all studied *Sorghum* genotypes (p<0.05). Maximum inhibition of shoot development (from 33.3% to 59.1% in comparison with the control) was reported after the combined treatment with Cycocel 750 SL at a dose of 1.5% w/v in all tested species. At the same time, the treatment with this growth regulator exhibit a stimulatory effect on the root elongation most pronounced at *S. sudanense* with 99.5% average enhancement of root length, followed by *S. bicolor* with 76.4%, whereas *S. vulgare* var. *technicum* was less influenced - 27.7%. Maximum stimulatory effect of the combined application of Cycocel 750 SL on the initial development of *Sorghum* seedlings was observed in *S. sudanense* (48.4% at 3.0% w/v dose), followed by *S. bicolor* (34.3% at 2.6% w/v dose) and *S. vulgare* var. *technicum* (24.3% at 2.6% w/v dose) when compared to the control (p<0.05).

Therefore, we could recommend the priming of *Sorghum* seeds with Cycocel 750 SL as an effective practice to enhance the germination and to reduce the biologically delayed seed emergence which will increase their competitiveness against weed infestation. The combined treatment of *Sorghum* genotypes with this growth regulator (seed priming and foliar application) at 1.5-2.6% w/v concentrations could enhance the biomass synthesis and initial development of seedlings with more than 50% (dependent to the genotype response).

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Alien Fishes in Some Tributaries of the Maritsa River in Bulgaria

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Abstract. The paper presents a study of alien fishes in the fish fauna of the Maritsa River tributaries' upper and middle zones. The study uses a large ichthyological sample, taken from four tributaries of the Maritsa River: the rivers Topolnitsa, Luda Yana, Stryama and Chepinska. The sample was collected in the period 2005-2011. Analysis identifies that out of 18 fishes, established in the studied rivers, three are invasive: the Prussian carp (*Carassius gibelio*), the Topmoouth gudgeon (*Pseudorasbora parva*), the Pumpkinseed sunfish (*Lepomis gibbosus*), and one is introduced: the Rainbow trout (*Oncorhynchus mykiss*). Alien species do not exceed 3% of the ichthyofauna composition of the studied rivers. It is identified that this pattern is partially caused by the fast water flow and absence of suitable habitats for these species. *O. mykiss* is only present in proximity to fish firms, wherefrom they have been accidentally released. This species comprises less than 0.2% of the total sample. *C. gibelio* and *L. gibbosus* are distributed in all studied water courses and are the most numerous (1.2% and respectively 0.7% of the sample*). P. parva* is found only in the rivers Topolnitsa and Luda Yana and represents 0.5% of the sample. In general, the number of non-native fish in the studied rivers is relatively small and this is why they are expected the impact on local fish fauna was small. However human activities can cause an increase in the population density of alien fishes in the Maritsa River tributaries.

Key words: Non-indigenous fish, Middle and Upper river zone, Topolnitsa, Luda Yana, Stryama, Chepinska river.

Introduction

Continental waters are surrounded by land and represent isolated basins. The colonization of such waters by new fish requires that the latter overcome large land and sea barriers (Haury & Patee, 1997). Often the distribution of aquatic organisms, in particular fish, has taken place via dispersal by birds (e.g. fish eggs may stick to water birds' feathers or feet), tornadoes, or trapping of one river by another in past geological times, such as the Danube and Rhine Rivers, Rhine and Rona Rivers. (Keith & Allardi, 1997). Fish species dispersal continues today, and European fauna continues to be enriched with species from Asia such as *Carassius gibelio* (Bloch, 1782) (Arcadievitch, 2006) and *Perccottus glenii* (Dybowski, 1877) (Jurajda et al., 2006). Human activities are also an important pathway for alien fishes' dispersal. For example, fish's introduction has been traced back to antiquity.

Consequently, the number of alien fishes in many European countries has gradually increased. For example, the inland waters of France are inhabited by 27 nonindigenous fishes (Keith & Allardi 1997).

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In Italy there are 30 species of alien fishes, which is almost half of the Italian fresh water fish fauna (Marcanto et al., 2000). In the inland waters of Greece, alien fishes amount to 25 species (Koutsikos et al. 2018).

The impact of alien species has not only been positive, but it has also posed a strong threat to local ichthyofauna. In many cases, they have proven to be food competitors to the native fish species. In this regard Marcanto et al. (2000) discovered that the acclimatization of species of the family Coregonidae in the Italian mountain lakes was the main reason for the decrease of *Alburnus arborella* (Bonapart, 1841) catches. Others fish, such as *Ameliurus melas* (Rafinesque, 1821), which was imported to France in the 19th century, became not only food competitors, but also dangerous predators, destroying the spawning and larvae of native fish (Bruslé & Quinquard, 2013). In some cases, food competition and predation by alien fish species have even led to the extinction of local fish fauna. The acclimatization of fresh water fishes to Morocco was considered as the main reason for the disappearance of the local trout species *Salmo pallaryi* (Pellegrin, 1824) (Azeroual, 2000). The introduction of alien fish species in Europe has also contributed to the transmission of dangerous new diseases to the native fish. Such was the introduction pathway of the pathogen parasite *Bothriocephalus acheilognathi* (Yamaguti 1934), which spread to European fish farms, cultivating grass carp *Ctenopharyngodon idella* (Valenciennes, 1844) (Ahmad et al., 2018).

In the early 20th century, an overview of the Bulgarian ichtyofauna by Kovachev (1923) and an examination of the fish fauna of the Aegean watershed by Shishkov (1939) presented no data about the presence of non-native fish species. Only during the 1970s did Michajlova discover two alien fishes in the above-mentioned Bulgarian watershed: *Gambusia holbrooki* (Baird & Girard, 1853) and *Oncorhynchus mykiss* (Walbaum, 1792). Inspecting the species composition of the Maritsa River's ichthyofauna, Velcheva & Mechterov (2005) found three non-native fish species: *Pseudorasbora parva* (Temminck & Schlegel, 1842), *C. guibelio* and *Lepomis gibbosus* (L., 1758). In the same year Apostolou (2005) reported that the same species have been found in the Mesta River. In an overview of the Bulgarian fish fauna composition Stephanof (2007) listed 24 alien fishes in the Aegean watershed in Bulgaria, including invasive species, introduced fishes for aquaculture and for sports fishing in the dams, in the fish farms and also in the rivers. In 2010, Apostolous et al.'s guide to the fish of the Mesta River referred to 7 non-native fishes on the territories of both Bulgaria and Greece. Four of these species were freshwater fish: *G. holbrooki, L. gibbosus, P. parva* and *C. gibelio*. In their studies of alien fishes in Bulgaria Uzunova & Zlatanova (2007), Yankova (2016) conclude that the main introductory pathway was aquaculture. The other authors (Koutsikos et al., 2018; Welcomme, 1988) confirm that the major entrance pathway for alien species in Europe has been aquaculture, with sports fishing coming in the second place; other factors have also been acknowledged as important. More recently Uzunova et al. (2019) reported the presence of *Micropterus salmoides* (Lacepede, 1802) in the Bulgarian stretch of the Struma River, and in some dams in the South-Western part of the country.

Bulgarian inland waters are connected to these of other European countries. Many alien fishes, which initially dispersed to other countries of Western or Eastern Europe, are now found in Bulgaria. Population pressures, such as food competition, predation and new diseases threatening native fish, are also be expected to take place. So, an assessment of the distribution of non-native species in the Bulgarian rivers is particularly important. In particular the invasion rate of alien species in the rivers Topolnitsa, Luda Yana, Stryama and Chepinska, has not yet been determined. Therefore, purpose of this study is to assess the presence, distribution and abundance of alien fishes in the local ichthyofauna of these four rivers.

Materials and Methods

Study area. The study area includes the four tributaries of the Maritsa River, the biggest river of the Aegean watershed. These are: the Chepinska River flowing from the Western Rhodope Mountains, and the rivers Topolnitsa, Stryama and Luda Yana originating from the Sredna Gora Mountain (Fig. 1).

Fig. 1 shows the catchment area of the Maritsa River. This river springs from the Rila, the highest Bulgarian Mountain. The river basin includes the Thracian valley, which is enclosed by two major mountain ranges. On the north the Maritsa watershed is bound by the Sredna Gora Mountain and on the south by the Rodopes Mts.

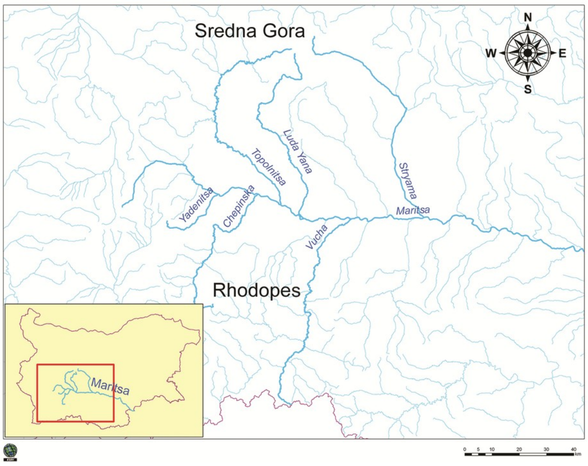

Fig. 1. Location of the study area.

The Chepinska River is a right tributary of the Maritsa River. The tributary's length is 81.7 km, with a catchment surface area of 899.6 km^2. The river originates from the Western Rhodopes Mountains (Hristova, 2012); it then flows into the Maritsa River near the village of Kovachevo (Pazardzhik).

The rivers Toponitsa, Luda Yana and Stryama are left tributaries of the Maritsa River. The Toponitsa is 154.8 km long and the surface of its catchment area is 1788.8 $km^{2.}$ The river springs from the Sredna Gora Mountain (Hristova, 2012). Three dams have been built on the Topolnitsa: Dushanci, Jekov vir and Topolnitsa. The river flows into the Maritsa River, west of the town of Pazardzhik.

The length of the Luda Yana River is 74 km and the surface of its catchment area is 685.3 km^2. The river springs from the Sredna Gora Mountain (Hristova, 2012); it flows into the Maritsa River near the village of Sinitovo.

The Stryama River is 110.1 km long. The surface of its catchment area is 1394.5 km^2. The stream Kavardjikliiska is generally considered as its source stream. The stream springs from the Stara Planina Mountain in the Teteven area. (Hristova, 2012). The river flows into the Maritsa River, near the village of Manole.

Taking into account their length, the Topolnitsa and Stryama rivers rank among the longest Bulgarian rivers, and the Chepinska and Luda Yana are classified as medium size rivers (Hristova, 2012). All four studied rivers are classified as a mountainous rivers (Protected Waters Act. 2012).

Study materials

Study material has been collected over a period of five years: 2005 – 2007 and 2010 - 2011. During this period, a sample of more than 3500 fish were caught in the rivers Topolnitsa, Luda Yana, Stryama and Chepinska by electrofishing (Kolev, 2010, 1913). This material is supplemented by data obtained during a study of density and biomass of *Salmo sp.* in 2008, in the upper zones of the same four rivers (Kolev, 2010). A SAMUS 725G converter was used, providing up to 640V direct current (DC), with a frequency of 50 Hz and output power reaching up to 200W. The catch was performed according to the EN 14011: 2004 instruction (Water quality – Sampling of fish with electricity). In order to collect the material for the study, 15 sampling areas were used (Table 1).

Each fish was measured in order to establish the following parameters: standard lengths (*L*) of the fish with a precision of 1 mm and net weight (*NW*) with a precision of 1 g. Total weight (TW) also was measured with a precision of 1 g. Total weight was used to estimate the alien-native fishes mass ratio. Fish age was determined by measuring fish scales, by using a microscope with a 40×

magnification. The identification of fish species was made according to the classification of Kottelat & Freyhof (2007).

The study uses the classification of alien species by Richardson et al. (2000).

Results

Among the 18 identified fish species of the rivers Topolnitsa, Luda Yana, Stryama and Chepinska (Kolev, 2010, 2013), three fishes we found to be invasive: C. *gibelio*, *P.*, *L. gibbosus*. One species – *O. mykiss* was identified as an introduced species (Bruslé & Quinquard, 2013; Jurajda et al., 2006; Kateřina, 2013; Van Kleef et al., 2008, see Table 1 and 2).

No alien fish species have been found in the upper parts of the studied rivers – i.e. in river stretches, located higher than 800 meters above the sea level. Hydrologic particularities of the middle zone of the surveyed rivers determined the strong dominance of two local ichthyofauna: *Barbus cyclolepis* (Heckel, 1848) and *Squalius orpheus* (Kottelat & Economidis, 2007). These two fishes accounted for over 85% of the local fish population. Each of the other fish species did not exceed 4% of the fish fauna. Overall, the four non-native species comprised 2.6% of the local fish fauna (Kolev, 2010, 2013).

Measuring 0.2% of the total fish sample, the abundance of *O. mykiss* was the smallest. The fish has been farmed in the trout farms "Kleptuza" (Velingrad) and "Dabene" (Karlovo) and was accidentally released into the rivers Chepinska and then Stryama. Consequently, the species was found only in the middle zone of these rivers (Table 1). Overall the rainbow trout is less abundant (Fig. 2). The specimen were one-year-old (1+), shorter than 300 mm and weighing less than 250g.

C. gibelio was widely distributed in all surveyed rivers (Fig.2). It was the most numerous fish amongst the nonnative species, comprising 1.2% of the total sample. *C. gibelio* was frequently detected in proximity to the confluence of the surveyed rivers and the Maritsa River. The fish was also present in the inert river materials extraction reservoir by the village of Lozen (Table 1). The age of the fish varies between 1 (0+) and 4 years. The biggest specimen, a four years old fish caught in Luda Yana, was 147 mm long and weighted 91g.

Table 1. Sampling areas. Presence of non-native fish species. *Legend:* "+" - the species was present; "-" - the species was not present; PrCp - prussian carp (*C. gibelio*); TmGd – topmoouth gudgeon (*P. parva*); Psun – pumpkinseed (*L. gibbosus*); sunfish; RbTr - rainbow trout (*O. mykiss*).

River	Location	PrCp	TmGd	PSun	RbTr
Chepinska	1. East of the village of Zlokuchene, near the road bridge of Belovo (Pazardzhik).	+	-	-	-
	2. A reservoir for inert materials extraction near the village of Lozen (Pazardzhik).	+	-	+	-
	3. Near the mineral baths of the Varvara village (Pazardzhik).	-	-	-	-
	4. Near the railway station M. Nikolov (Pazardzhik).	-	-	-	-
	5.Near the park "Kleptuza" (Velinfrad).	-	-	-	+
	6. On the road from Velingrad to Ablanitsa village (Pazardzhik).	-	-	-	-
Topolnitsa	7. Near the Boshulya village, next to the bridge under the Pazardjik-Septemvry road.	+	+	+	-
	8. On the road between the villages Lesichevo and Muhovo (Pazardzhik).	-	+	-	-
	9. Next to the dam wall of Topolnitsa reservoir, near	-	-	-	-

	the village of Muhovo (Pazardzhik).				
LudaYana	10. Next to the bridge under the Pazardzik-Plovdiv road.	+	+	+	-
	11. Next to the bridge under the Pazardzik-Chernogorovo village road.	-	-	+	-
	12. West of the the bridge under the Plovdiv – Rakovski village road.	-	-	-	-
Stryama	13. Near the Rajevo Konare village (Plovdiv).	-	-	+	-
	14. Next to the trout farm, near the Dabene village (Karlovo).	+	-	+	+
	15. West of the Rozino -Slatina villages road bridge (Plovdiv).	-	-	-	-

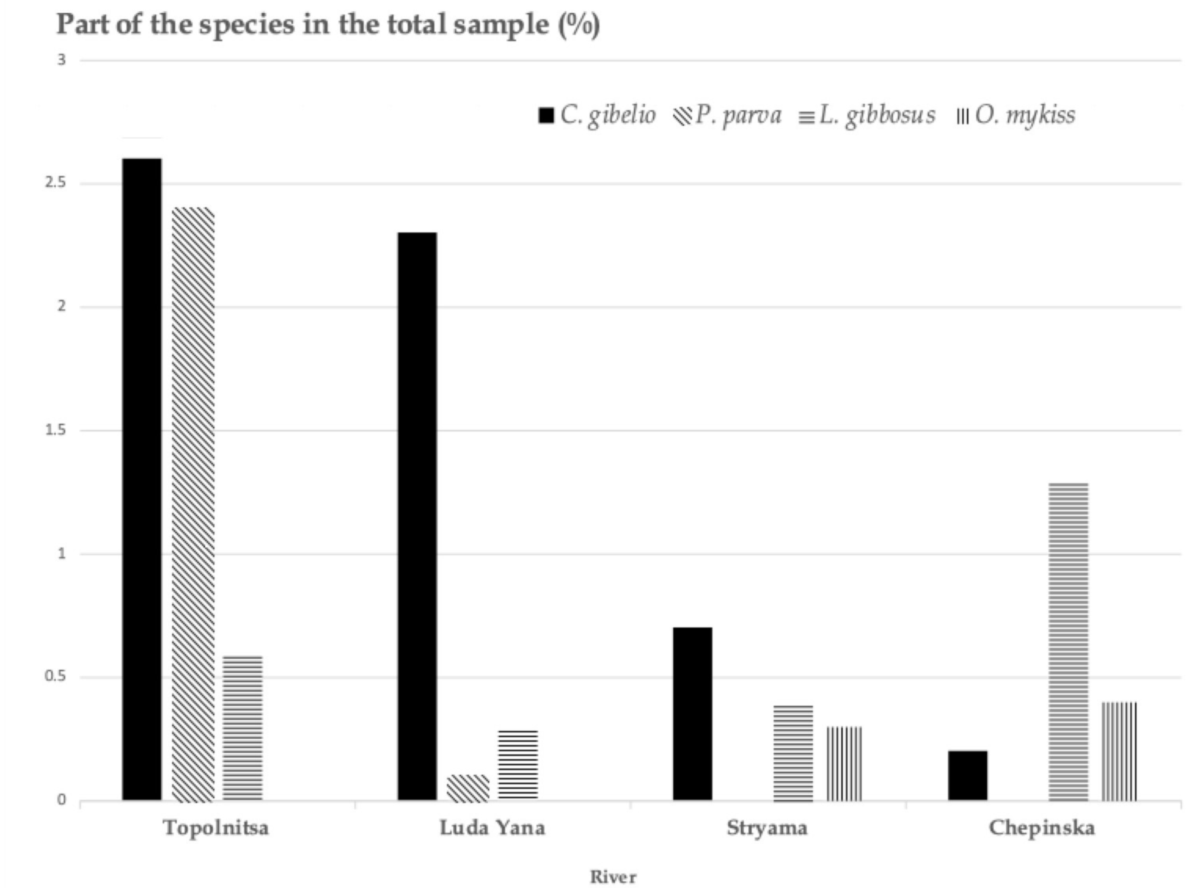

Fig. 2. Proportion of nonindigenous fishes of the total fish simple, caught in the rivers Topolnitsa, Luda Yana, Stryama and Chepinska.

L. gibbosus was the second most abundant alien fish in all four surveyed rivers; it accounted for 0.2% of the total fish population (Table 1). The sunfish was most numerous in the inert river materials extraction reservoir adjacent to the village of Lozen (Pazarzik). The biggest specimen caught in that water body, a five years' old (5+) sunfish, weighted 33g and reached up to 100 mm in length.

P. parva populations are established in the rivers Topolnitsa and Luda Yana, mainly in slow flow areas. The fish comprises 0.5% of the overall fish population. *P. parva* was most abundant in the Topolnitsa River (Fig. 2), wherein the catch of the biggest specimen took place: a four-year-old fish with a length of 80 mm and weighing 9.2 g.

Table 2 present data about the total weight of each fish, of each river. The table data was calculated by using the total weight of the alien and native fishes from all samples.

Table 2. Alien vs. native fishes mass ratio. Total fish weight (TW) (g) from samples 2005-2007, 2008, 2010-2011, collected in the upper and middle zone of the studied rivers. *Legend:* "*" – missing data, because of species higher conservation status.

Species / River	Topolnitsa	Stryama	Luda Yana	Chepinska	Total	%
Alien species weight (g)						
Carassius gibelio (Bloch, 1782)	315	105	331	171	*921*	
Oncorhynchus mykiss (Walbaum, 1792)	0	270	0	0	*270*	
Lepomis gibbosus (L., 1758)	41	67	15	119	*241*	
Pseudorasbora parva (Temminck & Schlegel, 1842)	74	0	5	0	*78*	
Total alien species weight (g)					1570	2
Native species weight (g)						
Squalius orpheus (Kottelat & Economidis, 2007)	10156	13768	3800	6350	*34074*	
Barbus cyclolepis (Heckel, 1848)	5651	6825	3570	6671	*22718*	
Salmo sp.	485	453	353	5150	*6441*	
Rutilus rutilus (L., 1758)	339	55	857	558	*1801*	
Chondrostoma vardarense (Karaman, 1928)	251	24	0	365	*639*	
Gobio bulgaricus (Drensky, 1926)	255	107	77	142	*581*	
Esox lucius (L., 1758)	0	300	0	0	*300*	
Perca fluviatilis (L., 1758)	0	31	101	132	*264*	
Alburnus alburnus (L., 1758)	30	25	1	160	*215*	
Vimba melanops (Heckel, 1837)	6	152	0	16	*174*	
Phoxinus phoxinus (L., 1758)	0	23	0	0	*23*	
Rodeus amarus (Bloch, 1782)	*	*	*	*	*	
Cobitis strumicae (Karaman, 1955)	*	*	*	*	*	
Sabanejewia balcanica (Karaman, 1922)	*	*	*	*	*	
Total native species weight (g)					67230	98
Total all species weight (g)					**68800**	100

Alien fishes' part of the weight of the total fish biomass in all catches remained negligible, less than 2 %. More than half of alien fishes' mass belonged to *C. gibelio*.

Discussion

Non-indigenous species percentiles of the ichthyofaunal populations of the rivers Topolnitsa, Luda Yana, Stryama and Chepinska are relatively small. The middle zone of the Maritsa River tributaries is strongly dominated by two fishes: *B. cyclolepis* and *S. orpheus*, which together comprise more than 86% of the local fish populations (Kolev, 2010, 2013). This fact is also strongly supported by the alien vs. native fishes mass ratio (Table 2). However, *C. gibelio*, *L. gibbosus*, *P. parva and O. mykiss* comprise about one fifth (18%) of the total fish population, with the remaining 15 fish species comprising altogether the remaining 82% (Kolev, 2010, 2013). Alien fishes are most abundant in the rivers Topolnitsa and Luda Yana, amounting to 5.7% and 2.7%, respectively, of the fish population. There are fewer alien fishes in the rivers Chepinska and Stryama: 1.9% in the former and 1.4% in the latter.

Strong water flow in the upper and middle zones of the surveyed rivers represents unfavorable habitat conditions for *L. gibbusus* and therefore it has a limiting impact on its distribution and abundance (Fedonenko & Marenkov, 2013). Consequently, the resultant small species density diminishes the negative impact of the *L. gibbusus* presence on local ichthyofauna. However, in areas with slow water speed, such as the inert river materials extraction reservoir, in proximity to the village of Lozen (Pazarzik), the numbers of *L. gibbusus* rapidly increase. High *L. gibbusus* density in the reservoir confirms the opinion of some authors (Uzunova et al., 2012; Van Kleef et al., 2008), that aggregates extraction reservoirs are one of the favorite habitats of this species. The study discovered that in the Lozen reservoir *L. gibbusus* has more significant influence on the rest of the fish community. This impact may be expressed mainly in competition for food, reduction of planktonic and benthic food and in the direct destruction of other fish's caviar (Uzunova et al., 2012). The study also found that many adult specimen inhabiting the Lozen gravel reservoir had length greater than 75 mm. These adult fish are fully capable of ensuring the reproduction of the *L. gibbusus* population at that location (Fedonenko & Marenkov, 2013).

The higher *C. gibelio* density in all the surveyed rivers is in full accordance with its widespread distribution in all Bulgarian water bodies (Apostolou, 2005; Apostolou et al., 2010; Boyadjiev, 1969; Karapetkova & Dikov, 1986; Stefanov & Trichkova, 2006; Stefanov, 2007). This is due to the great adaptability, reproductive potential and omnivorous nature of the species (Lorenzoni et al., 2007), even though its density in the middle zone of the Maritsa River's tributaries is lower than in standing water bodies (Boyadjiev, 1969). The present study found most of this species in zones, characterized by anthropogenic modifications, or so-called corrections, of the river bed, as well as the construction of dykes along the shores of the rivers Topolnitsa and Luda Yana, right before their confluence with the Maritsa River. *C. gibelio* competes for food with native fish species. Behavior, such as caviar predation and destruction, are very rare in this species. *C. gibelio* preferred habitats are different from these of the main fish species, inhabiting the area (Arkadievitch, 2006, Ulianovsk, Russia – pers. com.). *C. gibelio*'s, lack of suitable breeding sites, as well as sports fishing do not allow the fish to increase its abundance in the middle zone of the Maritsa River. In consequence, its impact on local fauna is not significant.

P. parva is more abundant in the Topolnitsa River, wherein this fish was introduced by the water outflow of the Topolnitsa dam. *P. parva* is most commonly introduced with the larvae of *Hypophtalmichthys molitrix* (Valenciennes, 1844) and *Hypophtalmichthys nobilis* (Richrdson, 1845) (Boltachov et al., 2006; Yankova, 2016). Fish stocking with these species in the Topolnitsa Dam (unpublished data from local forestry officials) have introduced *P. parva* there. The fishes are absent in the section of the river, located directly below the dam wall, which is adjacent to the village of Muhovo, because of a strong water current found there. Adapted to calmer waters (Kotovska & Hristenko, 2013) *P. parva* migrates to calm areas of the water course. These are the lower parts of the rivers Topolnitsa and Luna Yana, right before their inflow into the Maritsa River. Since this fish has a very large nutritional spectrum (Kotovska & Hristenko, 2013), it competes for food with native fish species, especially with the smallest-size fish groups. In three of the four studied sites, wherein *P. parva* is established, this fish co-inhabits with the *C. gibelio* (Table1). According to Didenko (2013), *P. parva* prefers areas, inhabited by *C. gibelio*. The fish not only enters into food competition with *P. parva*, but also parasites on it. Parasitic behaviour causing damage to the skin and gnawing on fins (Abramenko, 2012; Boltachev et al., 2006). It is also probable that caviar predation of *P. parva* also takes place in the surveyed rivers, as described in other water bodies by Kotovska & Hristenko (2013). The study found that the length of a high number of specimen from the rivers Topolnitsa and Luda Yana exceeds 30 mm, so the specimen represent fish, which has probably already matured (Boltachev et al., 2006). The high ecological plasticity, eurythermality, early sexual maturation and high reproductive potential of *P. parva* (Boltachev et al., 2006) make it a serious potential threat to native fish species in the middle zone of the Maritsa River. Koutsikos et al. (2018) assess the presence of *P. parva* and *L. gibbosus* in Greece, as particularly threatening to the local ichthyofauna.

O. mykiss has a limited spread and very small abundance in the Maritsa River tributaries. Its presence is due to accidental release from trout farms. The trout's negative impact is expressed in food competition with the *B. cyclolepis*, *S. orpheus*, *P. fluviatilis* and *E. lucius*. *O. mykiss* supplants other fish from their habitats and destroys their offspring. However, due to its low abundance, the negative impact of this fish is negligible. Domesticated in fish farms, the *O. mykiss* has now lost its instinct for self-preservation (Plasseraud, 1990) and the fish has become an easy catch for anglers. Sports fishing and absence of natural reproduction, observed only in the Rila Mountain by Konstantinov (1964), explain the low survival rate of this species in the Maritsa River tributaries. Thus, the presence and impact of *O. mykiss* in these rivers is fully controllable.

Currently, the small biomass of alien fish species still does not allow them to have a significant negative impact on the local ichthyofauna. Predation and food competition remain negligible. Transmission of new diseases to local fish has not yet been reported. At the same times, the most commonly represented species, both in abundance and in mass: *C. gibelio,* is also a desirable object for sports fishing, which suppresses its population increase..

Conclusions

Of the four non-native fishes, discovered in the middle zone of the Maritsa River tributaries, the most widespread are *C gibelio* (1.2% of the sample) and *L. gibbosus* (0.7% of the sample). Both species are observed in areas with slow water flow or standing water, such as river stretches with corrected riverbeds, as well as habitats with the river run gravel. However, the faster water flow speed and the dominant habitat of gravel riverbeds limit the penetration of these species to the majority of river beds and limit their impact on local fish fauna. The presence of *P. parva* is linked to fish stocking with *H. molitrix* and *H. nobilis*, and presents a significant potential threat to the local ichthyofauna. Overall, the habitat conditions in the upper zone of the Maritsa River tributaries are completely inappropriate for the four alien species and non-indigenous fish have not been able to penetrate there. The spread of alien species in the investigated rivers has been particularly facilitated by human activity.

Recommendations

Limiting the negative impact of *C. gibelio* and *L. gibbosus* in the Maritsa River tributaries requires a cessation of the anthropogenic river bed alterations and restoration of their primary appearance and water flow.

Strengthening the dominant habitat of control of the existing fish farms can prevent further increase in Rainbow trout abundance in the surveyed rivers.

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Comparison of Endophytic Colonization of Bulgarian Variety of Tobacco by Enthomopathogenic Fungi - Beauveria bassiana and Beauveria brongniartii

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Abstract. In the modern breeding programs, the application and utilization of endophytic potential of microorganism is an opportunity to reduce damage from different pests and viruses on tobacco plants. In the present study, 48 plants of 56 day seedlings of oriental tobacco (Krumovgrad 58) plants treated with 2 strains 538 and 730 of the entomopathogenic fungi *Beauveria bassiana* and a strain 646 of *Beauveria brongniartii*. Two different inoculation techniques were applied by spraying the leaves and by directly placing the inoculant in the soil near the root of the plants. In order to compare the effectiveness of the colonization techniques of different tobacco tissues roots, stems and leaves, samples were taken for analysis on 7, 21 and 28 days after inoculation. Results have proven that all three strains of *Beauveria* endophyticaly colonize different tobacco tissues within 28 days after inoculation. The outcomes of the present study show the potential of *B. bassiana* and *B. brongniartii* to use for prevention and protection of tobacco plants.

Key words: *Beauveria bassiana*, *Beauveria brongniartii*, *Nicotiana tabaccum*, endophyte.

Introduction

Tobacco production is of great importance for the Bulgarian economy. Unlike other agricultural crops, tobacco occupies relatively small areas, and the products obtained from it have a great economic significance (Dimitrov et al., 2005). One of the main reasons for reducing tobacco crops is damage and attack from various diseases, pests, weeds and parasites. In addition to the application of various pesticides and herbicides today, a number of effective and more environmentally friendly methods for combating tobacco diseases are sought (Dimitrov, 2003). Trends in plant breeding require a more profound

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understanding of the interactions between different components of the ecosystem and the use of this knowledge to applying new strategies in diseases and pests control (Vega et al., 2009). A key role in this relatively new approach to plant protection involves entomopathogenic fungi. Those microorganisms are usually endophytes, which mean that they exist inside plant tissue without causing any symptoms to the infected plant (Russo et al., 2015). The microorganisms can produce a series of chitinases, some of which act synergistically with proteases degrading the chitin shell of insects (Fan et al., 2013). One of the best studied and commonly used endophyte fungi are *B. bassiana* and *B. brongniartii*. Different techniques for introducing these endophytes in plants and soils have being conducted. As well as colonization rate of fungi has been evaluated. Considering the fact that endophytes have an antagonistic effect on specific insects and plant pathogens, the ultimate goal is to use them as a biological control agent against certain pests (Vega et al., 2009).

In addition, to pest and disease control a positive role of four *B. bassiana* strains has been published to decrease Zucchini yellow mosaic virus (ZYMV) infection in pumpkins (Jaber and Salem, 2014) has also been demonstrated. *B. bassiana* and *B. brongniartii* are effective against larvae of *Paraproba pendula* (Tajuddin et al., 2010), Stachys affinis (Goble et al., 2014; Konstantopoulou & Mazomenos, 2005) and the adults of *Tenebrio molitor* (L.), *Ceratitis capitate* and *Bactrocera oleae*. *B. brongniartii* produces secondary metabolites by which it kills the larvae of *Dendrolimus tabulaeformis* (Fan et al., 2013).

The aforementioned properties of *B. bassiana* and *B. brongniartii* are the reason for their artificial introduction into various economically important crop plants. The endophyte nature of insect pathogenic fungi in economically important plants such as *Vicia faba* (Jaber and Enkerli, 2016) plants has been proven. The study of C-sources utilization of isolates, collected from different regions of Bulgaria, has been studied and is compared, which included their phenotypic characterization and differentiation on their biochemical profiles. Each fungal isolate has been shown to exhibit a different specific profile, but sucrose, maltose and trehalose are assimilated to a higher degree than esculin, arabinose and dulcitol (Canfora et al., 2016).

In tobacco seedlings, foliar treatment results in 100% colonization of the leaves seven days after inoculation and decreases at the 28th day after inoculation. In maize, wheat and soybean, significant differences ($p < 0.001$) in endophyte colonization between different foliar, root and seed inoculation techniques (Russo et al., 2015) have been observed. Besides specifying the best inoculation technique for a given crop, another important aspect for maximally effective use of enthomopathogenic fungi is to determine the length of colonization of the fungus in the tissues. In a banana, *B. bassiana* was able to colonize the plant tissues for 4 months after the tissue-cultured plants were immersed in a spore suspension (Akello et al., 2009). The purpose of the study was to determine the ability of two *B. bassiana* strains (538 and 730) and a strain of *B. brongniartii* (646) to colonize different parts of the tobacco up to 28th day after inoculation and to compare the effectiveness of colonized leafy and soil inoculation of *Nicotiana tabacco* plants.

Materials and Methods

Plant material. The present study was conducted with 56 day seedlings from oriental tobacco (variety Krumovgrad 58, botanical classification: *N. tabacum*, *Basma*). The vegetative period, from planting to mass flowering, is 70-80 days. Each pot contains 400 g of peat mixture. All pot plants were watered with 50 mL of spring water in the pads to avoid inoculum loss in the soil-treated plants.

Fungal isolates. The fungal isolates were provided by prof. Slavimira Draganova Agricultural Academy – Bulgaria, Institute of Soil Science, Agrotechnologies and Plant Protection (ISSAPP). The strains 538 and 730 of *B. bassiana* (Bals.) Vuill from Moniliaceae family, order *Moniliales*, class *Deuteromycetes*, were isolated from larvae of the *Coleoptera* family *Chrysomelidae* spp. Strain 646 strain of *B. brongniartii* was isolated from *Coleoptera* species (*Hylurgops palliatus* Gyll.) of the family *Curculionidae*. Fungal cultures, starting from dry conidia, were grown on Sabouraud's dextrose agar in dark at 22 °C.

Morphological evaluation of the isolates. The fungal isolates were cultured on Yeast extract agar (YEA) plate (0.5 g yeast extract, 10 g glucose, 20 g agar and 1000 ml distilled water) and were maintained in an incubation chamber at 25°C. After an 8-day period, the macroscopic characteristics of each colony were described through the observation of the following parameters: growth rate considering the colony diameter, aspect and color of conidial and reverse masses, and exudate production.

Molecular identification. DNA was isolated with HiPurA™ Fungal DNA Purification Kit (HiMedia, India). The control of purity and concentrations of genomic DNA was conducted by electrophoresis in an agarose gel. ITS1-5.8-ITS2 region of the nuclear ribosomal DNA was amplified with ITS1 and ITS4 universal primers (White et al., 1990). PCR analysis was performed in 20 µl reaction final volumes containing 1 µl (30-50 ng) of DNA and 2 µL 10 × reaction PCR buffer mixture, containing 200 nM solution of dNTPs, 5 µM $MgCl_2$, 1 µl of 10 µM primers and 0.25 µl of 5 U / µl of Red-Taq DNA polymerase (Canvax, Spain). The amplification reaction conditions consisted of 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C with a final extension of 5 min at 72°C. Expected amplicons of 500 bp are excised from the gel and purified with a Gel isolation kit (Exgene Cells SV, mini, Gene All, U.K.). PCR products were separated on 1% agarose gel stained with SafeView (NBS Biologicals, UK) at 100 V for 50 minutes using a VWR Mini Electrophoreis System for gel visualization. Gene Ruler 1 kb plus (Bioneer, South Korea) is used as a molecular marker. The resulting sequences were analyzed with BLAST software (Altschul et al., 1990) and compared with nucleotide sequences in the gene bank database ncbi.nlm.nih.gov).

Conidial suspension. Conidia were obtained from cultures grown on YEA after incubation for 10 days at 25°C in darkness. Conidia were harvested with glass cell scrapers and placed in test tubes containing 0.01% (v/v) Tween 80 (polyoxyethylene sorbitan monolaurate) (Merck® KGaA, USA). Suspensions were vortexed for 2 min, filtered through four layers of sterile muslin, and adjusted to 1 × 108 conidia ml–1 (Gurulingappa et al., 2010) after cell counting by camera. Conidial viability was assessed before every experiment (Goettel and Inglis, 1997). This germination test was repeated for each stock suspension to maintain the constancy of the viability assessments. In all cases, the average viability of the conidia was over 90% for isolate 538, 95% for isolate 646, and 98% for isolate 730.

Inoculation techniques. Soil inoculation was performed by using a total of 36 plants with a 10 mL a conidia suspension obtained from each of examined strains with a concentration of 1 × 10^8 and was placed in the soil in close proximity to the plant roots (Fig. 1). Control plants were free of inoculum treatment. The foliar treatment was performed with 10 mL conidia suspension with concentration of 1 × 10^8. Aluminum foil was also placed to prevent inoculation from the soil and the roots of the plants. Isolations of leaf-treated plants were removed 12 hours after inoculation (Fig. 1).

Endophytic activity evaluation. On the 7th, 21st and 28th days post tobacco inoculation, samples of treated plants were taken to detect the presence of *B. bassiana* and *B. brongniartii* by inoculation of leaf, stem, and root explants of YEA medium. Two whole plants (i.e., root, stem and leaf) treated with the 3 different strains and 3 control plants were taken from the soil drench tobacco. Plants were removed from the soil and washed with dH_2O. Prior to introducing the explants into an in vitro medium, surface sterilization of the leaves, stems and roots was performed for 3 minutes in 0.008% Tween 80 w/v, 3 min in sodium hypochlorite NaOCl solution, 1 min in 70% ethanol and three times rinsed with sterile dH_2O for 50 s. To control the quality of antiseptic inoculation an antibiotic broth was made to the last washing sterile dH_2O (used in sterilization of the explants) to control the sterilization performed and to prove that the grown colonies of the fungus in the explants placed were not due to the surface layer of the plants. Six leaf disks of approximately 1 cm3 were incubated in culture medium with antibiotics added of concentration 0.02 g ampicillin, streptomycin and tetracycline. The presence of *Beauveria* fungi was recorded 10 day post incubation at 27 °C in the dark.

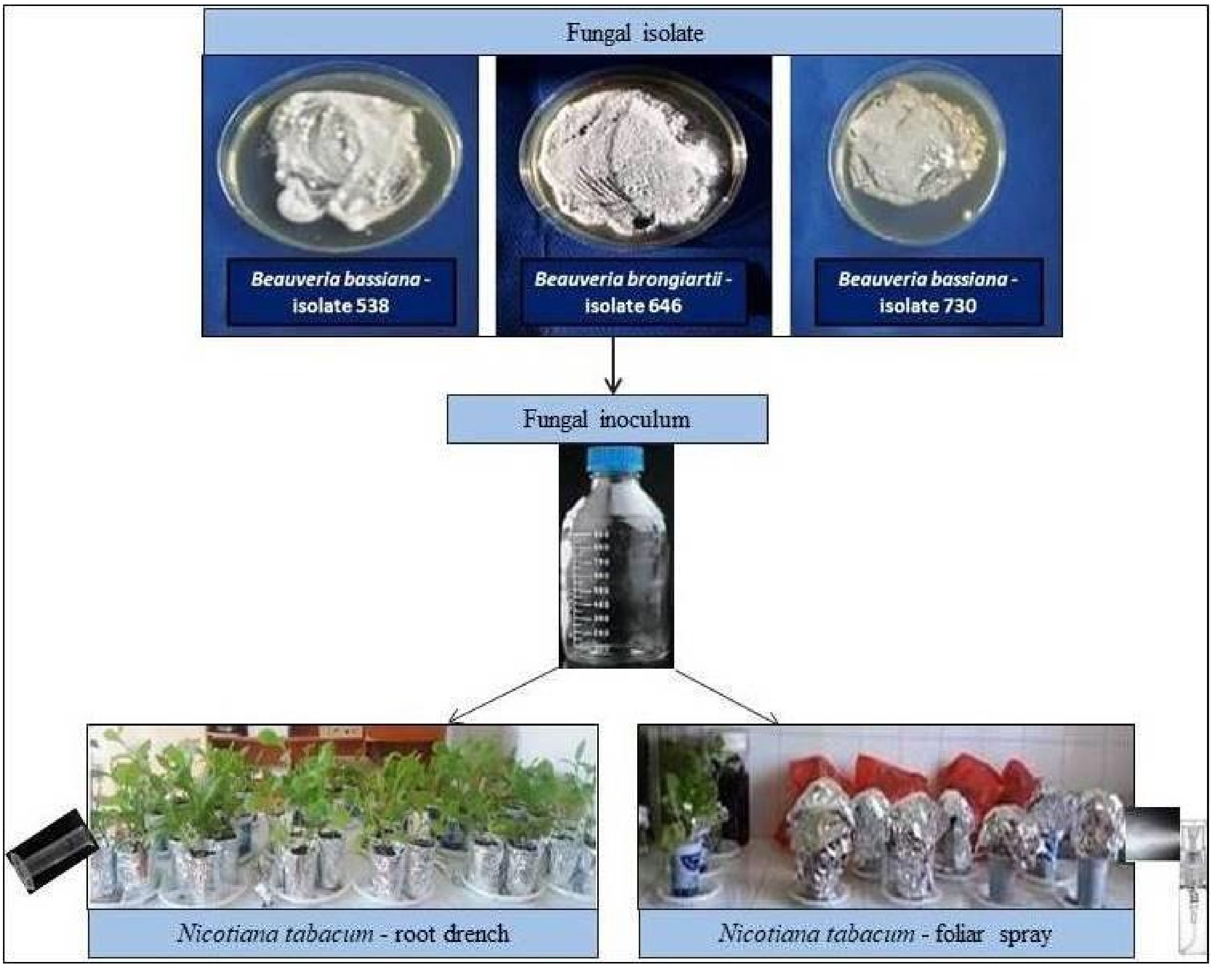

Fig. 1. Different inoculation techniques – soil drench and foliar spray with strains 730, 646 and 538.

Statistical processing of the results obtained. Isolation frequency (IF) and the degree of colonization (CR) of the *Beauveria* strains are calculated using the following formula: Isolation frequency (IF) = Ni/ Nt × 100 Colonization coefficient (CR) = Nc/ Nt × 100 Where Ni is the number of segments from which a fungi has been isolated; Nc is the total number of segments from which mushroom fungi were isolated from a sample and Nt is the total number of segments from which strains 538 and 730 of *B. bassiana* and strain 646 of *B. brongniartii* were isolated (Sun et al., 2011; Russo et al., 2015).

Results and Discussion

Morphological evaluation and molecular identification of the f isolates. The extensive overlap in conidia shape and dimensions among *Beauveria* species has limited their utility as key taxonomic structures (De Hoog, 1972; Parsa et al., 2013). Isolates could be divided into *B. bassiana* and *B. brongniartii* based on conidial dimensions; isolates with conidia longer than 3 µm were classified as *B. brongniartii*, isolates with shorter, spherical conidia were *B. bassiana*. On YEA *B. bassiana* grows slowly as a white mould with dry, powdery conidia in distinctive white spore balls. Each spore ball is composed of a cluster of conidiogenous cells, resulting in a long zig-zag extension. The fungi are characterized morphologically by globular to subglobular conidia. Although was strain 646 is determined morphologically as *Beauveria brongniartii* by its ellipsoidal conidia.

After processing the sequencing results and performing BLAST analysis with available data in GenBank the strain species identity was determined. Based on 18S gene sequences were compared with available in the database for the genera *Beauveria*. Nucletide sequence of 500 bp PCR fragments were used to define genetic similarity of the isolates with Mega 7.0 program by using neighbour-joining analysis (Kumar et al., 2016). After analysis, similarity between *B. bassiana* and *B. brongniartii* was very high (Fig. 2). Isolate 538 showed the high percent similarity with *B. bassiana* MG642849.1 (Vu et al., 2019; Mukawa et al., 2011). Strain 646 was established as *B. brongniartii*. Significant isolation-by-distance relationship was found (r = 0.33). Neighbor - joining analysis results showed that all the studied populations were divided into two discrete genetic groups with significant separation insignificant separation between two forms of *Beauveria* fungi. The sequencing of the ITS1-5.8 S - ITS2 rDNA regions also showed the insignificant separation of the two strains 730 and 538 of *B. bassiana*.

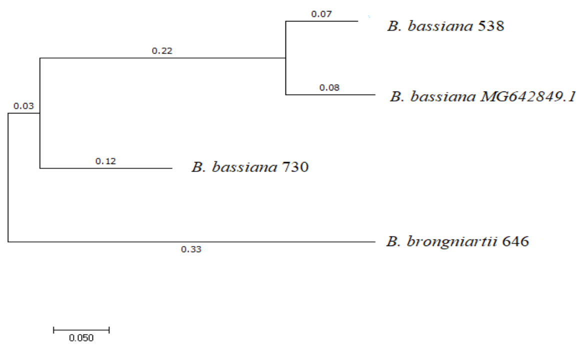

Fig. 2. Phylogenetic tree derived from neighbor-joining analysis depicting members of the *Beauveria* clades and a reference species. Branch lengths are proportional to the number of nucleotide differences. The marker bar denotes relative branch lengths.

Investigation of the frequency of fungal colonization. The frequency of colonization of the three isolates of insect pathogenic fungi (538, 646 and 730) was studied in a total of 36 soil treated plants and 12 leaf spraying plants. *B. bassiana* and *B. brongniartii* have not been applied to control plants.

On the seventh day after treatment of tobacco plants with 1×10^8 spore suspensions of *B. bassiana* strain 538, 730 and strain 646 of *B. brongniartii*, using the direct inoculation technique in the soil, the highest colonization rate was recorded in *B. bassiana* strain 538 - 16.6%, followed by strain 730 - 15.3%. *B. brongniartii* 646 has the lower colonization rate of tobacco roots around 7.14% (Fig. 3). The endophytic colonization of leaf explants on YEA media were relatively low - from 6.6% to 16.6%. Significantly high colonization of steam was recorded with root drench with strain 538 – 41.66%. Compared to this result when leaves were plated on YEA medium the amount of the inoculum was low - 6.6%.

In the first week after foliar spraying of the aerial parts of the plants, high colonization values of the leaves were found 27,20 - 49,65% and less of the stems 12,48 % - 33,33%. As a result of soil isolation during leaf inoculation, there is no development of the fungus in the roots of the test plants.

On day 7 after soil drench, the highest colonization rate shows *B. bassiana* 538, and *B. bassiana* 730 strains (Fig. 3).

On 21 days after the spore suspension was introduced into the soil, was observed an increase in the colonization rate of the fungal isolates of the three strains in the tobacco stems and leaves (Fig. 4). Similar results are also found with foliar treatment. There was a slight increase in stem and leaf colonization percentages in all strains tested. In order to better elucidate the vertical movement of mushroom endophytes, samples were taken from the lower and young upper leaves. It is particularly interesting that the old leaves have a higher colonization than the upper leaves. Results determined three times higher allocation of spores of isolate 538 and isolate 730 to the root in leaf-spaying treated plants on 21 DAT. In contract, when *B. brongniartii* isolate 646 was applied by leaf spraying, the root colonization was the two times lower compare to soil-inoculated plants.

On the 28th day after treatment, colonization was reduced in both inoculum delivery techniques as compared to the 21-day colonization rate (Fig. 5). In soil treatment, *B. brongniartii* strain 646 has the highest value. Foliar spraying of the plants showed he highest activity with *B. bassiana* strain 538. In leaf treatment, the colonization factor is higher on the lower leaf and decreases in the study of the upper young leaves.

Control plants are pure from colonization by *Beauveria bassiana* and *Beauveria brongniartii*.

Calculation of a colonization factor (CR). Fig. 6 presents the results of calculating a colonization factor (CR). On day 7 after soil introduction of inoculum from *B. bassiana* and *B. brongniartii* strain 538 was the most effective, followed by strain 646, and lowest value was determined at strain 730. In foliar treatment, highest rates of colonization were recorded when *B. bassiana* strain 730 was applied and lowest when strain 538 was used. *B. brongniartii* 646 is equally effective in soil drench application and foliar spraying inoculation till 28 day after treatment.

At the second and fourth weeks after treatment, the three *Beauveria* strains showed similar endophytic activity in both inoculum modes. On day 21, *B. brongniartii* 646 has with the highest colonization coefficient of 92.01% for soil treatment and 89.16% for leaf spraying. Analogous to the data in Fig. 6, at 28 days after the application of the fungal isolates, a significant reduction in colonization of tobacco plants was observed, the lowest being in soil treatment with strain 538 was 14.32%.

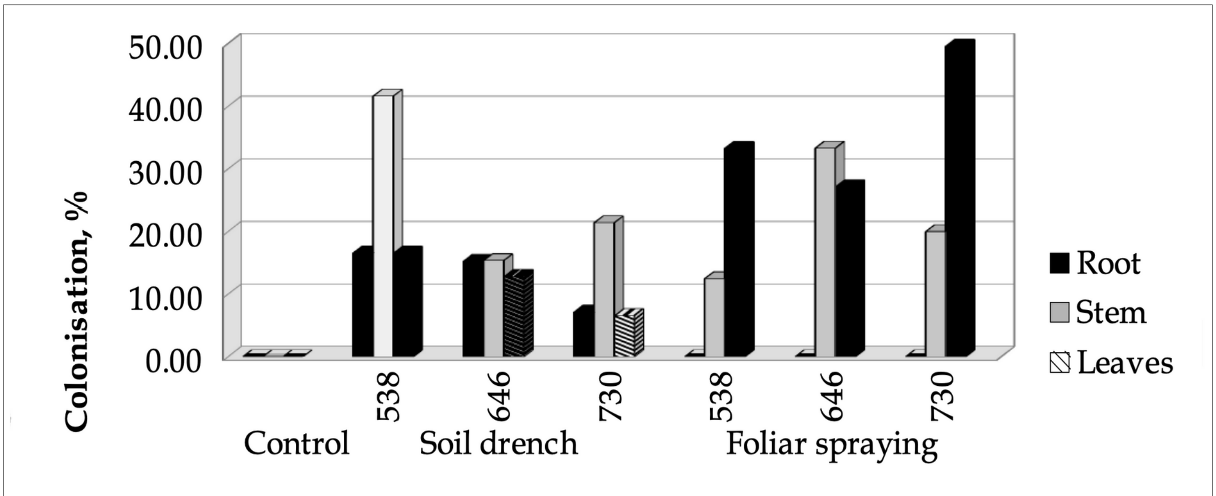

Fig. 3. Recovery percentage of *Beauveria* strains colonization of tobacco plants by soil drench and foliar spraying on 7th day post inoculation.

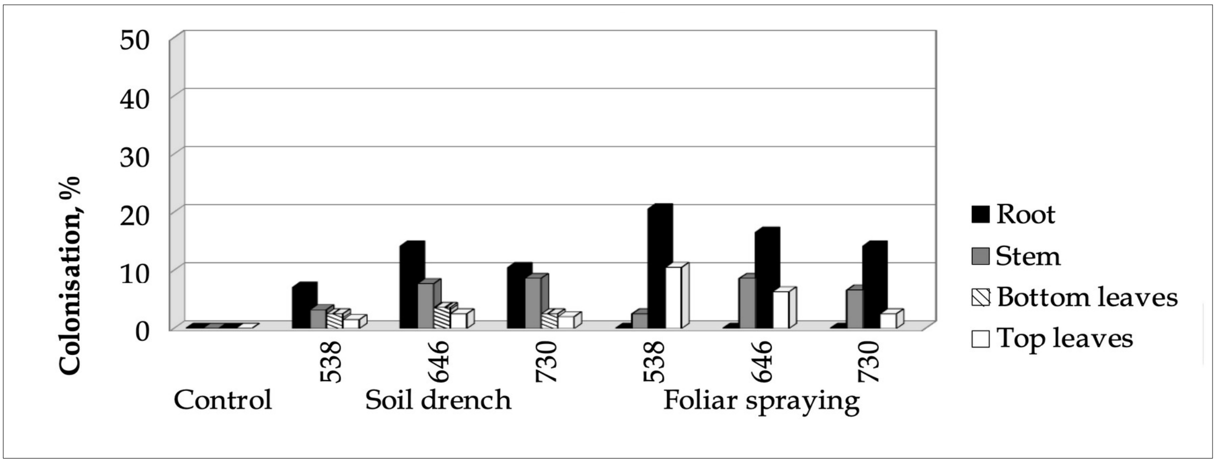

Fig. 4. Recovery percentage of *Beauveria* strains colonization of tobacco plants by soil drench and foliar spraying method on 21st day post inoculation

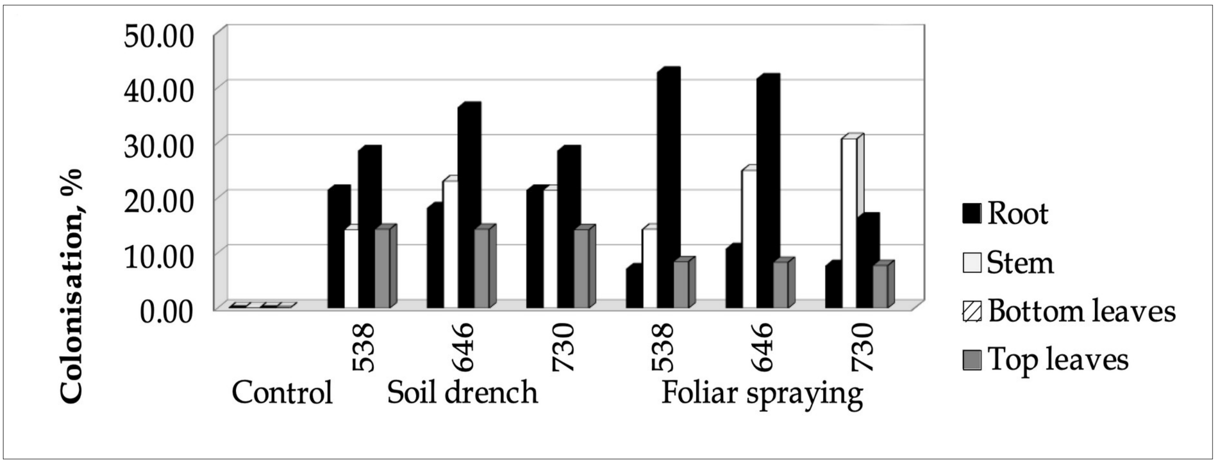

Fig. 5. Recovery percentage of *Beauveria* strains colonization of tobacco plants by soil drench and foliar spraying on 28th day post inoculation.

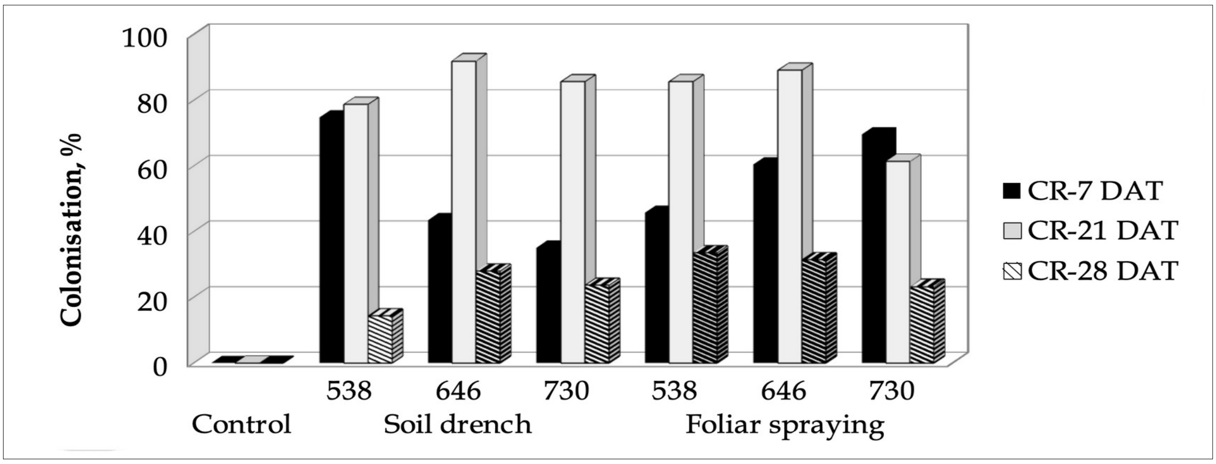

Fig. 6. Recovery percentage of CR of *Beauveria* fungi by different inoculation technique at 7, 21 and 28 days after soil drench or foliar spraying.

The present study found that *B. bassiana* and *B. brongniartii* successfully colonized tobacco plants. This result is similar to previous studies conducted with tobacco, corn soybean (Russo et al., 2015), opium (Quesada-Moraga et al., 2006) and tomatoes (Ownley et al., 2004). When different inoculation techniques were applied to several plants, alterations were observed and the date of the highest rate of colonization was recorded. In wheat, the highest percentage of colonization was achieved by leaf treatment on 14 day, and in root immersion and seed inoculation, the highest colonization was obtained on the seven day. In maize, the highest rate of colonization was achieved by foliar treatment on days 7 and 14 days (Russo et al., 2015).

According to Russo et al. (2015) data from the method of inoculation of tobacco plants with *B. bassiana*, the highest percentage of colonization was achieved by foliar treatment and the highest rate of colonization was recorded on 7 day. In contrast to the data obtained by Russo et al. (2015), current experiment shows relatively uniform rates of colonization with both applied techniques, with the exception that the leaf-treated plants with *Beauveria* was established root infection only on 21 day. The highest activity was recorded on the 7th day after inoculation, and in the present study, the highest colonization rates were 21 days after inoculation. Possible explanation is the activity of those fungi isolates, the type of inoculation and climatic conditions in the country. In support of this explanation is the result observed in two of the applied strains of *B. bassiana*. As a result of soil drench application of strain 538 on tobacco Krumovgrad 58 the percentage of the colonization rates were with similar values on 7th and 21st day. In leaf treated plants with strain 730 again the highest colonization rate was recorded on 7 days. Only in strain 646, distinguished by sequencing analysis as *B. brongniartii*, the highest colonization rate was found at 21st day for both inoculation methods. The results obtained by Russo et al. (2015) and current results demonstrated the tendency to reduce the colonization rate after the 21st day of treatment. In current experiment the endophyte activity of the fungus on day 28 was greatly reduced.

Most studies in other crops tend to reduce the fungal colonization of the various tissues of the plant over time (Greenfield et al., 2016), although vertical transmission of *B. bassiana* to the generations of endophyte colonized mother plants (Quesada-Moraga et al., 2014).

Conclusions

All three tested strains of *Beauveria* (646, 730 and 538) exhibit endophyte nature in the tobacco. Present study observed differences in efficacy among the two inoculation techniques. Both applied techniques with the three strains under examination have been found to colonize the different parts of the plants. When root immersion was used, the highest percentage of colonization of tobacco was detected at 21st day for the all the *Beauveria* strains applied. Leaf treatment with fungal strains 538 confirmed the highest percentage of colonization of tobacco on 7 day till 21 day after colonization. For strain 730 the highest percentage was recorded on 7 day post inoculation. There was no difference in colonization efficiency when applying the two different inoculation techniques. The strains of *B. bassiana* and *B. brongniartii* have been shown to be preserved in the different parts of the tobacco until 28 day, but the percentage of the inoculum decreases on the 28 day. Plant colonization does not affect the normal physiological development of tobacco. However, there is an obligation for better understanding of the biology of the entomopathogenic fungi in order to use them as biocontrol agents.

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Assessment of Trees Vitality in Urban Landscape of Steppe Zone

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Abstract. During planning of green planting in cities, vital status of plants which make up the groups is taken into account first. The methods of assessment of morphological and physiological parameters of the vital status are the most widely used ones. In the process of work, the vitality of woody plants was evaluated under the impact of anthropogenic pressure in the framework of the physiological state, which is determined to a great extent by the content of chlorophylls in the leaves of trees. The content of green and yellow pigments was studied in the drought conditions of vegetative period for 8 species of trees which are common in green planting of cities in the South-East of Ukraine. Stable species (*Populus simonii* Carriér, P*opulus* × *canadensis* Moench) respond by increase in the content of chlorophylls at maximum air temperatures during the growing season. Nonsufficient-stable species show significant effect of the amount of rainfall on the changes in chlorophyll content in leaves, which is the evidence of their adaptive capacity. Deterioration of the functional state of trees in street and park planting compared to suburban territories was revealed, confirmed by reduced chlorophyll content and increase in carotenoids. For the further introduction in the urban greening of industrial cities of steppe zone, the models of influence of the meteorological factors on the content of green pigments in the leaves of *Polulus simonii* and *Acer pseudoplatanus* L. are proposed.

Key words: trees, urban environment, total chlorophylls, carotenoids, meteorological factors, exhaust gases.

Introduction

Trees as a part of urban plantation perform a number of functions: they decontaminate the air, absorb CO_2, enrich the atmosphere with oxygen etc. However, in the conditions of big industrial cities, trees are affected by environmental pollutants. The air pollution of the territories of industrial-urban centres has on a complex character. In the residential areas of the city the main source of pollution are motor cars, in the industrial zones - the enterprises of thermal power, metallurgical, chemical, processing and mining industry. The result of car engines' running is the release of exhaust gases CO_2, N_xO_y, SO_2, hydrocarbons, aldehydes, soot and benzo(a)pyrene (Hopke, 2009). Among industrial pollutants, sulfur dioxide and nitrogen oxides represent the gravest hazard for plants. Premature fall of leaves and needles occurs in woody plants affected by sulfur dioxide (Palau et all., 2009). The action of nitrogen oxides leads to chloroses of various types: brownish-black sections appear on the top and at the edges of leaves (Spellberg, 1998). The level of pollution of a certain territory of the city is determined by the distance from the main sources of contamination, the major directions of the wind rose, the topographic features and the city planning situation (Korshikov et al., 2005).

Specific conditions of the southeastern steppe with high temperatures in summer and low temperatures in winter seasons, strong winds and low rainfall are exacerbated in the urban areas. Almost all environmental components in the big city – atmosphere, flora, soil, relief, hydrographic network, groundwaters, – undergo changes. Anthropogenic factors affect the indicators of temperature, humidity, the formation of air masses and light conditions, which in general leads to the emergence of peculiar microclimate. Thermal, chemical, radiation, electromagnetic, light, sound, vibration factors etc. are the main negative urban factors. In the cities, they often act simultaneously, particularly with regard to the motorways with heavy traffic (Zhang et al., 2007; Nowak et al., 2006; Morgenroth & Östberg; Iqbal et al., 2015).

In Ukraine, the large number of major cities with powerful enterprises are situated on the southeast. It should be noted that specific conditions of the southeastern part of steppe zone of Ukraine with high temperatures in summer and low temperatures in winter seasons, strong winds and low rainfall and drought are additional stressed factors for plants at urban territories. In these settings, the durability and efficiency of urban tree plantations are diminished, that has negative environmental and economic impacts (Korshikov & Vinogradova, 2005). In this connection, the urgent task of ecological research is to monitor and diagnose the living condition of urban-steppe landscapes plantations of the steppe zone with the complex influencing negative anthropogenic and natural and climatic factors.

In order to determine the vitality of trees, different approaches are used (Johnstone et al., 2013) – from ocular estimate method of the general conditions of the individual on point scales (Suslova et al., 2013), biomorphological assessments of growth, development, phenorhythm types and reproductive ability (Korshikov & Krasnoshtan, 2012) to physiological and biochemical indicators of the activity of metabolic processes and concentration of functionally important compounds and metabolites in the leaves - plastid pigments, total nitrogen, soluble proteins, non-structural pigments, starch, lipids, etc. (Glibovytska et al., 2017). A wide range of these methods is able to give definitely comprehensive assessment of the vital state of plants. At the same time, in ecological investigations preferences are disposed to the separate test-parameters and express-methods, which characterize the most adequately the state of plants under specific conditions depends on the performance targets.

Besides this, the parameter of photosynthetic pigment concentration is widely used in the conditions of industrial contamination of the functional state of the woody plants. The similar studies are conducted to determine the drought resistance of plants thereby the plant reaction to the pollution is the same as the reaction of dehydration factor. Photosystems of chloroplast especially photosystem II, that contains light harvesting complexes of photosynthetic pigments, are too sensitive systems of deferential mesophyll cells, and their adaptability ensures the preservation of the photosynthetic apparatus and its restoration in case of damage (Morgenroth & Östberg, 2017). According to Bessonova et al. (2015), sum of chlorophylls is reduced by the action of car exhausts. Changes of the concentration of chlorophylls and carotenoids indicate the decrease in the plants' metabolic processes under stress (Uhrin & Supuka, 2016). For quantity assessment of plants gas resistance has been suggested air pollution tolerance index, for the calculation of which, in addition to the leaf water content indexes, pH of leaf tissue extract, the parameter of chlorophyll concentration is used (Tripathi et al., 2009).

The physiological aspects of vital status of woody plants under the stressed hydrothermal urban conditions during the vegetative period are not yet fully understood. The easternmost continental area of the steppe zone of Ukraine – Donetsk city draws the biggest attention from this side. A full-scale study of the viability of the urban tree plantations was conducted here ifn through inventory using the ocular estimate method (Suslova, 2013). In some works, the physiological indicators of trees' vitality in the cities of southeastern Ukraine were determined. For example, the vitality for certain species of the *Acer* genus was determined with the use of chlorophyll concentration without taking into account meteorological factors of the period under study (Korshikov & Vinogradova, 2005). Therefore, the study of the impact of the urban environment on the functional state of trees and prediction of its subsequent changes is relevant for the proper management and planning of green building in the steppe zone.

The goal of the work was the assessment of the vitality of trees in urban and industrial territories in the conditions of continental drought climate of the steppe zone depending on the dynamics of changes and quantitative determination of plastid pigments.

Materials and Methods

Research was conducted during two years (2012, 2013) in one of the largest industrial cities of southeastern Ukraine – Donetsk. The city is located between 48° 02′ north latitude and 37° 48′ east longitude. Monitoring sites 1 and 2 (m.s. 1, m.s. 2) are situated in the residential area of the city with inherent level of air pollution, which is formed by the fumes from mining, processing and metallurgical enterprises, but they differ in the content of individual ingredients.

M.s. 1 is located on the Kyivskyi avenue with intensive with heavy traffic and higher pollution, m.s. 2 – in the large green area of the Recreation and Entertainment Park with less level of pollution. According to the Sectoral State Archive of the Hydro-meteorological Service of Ukraine (Ukrainian hydrometeorological center), the total average daily concentration of SO_2, NO_2, dust, CO, formaldehyde at m.s. 1 was 2.175 and 2.102 mg/m^3 in the years of research, whereas mt.2 – 1.712 and 2.006 mg/m^3. It should be noted that the level of SO_2 content at the monitoring points in both years was within 0.2 MPC.

Higher total level of pollution on m.s. 1 was achieved due to the increased content of the basic ingredients of exhaust gases in both years of research: CO - 1,6925 and 1,6515 mg/m^3 and, particularly, NO_2 - 0,1771 and 0,1731 mg/m^3, which was 4.43 and 4.33 MPC, respectively. At m.s. 2, the CO content was 1.2451 and 1.5234 mg/m^3; NO_2 - 0.0967 and 0.1575 mg/m^3 (2.42 and 3.94 MPC). Besides this, at m.s. 1 it was established increased average daily concentrations of ammonia - 0.0730 mg/m^3 (1.82 MPC), phenol mg/m^3 - 0.0036 (1.20 MPC). All this testifies to a higher level of air pollution on the m.s. 1 - the central city highway, compared to the city park area (m.s. 2).

Control monitoring site (m.s. 3) is placed at a distance of 15 km from the town in the village of Tonenke. There are no urban and industrial impacts on the environment, and it is characterized by a small (background to this region) content of ingredients such as SO_2 (0.0050 and 0.0100 mg/m^3) and NO_2 (0.093 and 0.1060 mg/m^3).

Species widely used in greening of the city were studied during the growing season in 2012 and 2013 (from May to October), which differ on the hydrothermal conditions. As illustrated in Fig.1, the first year was prosperous for plants due to the temperature regime and falls amount, except of the drought autumn period, which was not typical for the steppe zone. The second year was characterized by the droughts in spring and summer, which is common for the climate of the South-East of Ukraine and less suitable for normal growth and development of trees. Monthly average temperatures, air humidity and rainfall were taken from the reports of the National Hydro-meteorological Center of Ukraine (Ukrainian hydrometeorological center).

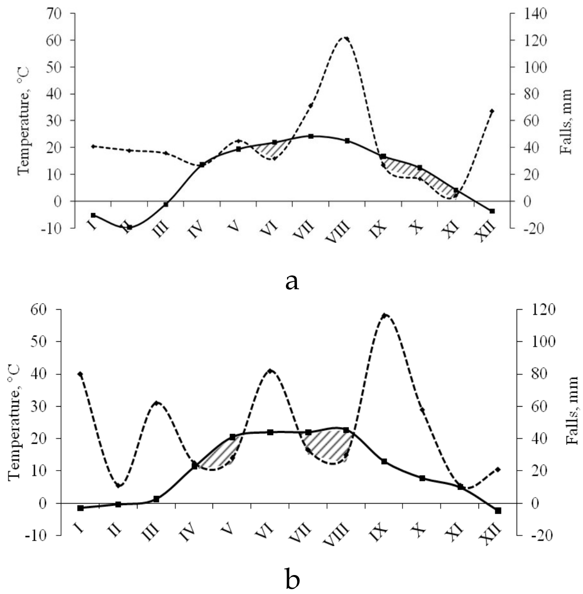

Fig.1. Hydrothermal conditions in the years of research in Donetsk: a – 2012, b – 2013.
- drought periods,
- dynamic of temperature during the year,
- dynamic of falls during the year.

Eight tree species of the *Aceracea*, *Salicaceae*, *Fabacea*, *Malvaceae* and *Oleaceae* genera were studied at three monitoring sites in different types of plantations: in the linear street plantations on the Kyivskyi avenue (m.s. 1), in the massive plantations in the Recreation and Entertainment Park (m.s. 2), plots in the field shelter belts and village plantations of v.Tonenke at control site (m.s. 3). We selected 7-8 even-aged model trees of each species with the most typical vital status for the plantations of monitoring sites.

General vitality of the plants was established under the ocular 8-point scale by Savelieva (1975), which is commonly used in the investigated area (Suslova et al., 2013; Polyakov et al., 2012). The scale is based on the criteria of the trunk and crown damages, inhibition of apical and lateral growths, drying of branches, dry tops, the presence of adventitious shoots, vegetative shoots on the trunk, coppice shoots, development on the trunk of trees fungi. We evaluated the absence of these criteria in plants as 8points. Their appearance and impact on the trees reduces the vitality of the plant from 7 to 1 point, complete drying of the whole tree and root system, the absence of shoots from the stump was assessed as 0 point.

We collected plants materials monthly in sunny windless weather from May to October. With the purpose of obtaining the average sample, leaves were sampled from 7-8 trees.

Chlorophylls and carotenoids were extracted in 80 % acetone in triplicate repetition. We determined solution absorbency (D) by spectrophotometer ULAB 108UV under the different wave length: 663 nm; 646 nm and 470 nm. Concentration of pigments of the extract (mg/l) was calculated by the Lichtenthaler equations (Shlyk, 1975), with the special extraction coefficient for each component:

$$C_{chl.a} = 12{,}21\ D_{663} - 2{,}81\ D_{646};$$
$$C_{chl.b} = 20{,}13\ D_{646} - 5{,}03\ D_{663};$$
$$C_{car} = (1000\ D_{470} - 3{,}27\ C_{chl.a} - 100\ C_{chl.b})/229.$$

We calculated quantity content of the chlorophyls a (*chl a*), chlorophyls b (*chl b*) and carotenoids (*car*) of plants material with counting of obtained concentration of pigments in the extract under volume of the extract (25 ml) and weighted amount of plant material (0.1-0.2) g. The result was shown in mg/g of wet weight.

Mathematical data processing was performed using one-way analysis of variance (ANOVA) with the Tukey's a posterior analysis under Levene's test. The relationship between monthly average meteo-indicators and pigment concentrations was determined by multiple regression analysis, followed by the calculation of Durbin-Watson statistics and serial correlation in the program Statistica 10.0 (StatSoft Inc. 2011).

Results

At the beginning of the experiment, the observation of planting conditions in the city were conducted. Taking into account the lack of sensitivity of the method of visual estimation, the general vital status of the even-aged trees of each species was determined on m.s. 1 and m.s. 2. The following groups were identified among the plant species under study: the first group (8 points) – the stable species *Robinia pseudoacacia* L., *Populus simonii* Carriér; the second group (6-7 points) medium-stable species *Acer platanoides* L., *Populus ×canadensis* Moench, *Syringa vulgaris* L., the third group (4-5 points) nonsufficient stable - *Acer pseudoplatanus* L., *Acer negundo* L., *Tilia cordata* Mill.

In order to determine the physiological aspects of the plant's vitality due to the different hydrothermal conditions of the vegetation period of two years of research, we analyzed the pigment content in the leaves of the investigated species in the control, without influence of urban factors. High level of chlorophyls sum was established in the conditions of July and September 2012 which were prosperous to the plants, mainly owing to chlorophyll *a (*Fig. 2a*).* This reaction is principally shown in the species of the third group - *Acer pseudoplatanus* L., *Acer negundo* L., *Syringa vulgaris* L., as well as in the trees of the second group - *Acer platanoides* L. Amount of rainfall in these months exceeds the limit by 74 % in July and 189 % in August of 2012. It should be noted that the concentration of green pigments of these species under the conditions of severe drought in September decreases extremely, except for *Syringa vulgaris* L., that characterizes with increasing of *chl a* and *chl b* amount. It is evidenced about the adaptation potential of *Syringa vulgaris*. The high chlorophyll concentration in more stable to the urban conditions species of the first and second groups were estimated on the start of vegetation in the May-June under the lack of humidifying.

It is found out the dynamic of chlorophyll concentration of two species *Populus simonii* and *Acer pseudoplatanus,* that represent the groups with the most and least stable species, under the influence of stress hydrothermal conditions in the growing season 2013 (Fig.2b).

As a result of multiple regression analysis the rainfall and air humidity are determined as the main factors influencing the content of green pigments in the leaves of species from the second and third groups in the control conditions (Table 1a). However *chl a+b* of species where the maximum content of green pigments is observed in drought periods of the year depends on the air temperature. For example, for *Populus simonii* (stable species) correlation with air temperature is established in the same way as for the medium-stable species *Syringa vulgaris* (R^2=0.81).

Sum of chlorophylls for the leaves of *Syringa vulgaris* in 2012 is described by linear equation:

$$y = 13.1192+0.0356 \cdot x_1-0,4561 \cdot x_2,$$

where y – sum of chlorophylls (mg/g), x_1 – amount of rainfall (mm), x_2 – temperature (°C).

Content of carotenoids in all investigated species in the control increases from May to October, however, the rate of its growth varies (Fig. 3). The most significant difference between carotenoids content at the beginning and the end of vegetation is established for *Acer platanoides* (4 times in October compared to May figure). Increase in the content of yellow pigments at the end of vegetation relates to the preparation of plants for winter and destruction of green pigments. The negative significant influence of temperature was revealed for this species (Table 3).

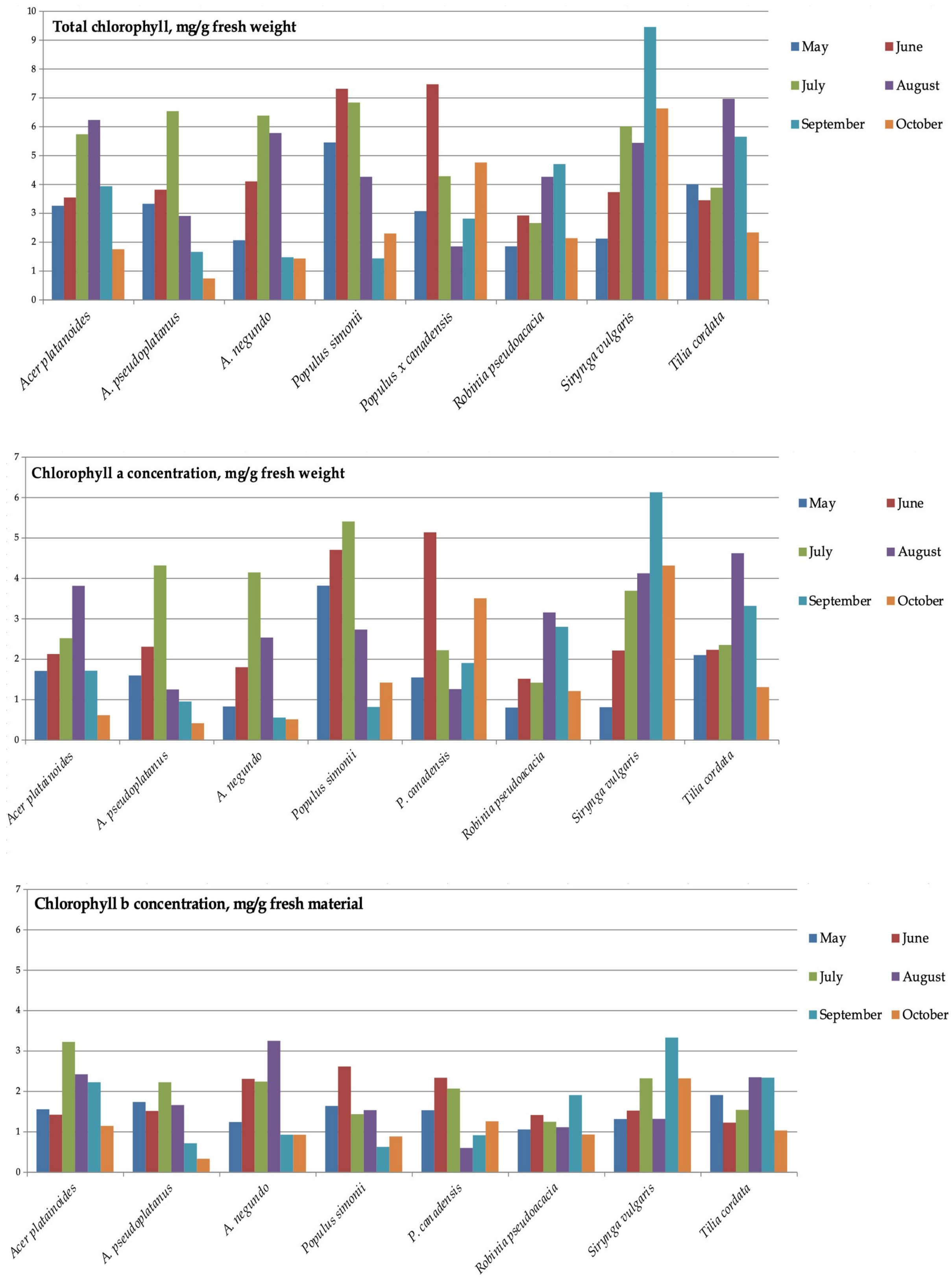

Fig. 2a. The chlorophyll concentration (total, *chl a* and *chl b*) in leaves of the investigated species from May to October at monitoring site 3 (Tonenke) – 2012.

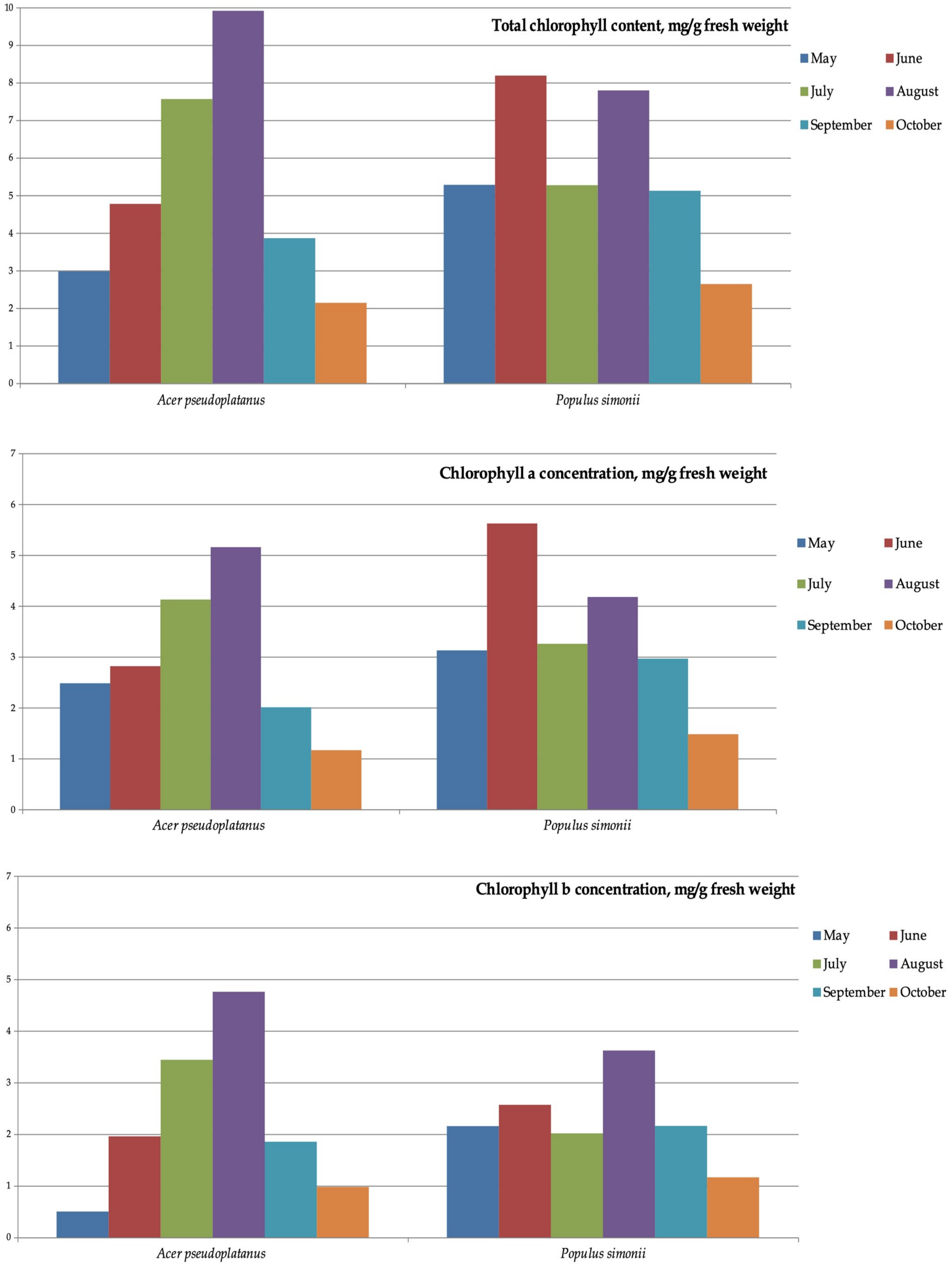

Fig. 2b. The chlorophyll concentration (total, *chl a* and *chl b*) in leaves of the investigated species from May to October at monitoring site 3 (Tonenke) – 2013.

Populus simonii and *Populus* × *canadensis* are characterized by the absence of the autumn leaf color at the end of vegetation, unlike the other tree species. In connection with this the content of carotenoids in the leaves of this species in October remains unchanged compared to May (Fig. 3). It should be noted that in June during the drought the content of carotenoids in *P. simonii* increases, indicating the protective role of carotenoids.

For stable species *Populus simonii* and nonsufficient stable species *Acer pseudoplatanus* (Fig.2b) it is found that the dynamics of chlorophyll content differs from year to year, but air temperature remains the main factor (Table 2).

The tendency of the pigment content changes during the growing season at m.s. 1 and m.s. 2 corresponds to the dynamics in control (m.s. 3), so the results are presented for one month of each season (Table 4). In summer and autumn, June and October are selected as the drought periods with unfavorable conditions for the growth and development of trees (Table 4).

Analysis of obtained results (Table 4) shows that reduction of chlorophyll content is observed in the leaves of species under study in street and park plantations in the conditions of contamination and urbanization compared to control against the same background of the hydrothermal conditions. For example, in June the sum of chlorophylls in *Tilia cordata* decreases by 39.2 % at m.s. 1 and by 25.8% at m.s. 2, indicating the physiological changes which occur in the plant body under action of pollutants.

More intensive reduction of the sum of chlorophylls in the leaves of species under study is recorded at m.s. 1 compared to m.s. 2 and control. The significant difference was recorded in May, under favorable weather conditions, in young leaves, which are more sensitive to airborne pollutants, of all species except *Syringa vulgaris*. For example, in the leaves of *Acer negundo* the chlorophyll content decreased by 51.7 % at m.s. 1 and by 31.7 % at m.s. 2 compared to control. The lowest sensitivity to pollutants was found for the leaves of *Robinia pseudoacacia* and *Syringa vulgaris.* Their sum of chlorophylls has grown by 54.9 % and 18.5 % at m.s. 1 respectively.

During the first draught in June *A. pseudoplatanus* (decrease in sum of chlorophylls by 57.9% and 28.7 % at m.s.1 and m.s.2 respectively), *Populus simonii* (62.5 % and 46.4 % m.s. 1 and m.s. 2), *P.* x *canadensis* (25.2 % and 72.4 % at m.s. 1 and m.s. 2) are more sensitive to contamination. *Acer platanoides, A. negundo, Syringa vulgaris* L. and *Tilia cordata* are less sensitive to contamination; chlorophyll content in them decreases from 22.0 % to 39.2 % depending on species at m.s. 1 compared to control. *Robinia pseudoacacia* is the most resistant to contamination during drought in June; chlorophyll content increases by 15 % at m.s. 1 and m.s. 2 respectively.

During the drought in October it is found at m.s. 1 that contamination has less significant impact on the condition of photosynthetic apparatus in all species, due to lower sensitivity of old leaves to adverse abiotic and anthropogenic factors of the environment. High endurance against contamination and prolonged drought was demonstrated by *A. pseudoplatanus* L., *Populus simonii, P.* x *canadensis, Robinia pseudoacacia* and *Syringa vulgaris.*

According to the results of studies it is established that at m.s. 1 and m.s. 2 the carotenoid content in the leaves of model specimens increases compared to the control, but the degree of increase depends on plant resistance, as can be seen from the dynamics of carotenoid content in June (Table 5).

The content of carotenoids in stable species (*P. simonii*, *P.* × *canadensis*, *Robinia pseudoacacia*) features almost no difference on Kyivskyi avenue (m.s. 1) and in the Recreation and Entertainment Park (m.s. 2) during vegetation. The established pattern indicates high resistance of these woody plants to conditions of the urban environment. Concentration of yellow pigments in *Acer pseudoplatanus* and *A. negundo*, growing in the park, increases by 30–35 % compared to m.s. 3, showing the sufficient level of adaptation of these species to conditions of the urban environment. Considerable increase in carotenoids in street and park plantations is recorded in *Acer platanoides*, *Syringa vulgaris* and *Tilia cordata* which are less stable.

The ratio of sum of chlorophylls to carotenoids (a+b/car.), as a rule, is constant in the normal conditions and promptly responds to changes in the environment, so it is used as an indicator of plants' adaptation to extreme conditions (Petrova et al., 2014). In order to understand the simultaneous action of abiotic stress – drought and anthropogenic pressure, the ratio a+b/car. was calculated for June 2012.

In most species a+b/car. varied from 4.86 (*Acer platanoides*, m.s. 1) to 9.05 (*Tilia cordata*, m.s. 3) (Fig.4).

Table 1. Stepwise regression analysis for sum of chlorophylls of woody plants in the vegetation period of 2012 (Tonenke, m.s. 3). Negatively correlated variables are highlighted in bold. Statistical significance, p-value: < 0,05 = *; < 0,001 = **; < 0.0001***.

Species	**Step 1**	**Step 2**	**Step 3**
Populus × canadensis	Falls 0.09***	Falls Temp 0.69***	-
Populus simonii	Temp 0.54***	Temp Falls 0.06***	Temp Falls Hum 0.14*
Tilia cordata	Falls 0,04***	Falls Hum 0.05**	
Robinia pseudoacacia	Falls 0.008*	Falls Hum 0.05**	-
Acer negundo	-	Hum 0.11*	
Acer platanoides	Falls 0.02***	-	-
Acer pseudoplatanus	Hum 0.30***	Hum Falls 0.03***	Hum Falls Temp 0.14**
Syringa vulgaris	Temp 0.46**	Temp Falls0.04*	

Table 2. Stepwise regression analysis for sum of chlorophylls of woody plants in the vegetation period of 2013 (Tonenke, m.s. 3). Negatively correlated variables are highlighted in bold. Statistical significance, p-value: < 0.05 = *; < 0.001 = **; < 0.0001***.

Species	**Step 1**	**Step 2**	**Step 3**
Acer pseudoplatanus	Temp 0.22***	**-**	**-**
Populus simonii	**Temp 0.66*** **	**-**	**-**

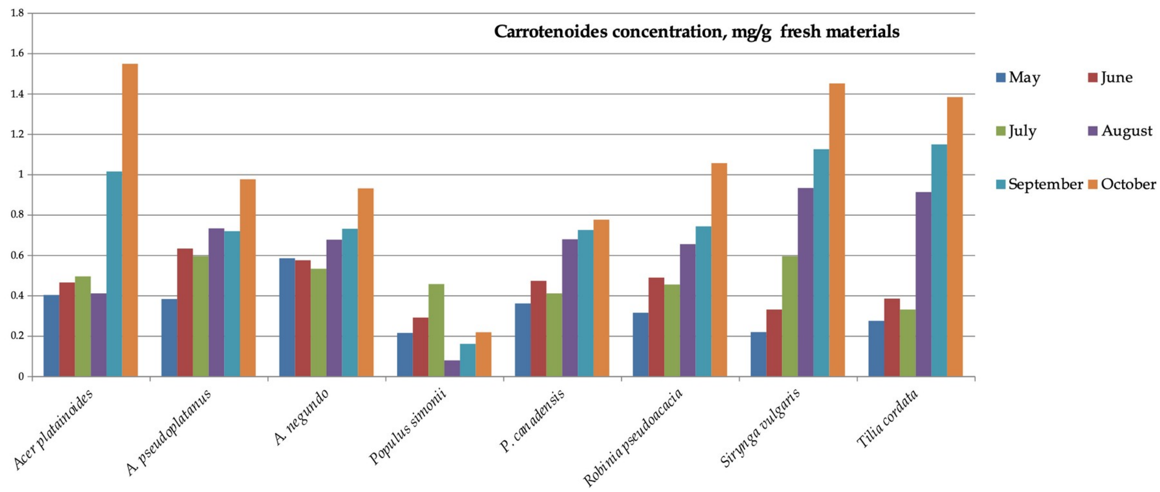

Fig. 3. The carotenoids concentration in leaves of the investigated species from May to October at monitoring site 3 (Tonenke) in 2012.

Table 3. Stepwise regression analysis for carotenoids concentration of woody plants in the vegetation period of 2012 on the control site. Negatively correlated variables are highlighted in bold. Statistical significance, p-value: < 0.05 = *; < 0.001 = **; <0.0001= ***.

Species	Step 1	Step 2	Step 3
P. × canadensis	Hum 0.02***	HumFalls 0.005***	HumFallsTemp 0.02**
P. simonii	Hum 0.02***	HumFalls 0.004***	-
Tilia cordata	Hum 0.04***	HumFalls 0.01***	HumFallsTemp 0.09***
Robinia pseudoacacia	Hum 0.02*	HumFalls 0.006***	HumFallsTemp 0.05***
Acer negundo	Temp 0.03***	TempFalls 0.003***	TempFallsHum 0.01***
A. platanoides	Temp 0.12***	TempFalls 0.004**	
A. pseudoplatanus	Hum 0.02*	HumFalls 0.004**	HumFallsTemp 0.03*
Syringa vulgaris	Temp 0.12***	TempFalls 0.01***	-

Table 4. Sum of chlorophylls of the species leaves at experimental sites during the vegetation period. Statistical significance: p-value: < 0,05 = *; < 0,001 = **; <0.0001= *** compared to control.

Species	May			June			October		
	m.s. 1	m.s. 2	m.s. 3	m.s. 1	m.s. 2	m.s. 3	m.s. 1	m.s. 2	m.s. 3
Acer platanoides	1.43 ±0.05***	1.32 ±0.04***	3.25 ±0.05	2.72 ±0.02**	3.14 ±0.05	3.53 ±0.02	1.13 ±0.06	1.15 ±0.01	1.74 ±0.01
A. pseudoplatanus	1.49 ±0.11***	2.48 ±0.25***	3.32 ±0.05	1.60 ±0.03***	2.71 ±0.07**	3.80 ±0.03	0.72 ±0.08	1.01 ±0.01	0.73 ±0.02
A. negundo	0.99 ±0.11***	1.40 ±0.07**	2.05 ±0.05	3.19 ±0.02	3.67 ±0.11	4.09 ±0.01	0.84 ±0.03	0.27 ±0.01	1.42 ±0.01
Populus simonii	1.44 ±0.08***	2.34 ±0.03***	5.44 ±0.04	2.77 ±0.05***	3.91 ±0.12***	7.30 ±0.02	1.43 ±0.06	0.81 ±0.01**	2.29 ±0.02
P. x *canadensis*	0.99 ±0.06***	1.93 ±0.06***	3.06 ±0.03	2.56 ±0.03***	6.36 ±0.05	7.46 ±0.04	3.55 ±0.02	4.31 ±0.01	4.75 ±0.02
Robinia pseudoacacia	2.85 ±0.07**	2.43 ±0.05**	1.84 ±0.02	3.35 ±0.03	3.37 ±0.02	2.91 ±0.01	1.37 ±0.03	1.64 ±0.02	2.13 ±0.02
Syringa vulgaris	2.50 ±0.06	2.85 ±0.09	2.11 ±0.02	2.57 ±0.03**	3.12 ±0.07	3.72 ±0.01	4.79 ±0.03	4.71 ±0.02	6.62 ±0.02
Tilia cordata	1.66 ±0.05***	1.95 ±0.04***	3.99 ±0.03***	2.09 ±0.04**	2.55 ±0.04*	3.44 ±0.03	1.25 ±0.03*	1.15 ±0.02	2.32 ±0.03

Table 5. Carotenoids content in some months of 2012 at experimental sites. Statistical significance: p-value: < 0,05 = *; < 0,001 = **; <0.0001= *** compared to control.

Species	May			June			October		
	m.s. 1	m.s. 2	m.s. 3	m.s. 1	m.s. 2	m.s. 3	m.s. 1	m.s. 2	m.s. 3
Acer platanoides	0.65 ±0.01***	0.53 ±0.02**	0.40 ±0.01	0.56 ±0.02	0.51 ±0.01	0.46 ±0.01	2.70 ±0.01***	2.13 ±0.01**	1.55 ±0.01
A. pseudoplatanus	0.55 ±0.02***	0.43 ±0.01	0.38 ±0.01	0.73 ±0.01**	0.68 ±0.01	0.63 ±0.01	1.45 ±0.01***	1.15 ±0.01	0.98 ±0.02
A. negundo	0.75 ±0.02***	0.62 ±0.02**	0.58 ±0.01	0.75 ±0.02***	0.66** ±0.02	0.57 ±0.01	1.23 ±0.02**	1.01 ±0.01	0.93 ±0.02
Populus simonii	0.31 ±0.01	0.27 ±0.01	0.21 ±0.01	0.38 ±0.01***	0.33 ±0.01	0.29 ±0.01	0.31 ±0.01	0.27 ±0.01	0.22 ±0.01
P. x *canadensis*	0.56 ±0.02***	0.49 ±0.03	0.36 ±0.01	0.60 ±0.01***	0.51**= ±0.01	0.47 ±0.01	0.95 ±0.01*	0.81 ±0.01	0.78 ±0.01
Robinia pseudoacacia	0.46 ±0.02***	0.39 ±0.01	0.31 ±0.01	0.61 ±0.02***	0.52* ±0.02	0.49 ±0.01	1.48 ±0.02**	1.31 ±0.02	1.06 ±0.03
Syringa vulgaris	0.33 ±0.01***	0.29 ±0.01	0.22 ±0.01	0.42 ±0.01***	0.39 ±0.01	0.33 ±0.01	2.23 ±0.01***	1.64 ±0.02	1.45 ±0.01
Tilia cordata	0.37 ±0.01**	0.33 ±0.02	0.27 ±0.01	0.47 ±0.01***	0.43 ±0.01	0.38 ±0.01	1.92 ±0.01**	1.71 ±0.02	1.38 ±0.02

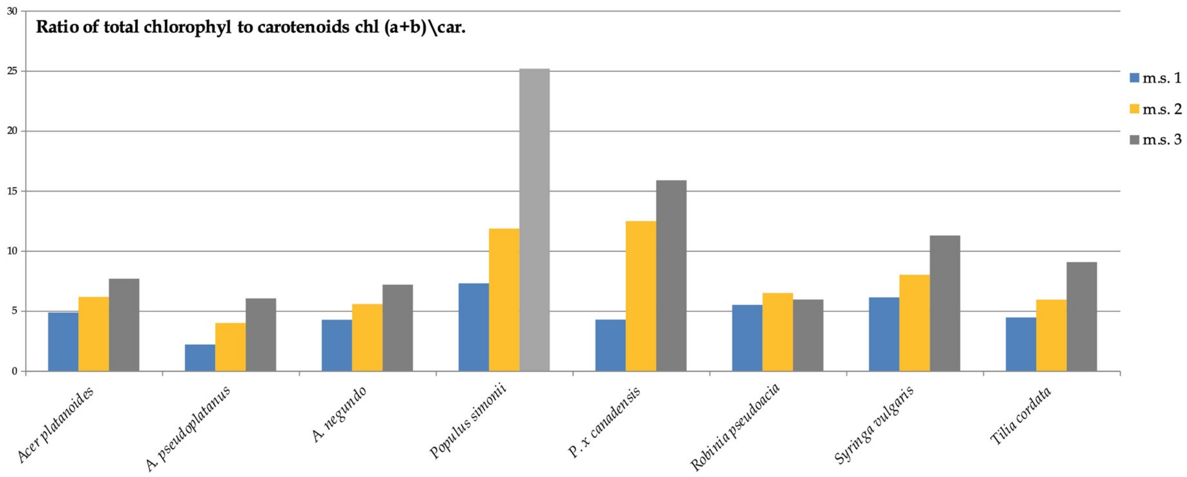

Fig. 4. The ratio of total chlorophylls to carotenoids in June, 2012 during the drought at monitoring sites.

However, this indicator in representatives of *Populus* and *Syringa vulgaris* genera exceeds the general range. For example, for *Populus simonii* and *Populus × canadensis* the maximum level of the ratio in the control (25.17 and 15.87 respectively) is established, and for *Syringa vulgaris* it makes 11.27, because of high chlorophyll concentration and low content of carotenoids. The tendency to growth of the concentration of green pigments and reduction of yellow ones in the control is maintained for the other species, except *Robinia pseudoacacia.* The highest ratio *chl a+b/car* is found in this species for the plants in park plantations, exceeding this indicator at m.s. 1 and m.s. 3 by 1.1 times only.

Therefore, contamination in the park plantations affects the plants which fact is confirmed by changes in the composition of the pigment apparatus of leaves, but in street plantations in the conditions of heavy traffic the reduction of chlorophyll content and increase of carotenoids in the investigated species is more significant. In stable species of trees (*Populus simonii, P.* × *canadensis, Robinia pseudoacacia*) these changes are less critical compared to medium-stable and nonsufficient stable species (*Acer pseudoplatanus, A. negundo, A. platanoides, Syringa vulgaris* and *Tilia cordata*).

Discussion

As a result of our study it is found that concentration of chlorophylls and carotenoids in woody plants varies depending on the meteorological factors during the season and on the level of contamination of urban areas. The growth of chlorophyll content in the leaves of trees under study from May to September is established, which is consistent with the literature sources (Petrova et al., 2017; Iusypiva & Vegerich, 2014). It is determined that the main factor influencing the chlorophyll formation is the air temperature. Stable and medium-stable species *Robinia pseudoacacia, Populus simonii, Acer platanoides, Populus* × *canadensis, Tilia cordata* have the largest chlorophyll amount in drought periods. As it is stated by Ballester et al. (2017), fruit species of trees also demonstrate high dependence on the formation of green pigments on the air temperature. During experiments for *R. pseudoacia*, *Tilia cordata*, and *A. platanoides* direct dependence on rainfall is established which is consistent with the results of Iusypiva & Vegerich (2014) for Dnipro City in 2012 for *R. pseudoacia*, in the leaves of which the maximum sum of chlorophylls was recorded in the month with the highest amount of rainfalls as well. The established pattern indicates less adaptive capacity of the representatives of *Robinia, Tilia* and *Acer* genera to the conditions of hydrothermal stress.

Under action of drought in the leaves of representatives of *Acer* genus (Swozcerna et al., 2010) the chlorophyll content increases, which agrees with the results of our experiments for *A. pseudoplatanus*, when at high temperatures and low rainfall in August 2013 the maximum amount of chlorophylls in the leaves of this species was recorded. This pattern indicates high adaptive capacity of sycamore maple in the conditions of South-East of Ukraine. Another species *Populus simonii* is defined by a high concentration of pigments under stress conditions at the start of vegetation period, a more stable level of chlorophyll content during the growing season. It should be noted that under the more prosperous conditions in June maximum is being reached by *chl a* fraction, under the stress conditions in August - due to the sharp increase in the *chl b* fraction, as well as it is found out a higher relative content of *chl b* at the end of the growing season. The obtained results show that the various in stability species in urban environment differ in the ratio of *chl a* and *chl b* under the action of hydrothermal factors of different tensions, that can be one of the part of the demonstration of species adaptive strategy.

Increase in sum of chlorophylls in *A. platanoides* and *A. negundo* in July and August 2012, when the amount of rainfall was higher in 74 and 190 % to the normal value accordingly, occurred due to increase in the proportion of chlorophyll *b*, which plays the protective role in addition to its main function. Chlorophyll *b* absorbs some of the rays, expanding the range of photoactivity which is particularly important in the conditions of considerable cloudiness in rainy weather. Chlorophyll *a* is more resistant to adverse environmental factors compared to chlorophyll *b* (Conzales-Dugo, 2017; Bessonova et al., 2006). Increase in the sum of chlorophylls during drought in *Acer pseudoplatanus, Populus* x *canadensis, P. simonii, Robinia pseudoacacia, Syringa vulgaris* at the expense of chlorophyll *a* indicates the normal development of the photosynthetic apparatus of these species and their high adaptive reactions under conditions of hydrothermal stress.

The linear equations of total chlorophyll and meteorological factors (falls and temperature) for both years were constructed for two investigates species:

Acer pseudoplatanus $y = -5.1278-0.0258 \cdot x_1+0.4658 \cdot x_2$ with $R^2 = 0.98$, in 2012 and $y = -6.8888-0.0529 \cdot x_1+0.6888 \cdot x_2$ with $R^2 = 0.81$ in 2013;

Populus simonii $y = -6.8888-0.0529 \cdot x_1+0.6888 \cdot x_2$ with $R^2 = 0.60$, in 2012 and $y = -1.2985+0.058 \cdot x_1-0.3181 \cdot x_2$ with $R^2 = 0.77$, in 2013, where y – sum of chlorophylls (mg/g), x_1 – amount of rainfall (mm), x_2 – temperature (ºC).

As can be seen from the linear equations, general tendency of pigments amount for change in the leaves despite the different weather conditions remains unchanged. Linear equations obtained for *Acer pseudoplatanus* confirm the inverse relationship between air temperature, rainfall, and sum of chlorophylls during the growing season in 2012.

Maximum chlorophyll content in the leaves of *Acer pseudoplatanus* falls on hot months, both in conditions of excessive humidity (July, 2012) and during the drought period (August, 2013). For *Populus simonii* the highest chlorophyll content is recorded in June, at high temperatures but varying amount of rainfall. The established pattern indicates high adaptive capacity of *A. pseudoplatanus* and *Populus simonii* during the drought.

Carotenoids fulfill the protective function by preventing the destruction of chlorophyll molecules in the light in the process of photo-oxidation. Photo-protective function lies in the fact that yellow pigments protect the reaction center from massive energy fluxes at high light intensities (Britton et al., 2008, Bessonova, 2006). As shown by the results of our study, in summer months the content of carotenoids in species under study increases. The highest indicators of carotenoids are found in autumn during the plants' preparation for winter.

During vegetation on the experimental sites (m.s. 1 and m.s. 2) the content of green pigments increases in late spring and during summer with the further decrease in concentration in autumn, which corresponds to the dynamics of chlorophyll change in the control. According to Zayika & Bondarenko (2018), the content of chlorophyll is reduced in the leaves of deciduous species in the area of the western forest-steppe of Ukraine in autumn which is agreed with our results for the southeastern steppe.

Sum of chlorophylls in most species at the monitoring sites is composed by chlorophyll *a*, except for *A. platanoides* and *A. negundo*, as in control. Formation of chlorophyll *b* molecules is reduced because of inhibition of enzymes of its synthesis under action of xenobiotics (Petrova et al., 2017; Bessonova et al., 2015).

Covering of the question regarding changes in carotenoids in the leaves of trees of street plantations in the literature is ambiguous. For example, Petrova et al., 2017, for *Acer heldreichii* Boiss. & Heldr. and *Tilia tomentosa* Moench point to the decrease in carotenoids at the contaminated site, however for *Fraxinus excelsior* – to growth of their content in the conditions of impact of car exhausts. Reduced content of carotenoids under impact of car exhausts is also recorded by Joshi & Swami (2009). Increase in concentration of the yellow pigments under action of car exhausts is established in the studies of Sillanpää et al. (2008) for *Betula pendula* Roth, which may suggest different adaptive capacity of trees and varying levels of contamination. Korshikov & Vinogradova (2013) note the increase of carotenoids in damaged leaves of *Acer pseudoplatanus* and *Acer platanoides* under action of pollutants from the work of internal combustion engines in Donetsk.

According to results of our study, the functional state of trees in street and park plantations is worse compared to control, which is confirmed by the decrease in chlorophyll content and growth of carotenoids. Carotenoid content at m.s. 2 increases to 30 %, and at m.s. 1 – to 50 %, indicating the adaptive capacity of plants, since carotenoids possess high antioxidant power. The total number of chlorophylls is reduced by 20 to 30% in the trees of park plantations, and by 40–50% in the street plantations, compared to the clean site.

It is to be noted that the representatives of the first group (stable species), such as *Robinia pseudoacacia*, shows the exceedence of sum of chlorophyls during the drought periode at m.s. 1 and m.s. 2. At the same time the species of the second group (medium-stable species), for example, *Syringa vulgaris* decreases level of chlorophyls content under the unfavorable urban and hydrothermal conditions, as well as, Tilia cordata from the third (nonsufficient stable) groups decreases amount of chlorophylls and increased carotenoid content. The obtained predicted pain evidences the adaptive strategies of the studied species under anthropogenic and hydrothermal stress.

Therefore, it is found that contamination in the park plantations affects the plants which fact is confirmed by changes in the composition of the pigment apparatus of leaves, but in street plantations the reduction of chlorophyll content and increase of carotenoids in the investigated species is more significant. In stable tree species (*Populus simonii, P. × canadensis, Robinia pseudoacacia*) these changes are less critical than in less stable species (*Acer pseudoplatanus, A. negundo, A. platanoides, Syringa vulgaris* and *Tilia cordata*).

Conclusion

As a result of the study, the functional state of trees in street and parks plantations was found to be weakened, which indicates a decrease in the content of green pigments and an increase in the concentration of carotenoids in the area of automobile vehicle exhaust. The impact of hydrothermal conditions and factors of urban environment determines the corresponding changes in the concentration of photosynthetic pigments in woody plants differentiated by vitality. It is established among meteorological factors air temperature as the main factor. Changes in the concentration of green pigments in the leaves of species under study, depending on the temperature and rainfall, are described by linear equations, which is important for predicting the growth and development of plants during green building on urban territories in the conditions of the steppe zone.

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Air Quality Formation Factors of Urban Areas (with the Example of the Odessa City)

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Abstract. Urban areas are characterized by a concentration of the technogenic sources of pollution, and their functioning is a major factor in the formation of the air basin quality. Odessa is characterized by the high levels of the air basin pollution, a significant dominance of the mobile sources of the air basin pollution, an insufficient landscaping level and public use green areas in some city districts, an unsatisfactory condition of the city trees and a presence of the unwanted species (sources of poplar fluff) and weeds (ragweed etc.). The level of a technogenic load on the Odessa air basin from the stationary sources is much higher than the one in the region. Reduction of the air basin pollution is possible due to redistribution and regulation of the traffic flows, increasing motor transport environmental friendliness, reorientation of the industrial production to the suburban territories, further improvement of an ambient air monitoring system, increasing the greenery share, stimulating the energy efficiency and reducing a resource consumption in the production and housing services, attracting the use of the alternative energy sources.

Key words: air basin, urban areas, pollution, a technogenic load.

Introduction

Urban areas are detached sources of pollution and are one of the main factors of environmental changes. The combination of ideas about the urban areas condition and quality is based on studying its most vulnerable natural part, whose components are indicators of an anthropogenic impact on the urban environment. The authors of the work (Albert, 2018) note that exposure to air pollution is the fifth ranking human health risk factor. According to WHO, more than 80% of urban areas are characterized by high levels of pollution. For low and middle income countries that figure rockets to 98% (Notman, 2017).

Odessa is not only a large multifunctional city in the south of Ukraine, but it is also a territory that causes an adverse environmental situation. Air basin pollution is a priority area among the current environmental problems of Odessa (Safranov et al., 2007; Glushkov et al., 2017). According to the Central Geophysical Observatory named after B. Sreznevsky (2018) (CGO, 2019), by the value of complex *API* Odessa belongs to the five most polluted cities in Ukraine.

The aim of the work is to evaluate and analyze a level of air pollution in Odessa to substantiate the measures to improve the air basin condition. To accomplish this, we must

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complete the following tasks: summarize the long-term data of the air pollution indicators in Odessa; analyze the main factors that cause a current condition of the Odessa air basin.

Materials and Methods

Assessment and analysis of an air pollution level in Odessa were performed over the multi-year period 2003 – 2018. The materials of the Black Sea and Azov Sea Hydrometeorological Center from monitoring the air quality of the city at the network of the constant observation points, as well as the Regional reports on the state of the environment and Ecological passports of the Odessa region were used for the evaluation. The data included the results of the observations at a network of 8 stationary observation points in the city. The observations of the individual impurities content in the air at this time are carried out according to the short or full programs, which provide air sampling daily 2 (07.00 and 19.00) - 4 (01.00, 07.00, 13.00 and 19.00) times a day, followed by the analysis in a chemical laboratory. At the same time, certain meteorological parameters, which can be used as the influencing factors in predicting the air pollution in the future are determined.

To assess a level of air pollution, a method of estimation based on calculating *API* and complex *API* (*CAPI*) was used. *API* is calculated as a separate admixture by the formula (Bezuglaya, 1986):

$$API = \left(\frac{\bar{q}}{MPC_{da}}\right)C_i \quad , \qquad (1)$$

where MPC_{da} is daily average maximum permissible concentration; *Ci* is a constant that acquires values of 1,7; 1,3; 1,0; 0,9 respectively for 1; 2; 3; 4th grade of a substance harm and allows you to bring a degree of harmfulness of the *i*-th substance to a degree of dioxide sulfur harm.

According to the methodology if $API \leq 1$ a quality of air on the content of a separate pollutant meets sanitary and hygiene requirements.

Complex *API* is a quantitative description of an air pollution level formed by *n* substances which are present in the atmosphere of the city. *CAPI* is calculated by the formula (Bezuglaya, 1986):

$$CAPI = \sum_{i=1}^{n} API_i \quad . \qquad (2)$$

For the integral estimation of an air pollution level with the help of *CAPI*, one can use the values of individual air pollution indexes of the 5 pollutants for which these values are the greatest. The equation is (Bezuglaya, 1986):

$$I_5 = \sum_{i=1}^{5} API_i \quad . \qquad (3)$$

The I_5 value of less than 2.5 corresponds to a clean atmosphere; from 2,5 to 7,5 – slightly polluted; from 7.6 to 12.5 – contaminated; from 12.6 to 22.5 – heavily polluted; from 22,6 to 52,5 – highly polluted; more than 52.5 – extremely polluted atmosphere (Bezuglaya, 1986).

Results and Discussion

Analysis of the initial information showed that in Odessa, observations are generally made on the content of 11 pollutants. We used information about the content of 7 pollutants, which were constantly present in the information sources. These are substances such as dust (total suspended particles), soot, carbon monoxide, nitrogen dioxide, phenol, hydrogen fluoride and formaldehyde. It should also be noted that the content of the other 4 pollutants is much lower than the *MPC* and does not significantly affect the formation of a general level of atmospheric pollution in the city.

Fig. 1 presents the data on the dynamics of changing the average annual pollutant concentrations in the air of Odessa. As it can be seen, the concentrations of almost all substances exceed the value of the daily average maximum permissible concentrations (MPC_{da}) by an average of 1.5 - 2 times. The maximum concentrations are indicated by the content of formaldehyde (3.3 MPC_{da} and more). The content of phenol, soot, hydrogen fluoride and formaldehyde tends to decrease in concentrations, a slight increase is observed in the carbon monoxide content.

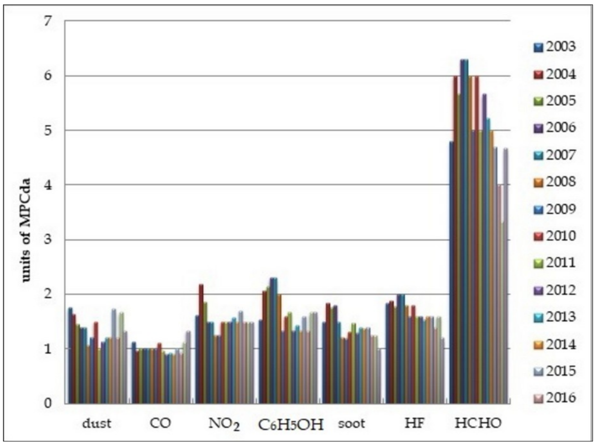

Fig. 1. Dynamics of changing the average annual pollutants concentrations in the air of Odessa in 2003 – 2018.

Fig. 2 shows the results of the *CAPI* and I_5 calculations. It should be noted that in the period 2003 – 2017, the content of 4 pollutants, namely nitrogen dioxide, phenol, hydrogen fluoride and formaldehyde, was constantly taken into account while calculating I_5. The fifth indicator was the content of soot (most often) or dust. In 2018, I_5 was calculated on the content of dust, carbon monoxide, nitrogen dioxide, phenol and formaldehyde.

Fig. 2 shows that during the study period a general tendency of decreasing the level of air pollution in Odessa is manifested. Highs were recorded in 2004, 2006 – 2007 by increasing the formaldehyde content.

According to the value of I_5, a level of air pollution in Odessa can be classified as: 2003 – 2015 – "heavily polluted"; 2016 – 2017 – "contaminated"; 2018 – "heavily polluted".

It should be noted that the obtained results do not correspond to the data from the results of monitoring at the mobile posts in 2009 – 2011. A comparative analysis of the level of air pollution according to the data of stationary and mobile points was performed in the work (Chugai et al., 2012). Thus, according to the mobile observations, the content of individual pollutants was somewhat lower, but the data was matched, i.e. the order of the concentrations was uniform.

The principle of calculating a technogenic load module (M_T), which is defined as a sum of weight units of all types of wastes (solid, liquid, gaseous) from the industrial, agricultural and municipal objects for a time interval of 1 year to the area of the administrative district or region within which these objects are situated was applied to estimate a technogenic load on the air basin, measured in thousands of t/km^2 per year (Adamenko & Rudko, 1997). Based on the principle of M_T determination, the assessment of the anthropogenic load level on the air basin was performed on the basis of calculating an anthropogenic load module on the air basin (M_{AB}), which is defined as the amount of pollutants emissions into the air in thousands of t/km^2 per year.

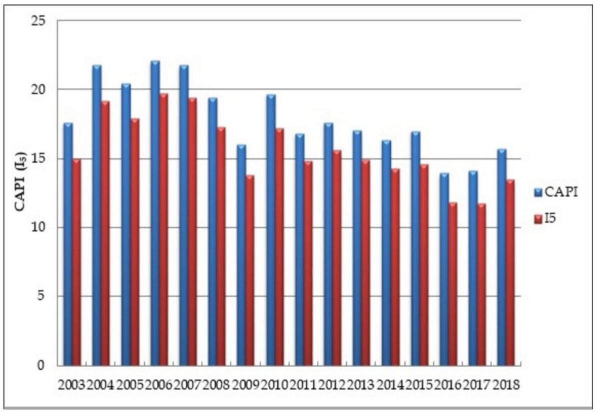

Fig. 2. The value of *CAPI* and I_5 in Odessa in 2003 – 2018.

The estimation of an anthropogenic load on the air basin of the Odessa region and Odessa was performed according to the data of the pollutants emissions from the stationary and mobile sources given in the regional reports by Odessa Regional State Administration (DENR-ORSA, 2010-2019). It should be noted that the mobile sources are the predominant sources of emissions for both Odessa and the Odessa region. Fig. 3 shows the results of calculating the M_{AB} indicator for the Odessa region.

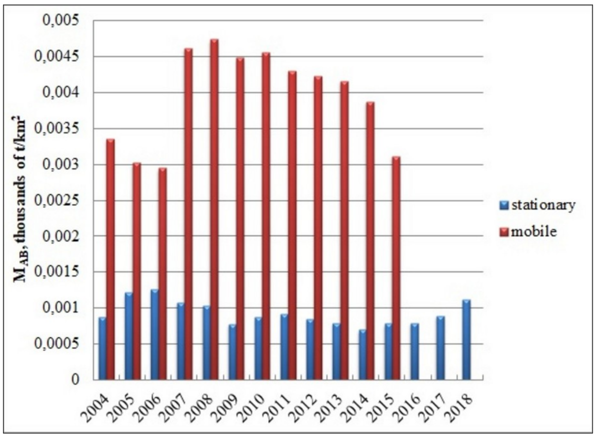

Fig. 3. The value of the M_{AB} indicator for the Odessa region.

Fig. 3 also shows that the level of loading from the mobile sources is significantly higher than the one from the stationary sources. For the mobile sources in the region a sharp increase in the M_{AB} index in 2007 with a further decrease was determined. Also, as of 2016, the official data lack the information on the emissions from the mobile sources and it complicates the analysis considerably. For the stationary sources, a level of loading during the study period was slightly varying. In 2018 it is in line with 2005 – 2007.

A level of technogenic load on the Odessa air basin from the stationary sources is much higher than the one in the region (see Fig. 4). This is quite natural, since the amount of emissions in the city of Odessa in different years was from 30 to 50 % in the region as a whole. There was a downward tendency in the load from 2007 to 2017, however, in 2018, the M_{AB} indicator increased significantly. In our opinion, this is a consequence, first of all, of non-compliance with the requirements for the limits of pollutants emission into the atmosphere.

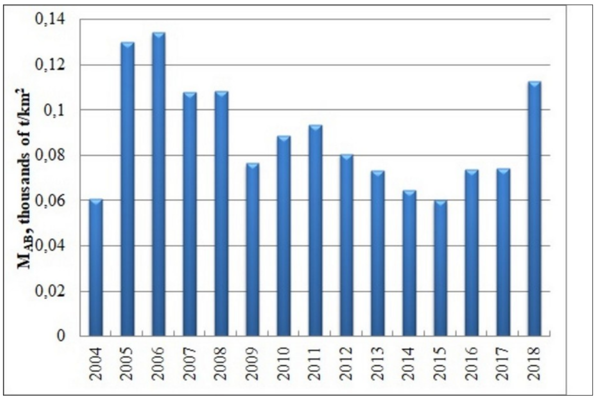

Fig. 4. The value of the M_{AB} indicator for Odessa (stationary sources).

The network of 8 stationary points for the air quality covers mainly the central and northern parts of Odessa (Fig. 5), and 23 route posts are located throughout the city. But most of the stationary posts are located in the areas which are heavily influenced by the stationary and mobile sources of the air pollution, and therefore the mean values of *API* do not give a real idea of the difference between technogenically-loaded and rural-recreational zones (respectively 25 % and 75 % of the city area territory). The most stationary sites are located in the residential area, and the main industrial pollution sources are located in the northwestern part of the city. Since the northwestern winds dominate, it negatively affects the state of the air basin at the locations of the stationary posts. Therefore, a network of the stationary air quality stations in Odessa should be updated and optimized.

Most of the stationary posts for air pollution monitoring are located on the windward side, and this is negatively reflected in the indicators of the air condition in the historic and residential parts of the city.

A level of environmental safety of the stationary pollution sources can be increased by improving air protection measures and technological processes, streamlining a configuration of the sanitary protection zones, etc., but a dominance of the mobile air pollution sources complicates the situation, since these measures should be applied to a large number of mobile pollution sources (for example, in 2013, 84.1 % of all the pollutants entered the city's air basin from the road, rail, air and water transport, as well as from the manufacturing equipment). Measures to reduce air pollution by redistributing and regulating traffic flows, as well as improving the environmental performance of vehicles are inefficient. This is due to the fact that a state of Ukrainian cars is poor (27 % of cars are over 30 years and 47 % of cars are between 10 and 30 years).

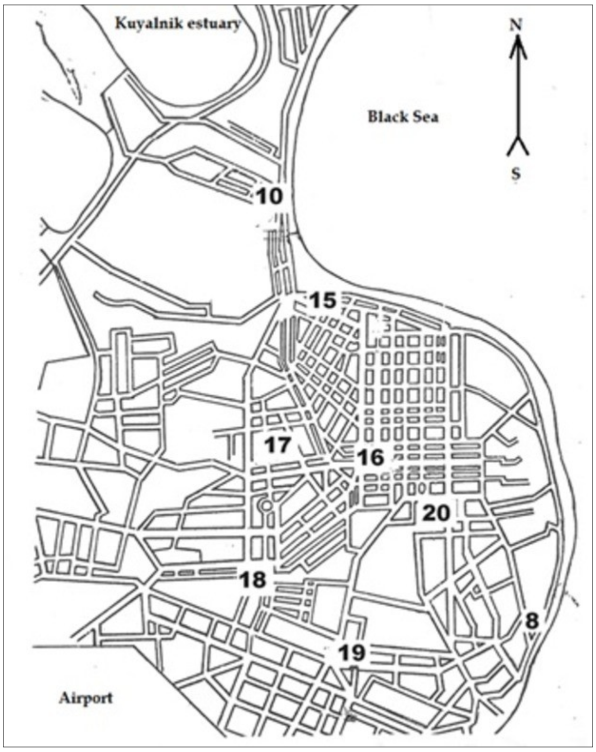

Fig. 5. Locations of the stationary points in Odessa.

In addition, the list of the pollutants monitored in Odessa does not meet the urgent needs, namely: there is no distribution of solids (*PM*) by a diameter of 10 µm and less (PM_{10}) and a diameter of 2.5 µm and less ($PM_{2.5}$). A number of harmful pollutants (*As*, *Cd*, *Hg*, *Ni*, polycyclic aromatic hydrocarbons, volatile organic compounds) are ignored (Annoumous, 2020). In the EU, the monitoring program is based on the threshold levels, the excess of which determines a need for some type of monitoring. This makes it possible not to measure a large number of substances, focusing instead on the key pollutants. The methods of measuring pollutants concentrations that are currently being monitored in Odessa also require improvements.

In August 2019, Ukraine approved a new "Procedure for State Monitoring in the Field of Air Protection" (Ukranian Ministry, 2019). This Procedure is a step towards the implementation of the model of air monitoring in EU countries. Unlike, for example, according to this document it is planned to isolate $PM_{2.5}$ and PM_{10} from the monitoring system operating in Ukraine.

Continuous monitoring of meteorological parameters and individual indicators of the air quality is carried out at the stationary post of Odessa State Environmental University (OSENU) since May 2019 using the Air Quality Transmitter AQT420 of Vaisala Oyj (Republic of Finland), which was acquired within the Erasmus International Project in 2018 + 561975-EPP-1-2015-1-FI-EPPKA2-CBHE-JP (ECOIMPACT) (e-impact.net). The list of pollutants determined by this apparatus includes nitrogen dioxide, sulfur dioxide, carbon monoxide, ozone and particulate matter.

We compared the observations of a content of the individual pollutants at OSENU with the long-term observations in the city as a whole. Note that according to WHO recommendations (WHO, 2006), the PM_{10} content is used as a primary indicator of the suspended substances content. The nitrogen dioxide content and the carbon monoxide content were also evaluated. Fig. 6 shows the *API* values for these substances in the summer-autumn period according to the observations at the OSENU post.

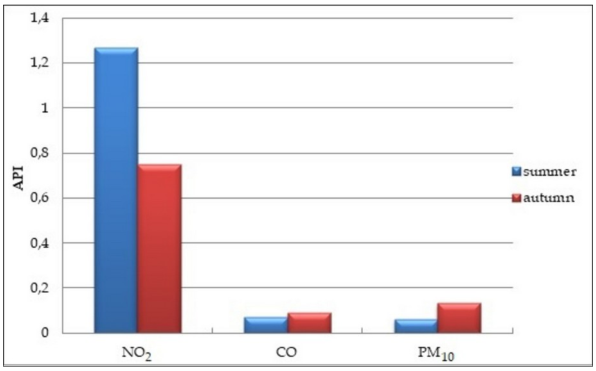

Fig. 6. The individual pollutants *API* values according to the observations at the OSENU post (2019).

The analysis of Fig. 6 and Fig. 1 shows that the nitrogen dioxide content generally corresponds to the average long-term values in the city as a whole. The carbon monoxide content is two orders of magnitude lower, according to the observations at a network of posts in the city, the PM_{10} content is one order of magnitude lower than the dust concentrations. There are no industrial sources of pollutant emissions near the observation point at OSENU, the most important sources are road transport. The territory OSENU is located in close proximity to the Black Sea coasts.

In order to obtain reliable estimates of the air quality, there is an urgent need to align the current monitoring system in Ukraine to the requirements of the new "State Monitoring Procedure in the field of air protection" (Ukranian Ministry, 2019) and to conduct the observations with a single metrological and methodological support.

An important factor influencing the air quality is a state of greenery. The city's green area is 742 hectares, which is in terms of one resident is 7.4 m^2/person and is 61.7 % for the city. If in the center of the city there are about 4 m^2 of greenery on average per person (at the norm of 12 m^2), then in the large residential areas there is no more than 1 m^2 of plantations per capita. In some areas the number of green spaces per inhabitant is only 17 % of the national standard. It should be noted that WHO is proposing the even higher figure – 50 m^2 per urban dweller – as a necessary norm for ensuring public health (Russo & Cirella, 2018).

The area of green space in recent years has decreased by about 25 %, and it is very important to expand a recreational zone of the city by creating the "Odessa Green Belt" (Halin, 2020), which implies a creation of a continuous ring of parks, squares and green corridors around the historic city center. In this ring it is proposed to create the conditions for a convenient movement of pedestrians and cyclists, as well as to place several intermodal electric transport stop complexes (a city train, a tram, a monorail). The conditions created here and a new transport infrastructure will attract investors' interest to construct residential complexes on its border. The areas around the railway are the most promising for creating a natural frame. The railway branch enters the city from the north and skirts its historic part. At present it is a strip of depressed territories: idle industrial enterprises, warehouses, a low-rise residential sector. The green space will help to clean the city's air basin from the harmful gas impurities, to retain dust particles, to curb wind gusts, to absorb noise and to enrich the air with oxygen.

It should be noted that less than 10 species of native shrubs are involved in the species composition of the city's flora, but in the landscaping of the city about 800 species and forms imported from other countries and continents, which are decorative, and at the same time well tolerate the air pollution and paving the streets and also are capable of catching dust and harmful fumes, are usually used. Since female poplar individuals produce fluff, it is advisable to use only male specimens in the city's landscaping. Among about 900 species of city's grassland, over one third are weeds.

During flowering a part of them is an allergen (for example, *Ambrosia artemisiifolia*). Therefore, for the accumulation of harmful impurities, it is proposed to plant also the resistant varieties of shrubs. The work (Vasilieva et al., 1998) substantiates a need to treat the plants carefully, to select their correct assortment, as well as to care of them properly. Based on the study of the authors (Bonetsky, 1998), in order to neutralize a number of harmful impurities in the urban air, it is recommended to plant such shrubs as horse chestnut, nanking cherry, American sycamore, *Platycladus orientalis*, and atlas cedar, as well as essential oil plants-terpenoproducers.

In Odessa, an increase in air temperature (maximum, minimum, average), a change in the nature of precipitation, a change in the duration of the growing season, the shift of climatic seasons, etc., were recorded. In addition, the city's population structure, a poor infrastructure, an inadequate funding, high levels of air pollution, etc. increase the city's vulnerability to the potential negative effects of climate change significantly (Shevchenko et al., 2014). Since the green areas are the most vulnerable ones in the city, when developing a city adaptation plan a significant part of the measures should be aimed at reducing the vulnerability of urban greening. One of the ways of the Odessa's adaptation to the climate changes (reducing the effect of a climate urbanization) is to increase the area of green space.

Conclusions

As a result of the conducted research we can draw the following conclusions:

1) The concentrations of almost all pollutants exceed the average daily *MPC* by 1.5 – 2 times; the content of phenol, soot, hydrogen fluoride and formaldehyde tends to decrease in the concentrations, the carbon monoxide content is slightly increasing.

2) By the I_5 value , the air pollution level in Odessa can be classified as: 2003 – 2015 – "heavily polluted"; 2016 – 2017 – "contaminated"; 2018 – "heavily polluted".

3) The level of a technogenic load on the Odessa air basin from the constant sources is much higher than the one in the region; there was a downward trend in loading from 2007 to 2017, however, in 2018, the M_{AB} indicator increased significantly, which is a consequence of non-compliance with the requirements for the air emission limits.

4) A list of pollutants monitored in Odessa does not meet the urgent needs, namely: there is no distribution of PM_{10} and $PM_{2.5}$; a number of harmful pollutants are remained unaddressed; the methods for measuring the pollutants concentrations need to be improved.

5) The comparison of these observations data according the individual pollutants content at the OSENU posts with the data of the long-term observations in the city showed that the NO_2 content corresponds to the average multiple-year observations in the city as a whole, the *CO* content is two orders of magnitude lower than the observations data at a network of posts in the city, the PM_{10} content is one order of magnitude lower than dust concentrations; a network of the stationary observation points for air quality in Odessa should be updated and optimized.

6) A network of 8 stationary points covers mainly the central and northern parts of Odessa, and 23 route posts are located throughout the city. But most of the points are located in the areas that are heavily influenced by the stationary and mobile sources of air pollution, and therefore the mean *API* values do not give a real idea of the difference between the technogenically-loaded and the rural-recreational zones (25 % and 75 % of urban area).

7) The level of environmental safety of the stationary sources of pollution can be increased by improving the air protection measures and technological processes, streamlining the configuration of the sanitary protection zones, etc., but a dominance of the mobile sources of the air basin pollution complicates the situation, since these measures should be applied to a large number of poor state vehicles.

8) An important factor influencing the air quality is a state of greenery. The city's green area is 742 hectares, which is in terms of one resident is 7.4 m^2/person (at the state standard of 12 m^2/person for the cities of the Odessa level and also the WHO standard of 50 m^2 for one urban resident).

9) The green area has decreased by about 25 % in recent years that is why it is very important to expand a recreational zone of the city by creating a "Green Belt of Odessa" (a continuous ring of parks, squares and green corridors).

10) In order to neutralize a number of harmful impurities in the urban air, it is recommended to plant shrubs such as horse chestnut, Nanking cherry, American sycamore, *Platycladus orientalis*, and atlas cedar, as well as essential oil plants-terpenoproducers.

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Effect of Main Climatic Parameters on Some Morphological and Qualitative Characteristics of Doubled Haploid Sunflower Lines

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Abstract. The dynamics of changing climatic factors and the study of the impact they have on cultivated crops is an area that needs to be carefully and thoroughly researched in order to adequately address the future challenges of human nutrition. The present study concerned the influence of major environmental factors on some morphological and economic characteristics of sunflower (*Helianthus annuus* L.), which is a main oil crop in Bulgaria. Development and selection of parental lines with stabilized morphological and economic parameters is a main point of the heterosis breeding in sunflower. This study was carried out during 2009-2011 at Dobrudzha Agricultural Institute – General Toshevo. The three years of the investigation differed by the sum of vegetative rainfalls. The investigation involved 10 doubled haploid fertility restorer lines. The analysis of the results showed that the year conditions and the genotype of the investigated lines had a significant effect (p=0.001) on the studied morphological traits and on the oil content in seeds. The combined influence of these two factors was not significant on plant height and oil content. The head diameter was influenced to a much higher degree by the year conditions than the plant height. The oil content in seed was the trait with lowest variation during the period of investigation in comparison to the two morphological traits. The highest values of the morphological traits and the content of oil in seed were determined in the warm and humid year 2010.

Key words: sunflower, doubled haploid lines, morphological traits, oil content in seed.

Introduction

Sunflower (*Helianthus annuus* L.) is one of the main oil seed crops both in Bulgaria and worldwide. The leading trend nowadays is heterosis breeding of sunflower, its major reference points being high plasticity and adaptability of the sunflower plant ensuring sustainable yields and seed quality under changing climatic factors. To produce a good sunflower hybrid, it is necessary to develop suitable parental components, which, when crossed, would give hybrid varieties with high productivity and very good resistance to biotic and abiotic factors (Cheres et al., 2000; Škorić, 2009). In the last 18 years, together with the conventional breeding methods, a number of biotechnology methods for developing sunflower lines with valuable economic properties are being extensively utilized for the needs of heterosis breeding. By using gamma-induced parthenogenesis in combination with conventional breeding methods of evaluation and selection, the developing time for new sunflower lines is shortened considerably (Todorova et al., 1997a; Todorova et al., 1997b; Drumeva et al., 2005; Drumeva, 2012b). The qualitative traits of the lines are dependent on the gene plasma of the initial hybrids these lines were derived from.

The production of hybrid sunflower seeds is preceded by the seed production of their parental lines, which should be of very high genetic purity. This process is rather specific and related to considerable financial investment; therefore knowing the potential of the individual lines is crucial (Georgiev et al., 2004).

Many factors influence the variation of the sunflower morphological indices, productivity and quality. The potential of the genotype plays a decisive role for the quantification of the separate traits, but its expression depends also on the effect of the abiotic factors of the environment, the main component of which are the climatic factors.

The most significant from the climate factors are the quantity of rainfall and the temperature over the vegetation (Hunyadi et al., 2007). Their interaction underlie the formation of quantity and quality of the yield-forming elements in sunflower (Johnston et al., 2002). Increasing of yield is influenced not only by total rainfalls but also by their uniform distribution during the vegetation period, i.e. during the growth stages, when the crop uses them effectively. The sunflower requires higher precipitation during the formation of the assimilatory apparatus with maximum in growth stage of flowering and formation of heads (Černý et al., 2014). The increasingly sharper variations of the climatic conditions put to test the grown crops and although there are different mechanisms for their mitigation, the selection of parental forms with complementing characteristics remains decisive (Škorić, 2009). Due to its ability to grow in different agroecological conditions and its moderate drought tolerance, sunflower may become the oil crop of preference in the future, especially in the light of global environmental changes (Miladinović et al., 2019).

Determining the range of variation and ecological plasticity of the separate traits allows their more precise assessment and successful performance of selection during the breeding process (Petakov, 1994).

The aim of this investigation was to study the effect of the main climatic parameters – rainfalls and temperature on the value of some morphological and qualitative characteristics of doubled haploid fertility restorer lines in sunflower.

Material and Methods

The experimental work encompassed the period 2009-2011 and was carried out in the experimental fields of Dobrudzha Agricultural Institute – General Toshevo. The investigation involved 10 doubled haploid lines restorers of fertility, which were obtained from different hybrid combinations: Al_r1 (Albena x 147 R x 600 Gy), Hel_r2 (Heliasol x 147 R x 600 Gy), Di_r3 (Diabolo x 147 R x 600 Gy), Br_r4 (Brio x 147 R x 600 Gy), Ar_r5 (Arena x 147 R x 600 Gy), Op_r6 (Opera x 147 R x 600 Gy), Gy), Br_r4 (Brio x 147 R x 600 Gy), Ar_r5 (Arena x 147 R x 600 Gy), Op_r6 (Opera x 147 R x 600 Gy), Br_r4 (Brio x 147 R x 600 Gy), Ar_r5 (Arena x 147 R x 600 Gy), Op_r6 (Opera x 147 R x 600 Gy).

Line 147 R, the father line of hybrid Albena, was used a control. The lines, included in this investigation, were obtained by the methodology of Todorova et al. (1997) through pollination of the initial hybrids with pollen preliminary irradiated with high gamma-ray doses in combination with embryo culture. The pollen was irradiated with dose 600 Gy using Cs 137 as a source of ionizing radiation. The isolation and cultivation of the obtained embryos was carried out according to Azpiroz et al. (1988).

The experiment was conducted in randomized block design, in three replications, the size of the experimental plot being 20 m^2.

Biometrical measurements were done on 10 plants from each line for the parameters plant height and head diameter. Plant height was measured from the surface of the soil to the place of head attachment to the stem. The reading was carried out at stage physiological maturity (15-20 days prior to harvesting). At the same time, the head diameter was also determined by doing two perpendicular measurements.

Oil content in seed was determined through nuclear magnetic resonance (Gronlund & Zimmerman, 1975).

Data were processed statistically with the help of the software package SPSS 16.0. (IBM Corp, 2007).

Results and Discussion

The three years of investigation differed by the sum of vegetative rainfalls; precipitation was highest in 2010 – 369.1 mm (Fig. 1). In 2009 and 2011 the rainfalls during April – September were with 16.5% and 5.9%, respectively, less than the norm.

The values of the mean monthly air temperatures are significantly important for the development of the plants and the formation of the yield components in sunflower. Among the three years of investigation, year 2010 was with the highest air temperature during the vegetative growth of plants – 18.2 °C. Values close to the average for the long term period were registered during the other two years, year 2009 being a little warmer than 2011.

In all three years of the investigation, the air temperature was within the range recommendable for sunflower, but the better moisture reserves in soil during 2010 were a prerequisite for the better development of the crop. During the vegetative growth season of sunflower, the effect of precipitation exceeded the effect of temperatures, but a synchronization between the metabolic requirements of the plant and the climatic factors is needed for the optimal occurrence of the biological processes in the plant cells (Kudrna, 1985).

The analysis of the results showed that the year conditions and the genotype of the studied doubled haploid lines influenced significantly (p=0.001) the investigated morphological traits, as well as the percentage of oil in seed (Table 1). The combined effect of these two factors was not significant on plant height and oil content.

Under the conditions of this investigation, the value of the traits plant height and oil content in seed was determined primarily by the genotype, the relative weight of this factor being 62.66% and 69.97%, while the determining significance of head diameter was that of the climatic year conditions (61.52%). The gene effect of head diameter is often minor compared to other agronomic traits, because it depends on the environmental condition and the vegetation period (Marinković & Škorić, 1990; Miller & Fick, 1997; Škorić, 2009). The combined effect of the investigated traits was with low relative weight, varying from 1.46% for plant height to 2.91% for oil content in seed, and reaching up to 6.29% for head diameter. The probable reason for the absence of statistical significance of the mutual effect of the two factors is the good plasticity of the sunflower plant, which demonstrated its adaptability according to the changes in the vegetative growth conditions over the years of study.

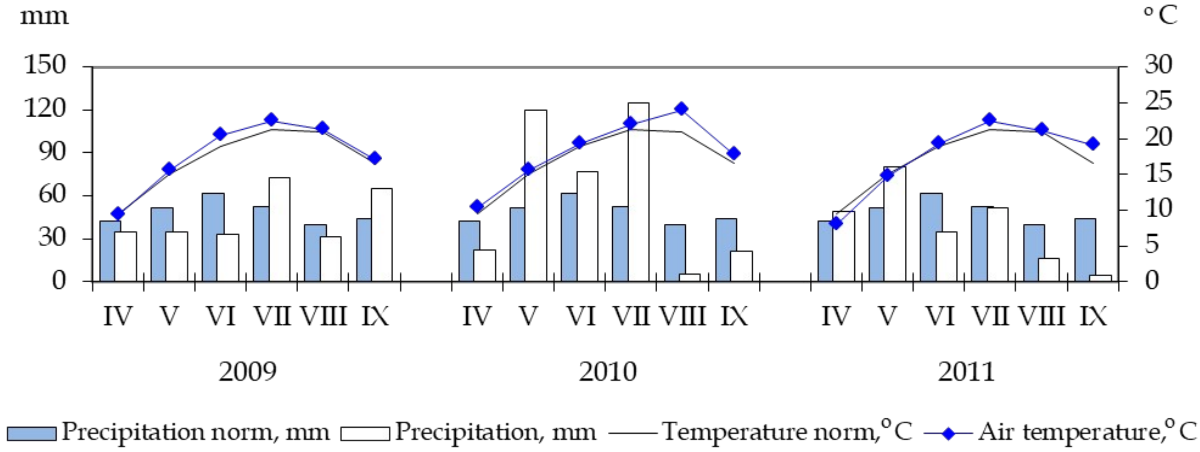

Fig. 1. Monthly values of rainfalls and air temperature from April till September during 2009-2011.

Table 1. Analysis of variance of year (A) $_x$ line (B) in sunflower for plant height, head diameter and seed oil content.

Indices	Source of variation	df	F	Sig
Plant height (cm)	Factor A. Years	2	422.354	.000
	Factor B. Lines	10	147.542	.000
	A $_x$ B	20	1.717	.053
Head diameter (mm)	Factor A. Years	2	593.385	.000
	Factor B. Lines	10	62.107	.000
	A $_x$ B	20	6.066	.000
Oil content (%)	Factor A. Years	2	132.912	.000
	Factor B. Lines	10	68.577	.000
	A $_x$ B	20	1.427	.142

Plant height is a varietal trait related to productivity, which is also influenced by the meteorological conditions, primarily by the vegetative rainfalls. The investigated lines differed by plant height; averaged for the three years of investigation, the lowest values were read in line Al_r1 – 126 cm, and the highest - in line Ba_r7 – 167 cm (Table 2). The differences in the value of this trait between the separate years of study were significant at various levels (Fig. 2).

Among the lines, included in the experiment, line Br_r4 was with the lowest variation coefficient, followed by lines Ba_r7 and Ol_r9, and line Ar_r5 was with the highest coefficient of variation. The different lines had different potential for overcoming of the unfavorable environmental factors and their response can be considered a result from the interaction of the genotype with the factors of the environment. In this investigation, the lines were grown under equal conditions and it can be assumed that the more narrow variation of the investigated parameters is due to the better adaptability of a given line to the ecological factor.

Table 2. Mean values of main morphological and qualitative traits of the lines during the period of study.

DHR lines	Plant height (cm) $\bar{x}$ ± SD	CV (%)	Head diameter (cm) $\bar{x}$ ± SD	CV (%)	Oil content in seed (%) $\bar{x}$ ± SD	CV (%)
147 R	144.0 ± 9.54	6.62	15.1 ± 2.15	14.24	37.5 ± 1.48	3.96
Al_r1	126.0 ± 9.54	7.57	13.2 ± 2.36	17.91	36.4 ± 0.92	2.52
Hel_r2	137.7 ± 9.61	6.98	15.4 ± 1.72	11.17	34.6 ± 1.15	3.33
Di_r3	132.7 ± 12.22	9.21	13.3 ± 2.25	16.93	35.3 ± 1.10	3.12
Br_r4	162.0 ± 8.00	4.94	16.4 ± 1.57	9.58	38.0 ± 1.51	3.97
Ar_r5	149.3 ± 14.01	9.38	13.8 ± 2.65	19.28	36.5 ± 1.55	4.25
Op_r6	154.0 ± 14.00	9.09	14.3 ± 3.10	21.74	37.5 ± 1.66	4.41
Ba_r7	167.0 ± 9.85	5.90	16.7 ± 1.31	7.80	35.8 ± 1.01	2.81
SL_r8	144.0 ± 11.53	8.01	15.7 ± 1.61	10.25	38.6 ± 1.36	3.51
Ol_r9	155.7 ± 10.07	6.47	15.9 ± 1.56	9.85	33.3 ± 0.71	2.13
As_r10	146.3 ± 11.50	7.86	15.0 ± 1.40	9.36	34.4 ± 1.20	3.49

The highest mean values according to this index were read in 2010 against the background of 348.1 mm precipitation during the vegetative growth of plants (April – August) and mean air temperature of 18.2 °C (Fig. 2). Under these conditions, the variation in plant height of the investigated fertility restorer lines was within the range 136-178 cm.The lowest mean values (117-159 cm) were registered in 2009 at 206.4 mm and 17.8 °C mean air temperature.The difference in the rainfalls between the experimental years was 141.7 mm, and this difference reduced the marginal values of the investigated trait with 19 cm during the dry year 2009. The value of the trait was within 125-164 cm in 2011 at 232.8 mm of rainfalls during the vegetative growth of plants and 17.1 °C mean air temperature.

Averaged for the three years, the fertility restorer lines demonstrated values of head diameter within the range from 13.2 to 16.7 cm (Table 2), which corresponded to values of head diameter in branched fertility restorers determined by other authors as well (Georgiev et al., 2004).

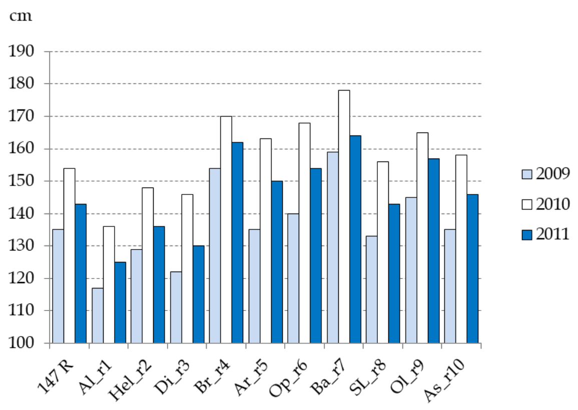

Fig. 2. Mean values of trait plant height (cm) of the lines during different years from the investigation. $p_{0.05}$=7.978; $p_{0.01}$=9.939; $p_{0.001}$=14.237

The value of this trait was highest in 2010, and lowest – in 2009, with differences significant at *p*=0.001 (Fig. 3). The lowest value of this parameter in 2010 was 15.4 cm in line Al_r1, and the highest was the value in 2009 – 15.7 cm in line Ba_r7. The minimal values of this parameter overlapped in 2010, with an insignificant difference of 0.3 cm with the maximal value of the trait in 2009.

These results showed that the head diameter was influenced to a much greater degree by the year conditions in comparison to plant height, which was confirmed also by the relatively higher coefficient of variation according to this trait in the greater part of the investigated lines (Table 2). Averaged for the three experimental years, the variation according to this trait among the investigated lines was within the range 7.80% – 21.74%, delineated by lines Ba_r7 and Op_r6. Line Ba_r7 was with the lowest coefficient of variation according to the trait, followed by lines As_r10 and Br_r4. Such results, proving the relatively wider variability of the trait in comparison to plant height, depending on the growing conditions, have been reported by other authors, as well (Dušanić et al., 2004; Škorić, et al., 2012). In their investigation, Kaya et al. (2016) found out that plant height was affected less by drought stress, while head diameter of plants decreased with up to 50% under drought stress conditions.

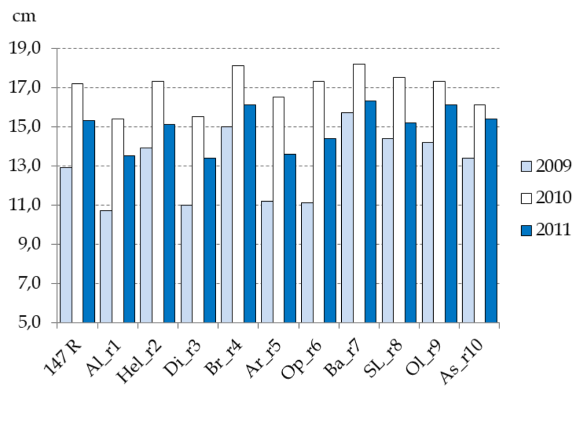

Fig. 3. Mean values of head diameter (cm) of the investigated lines in different years of investigation. $p_{0.05}$=1.778; $p_{0.01}$=2.290; $p_{0.001}$=3.412

Oil content in seed is a parameter of primary technical importance. The polygenic nature of the trait, in which dominance effects are observed, allows obtaining hybrids with high oil content even when only one of the lines is with high oil percentage. Since sunflower is an oil seed crop, this parameter is with high breeding value and a large part of the breeding programs related to developing oil seed sunflower hybrids, have to do with increasing the values of this parameter in the parental lines of such hybrids (Hladni et al., 2006; Hladni et al., 2008; Drumeva, 2012a). Therefore, negative deviations in its values are highly undesirable from a breeding point of view. Oil content in the seed of the investigated lines varied from 33.3% in Ol_r9 to 38.6% in SL_r8, averaged for the three years of investigation (Table 2). The most favorable conditions for accumulation of oil in seed were in the warm and humid year 2010, when a maximum of 39.9% oil was registered in the seeds of line SL_r8 (Fig. 4). The seed oil content of this line in 2011 was 37.2%, and in 2008 – 38.8%. The difference in the oil content determined in these two years was not significant. Lines Br_r4 and Op_r6 also possessed high oil content.

It is worth mentioning that the highest absolute percent of oil in the lines was registered in 2009, which was drier than 2011. The differences in the precipitation during the vegetative growth between these two years was 26 mm in favor of 2011 for the entire vegetation period, but the amount of rainfalls in July of the drier year 2009 significantly exceeded the amount of rainfalls during the same month of year 2011 (Fig. 1). This is the month, in which the moisture reserves in soil available to the plants are especially important because of the beginning of the grain filling stage. According to a number of authors (Černý et al., 2014; Škorić, 2009; Škorić et al., 2012; Stanojevic et al., 1992), the oil content in seed is determined by the genotype and the agro ecological conditions, the effect of which is especially evident at the seed filling stage. The insufficient precipitation has a strong effect on the metabolic processes of the sunflower plant, especially at the stage of increased demand for physiological moisture resulting in lower oil content accumulated in the seed. According to Škorić (2009), drought at this stage causes seed oil percentage to decrease by 7-8%. This was probably the reason for the averagely higher oil content of the lines read during the experimental year 2009, when the amount of rainfalls in July was 72.4 mm, and in 2011 – 51.8 mm. Regardless of the differences determined in the absolute values of the lines according to this index, the variation of the mean values was rather low, indicating considerable stability of the parameter. Oil percent in seed was the trait with lowest variation during the period of study in comparison to the two morphological traits (Table 2).

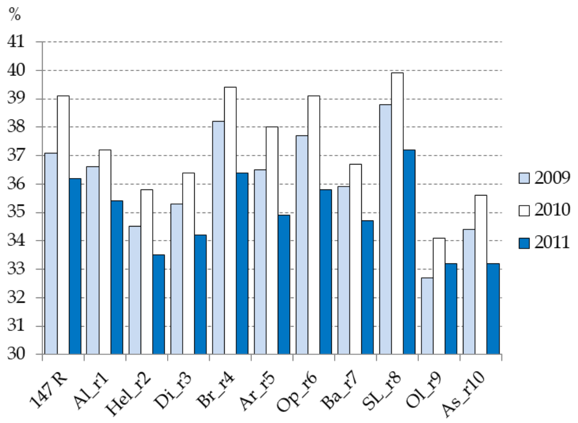

Fig. 4. Mean values of oil content in seed (%) of the investigated lines over years. $p_{0.05}=1.917$; $p_{0.01}=2.521$; $p_{0.001}=3.845$

Conclusions

The year conditions and the genotype of the investigated doubled haploid lines had a significant effect on the studied morphological traits and on the oil percentage in seed. The combined effect of these two factors was not significant on plant height and oil in seed. Under the conditions of this investigation, the values of the traits plant height and oil content in seed were determined mainly by the genotype, the relative weight of this factor being respectively 62.66% and 69.97%, while the climatic year conditions had decisive impact on head diameter (61.52%).

The highest values with regard to the morphological traits and oil percent in seed were read during the warm and humid year 2010.

Under the conditions of this investigation, line Br_r4 was with the best adaptability to the conditions of the environment; in it, the variation of the mean values of all three traits was relatively low for the three-year period of the investigation. The line also had high oil percentage, and together with lines SL_r8 and Op_r6, also with high oil percent, can be used in breeding programs for developing oil seed sunflower hybrids.

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Assessment of the Ecological Status of "Dalgachka" River in its Section within the Protected Site "Ovcharovo", (NE Bulgaria)

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Abstract. Hydrobiological monitoring and assessment of the ecological status of Dalgachka River and an ecological potential assessment based on abiotic metrics of Ovcharovo dam, was performed in the region of Protected Site "Ovcharovo" in 2017 and 2018. The ecological status assessment was based on the macroinvertebrate community as a bio-indicator and the measurement of the supportive physicochemical and hydrological parameters of the water (according Water frame directive and Bulgarian legislation). The water basins fall into Ecoregion 12 - Pontic province and Black Sea River Basin. To date no recorded indices/parameters or sample collection from Dalgachka River and dam Ovcharovo were obtained, despite such data are required for the purposes of the ecological status/potential assessment. In the present study we assessed for the first time the ecological status of the river section which passes through Protected Site "Ovcharovo" - BG0002093 and provide supportive physico-chemical parameters of the superficial waters in Ovcharovo dam. The river/dam ecosystem is of moderate ecological status, because of high concentration of nitrates and BOD_5 in the dam and phosphorus, phosphates and BOD_5 in the river section. The ecological status assessment conducted in the spring of 2017 at sample site 1 showed moderate status and at sample site 2 the ecological status was assessed as good, according to Biotic index. Additionally, we established a georeferenced database which can be used as a reference base for the further research on the protected site, but can also serve the purposes of the ecological decision making for the area.

Key words: ecological status assessment, macrozoobenthos, river dam system, protected area, water physicocemistry, water dependent Natura 2000 site.

Introduction

The Biological quality elements (BQE), which are used for ecological status (ES) assessment of rivers in Bulgaria are defined by the National water legislation. These are: phytoplankton, phytobenthos and macrophytes, macroinvertebrates and fish (Ordinance N4/2013). The following anthropologically induced hydromorphological changes have been identified to have a negative impact on river ecosystems: river flow regulation, sediment retention, water

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abstraction and others. The high concentration of nutrients and organic contamination from diffuse or local sources of pollution, riparian vegetation modification (for example from agricultural activity), biological pressures from alien species and others may put pressures on the fresh water basins (Pardo et al., 2012; RBMP of Black Sea Basin Directorate, appendix 4.1.1.s, 2016).

Macrozoobenthic fauna and ichthyofauna are among the most sensitive BQE to hydro-morphological alterations (Marzina et al., 2012) such as: quantity and dynamics of the water flow, fluctuations of the water level, cutting longitudinal river continuum with different damming facilities, artificial riverbed corrections, dikes and damaged connection between the main water course and adjacent wetlands, reduced biodiversity of micro- and meso-habitats (RBMP of Black Sea Basin Directorate, appendix 4.1.1.s, 2016). The most relevant BQE for assessment of organic pollution is the macrozoobenthos, (RBMP of Black Sea Basin Directorate, appendix 4.1.1.s, 2016), due to the fact that these organisms are numerous, but highly sensitive (Patang et al., 2018).

Macrozoobenthos is a well studied BQE, introduced by the Water Framework Directive 2000/60/EU (WFD) for the purposes of the ES assessment. Benthic communities proved to be an efficient indicator of water quality and ecosystem integrity, since they are weak migrators and respond to habitat and long-term ecosystem changes (Li et al., 2019). Many methodologies connected to ES, which are based on invertebrate ratio and biodiversity were introduced as national standards in countries like Germany, Chech Republic, Serbia, Bulgaria and others (Uzunov et al., 2013).

Factors, like the absence of fish fauna, the greater sensitivity of macroinvertebrate metrics to general degradation of the river and the high sensitivity of the BQE "macroinvertebrates" to all kind of anthropogenic impacts (RBMP; Marzina et al., 2012), determine this biological indicator as the most appropriate for the analysis of the ES of Dalgachka river. The National Environmental Monitoring System (NEMS) of Bulgaria had no monitoring points on Dalgachka river, nor on the Ovcharovo dam (RBMP of Black Sea Basin Directorate, appendix 4.1.1.a 2016). Other scientific data concerning the ecology of that river or the lentic ecosystem are lacking to date. The present research was the first one, which gathers data on that river-dam ecosystem.

Dalgachka river and Ovcharovo dam are part of one surface water system with code BG2KA800R033. Currently, 5 monitoring points for hydrobiological and physicochemical monitoring of that water body are available from the National Environmental Monitoring System (NEMS). None of these points provide data or takes into consideration the ecological conditions and ES/EP of the sections of both water bodies included in PS "Ovcharovo" – BG0002093 (RBMP of Black Sea Basin Directorate, 2016, Section 4, appendix 4.1.2).

The upper course of the river – 5.5 km long river stretch, to sample site 2 (Ss2) (including the dam built there) is in the range of Natura 2000 protected site (PS) BG0002093 "Ovcharovo" (natura2000.moew.government.bg). The PS was classified as SCI, under the jurisdiction of HD, because in the area, one of the identified habitats is listed in Appendix I of Directive 92/43/EEC – wet meadows on the northern slopes of Preslavska Mountain, around the tributaries of Kamchia river. As a SPA, Ovcharovo PS is important for protection of water dependent birds like the Corncrake (*Crex crex*) – listed in Appendix I of migrating birds from BD (natura2000.moew.government.bg).

The presence of suitable habitats, like wet meadows (Commandment RD-844/17.11.2008) and the fact that the site aims the protection of *C. crex*, should have required the inclusion of the surface waters of the PS Ovcharovo in the register for water protection in accordance to al. 6 of WFD. The PS should have been stated as "water-dependent Natura 2000 site" (European Commission, Links between the WFD (WFD 2000/60/EC) and Nature Directives, December 2011) - all surface waters within the PS should be subject of protection by WFD and the National water legislation. That would mean, that besides the aims of the Bird directive and the Habitat directive, the aims of WFD should have been reached, and GES for all surface waters within the margins of the site should have been achieved in 2015 (Directive 2000/60/EC). According to WFD CIS Guidance document (2003), water bodies such as BG2KA800R033, which enter into the Natura 2000 network in Bulgaria, should be divided into subsections, because of some additional requirements to the territory. That subdivision will help for the better management of the water units. For the proper assessment of the river/dam system status, data on the condition of the water body are needed. The data are also important to consider the ES in attempt to avoid further degradation of the water ecosystems.

The present survey was the first one, which provides hydro-biological data on river-dam ecosystem "Dalgachka river/ Ovcharovo dam". The main goal of the study was to assess the ES of the river forming the PS BG0002093 "Ovcharovo" and to gather data on the supportive physico-chemical parameters concerning the ecological potential (EP) of the dam, because of the identification of the PS as SCI and SPA.

Material and Methods

Study area. The investigated section of Dalgachka river and Ovcharovo dam is located in Targovishte district (N-E Bulgaria). The river and the dam are part of the Kamchia river watershed and are indicated as water body with code BG2KA800R033 named Kamchia river, III – section, river Dalgachka from the spring to the Kralevska river. (RBMP of Black Sea Basin Directorate, appendix 1.2.5, 2016). Our studies were provided in the lands of the villages Ovcharovo and Pevets.

According to the Bulgarian typology of the surface waters, Dalgachka river refers to semi-mountain river type R4, and the dam refers to small and medium semi-mountain lake types L12 - both in the Pontic province (RBMP of Black Sea Basin Directorate, appendix 1.2, 2016). The river gathers its water sources from the slopes of Preslavska mountain and flows into Ovcharovo dam, which was constructed in 1977 for the purposes of irrigation (see Permission № 200218/15.02.2005). Ten kilometers downstream, the waters are discharged into the Kralevska river.

Benthological samples were taken twice – on 14.05.2017 and 31.10.2018. The probes were obtained in different years and seasons in order to register variation in the hydrology regimes and the subsequent difference in the population of benthic organisms.

We selected the sample site at the dam (Ssd) located near the wall, because from that part of the reservoir (near the water tower), the water is discharged down the stream. The two sample sites (Ss) in the river are located in the sections along the river bed around which different types of agricultural activities were performed (Fig. 1, Tab. 1). Sample sites above the "Ovcharovo" dam were not considered, due to the lack of major agricultural or other human activity in that region of the PS. The territory above the dam is occupied by forest ecosystem and a survey of that river stretch will not provide information concerning the anthropogenic influence to the aquatic ecosystem.

Sample collection and processing. Water samples for physicochemical analysis were collected from the surface water of the dam, and Ss 1 and Ss 2 (Table 1), according to BNS EN ISO 5667-6:2016. The conductivity (Cond.) and temperatures (T) were measured *in situ* by portable conductometer WTW 196 LF (WTW Gmbh, Weilheim, Germany). According to Ordinance N4 (2013), obligatory parameters for ecological status assessment such as pH, dissolved oxygen (DO), total phosphorus (TP mg l^{-1}), phosphate as phosphorus (PO_4-P mg l^{-1}), nitrite nitrogen (NO_2-N mg l^{-1}), nitrate nitrogen (NO_3-N mg l^{-1}), ammonium nitrogen (NH_4-N mg l^{-1}) and Biological demand of oxygen for 5 days

(BOD_5 mg l^{-1}) have to be monitored. For our study, all these parameters were measured using HI 83200 Multiparameter Bench Photometer for Laboratories (Hanna Instruments, Woonsocket, Rhode Island, USA). Storage of water samples for BOD_5 was performed according to BNS EN 1899-2, 2004 (20 ± 2° C).

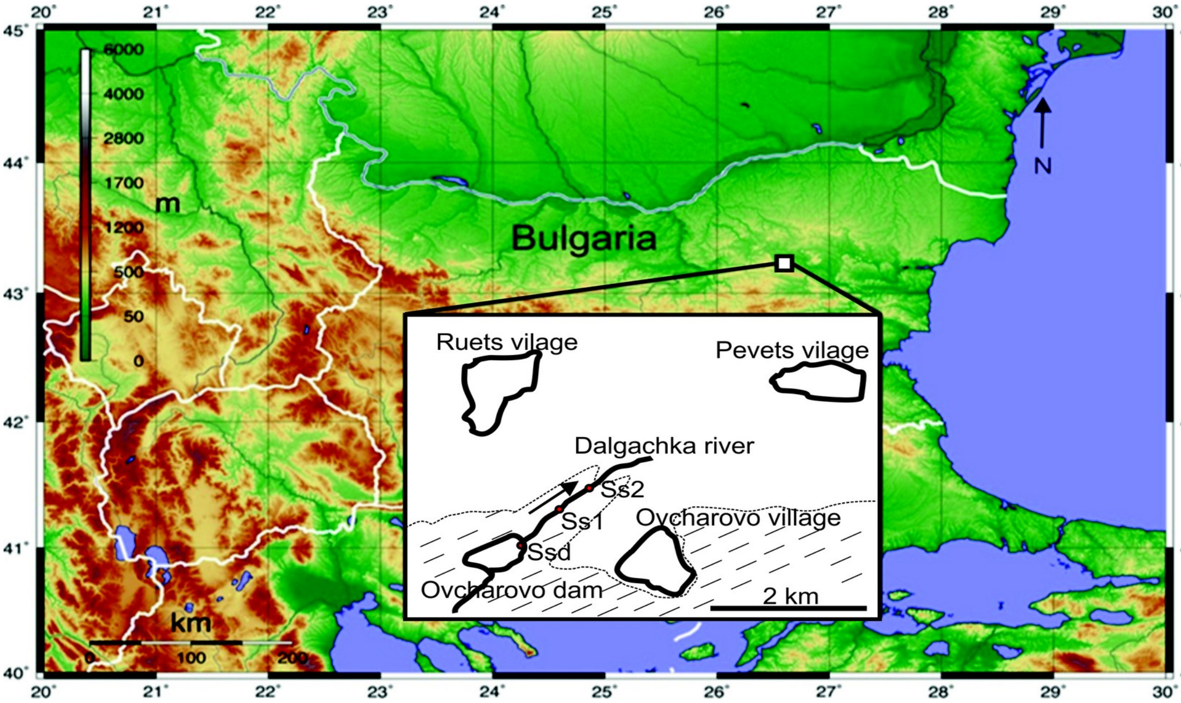

Fig. 1. Schematic map of the locations of the sampling sites; Ssd, position of the sample site at the dam "Ovcharovo"; Ss1, position of the sample site 1 at Dalgachka river; Ss2. position of the sample site 2 at Dalgachka river; the arrow indicates the direction of the water flow of the river; the margins of the PS BG0002093 "Ovcharovo" are indicated in striped code.

Table 1. Sample sites and basic information. Legend: Ssd – Sample site at the dam; Ss 1 – sample site 1 in the river; Ss 2- sample site 2 in the river.

	Water body type	Latitude N	Longitude E	Altitude (m a.s.l)	Distance to the dam
Ssd	L 12	43°11.212′	26°37.234′	300 m	-
Open lined channel	-	43°11.326′	26°37.784′	275 m	15 m After
Ss 1	R 4	43°11.513′	26°38.144′	259 m	700 m After
Ss 2	R 4	43°11.730′	26°38.474′	251 m	1300 m After

Two semi-quantitative composite macroinvertebrate samples were collected in 2017 and 2018 with a hand-net (500 µm mesh size) for about 5 minutes, according to Cheshmedjiev et al. (2011). Sample collection of invertebrates from the autumn of 2018 was performed during a period of low water levels. The water masses, released from the dam, were insufficient to form a stable surface runoff. The collection of organisms from Ss1 was impossible, because of the lack of superficial runoff in that particular riverbed section. The invertebrates were sorted in the laboratory and identified to family and order level for ecological status determination of the studied sites by two metrics – Total Taxon Number (TTN) and adapted version of Irish Biotic Index (BI) (Cheshmedjiev & Varadinova, 2013).

Quantity and dynamics of water outflow. The quantity of the water outflow from Ovcharovo dam was measured in open lined channel immediately after the dam wall (Table 1). We selected that point, because river sections with morphologically homogenous river bed create uniform water current with relatively permanent water velocity (V). That velocity remains unchanged on the full width of the channel and that makes it suitable to determine the flow rate (Gore, 2007). Maximum velocity (V max.) and average velocity (V av.) was measured with flowmeter FP 201, serial № 92389, Nibco – NSF – PW (Global Water Instrumentation Inc., - a Xylem brand, Gold River, California, USA) and the flow rate (Q) was determined by calculations based on the measurements and the width and depth of the water column.

Assessment of the ecological status/potential of the studied sites. EP assessment of the dam was based only on the one time annual measurement on physicochemical parameters of the surface water, which is enough to asses only the quality of the single samples (Ordinance N4/2013), while for ES assessment of the river, the bottom invertebrate fauna indices (according to national legislation) were used, considering BI as relevant. We used the type specific scale with the ranges provided for semi-mountain rivers of type R4 and for small and medium semi-mountain lake types L12 in Ponthic provinces provided by the Ordinance N4 (2013). It prescribes three ranges, based on abiotic metrics and five ranges (Ordinance N4/2013), based on the biological indicator visualized by the same color coding (see Table 2).

Results

Dalgachka river is a representative of R4 river type with a narrow river bed and mainly rocky bottom substrate. At Ss1 and Ss2 the river bed is formed by macrolithal (stones with diameter 40-20 cm), mesolithal (stones with diameter 20-6 cm), microlithal (gravel 6-2 cm), akal (fine to medium-sized gravel 0.2-2 cm) fractions and psammopelal (sand and mud). The ratio of distribution of the substrate at Ss1 is 40% psammopelal, to 20% akal, to 30% microlithal, to 10% mesolithal. The ratio at Ss2 is 50% mesolithal, to 25% microlithal, to 10% akal, 10% macrolithal, to 5% psammopelal.

The hydrological measurements of the outflow from the dam registered spring maximum and autumn minimum. The low runoff in 2018 (Table 3), the low velocity and the infiltration capacity of the river bed led to an absence of surface water at Ss1, but at Ss2 water appeared again (due to groundwater source, or because of reduced infiltration capacity of the river bed).

The data, regarding the physicochemical conditions of the surface water from the Ovcharovo dam in the spring of 2017 and the autumn of 2018 demonstrates different concentration levels, corresponding to different EP for the oxygen regime in respect to DO and BOD_5 and for TP, $P\text{-}PO_4$ and $N\text{-}NO_2$. For both years, the values for $N\text{-}NO_3$ were not in the range to ''achieve'' good ecological potential (Table 3).

The results from the measured physico-chemistry of the water at the studied river sections indicated that the majority of the elements pointed out for good ecological status. Data considering Ss1 were gathered solely from 2017. MoES was registered only for TP and $P\text{-}PO_4$. According to our measurements, at every sample collection on Ss2 was found one abiotic parameter with levels corresponding to moderate status (Table 3).

A comparison between Ss1 and Ss2 for the spring in 2017 was possible. The comparison indicated different concentrations levels for TP, corresponding with different ES (Table 3).

Taxonomic composition of the macrozoobenthos was performed to allow ES assessment. A total of 19 taxonomic groups were found at the studied sites. Ss2 had greater diversity and density of benthic organisms, compared to Ss1. These results provided the values for BI, which characterize Ss1 with MoES and Ss2 with GES in 2017, while in the autumn of 2018 was possible to assessed solely Ss 2 with MoES, because the river was dry at Ss1 (Table 4). The results from Ss2 in 2018 showed equality between the used indices TTN and BI - both indicated MoES of river Dalgachka.

Table 2. Color coding, according to National water legislation.

High ecological status/Maximum ecological potential (HES/MEP)	Good ecological status/potential (GES/GEP)	Moderate ecological status/potential (MoES/MoEP)	Poor ecological status/potential (PES/PEP)	Bad ecological status/potential (BES/BEP)

Table 3. Hydrological and physicochemical parameters. Legend: Q (m^3 s^{-1}); V av. (m s^{-1}); V max.; DO (mg l^{-1}); Cond. (μs cm^{-1}); N-NH_4 (mg l^{-1}); N-NO_3 (mg l^{-1}); N-NO_2 (mg l^{-1}); P-PO_4 -(mg l^{-1}); TP (mg l^{-1}); BOD_5 (mg l^{-1}).

	SsD	SsD	Ss1	Ss1	Ss2	Ss2
Season Year	Spring 2017	Autumn 2018	Spring 2017	Autumn 2018	Spring 2017	Autumn 2018
Q	-	-	0.046	-	0.046	0.004
V av.	-	-	0.34	-	0.57	0.25
V max.	-	-	0.5	-	0.6	0.3
DO	7.5	10.67	6.9	-	7.65	9.17
pH	8.4	8.38	8.4	-	8.2	7.92
Cond.	222	260	280	-	273	580
N-NH_4	0.02	0.01	0.16	-	0.12	0.05
N-NO_3	0.9	1.5	1	-	1	0.75
N-NO_2	0.01	0.02	0.01	-	0	0.01
P-PO_4	0.03	0	0.097	-	0.048	0.03
TP	0.03	0	0.1	-	0.05	0.03
BOD_5	3.25	1	2	-	2.35	3.7

Table 4. Taxonomic groups of macrozoobenthos. Legend: TNI – total number of individuals per square meter; TTN – total taxon number; BI – biotic index; (A) - Sensible macroinvertebrate taxon; (B) - Less sensible macroinvertebrate taxon; (C) - Relatively tolerant macroinvertebrate taxon; (D) - Tolerant macroinvertebrate taxon.

	Ss1 – 2017	**Ss1 – 2018**	**Ss2 – 2017**	**Ss2 – 2018**
Ephemoptera order				
Heptagonidae fam. (A)	60	-	115	8
Ephemeridae fam.				-
Ephemera sp.(B)	36	-	8	
Baetidae (C)	52	-	186	30
Odonata order				
Aeshnidae fam. (B)	-	-	7	-

Athericidae fam. (B)	6	-	20	-
Tricladida order				
Dugesidae fam.				
Dugesia gonocephala (B)	7	-	9	-
Plecoptera order				
Nemouridae fam. (B)	-	-	-	3
Trichoptera order				
Psychomyiidae fam. (C)	-	-	3	-
Hydropsyhidae fam.(C)	13	-	19	2
Philopotamidae fam.(C)	-	-	2	-
Rhyacophilidae fam. (C)	-	-	7	-
Limnephilidae (C)	15	-	9	1
Gammaridae fam.				
Gammarus sp.(C)	262	-	554	431
Empididae fam.(C)	8	-	-	-
Coleoptera				
Elmintidae fam. (C)	92	-	124	3
Tricladida order(C)	3	-	31	-
Tipulidae fam. (C)	-	-	-	1
Simulidae fam. (C)	-	-	-	1
Chironomidae fam. (D)	-	-	10	22
Rheotanytarsus sp.(B)	5	-	-	-
Oligochaeta subclass(D)	-	-	112	-
TNI	559	-	1216	502
TTN	12	-	16	10
BI	3	-	3-4	2-3

Discussion

Our research revealed that the physicochemical parameters in 2017 and 2018 for the Ovcharovo dam accomplished the goal of WFD and ''achieved'' values at the range of GEP for all the abiotic parameters (excluding N-NO_3 for both years and BOD_5 in 2017). The observed high concentrations of N-NO_3, corresponding to MoEP, in the epilimnium of Ovcharovo dam, are likely due to agriculture activities, such as excessive use of fertilizers, but could also be caused by enhanced soil erosion and surface runoff (Damyanova & Varadinova, 2018).

The measurements of the supportive physicochemical parameters of the river showed rather heterogeneous results for the various abiotic factors. The values of the parameter TP for example, was in the range of GES in 2017 and in 2018 only at Ss2, but the results at Ss1 for 2017 correspond to MoES and were 0.1 mg l^{-1}. GES and MoES values don't fit to the definition of unpolluted waters, according to Wetzel (2003), because mean concentration of total dissolved phosphorus in unpolluted waters should be about 0.025 mg l^{-1}. Every result with values below 0.025 mg l^{-1} for TP in that river type (R4), corresponds to HES, according to our legislation. The measured values of TP can easily be reached after a heavy rainfall, at initial stages of snowmelt or from agricultural runoff (Wetzel, 2003), considering the fact that a large proportion of TP is delivered to surface waters via runoff in dissolved or particulate forms (Fraterrigo, 2008). The territories located to the left of the river bed (around Ss1) were occupied by annual crops, which demand more intensive tillage, than the surroundings of Ss2, which are occupied by perennial raspberry plantation. Greater rate of soil disturbance in agricultural riparian area within 100 meters of open water is the part of the watershed which is the most sensitive to increase of phosphorus loads (Sorrano et al., 1996). Therefore, the differences in the concentration levels of TP at both sample sites could be due to hydromorphological variations of the bottom substrate, caused by agricultural pressure as a factor increasing erosion processes and as a factor of diffuse source of pollution.

BOD_5 has values in the range of MoES only during the low water period in the autumn of 2018. That result could be attributed to natural decomposition processes or can be a good indicator for organic pollution (Damyanova & Varadinova, 2018). The other six measured physicochemical parameters are with values characterizing the river sections with GES and HES.

Based on the BQE "macrozoobenthos" two comparisons were made. The river section from dam Ovcharovo to the boundary of the PS in 2017, according to BI varies from MoES at Ss1 to GES at Ss2 and according to TTN from GES at Ss1 to HES at Ss2 (Table 4). The ES at Ss2 demonstrate a drop of the value for BI from 3-4 (GES) to 3 (MoES) from 2017 to 2018 and for TTN from HES to MoES (Table 4). The decline of the ES at Ss2 can be attributed to the changing hydrological conditions such as e.g. the flow rate (Table 3), which influence the community structure of bottom dwelling invertebrate fauna (Li, et al., 2019). Better management of the dam can contribute for maintaining GES at the river section downstream from Ovcharovo dam through all seasons, by promoting a more constant flow rate.

In 2017, Ss1 indicated poorer ES compared to Ss2. These results correspond with the different community structure of the benthic invertebrate fauna at the two sample sites. The source for that difference could be the dominance of finer substrate as psammopelal and lower flow velocity at Ss1, because these hydro-morphological factors affect both directly, and indirectly the macrozoobenthic community (Li, et al., 2019). The greater portion of finer sediment in the bottom substrate at Ss1 was probably caused by enhanced surface runoff induced from agricultural activities. Local point sources of pollution were not present, which means that allochthonous input may be occurring at Ss1.

The present preliminary study indicates on pressure in the PS at all sample sites, due to agricultural activities near the water bodies and inappropriate flow rate management of Ovcharovo dam. These factors could lead to degradation of the aquatic ecosystem to MoES regarding both measured abiotic and hydrologic parameters, as well as biotic indicators. Our results can serve as a basis and reference for eventual future monitoring, which would identify the potential pressures and threats that may impact the site and cause further environmental degradation of that complex aquatic ecosystem. The information published in this research article may be used from Black Sea Region Basin Directorate (BSBD) in the preparation of new River Basin Management Plan, in order to accomplish more detailed and objective information concerning the ES of the water body BG2KA800R033.

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Seasonal Effects on Ecological Status/Potential Assessment in Lakes Based on Macrozoobenthos

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Abstract. Seasonal impact on the ecological status assessment through macrozoobenthos in representative lentic water bodies (lakes and dams) situated at Ecoregion 7 Eastern Balkans (South Bulgaria) was made. Data from twelve standing water bodies during three seasons (summer 2018, autumn 2018 and spring 2019) were analysed. The Total number of taxa index showed seasonal dependence. The Biotic index was not significantly changed across seasons in different types of stagnant waters. The seasonal effects on the ecological status/potential evaluation depended on the type of the standing water body (natural/heavily modified/artificial), environmental factors and different anthropogenic pressure which caused changes in the aquatic ecosystems.

Key words: macrozoobenthos, seasonality, ecological status/potential assessment.

Introduction

Water ecosystems conditions are shaped by environmental factors and anthropogenic impacts and directly affect the viability and distribution of the water communities. When characterizing the ecological situation in the aquatic ecosystems, it is essential to take into account the seasonal fluctuations of the environmental factors and their impact on the composition and structure of the bottom invertebrate fauna (Chen et al., 2018). Depending on their preferences to the aquatic environment, macrozoobenthos representatives are characterized by a different seasonal abundance and biomass in the aquatic ecosystems (Kotwicki, 1997; Živic et al., 2005). Higher taxonomic richness of macrozoobenthos during the rainy season compare with the dry season has been reported by Moreno & Callisto (2006). According Atobatele & Ugwumba (2010) in reservoirs larger numbers of taxa were recorded during the dry season than in the wet season. Study of Borisova et al. (2016) in Srebarna Biosphere Reserve revealed that Sörensen's similarity coefficient showed clear differences between the taxonomic composition of macrozoobenthos but in the main distinguished types of habitats. Species richness, dominant species and standing

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crops varied significantly in different seasons, and also in different areas (Yang et al., 2019). The development of an appropriate ecological status/potential assessment metrics based on a biological quality element macrozoobenthos has its importance in the context of the specific characteristics of the standing aquatic ecosystems as well as the human pressure on them. Some studies on standing waters demonstrated that diversity indices of the macrozoobenthos had no significant differences between seasons (Li & Ding, 2016; Gao et al., 2019) and, the abundance of dominant taxa indicated no clear seasonal changes (Cai et al., 2012).

Assessment based on Shannon index, Margalef index and Hilsenhoff biotic index showed that water quality in the Lake Nansi in spring and winter was slightly better than that in summer and autumn (Yang et al., 2019). Seasonal variations in quality indices were found to be site specific as some showed improvement in ecological status and macrozoobenthos quality while others revealed degradation from the dry season to the wet season (Kanhai & Juman, 2018). Both indices, %Oligochaeta and PETI, determined clear seasonal changes in ecological state assessment (Borisova et al., 2016).

The aim of the study is to make a comparative analysis of the ecological assessment in representative standing water bodies determined by macrozoobenthos during different seasons.

Material and Methods

The study was carried out in representative types (L1-Glacial high-mountain lakes/Alpine lakes, L4-Lowland and semi-mountain lakes and swamps, L6-Riverside wetlands, L11-Large deep reservoirs, L13-Medium-size and small semi-mountain reservoirs, L15 Large lowland reservoirs up to middle depth, L17- Small and medium size reservoirs) of standing water bodies (lakes and dams) situated in Ecoregion 7 Eastern Balkans, South Bulgaria (Fig. 1).

Macrozoobenthos samples from twelve standing water bodies (Bezbog Lake, Choklyovo Marsh, Bistraka Marsh, Dospat Dam, Stoykovtsi Dam, Drenov Dol Dam, Pchelina Dam, Dolna Dikanya Dam, Dyakovo Dam, Pyasachnik Dam, Ovcharitsa Dam, Ovchi Kladenets Dam) during two sampling seasons - autumn 2018 and spring 2019 were taken. Data from these studies were associated with results received in the summer of 2018 (see Smilyanov et al., 2018) in order to analyze the impact of seasonality on the ecological status/potential of the studied lakes and dams.

Methodologies applied for the purposes of biological monitoring in Bulgaria and reporting of the state of standing water in Bulgaria to the European Environment Agency were used. Multi-habitat sampling approach (Cheshmedjiev et al., 2011) with the standards BDS EN ISO 5667-1:2007 and BDS EN ISO 5667-3:2018 was applied during the macrozoobenthos collecting. The indices Total number of taxa (TNT) and Biotic index for slow-flow running waters (BI) with experimental scale (Varadinova, 2012; 2013; Cheshmedjiev & Varadinova, 2013) were used for the ecological status/potential assessment of the standing water bodies.

The map of the surveyed lentic water bodies was prepared with software product ArcGIS 9.1. The lake types, geographical coordinates and main features of the standing water bodies were presented in Smilyanov et al. (2018). Cluster analysis techniques from Statistica 7 was used to visualize the similarity in the taxonomic composition of the macrozoobenthos in the studied standing water bodies.

Results

Benthic communities showed greater similarity in taxonomic composition between analysed dams within a one season than the lakes. In contrast, seasonality does not play a determining role in the formation of the macrozoobenthos in the natural lentic water bodies. Macrozoobenthos found in reference lakes (Bezbog, Bistraka) demonstrated close resemblance in community composition through the three studied periods (Fig. 2).

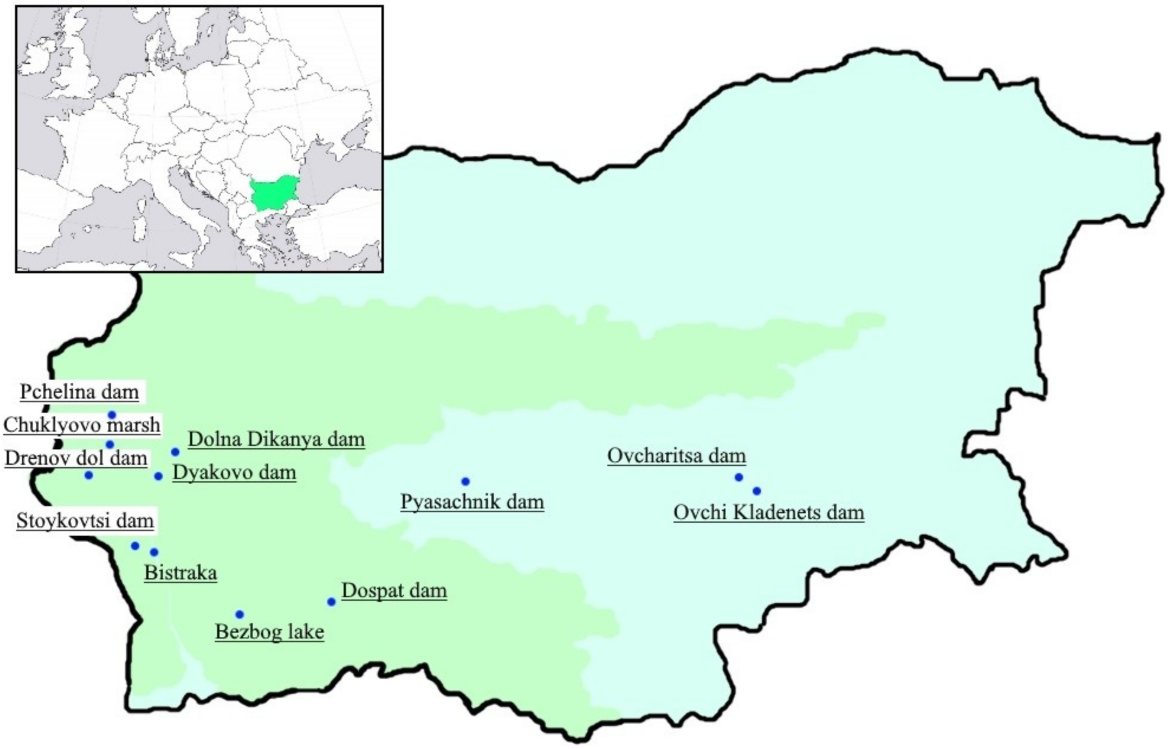

Fig 1. Distribution of the studied water bodies on the territory of Bulgaria.

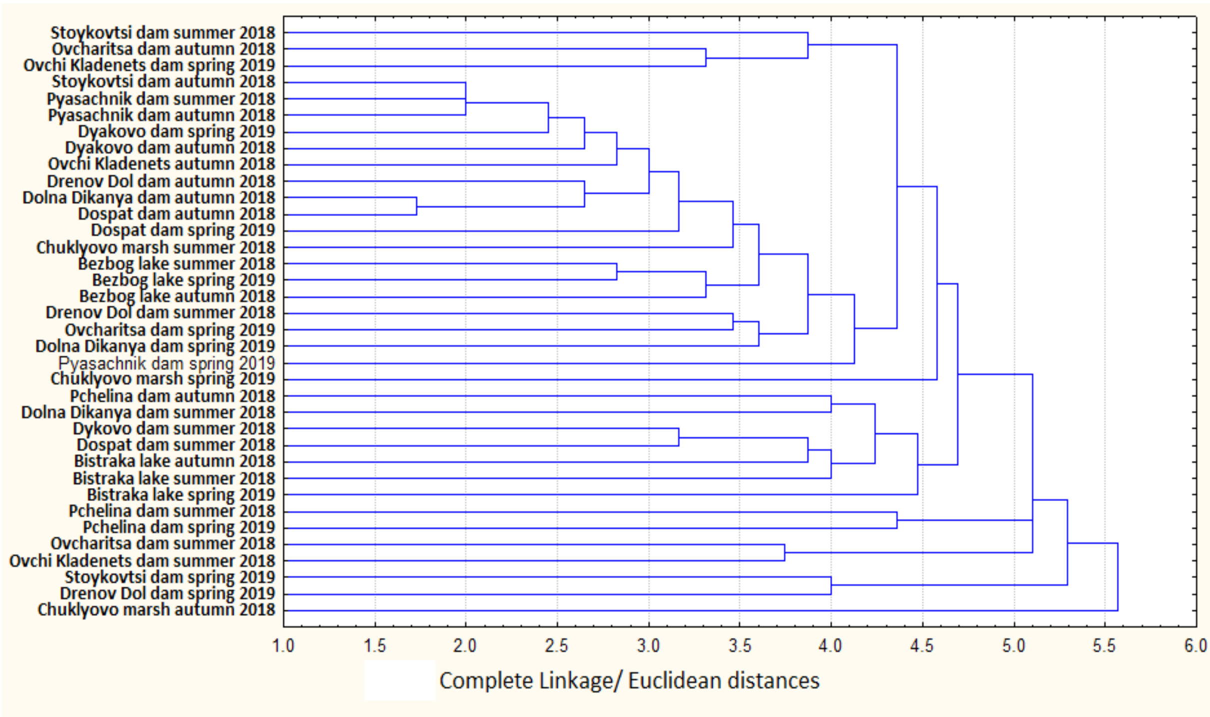

Fig. 2. Analysis of the similarity in the taxonomic composition of the macrozoobenthos between studied water bodies in three different seasons (summer 2018, autumn 2018, spring 2019).

The greatest taxonomic richness was recorded in the summer of 2018 in all standing water bodies (Fig. 3). The bigger diversity corresponded to the registered higher ecological assessment of the water bodies status based on TNT index. In the autumn 2018 and spring 2019, the number of taxa decreased (except for the Chuklyovo Marsh in the autumn) and accordingly, the ecological evaluation was worsened.

The ecological status/potential evaluated by BI didn't change over the seasons in the nearly half of the studied water bodies (Fig. 4). Thus, in Chucklyovo Marsh and Bistraka Lake, the values of the BI moved within the limits of good ecological status in all three seasons. Among the dams, Dospat, Pchelina, Ovcharitsa and Ovchi Klagenets were characterized by unchanged conditions (moderate potential for first two dams, poor for the third and fourth) in all three seasons. Unlike the evaluation defined by TNT, in the above water bodies there was no registered seasonal dependence in the ecological status/potential assessment determined by BI.

The values of BI changed within two (Drenov Dol, Dolna Dikanya and Dyakovo) or three (Pyasachnik, Stoykovtsi) adjacent ecological quality classes (good, moderate, bad) in the rest studied dams. The ecological status assessment of Bezbog Lake also shifted during the three different seasons, showing a deterioration in the spring 2019.

Discussion

Similar taxonomic composition of the bottom invertebrate fauna registered in natural water bodies (Bezbog, Bistraka) during the whole vegetation period could be explained by the reference nature of the two lakes and lack of significant human pressure. The change of seasons affected more considerable on the macrozoobenthos composition in the dams than in the natural water bodies (Fig.2). However, the reasons for these differences were not only the seasonal alteration but external unfavourable influences. Formation of the benthic communities composition in the standing waters are the result of a number of factors.

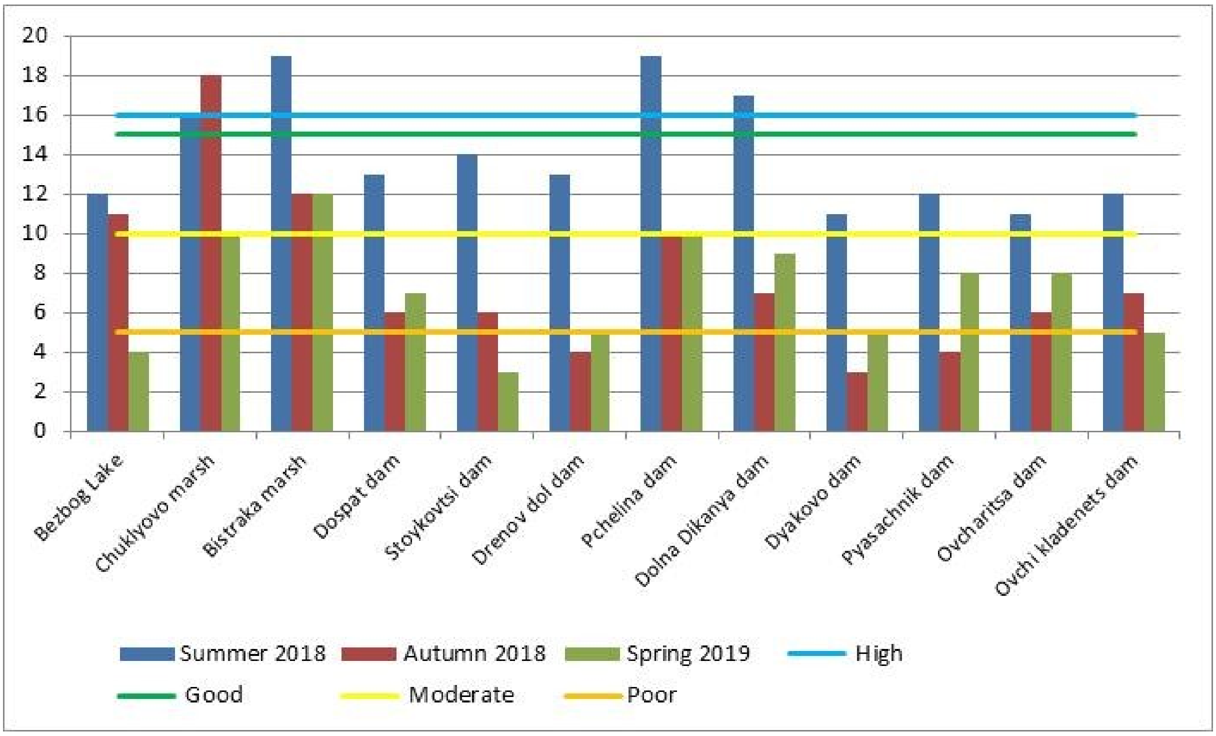

Fig. 3. Dynamics of the TNT index at the studied standing water bodies during the three seasons.

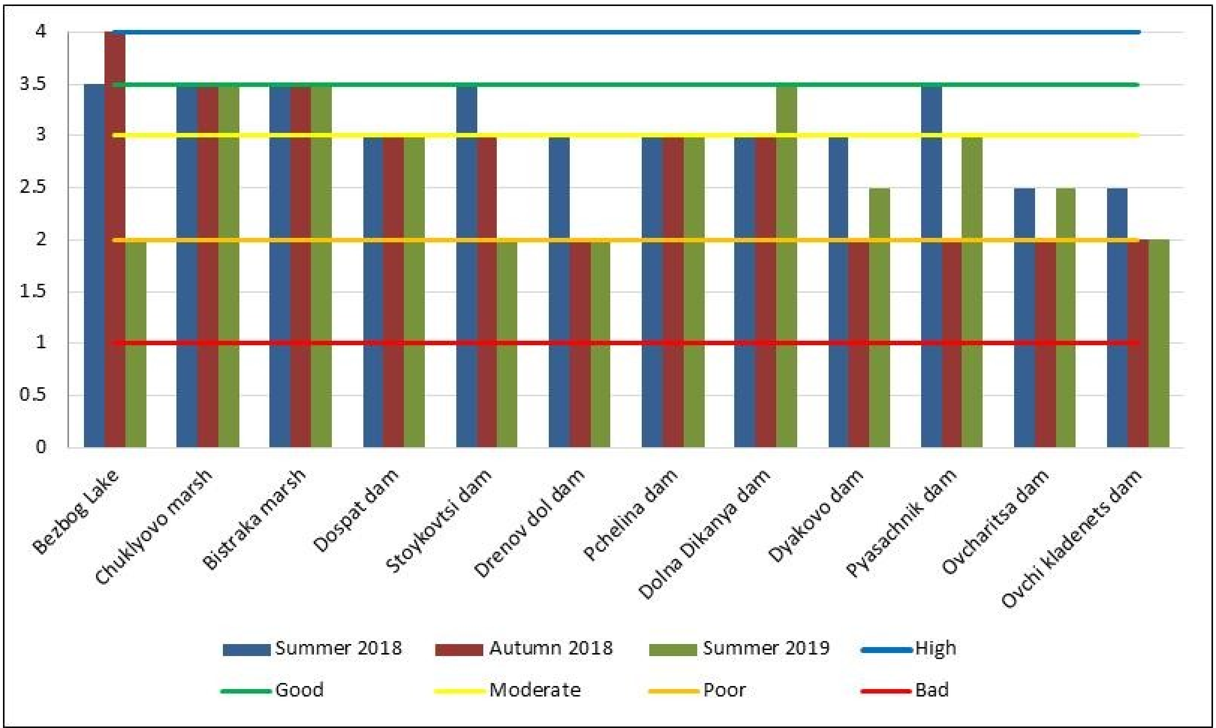

Fig. 4. Dynamics of the BI index at the studied standing water bodies during the three seasons.

Human activities have seriously destroyed the macrozoobenthos habitats and led to the decline in macrozoobenthos diversity (Li et al., 2019). Anthropogenic eutrophication was considered to have a negative influence on macrozoobenthos intolerant groups in lakes (Timm et al., 2006). Hydromorphological pressure (mainly water abstraction) also played adverse effect in dams and caused habitat degradation which led to deterioration of benthic communities biodiversity (Smilyanov et al., 2018). These impacts induced worsening of the environmental conditions and reflected negatively on the ecological status/potential of the standing water.

An important requirement for the ecological status/potential assessment is to take into account the type-specific characteristics of the studied lakes and dams (Varadinova et al., 2019). Studies on the application of the various indices based on macrozoobenthos did not give a definite answer as to whether seasonality affects the environmental assessment of standing water (Shou et al., 2009; Cai et al., 2012; Borisova et al., 2016; LI & Ding, 2016; Kanhai & Juman, 2018; Gao et al., 2019; Yang et al., 2019). It is essential to determine to what extent applied benthic indices could reflect different aspects of natural and anthropogenic impacts and characterised a real ecological situation in the aquatic ecosystem.

In this study the TNT index demonstrated seasonal dependence. For all standing water bodies, its values varied within the boundaries of good and high ecological status in the summer of 2018 (Fig. 3). A tendency of deterioration of the ecological evaluation in autumn 2018 and spring 2019 compared to summer 2018 was registered. This trend was observed not only in natural water bodies (including the reference ones) but also in dams in which the aquatic ecosystem was under permanent anthropogenic pressure of different nature such as disturbance in hydrological regime, allochthonous inputs, etc.(Smilyanov et al., 2018). Other studies revealed that macrozoobenthos diversity is higher in dry than in wet seasons (Atobatele et al., 2010), more species were found in the summer than in the winter season, (Mustafa et al., 2013) or species richness was more or less similar in both season

(Gao et al., 2019). Registered in our study higher number of taxa in summer, in all studied lakes and dams was the result of favourable environmental conditions with the defining role of the water temperature as the main environmental factor affecting the most contributing macrobenthos species (Chen et al., 2018).

To assess the influence of the seasonality on the on-going evaluation of the ecological status/potential in lakes and dams has to be recorded under relatively unaltered external conditions. However, for some dams, the impact of various anthropogenic factors (eg withdrawal for irrigation, water supply, active angling and aquaculture cultivation) could alter the conditions in the aquatic ecosystem during two consecutive seasons. This could provoke a response of biotic stress, which is particularly noticeable for the littoral macrozoobenthos where the samples were taken. The disturbance of the ecological health in aquatic habitat caused a negative effect on the ecological status of the water body, which was assessed by analysed biological quality element (Fig. 4). Changes in the status of the natural water body Bezbog are more likely due to other specific factors. Bezbog lake is located at more than 2000 m above sea level. This is a prerequisite for the shift of the biological seasons, later snowmelt and the effects of the subsequent activation of the mineralization processes of the accumulated organic matter on the benthic habitat conditions, which was a probable cause of the unfavourable assessment registered in the spring of 2019. Consideration should be given here to the fact that these are one-off studies conducted only over three seasons (excluding winter). A series of consecutive studies may provide a more accurate picture of the impact of seasonal changes on the correctness of the macrozoobenthos-based ecological assessment.

Conclusions

The analysis of the seasonality impact on the ecological status/potential assessment should be made in the relatively similar stagnant waters with similar type specific characteristics and environmental conditions. It is important to choose a representative evaluation approach and appropriate indices that, in different seasons, could adequately assess the effect of the natural and anthropogenic factors. The influence of seasonality depends on the type of standing water body (natural/heavily modified/artificial) and anthropogenic pressure (water abstraction, organic pollution, etc.). The diverse in nature and degree human impacts have different reflection on the aquatic ecosystems and make difficult to distinguish only the seasonal effect on the ecological assessment of the studied water bodies.

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Ecological Plasticity and Stability of Some Agronomical Performances in Triticale Varieties (x Triticosecale Wittm)

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Abstract. For the aims of the study were used three years data for the yield, plant height, test weight and mass of 1000 grains of triticale varieties with different genotypes (Kolorit, Musala and Trismart), cultivated on the experimental field of the Crop Science Department at the Agricultural University of Plovdiv, Bulgaria. In order to determine the ecological plasticity and stability of the tested parameters, the modified model of Eberhart and Russel was applied. The yield plasticity varies from 0.825 by Trismart variety to 1.189 by Musala variety. The Musala variety is distinguished with the highest values of the plasticity b_k and this variety significant is the most plastic. By the component yield with the lowest stability is the variety with the highest plasticity-Musala. The variety Trismart, who is distinguished with the highest stability, possesses also the lowest plasticity. According the component plant height all varieties manifest high stability and only the variety Musala can be accepted as ecological plastic regarding this parameter, because it possesses plasticity values of b_k>1. The yield plasticity coefficient correlates positive with the plant height and the mass of 1000 grains. According the yield stability coefficient by all examined components are determined negative proven correlations.

Key words: triticale, plasticity, stability, yield.

Introduction

Agriculture is the sector, which is mostly influenced by the climatic conditions. The meteorological conditions of the year are of primary importance for the correct growth and development of the agricultural crops (Xu, 2016). The formation of certain trends in the yield components is directly subordinated to the environmental conditions and the manifestation of their values in contrasting environmental conditions helps to distinguish those genotypes, which exhibit stability with respect to the yield and its components (Stoyanov & Baychev, 2018). The estimation of the relative contributions of the variety, the environmental conditions and the genotype x environment interaction to variety performance is required for determining the adaptation capacity (Subira et al., 2015). The sustainable agricultural development and changes in cultivation practices can ensure the adaptation of the sector to the climate changes (Brouziyne et al., 2018; Nastis et al., 2012). In this connection the imposition of cultures with higher resistance to unsuitable climatic condition as triticale could be a possible solution. Created artificially by crossing rye and wheat genome, triticale nowadays becomes increasingly popular, because of its high productive potential. Triticale is a crop with high economic efficiency especially in areas with extreme droughts, high temperatures, poor soil nutritional regime, insufficient rainfall, where other cereal crops cannot be grown successfully (Oral, 2018). The big genetic diversity among the created triticale varieties is the reason for the ecological plasticity, stability and productivity of the crop (Ferreira et al., 2015; Mühleisen et al., 2014).

The aim of the study is to determine the impact of the climatic conditions on the stability and plasticity of some agronomical performances in triticale varieties.

Material and Methods

In order to achieve the aims of the study, a field experiment with duration of three years was set on the experimental field of the Crop Science Department at the Agricultural University of Plovdiv, Bulgaria. The meteorological data during the investigation period are presented on Table 1.

Agrochemical analysis of the soil after harvesting of the predecessor, before sowing, determines the conditions of mineral nutrition of the plants after emergence until the onset of the spring vegetation (Table 2). During the three years of experience, the soil reaction is slightly alkaline with pH 7.78 (2016) 7.54 (2017) and 7.66 (2018), which is characteristic for the alluvial meadow soils, in particular the soil on the experimental field of the Crop science Department (Popova & Sevov 2010).

Table 1. Climate conditions during triticale vegetation.

Year	Temperature (monthly average, ^{0}C)								
	X	**XI**	**XII**	**I**	**II**	**III**	**IV**	**V**	**VI**
2016/2017	10.8	6.6	2.2	-3.9	3.2	9.7	12.7	17.6	23.7
2017/2018	13.3	8.2	4.9	2.9	3.9	7.1	16.4	19.4	22.6
2018/2019	13.7	7.3	2.8	2.5	4.7	10.6	12.6	18.2	23.4
Long-term average	12.9	7.2	2.2	-0.4	2.2	6.0	12.2	17.2	20.9
	Precipitation (sum, mm)								
2016/2017	5.6	32.9	2.4	70.1	11.1	47.9	26.1	52.7	15.4
2017/2018	70.4	47.6	23.7	21.7	96.7	45.5	24.9	112.3	14.4
2018/2019	34.3	62.5	17.9	30.9	17.2	8.8	76.5	21.3	13.2
Long- term average	40.1	48.4	44.3	42.1	32.7	38.2	45.1	65.3	63.4

Table 2. Agrochemical analysis of soil before sowing.

Year	pH	Mineral nitrogen, mg/kg soil	P_2O_5, mg/100 g	K_2O, mg/100 g
2016	7.78	15.24	79.41	65.66
2017	7.54	19.49	84.35	54.27
2018	7.66	18.87	78.76	64.23

There were used three triticale varieties with different genotypes– Kolorit, breeded at the Dobruja Agricultural Institute – Gen. Toshevo, Bulgaria (country standard), Musala, breeded at the Sadovo seed company - Bulgaria and Trismart, breeded at the Caussade semences - France. The genotypic plasticity and stability of the tested varieties are determined by the yield and some components of the yield as plant height (cm), test weight (kg/100 l grain) and mass of 1000 grains, g. The coefficients of plasticity (b_k) and stability (s_k) are determined according the model of Eberhart and Russel (Eberhart & Russel, 1966):

$$Yijk = Y.. + Gi + Pj + rij + eijk$$

where G is the effect of the genotype, and P the effect of the examined area.

The modified version of the model (Penchev & Stoeva, 2004), was also used for the calculations:

$$Yijk = Y..+ Gi + Qj + GQij + eijk$$

where G is the effect of the genotype, Q the effect of the climatic conditions and GQ the interaction between them.

Two-way analysis of variance (ANOVA) was applied to establish the statistically proven effect of the factors and their interaction. Correlation analysis was used to calculate the relationships between the traits studied. For the results processing is used the software product MS Excel Data analysis.

Results and Discussion

The regression coefficient and the deviations from the regression line were being estimated, so the applied dispersion analysis allows the diffraction to be determined (Table 3). The model of Eberhart and Russel can be applied only, if the interaction between the genotype and the climatic conditions is statistically significant, because future changes are based on genetics and plants will probably change the estimated parameter by different environmental conditions. According Eberhart & Russel (1966) the ecological plasticity is the average variety reaction to the environmental changes, and stability is the deviation of the empirical data from the average reaction at any environmental condition.

The average variety reaction to the changes in the climatic conditions is characterized by the coefficients b_k of the linear regressions (Table 4). Those coefficients indicate not only the plasticity of the varieties, but also allow to forecast the researched parameter in the range of the tested conditions. The regression coefficients b_k is an angular coefficient of the regression straight lines and it is established, that the variety is more responsive to the growing conditions by an increase in the values of b_k.

Normally the values of b_k are positive, but in some cases like for example yield decrease as a result of logging, the regression coefficient can acquire a negative value. When the values of the regression coefficients b_k are higher than one, it means, that the variety does not react to the changes in the environment.

The yield plasticity varies from 0.825 by Trismart variety to 1.189 by Musala variety. The Musala variety is distinguished with the highest values of the plasticity b_k and this variety proven is the most ecologically plastic. The standard Kolorit and Trismart variety manifest lower values, as the differences between them are statistically unproven and they are placed in the same statistical group. A specific genotypic response to the conditions during the harvest year and big variety fluctuation are have been reported by many authors (Barnett et al., 2006; Baychev & Mihova, 2014; Madic et al., 2018; Stoyanov & Baychev, 2018). According to the indicator plant height it can be concluded, that by the variety Musala the parameter is the most plastic, because the values are the highest (1.088), followed by the standard and Trismart variety, where the differences in the values of b_k are statistically non-significant. The plasticity b_k of the indicator test weight is with the highest values by the Trismart variety, followed by the standard Kolorit with values of 1.344 and at least Musala variety with values of 1.109. The differences between the varieties are significant and they are placed in different groups. As the most plastic in a relation to the parameter mass of 1000 grains is distinguished the standard, followed by the variety Musala, while the plasticity of the examined parameter by the variety Trismart is influenced in lower ratio by the environmental changes.

The dispersion S_k is the parameter, which assesses the ecological stability of the varieties. If the dispersion of the stability S_k tends more to the zero, then the empirical values of the signs distinguish less from the theoretical values, located on the regression line. According the applied model of Eberhart and Russel any variety can be accepted as ecological plastic and stable under condition that it possesses values of $b_k>1$ and of $S_k>0$. In the present research the dispersion rates are higher than zero by all tested varieties observing all examined parameters, what according the used model can determine them as stabile (Table 5). The tendency determined in previous studies with another triticale varieties, (Kirchev & Georgieva, 2017), that the stability values of the examined components can be counter proportional to the variety's plasticity, is established also in the present study. By the component "yield" with the lowest stability is the variety with the highest plasticity- Musala. The variety Trismart, who is distinguished with the highest stability, possesses also the lowest plasticity. According the component plant height all varieties manifest high stability and only the variety Musala can be accepted as ecological plastic regarding this parameter, because it possesses plasticity values of $b_k>1$. The stability of the component test weight is very low by all varieties, which can determine them as ecological plastic regarding this sign. The stability of the component test weight is the highest by the variety Musala. All varieties are stable regarding the last component- mass of 1000 grains, but only the standard can be determined also as ecological plastic with values above one. According many authors (Madic et al., 2018; Mihova et al., 2017; Stoyanov & Baychev, 2018), the values of the mass of 1000 grains remain relative stable in contrasting environmental conditions.

The relation between the yield plasticity and stability with the examined plant parameters is defined through correlation analysis (Table 6).

Table 3. The two-way ANOVA analysis of variance of triticale genotypes x climatic conditions. Legend: *significance at $p<0.05$.

Source of Variation	df	Yield		Pl. height		Test weight		Mass of 1000 grains	
F*; F-criteria		F	Fcrit	F	Fcrit	F	Fcrit	F	Fcrit
Genotype (G)	2	864.59*	3.55	25.42*	3.55	59.42*	3.55	88.83*	3.55
Climatic conditions (Q)	2	68.07*	3.55	7.72*	3.55	62.21*	3.55	23.52*	3.55
Interaction (G x Q)	4	10.71*	2.92	7.90*	2.92	12.71*	2.92	3.05*	2.92

Table 4. Ecological plasticity of yield and some yield components in triticale varieties. Legend: Values with the same letters do not differ significantly.

Indicators Varieties	Yield	Pl. height	Test weight	Mass of 1000 grains
Kolorit	0.984a	0.997a	1.344b	1.296c
Musala	1.189b	1.088a	1.109a	0.978b
Trismart	0.825a	0.974a	1.545c	0.724a
LSD 5%	0.17	0.01	0.19	0.26

Table 5. Ecological stability of yield and some yield components in triticale varieties. Legend: Values with the same letters do not differ significantly.

Indicators Varieties	Yield	Pl. height	Test weight	Mass of 1000 grains
Kolorit	0.145a	1.045a	0.265b	0.487a
Musala	0.124a	1.240b	0.350c	0.435a
Trismart	0.205b	1.114a	0.189a	0.629b
LSD 5%	0.01	0.05	0.06	0.06

Table 6. Correlations between yield and yield components plasticity and stability coefficients. Legend: *significance at $p<0.05$.

$b_{k;} s_k$	b_k			s_k		
Indicators	*1*	*2*	*3*	*1*	*2*	*3*
1.Yield	1			1		
2.Plant height	0,967*	1		-0,771*	1	
3.Test weight	-1,000*	-0,959*	1	-0,032	-0,612	1
4.Mass of 1000 grains	0,377	0,127	-0,402	-0,259	-0,416	0,974*

The yield plasticity coefficient correlates significantly positive with the plant height. The correlation with the yield and the mass of 1000 grains plasticity is also positive, but non-significant. There is a strong negative correlation between the grain yield plasticity and the test weight, which defines these two indicators as opposite to the environmental plasticity of the variety. A similar negative correlation was found between the plant height and the test weight. These results can summarize the relationships between the plasticity of a variety in terms of its yield and its height as negative with respect to one of the main quality indicators of grain in cereals – the test weight.

In the present research the ecological stability of the grain yield of the examined triticale varieties correlates negatively with all other investigated indicators. Statistically significant values are indicated only by the plant height (+0.771). A proven positive relationship by the stability of the studied parameters exists between the two quality indicators - the mass of 1000 grains and the test weight (+0.974).

Conclusion

The Musala variety can be defined as ecological plastic regarding the signs – yield, plant height and test weight. The standard manifest plasticity regarding the components test weight and mass of 1000 grains, while by the variety Trismart the ecological plasticity is established only in term of the component test weight. The positive dispersion rates by all tested varieties observing all examined parameters enable to determine them as stabile. From the performed correlation analysis, it was established, that the yield plasticity coefficient correlates positive with the plant height and the mass of 1000 grains.

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The Content of Heavy Metals, Radionuclides and Nitrates in the Fruiting Bodies of Oyster Mushroom Distributed within the Urban Ecosystem

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Abstract. The content of heavy metals, radionuclides, nitrates in edible mushrooms is a topical issue for many researchers in the world. However, such issues on edible mushrooms, in particular, *Pleurotus ostreatus*, which are found naturally within a large city, require more detailed studies because these mushrooms are used in human nutrition. It was found that in some areas, the cadmium content in the studied oyster mushroom specimens is three times higher than the maximum permissible concentration (0.092-0.095 mg / kg). The lead content in the fruiting bodies of oyster mushroom is in the range of maximum permissible concentration (MPC) and is 0.19-0.22 mg / kg. The content of mobile forms of copper exceeded the MPC only in two cases - 5.72 mg / kg and 5.06 mg / kg. The zinc content was 8.87-13.41 mg / kg with MPC being 10 mg / kg. The content of ^{137}Cs was in the range of 0.095-0.1 Bq / kg and that of ^{90}Sr was 0.3-0.4 Bq / kg, which does not exceed the MPC. The content of nitrates in the fruiting bodies of oyster mushrooms did not exceed the maximum permissible concentration of 50 mg / kg. Considering the data obtained on the content of heavy metals, radionuclides and nitrates in *Pleurotus ostreatus*, it should be noted that it is risky to eat such fruiting bodies that have developed in the urban-ecological environment.

Key words: oyster mushroom, heavy metals, radionuclides, nitrates, urboecosystem, city.

Introduction

The oyster mushroom is involved in the destruction of dead wood which is the main habitat of living organisms. (Fontes et al., 2013; Nnorom et al., 2012; Silva et al., 2012). The decomposition of wood creates particularly favorable conditions for the development of ground cover and the restoration of tree species. It is also essential for carbon accumulation and soil protection from erosion (Finley, 2006; Favero et al., 1990).

We studied the content of heavy metals, radionuclides and nitrates in the fruiting bodies of oyster mushroom which develops within the urban ecosystem of the city of Lviv. Lviv (a city with almost one million people located in the west of Ukraine) is the center of a large urban agglomeration. Increasing air pollution and xerophilization of the urban environment is a negative factor for the entire biota of the city's complex green zone. The diversity and species richness of the vegetation and macromycetes of the complex green zone of Lviv is associated with its geographical location, the history of the formation of the terrain relief and climatic changes in previous geological periods (Piskur et al., 2011; Troyan, 1989). The remains of ancient pre-glacial vegetation have almost completely disappeared under the influence of climate cooling in the glacial period, and in its place appeared the northern and northeastern vegetation. The following species were introduced into this region: from the east - steppe species, from the mountains of Central Europe - mountainous, from the west - lowland Atlantic species.

Modern flora of the green zone in Lviv is heterogeneous in composition. It is characterized by the following species (Bellettini et al., 2017; Kycheryavyi, 1999; Szafer 1914): boreal, or taiga species (Norway spruce, Scots pine, spindle tree, wintergreen, etc.), Central European, or of zones of broadleaved forests (European beech, common oak and sessile oak, silver fir, common hornbeam, maple sycamore, perennial liverleaf, etc.), mountain species which include: European mountain species, and among them the Carpathian species, including endemic species (violet (*Viola declinata*)), pontine, or steppe species (*Striated fescue, Feather grass,* etc.).

According to the data of Baysal et al. (2003) and Yang et al. (2013), the oyster mushroom is most common in fresh hazel, fresh hornbeam-oak, and in fresh hornbeam-beech forests, it is very rarely found in pine forest types. It should be noted that in forest conditions, oyster mushroom becomes food for various pests (Munoz et al., 2006), and intensive cultivation of edible mushrooms can often be disturbed by certain bacterial, mold and viral diseases, which often causes significant production losses. These infections are facilitated by special conditions under which mushroom cultivation is usually carried out, such as high temperatures, humidity, carbon dioxide levels (CO_2) and the presence of pests (Kycheryavyi et al., 2018).

Note that the natural forest stands in the territory of the city of Lviv were replaced by green plantings of parks, gardens, miniparks, boulevards, and street alleys (Fernandes et al., 2015; Fang et al., 2014), which affects the development of the *Pleurotus ostreatus* populations. Most urban parks are man-made parks. However, there are those that were transformed into parks from forests - Zalizna Voda, Pogulyanka. The forests of Bryukhovychy and Zavadiv, located on the elevated ridges of Roztochya, are being turned into forest parks (Bysko et al., 1982; Bazyuk-Dubey, 2012; Brunets, 2013).

Many well-known scientists conducted their studies to determine the peculiarities of heavy metals entry into plant products and identify the most pollution-resistant agricultural plants. The scientists (Mils & Parker, 1980) studied the effect of high levels of cadmium in soils on various plant species, adding it to the soil in various quantities. The scientist (Garmash, 1982) studied the intake of Pb and Cd from the soil by vegetable crops; for this, these substances were added to the soil in various quantities. The capacity of various wheat varieties to absorb heavy metals was studied. In so doing, wastewater sludge was introduced into the soil (Alekseev, 1987).

The knowledge of the accumulation of heavy metals in mushrooms is limited, as well as the knowledge of their bioavailability in humans. Selenium, arsenic and antimony are not found in high concentrations. Cultivated species, especially *Agaricus bisporus* and *Pleurotus ostreatus*, contain only low levels of trace elements. Very scarce is information on metal losses during the preservation and cooking of mushrooms (Alananbeh et al., 2014).

The distribution of arsenic, cadmium, lead, mercury and selenium was investigated in 1,194 samples of 60 species of common edible mushrooms, collected mainly in the province of Reggio Emilia, Italy. The average amount of lead present in all samples was generally below the maximum permissible concentration. The high Hg content was in the range of 5-10 mg/kg of dry weight. The mushrooms were rich in selenium. The accumulation of heavy metals may be specific to species and, thus, take a taxonomic role, but in these studies it has proved to be unreliable as an ecological index (Demirbas, 2001).

The scientists in (Pavlik, 2005; Pavlik & Pavlik, 2013) present the results of studies on lead content in 238 samples of 28 species of edible mushrooms collected from different sites in the province of Lugo (Spain) during 2005 and 2006. The highest average lead content varied in the range of 2.2–4.1 mg / kg (in the oyster mushroom usually less than 1 mg / kg, which coincides with our data). No statistically significant differences were found between lead levels in hymenophore and in fruiting bodies. The authors proved that the consumption of the mushrooms under study cannot be regarded as a toxicological risk in terms of lead content, and they provide necessary nutritional profile.

Eighteen different types of forest mushrooms (*Agaricus bisporus, Agaricus silvicola, Amanita muscaria, Amanita rubescens, Amanita vaginata, Boletus sp., Hydnum repandum, Hypholoma fasciculare, Laccaria lacceta, Lactarius piperatus, Lactarius sp., Lactarius volemus, Pleurotus ostreatus, Russula cyanoxantha, Russula sp., Russula delica, Russula foetens, Tricholoma terreum*) were analyzed for heavy metal content (Pb, Cd, Hg, Cu, Mn, Zn, Fe, Co, As, Ca, Na, K, Mg, Ba, Ni, Ti, Cr, Al, Bi, Sb, Ag) (Kalac & Svoboda, 2000). The heavy metal content for oyster mushrooms was high and amounted to (mg / kg): Pb – 3.24, Cd – 1.18, Hg – 0.42, Cu – 13.6, Mn – 6.27, Fe – 86.1, Zn – 29.8, which exceeds the figures obtained by the researchers of this work.

The studies (Kalac, 2009) determined the content of heavy metals in 10 species of mushroom. Heavy metal content levels are significantly lower in species such as *P. squamasus, P.ostreatus, B. badius, M. esculenta*, and *M. vulgaris* than in other mushrooms. The levels of Cd, Pb, Zn, and Mn comply with FAO / WHO standards (1976).

It was found that the accumulation level of Hg in *P. ostreatus* mushrooms was low and was different in various sites. *P. ostreatus* can be described as a weak Hg collector. Also, such a conclusion was made by the scientists in the work**.** However, if the oyster mushroom is cultivated on substrates polluted with industrial wastewater, this can lead to risks to human health. It is established that high concentrations of Hg greatly reduce the growth of mycelium. The range of the accumulation coefficient was 65-140, that is, very pronounced (Bellettini et al., 2019).

The concentration of cadmium in the fruiting bodies of oyster mushrooms is related to the level of cadmium in the substrate. This metal is present at higher levels in the cap of the mushroom (22-56 mg / kg of dry weight) than in the stipes (13-36 mg / kg of dry weight). The concentration factor (CF) is very low on the control (about 2). The work suggests the presence of a cadmium control mechanism in these types of mushroom. However, the level of cadmium in the fruiting body may pose a risk to consumers of *P. ostreatus* in accordance with the limitations of FAO / WHO (1972) (Favero et al., 1990).

The study investigated the content of heavy metals such as Pb, Cd, As, Hg in rice and edible mushrooms in China to assess the level of pollution and edible safety. Ninety-two rice samples were collected from the main rice cultivation regions in China, and 38 fresh and 21 dried mushroom samples (including oyster mushrooms) were collected at typical markets in Nanjing. It was found that the content of Pb, Cd and As in 4.3%, 3.3% and 2.2% of rice samples, respectively, was higher than the maximum permissible concentration. In fresh edible mushrooms, the content of Pb and Hg in 2.6% of samples was higher than MPC.

However, only the Hg content in 4.8% of dry edible mushroom samples was higher than its MPC. It was concluded that more than 95% of the rice and edible mushroom samples in our testing had high food safety.

The content of phenols, antioxidant activity and the content of metals in *P. ostreatus* grown on coffee chaff enriched with iron (Fe), zinc (Zn) and lithium (Li) were studied. The content of phenolic compounds was different in mushrooms enriched with Fe, Zn or Li. Mineral enrichment contributed to lowering Fe content in three samples. Changes in the content of Zn in the samples did not occur. Li accumulation was observed in a sample enriched with LiCl and in *P. ostreatus* enriched with Fe, Zn, Li. Heavy metals such as Ni, Cr and Cd were not detected, and only low levels of Pb and Al were observed. The study (Fang Y et al., 2014) was the first report to demonstrate an understanding of how the mineral supplements reduce the antioxidant activity in *P. ostreatus* enriched with iron, zinc or lithium.

When growing oyster mushroom on the waste of palm leaves (25%) and the waste of agricultural products (75%), it was found that the content of K was the highest, followed by Na, Mg, Ca, and Zn. This is the first study that reported the success of growing mushrooms on palm leaf waste mixed with other agro-waste (Garcia et al., 2009)**.** The studies of (Gregori et al., 2007), were devoted to the intensive cultivation of oyster mushroom on waste paper, supplemented with peat, chicken droppings and rice husk. It was found that an increase in the rice husk content in the substrate contributed to the acceleration of cultivation, the formation of the cap and fruiting body of mushrooms, which led to an increase in yield, while an increase in peat and chicken manure had a negative effect on the growth. In general, the studies show that the chemical composition of oyster mushrooms grown on paper-derived waste was very satisfactory. The results obtained confirmed the nutritional characteristics of the samples, placing emphasis on effective means for recycling paper.

The mushrooms (*Pleurotus ostreatus*) were cultivated (Černy, 1989) on a basal substrate of rice straw, a basal substrate of wheat straw, a basal substrate of cotton shell, a substrate of mixture of wheat straw and rice straw with different proportions (15%, 30% and 45% in rice straw, 20%, 30% and 40% in wheat straw) to find a cost-effective substrate. It was found that the addition of cotton seeds in rice straw and wheat straw enhanced the launch of mycelium, the initial development and formation of the fruiting body. Compared to the sterilized substrate, the unsterilized substrate had a comparatively higher mycelium growth rate, a shorter period of general colonization and days from the opening of the bag to the formation of primodia. However, the unsterilized substrate did not give a significantly higher yield of mushrooms and biological efficiency than the sterilized substrate; some undesirable characteristics were observed, that is, a smaller diameter of the mushroom cap and a relatively long stipe (Pasternak, 2010; Popovych & Les, 2014).

At the same time, the authors (Huang et al., 2010) show that the fruiting bodies of oyster mushroom, grown on the substrate with bamboo sawdust, contain more free amino acids than those from the control group with coniferous sawdust. It is concluded that bamboo sawdust can be used as the main material for the cultivation of mushrooms. In the future, bamboo sawdust can be used in Malaysia, Thailand and Indonesia with their subtropical climate in order to expand the cultivation of oyster mushrooms.

The cultivation of mushrooms (Gregori et al., 2007)**,** enriched with Se in the substrate of the coffee chaff, was effective, showing increased biological efficiency and Se absorption. Even the lowest concentration of Se, added to the coffee chaff, 3.2 mg / kg, led to the fact that the *P. ostreatus* mushrooms, which contain a sufficient amount of Se, provided the recommended daily intake of Se for adults. These results demonstrate the great potential of coffee chaff in the production of Se-enriched mushrooms and demonstrate the ability of this mushroom to absorb and biomagnetize Se. Selenium (Se) is important for human diet, and it is in a low concentration in the soil, and hence in food. The introduction of Se into fungal proteins shows great potential for improving the nutritional value of the mushroom**.** Selenium has several physiological functions in protein activity, it increases the function of the immune system, reduces the risk of developing cancer (Isildak et al., 2004).

Mycorhization is the subject of research which found that *Pleurotus ostreatus* can be used as a promising option for the removal of heavy metals from wastewater of the coal industry. The effectiveness of *Pleurotus ostreatus* for absorbing heavy metals was the highest in 50% dilution of wastewater (57.2% Mn, 82.6% Zn, 98.0% Ni, 99.9% Cu, 99.3% Co, 99.1 % Cr, 89.2% Fe and 35.6% Pb) and 25% dilution of effluent (33.0% Mn, 55.1% Zn, 97.8% Ni, 99.7% Cu, 97% Co, 84 , 4% Cr, 87.1% Fe and 73.4% Pb), and raw materials (23.3% Mn, 73.1%)% Zn, 78.7% Ni, 87.5% Cu, 59.3% Co, 64.6% Cr, 34.6% Fe, and 11.3% Pb (Melgar et al., 2009).

Thus, the study of heavy metals, radionuclides, nitrates in edible mushrooms is a topical issue for many researchers in the world. However, the studies on the content of heavy metals in edible mushrooms, in particular, *Pleurotus ostreatus*, which develop naturally within a large city, require detailed investigation since they are used in human nutrition.

Materials and Methods

In the area of green plantings in Lviv, landscape gardening objects (street plantings, miniparks, boulevards, parks) with the presence of trees of different species composition were selected. The distribution and occurrence of the oyster mushroom species was studied using the transect route method (Dudka & Vasser, 1987). The studies were conducted in the spring (April-May) and autumn (October-November) 2014-2018. The fruiting bodies of the oyster mushroom were found in 7 urban environments (Fig. 1). The abundance and growth patterns of oyster mushrooms in urban areas of the city of Lviv are given in Table 1.

The content of heavy metals in the fruiting bodies of mushrooms was determined by the method (Kalac, 2013) in the hydrochloric acid extract (Balyk, 2005). Preparation of mushrooms for laboratory tests included drying of the raw material, grinding and dry ashing in a muffle furnace (t = + 450- + 550 ° C), dissolving the ash in a 10% HCl solution, followed by identification of mobile forms of heavy metals on an atomic absorption spectrophotometer (Baliuk et al., 1999). The chemical analyses of the mushrooms were carried out in a certified laboratory of the Volyn branch of the state institution *Institute of Soil Conservation in Ukraine* (Lutsk, Volyn Region).

The radionuclides were determined by a scintillation method using the SEG-001 "AKP-S" gamma-ray energy spectrometer and the SEB-01 beta-radiation with digital data calculation software (the average value of the relative measurement error at a confidence level of 95% does not exceed 25%).

The content of nitrates in the fruiting bodies of the mushrooms was determined using the eco-tester of the environment "Soeks".

Table 1. Locations of *Pleurotus ostreatus* L. occurrence within the urban environment of Lviv City.

Sample No.	The name of the street in the city of Lviv where the natural growth of oyster mushroom was found	Abundance (according to O.Drude)	Growth pattern (according to Brown Blanke)
1	Valova Str. (minipark)	sparsae	2
2	29b, Golovaty Str.	sparsae	2
3	180, Shevchenko Str.	copiosae 3	4
4	109, Zelena Str.	solitariae	1
5	Snopkivsky park	sparsae	2
6	Chervona Kalyna ave.	copiosae 3	4
7	Shevchenkivsky grove park	copiosae 3	4

Valova Str. (minipark)

29 b, Golovaty Str.

180, Shevchenko Str.

109, Zelena Str.

Snopkivsky park

5, Chervona Kalyna ave.

Shevchenkivsky grove park

Fig. 1. Identified places of oyster mushroom growth in the city of Lviv. Photos: Mikhail Les. 2014-2018.

Results and Discussion

The content of heavy metals — cadmium, lead, copper, and zinc — in oyster mushroom fruiting bodies was investigated. All the mushroom individuals developed on trees within the city of Lviv, and along the motor roads - samples 2, 3, 4, 6; in gardens and parks - samples 1, 5, 7. It is found that in some areas, the cadmium content in the test specimens of oyster mushroom is three times higher than the maximum permissible concentrations and amounts to 0.03 mg / kg. It is stated that within the city limits, the excess of this heavy metal in the examined oyster mushroom specimens is uneven and is obviously dependent on the contamination of the soil environment and donor wood. Figure 2 shows data on the accumulation of cadmium by oyster mushroom individuals within the city.

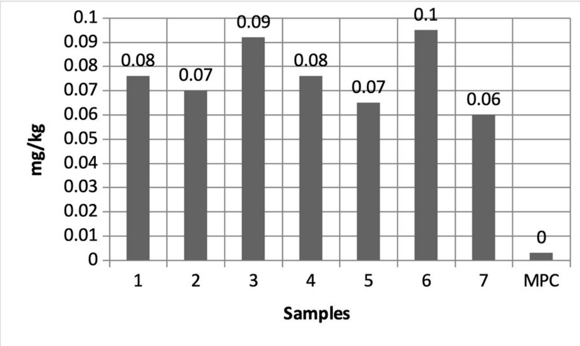


Fig. 2. Cadmium content in the fruiting bodies of oyster mushroom.

The lead content in the fruiting bodies of oyster mushroom is within the maximum permissible concentration (MPC) and is 0.19-0.22 mg / kg. The content of mobile forms of copper exceeded the MPC in only two cases and amounted to 5.72 mg / kg and 5.06 mg / kg. A very different and disappointing situation was with zinc. The studies have shown that the content of this metal in the samples was 8.87-13.41 mg / kg with MPC of 10 mg / kg (Fig.3).

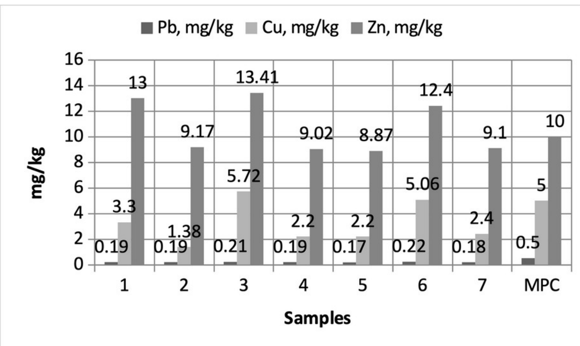


Fig. 3. The content of lead, copper and zinc in the fruiting bodies of oyster mushroom.

In general, as shown by the studies (Pylypets, 2000, pers. comm, Ukraine) on the migration of heavy metals in the soils of Lviv, our data (Cd > Pb > Cu > Zn) basically confirm the author's data (Cd > Pb > Cu > Zn).

The data obtained indicate that the components of forest and other terrestrial ecosystems, which absorb impurities from the atmosphere and accumulate, transport and transfer them, are "sinks" of heavy metals. As is known, the main absorbers of impurities entering park and forest-park biogeocoenoses are soil and plants. Regarding the pollutants such as persistent particles of heavy metals, their storage functions in plants and soil are closely related, since some of the heavy metals such as Cd, Pb, Cu, Zn studied by us, enter the soil through vegetation which supplies litter to the forest floor and litter to park-and-garden plantings.

Scientists estimate that excessive accumulation of Cd, Pb, Cu, and Zn in the organogenic horizon of forest ecosystems slows down the rate of decomposition processes due to the binding of the heavy metals by colloids of organic substances, ions of heavy metals. In their opinion, these processes can have a direct toxic effect on microorganisms-decomposers, or on the enzymes they produce.

In each case, the accumulation of heavy metals is a serious problem from the standpoint of biochemical cycles, and exceeding the MPC, which is found in the samples we studied, is undesirable and harmful from the sanitary and hygienic point of view.

The studies on the radionuclide content in the fruiting bodies of oyster mushroom showed that the contents of ^{137}Cs and ^{90}Sr do not exceed the maximum permissible concentration (GN 6.6.1.1-130-2006). The same conclusions were reached by the researcher in (Cocchi et al., 2006). He found that wild-growing mushrooms accumulated ^{134}Cs and ^{137}Cs, but this did not pose a threat to the human body. The cultivated mushrooms had low radioactivity content caused by natural isotope ^{40}K. The results of our studies are shown in (Fig. 4).

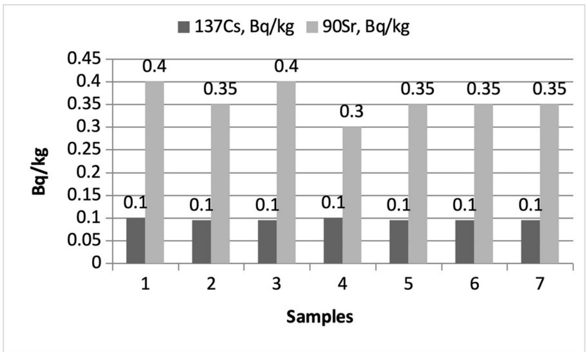


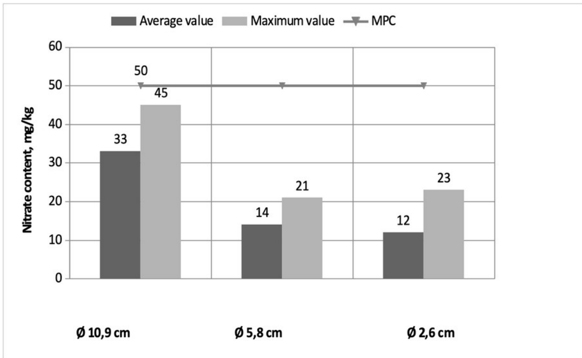
Fig. 4. The radionuclide content in the fruiting bodies of oyster mushrooms.

It should be noted that in plant products, the maximum permissible concentration for ^{137}Cs is 500 Bq / kg, and for ^{90}Sr - 50 Bq / kg.

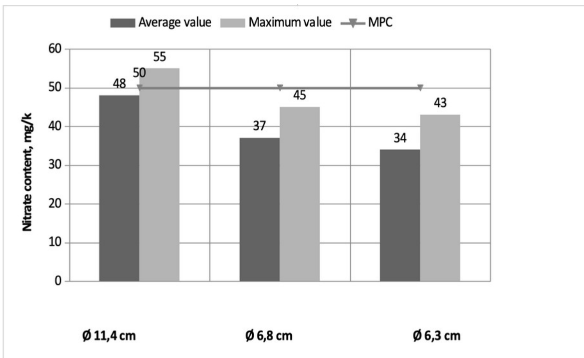
In the process of the nitrogen cycle, a gradual decomposition of organic compounds occurs, as a result of which nitrogen passes into the nitrate form. The nitrates formed in the soil are quickly assimilated by tree roots and are accumulated by both xylophytes and saprophytes (Bressa et al., 1988). Thus, natural nitrates are formed which are included in the subsequent cycle of nitrogen (Vaseem et al., 2017). However, owing to the growing burning of fuels (factories, internal combustion engines), nitrogen oxides are formed in the atmosphere, and they enter the soil with rain in large quantities and are converted there with the help of nitrogen-fixing bacteria into nitrates. Excessive concentration of nitrates in plant products is harmful to people, especially in childhood (Kycheryavyi, 2010). Therefore, when collecting mushrooms and passing them to the consumer, we must ensure that the maximum permissible concentrations are not exceeded.

Our observations, conducted in urban parks, have found that in the same urban environment, as the oyster mushroom, the honey mushroom (*Armillariella mellea*) develops. These species fructify at the same period of time. Therefore, it was decided to collect samples of the fruiting bodies of individuals of both species and measure the content of nitrates in them (Kycheryavyi et al., 2016).

It was found that high levels of nitrate content are characteristic of those fruiting bodies of *Pleurotus ostreatus* and *Armillariella mellea* specimens which have the largest cap diameters. In oyster mushroom with different cap diameters, the nitrate content did not exceed the maximum concentration which is 50 mg / kg (fig. 5a). As can be seen from (fig. 5b), in all three variants of the experiments (the size of the caps was different), the average and maximum rates of nitrate accumulation were higher in honey mushrooms. At the same time, the honey mushroom sample with the largest cap diameter (11.4 cm) was found to exceed the maximum permissible concentration by 5 mg / kg.



a)



b)

Fig. 5. The nitrate content in the fruiting bodies of the oyster mushroom *Pleurotus ostreatus* (a) and the honey mushroom *Armillariella mellea* (b).

The obtained results are in favor of the oyster mushroom which assimilates less nitrates and is safer for the consumer.

Conclusions

As a result of studying the content of heavy metals in the fruiting bodies of oyster mushroom, which develops within the urban ecosystem of Lviv, it has been found: in some areas, the content of cadmium in the investigated individuals is three times higher than the maximum permissible concentration and amounted to 0.092-0.095 mg/kg; the lead content in the fruiting bodies is within the maximum permissible concentration and is 0.19-0.22 mg/kg; the copper content exceeds the MPC in two cases and amounted to 5.72 mg/kg and 5.06 mg/kg.

The studies on the radionuclide content in oyster mushroom fruiting bodies have established that the contents of ^{137}Cs and ^{90}Sr do not exceed the maximum permissible concentrations. The ^{137}Cs content was in the range of 0.095-0.1 Bq/kg, and 90Sr - 0.3-0.4 Bq/kg.

The highest nitrate levels are found in those fruiting bodies of *Pleurotus ostreatus* and *Armillariella mellea* which have the largest cap diameters. In oyster mushroom with different cap diameters, the nitrate content did not exceed the maximum concentration which is 50 mg/ kg.

Taking into account the data obtained on the contents of heavy metals, raiodnuclides and nitrates in *Pleurotus ostreatus*, it should be noted that to eat such fruiting bodies that have developed in the urban-ecological environment of Lviv is risky.

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Soilless Propagation of Haberlea rhodopensis Friv. Using Different Hydroponic Systems and Substrata

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Abstract. *Haberlea rhodopensis* (Gesneriaceae) is a tertiary relict with high conservation value, endemic to the Balkan Peninsula. The interest to this species is due mainly to its resurrection ability and multiple pharmacological activities, although it is valued also as an ornamental plant for use in rock gardens. *H. rhodopensis* plants are very slow-growing and no efficient method for their mass propagation has been set up until now. The present study reports the first trials on soilless cultivation of the species. Two aero-hydroponic systems with vertical and horizontal arrangements were used, studying the impact of different inert substrata, together with either leaf treatment with Indole-3-butyric acid (IBA) or leaf age and status, on rosette formation. In addition, plant propagation from seeds was tested on a small hydroponic system. Leaf rooting and survival were relatively high, up to 86.7%; however variants differed by root quality and time for root formation. Best results were obtained when IBA-treated leaves were either immediately put in perlite/agrolava substrate on the vertical system (46.7% leaves with rosettes, 2.9 well-shaped rosettes per leaf) or rooted in wet perlite prior to cultivation on the horizontal system in agrolava fractions (85.0% leaves with rosettes, 2.2 well-shaped rosettes per leaf). Rosettes were transferred to soil mixture and acclimatized in a greenhouse, and the largest 47 of them reached 10 cm in diameter for one year. Possible procedure improvements are discussed aiming at enhancement of *H. rhodopensis* soilless propagation.

Key words: endemics, Orpheus flower, resurrection plants, soilless cultivation, plant reproduction.

Introduction

Haberlea rhodopensis Friv. (Gesneriaceae) is a tertiary relict and endemic species to the Balkan Peninsula (Szeląg & Somlyay, 2009; Petrova & Vladimirov, 2010). In the tertiary period it was widely distributed in Europe and Asia; however, after the last glaciations its area shrank only to the territories of Bulgaria and Greece. This perennial herbaceous plant with leaf rosette and blue-violet flowers is growing on shady humid places, in crevices of limestone and silicate rocks in the zone of beech and pine forests at altitude from 150 to 1500 m. It is called "resurrection plant" as its vegetative parts are able to withstand up to 30 months of almost complete water loss and to recover fast under normal conditions (Gantschev, 1950). Known also as "Flower of Orpheus", *H. rhodopensis* was used by Thracians as a medicinal plant for treatment of many diseases. The species is protected in Bulgaria by the Biodiversity Act (2002) and the Medicinal Plants Act (2000), and is listed with the category "Least concern" in the Red List of Vascular Plants of Bulgaria (Petrova & Vladimirov, 2009). In the Red Data Book of Greece, it is listed under category "Vulnerable" (Theodoropulos et al., 2009) and is protected under the Greek Law Presidential Degree 67/81. Its natural localities fall in five Natura 2000 sites (Bazos & Petrova, 2013).

Currently *H. rhodopensis* is used as an ornamental plant at very small scale (Bazos & Petrova, 2013) because of its limited distribution. The increasing interest to the species is due mainly to its ability for resurrection (Georgieva et al., 2012) and its multiple pharmacological activities and potential rejuvenation effect (Popov et al., 2011; Berkov et al., 2011; Radev et al., 2012). However, the gathering of *H. rhodopensis* plants is forbidden because of its limited resources. The habitat is not threatened and the overall population trend is stable being mostly in inaccessible areas. Cultivation of the species is recommended for *ex situ* conservation in botanic gardens (Bazos & Petrova, 2013). Along with the phytochemical analyses there are some trials oriented to plant propagation. In this relation, the age structure of the populations has been studied as well as the seed germination under laboratory conditions and the propagation peculiarities (Bogacheva-Milkoteva et al., 2013). Experiments on *in vitro* propagation of *H. rhodopensis* were carried out as well (Djilianov et al., 2005). Monitoring of the species in Bulgaria determined nine populations with 67 localities, and a representative gene bank of *in vitro* plants originating from different populations has been established aiming at further reinforcement of some localities. However, authors reported many difficulties related to the low percentage of seedling survival and their very slow growth (Daskalova et al., 2011a, 2011b).

An alternative propagation of *H. rhodopensis* could be by hydroponic techniques, i.e. by using water solutions of mineral nutrients instead of soil, with or without artificial medium consisting of some inert material such as perlite, gravel, clay pebbles for mechanical support of the plants. Soilless cultivation has many advantages, among them most important being the crop yields, which increase significantly as plants are cultivated in greenhouse-type facilities under controlled ambient conditions and receive balanced nutrients according to their specific needs (Texier, 2013). Thus, crop productivity does not depend on the seasons, weather, precipitations, pest infestations, neither on the soil type, fertility, salinity, pH, and soil-related weeds, diseases and pests. Soilless technologies are environmentally friendly, as the use of water is minimized owing to the surplus solution recycling. There are different types of hydroponic systems; among them aeroponic ones are newer and of higher technology, where roots are not submerged in the solution and their humidification is ensured by mist; they are appropriate also for commercial level of plant growing (Mugundhan et al., 2011). In the aero-hydroponic systems no mist is used, and the roots are in meshy pots constantly or periodically sprinkled with nutrient solution by means of a pump. Until now, hydroponics has been applied mainly for vegetables; however, it could be very effective for rare species with conservation importance and medicinal and aromatic plants with resource deficiency, and there are some examples such as *Mentha, Stevia, Arnica, Ocimum* (Giurgiu et al., 2014). Smaller plants like *H. rhodopensis* could be grown in two or more shelfs, thus allowing establishment of vertical farming and additional increase of the cultivation efficiency.

The main goal of the present study was to stimulate the rooting of *H. rhodopensis* separate leaves and the formation of rosettes by applying different aero-hydroponic systems and substrata, as a first step of its successful propagation. Another challenge was to test the opportunity to obtain plants via hydroponically germinated seeds.

Materials and methods

Plant material. Leaves and seed capsules were collected from randomly chosen *H. rhodopensis* plants of the population in the Rhodope Mountains, near the village of Sitovo, Plovdiv district, in October 2017.

Leaf rooting and rosette formation. Leaf rooting was studied by two aero-hydroponic systems: vertical Green Diamond (GHE), and horizontal Aeroflo-20 (GHE) with 120 and 20 meshy pots (all 8 cm in diameter), respectively. As a control, 36 fresh leaves were put into a flower box; stalks dipped in wet perlite, covered with a glace to keep air humidity high, and left in the greenhouse.

Two factors with possible influence on the rooting: substrate and indole-3-butyric acid (IBA) were examined on the vertical system. The substrate consisted of fine material (peat cubes, mineral wool, or perlite, in a semi-permeable tissue), surrounded by larger particles (agrolava pebbles or keramzite pellets, medium fractions). The treatment with the auxin was done by dipping the stalks of the leaves into 25% IBA powder (Rhizopon BV, The Netherlands) prior to their embedding in the substrate. The combination of substrata and IBA-treated or control non-treated leaves resulted in eight parallel variants distributed in four pairs: peat cubes surrounded by agrolava pebbles, with IBA-treated leaves (variant PC-AP-IBA) or with control leaves (variant PC-AP-C); mineral wool surrounded by agrolava pebbles, with IBA-treated leaves (variant MW-AP-IBA) or with control leaves (variant MW-AP-C); perlite surrounded by agrolava pebbles, with IBA-treated leaves (variant P-AP-IBA) or with control leaves (variant P-AP-C); and perlite surrounded by keramzite pellets, with IBA-treated leaves (variant P-KP-IBA) or with control leaves (variant P-KP-C). Total 15 leaves freshly gathered from the native plants were used per variant.

On the Aeroflo-20 horizontal system three variants were tested consecutively, with 20 leaves per variant, thus studying the impact of the substrate and the leaf age and status. Two different substrata were used: perlite in a semi-permeable tissue surrounded by agrolava pebbles, and agrolava pebbles alone. All leaves were treated with IBA powder as described for the vertical system. The first trial was with substrate perlite surrounded by agrolava pebbles and leaves freshly gathered from the native plants, (variant P-AP-FL). Leaves of the second trial were stored in wet perlite during the 6 months of the first trial, and they formed meantime short roots and some nucleus of rosettes. The substrate used was agrolava pebbles (variant AP-6m-L). Leaves for the last trial were taken from entire *H. rhodopensis* plants after their wintering in the greenhouse, and put in wet perlite for one month prior to the beginning of the experiment. The substrate was agrolava pebbles (variant AP-1m-L).

The nutrient solution comprised distilled and tap water in proportion 3:1, supplemented with Flora Micro, Flora Grow, and Flora Bloom (GHE), and with the bacterium *Trichoderma harzianum* (0.1 g/L). This bacterium was added preventively as a biocontrol agent against some fungal pathogens and for enhancement of the root growth (Harman, 2000). The pH was maintained between 5.5 and 6.5, and electrical conductivity (EC) between 0.40 and 0.98 $mS.cm^{-1}$. The substrate wetting on the two systems was constant but the solution was running in a different way: strong streams of the horizontal system were laterally sprayed directly on the substrate, whereas the substrata in the vertical system were gently sprinkled by drops falling obliquely from about 50 cm of distance. The experiments were conducted in a room phytotron with mixed daylight and artificial light (Metal Halide Superveg lamps, 250 W) 16/8 h photoperiod, between 50 and 54 $\mu mol\ m^{-2}\ s^{-1}$, and variations around-the-clock of the temperature (23 ± 4 ºC) and air humidity (32 to 68%).

Criteria for selection of the best variant were: leaf rooting and survival rate, leaf capacity to form rosettes, and number of well-shaped large rosettes at least 3 cm in diameter.

Propagation by seeds. Seeds were germinated on Cutting Board hydroponic system (GHE) with 27 meshy pots, 6 cm in diameter. Fresh dust-like seeds were stratified at 6 °C for 2 weeks before sowing on the pots which were filled in with peat cubes surrounded by agrolava pebbles and wetted with standard nutrient solution (pH 5.5-6.5; EC 0.4-0.6 $mS.cm^{-1}$) through bubbles formed during the permanent pump stream of the solution. Seeds were darkened during the first month, and after the beginning of germination they were exposed to mixed daily and artificial light between 22 and 27 $\mu mol\ m^{-2}\ s^{-1}$, with light/dark photoperiod 16/8 h. Seedlings with 5-10 mm diameter of the rosettes were transferred to a tray with soil mixture and perlite in proportion 1:1 (v/v), and grown in the phytotron, until they grew enough to be transferred in pots. In addition, other seeds were put on 10 water damped coco-fiber cubes, in a tray with transparent cover, periodically removed after seed germination, when condense appeared, in order to control air humidity.

Rosettes acclimation in a greenhouse. All rosettes obtained in different ways were transferred in pots (9 cm in diameter) with soil mixture (Biobizz worldwide) containing peat moss, sphagnum peat and perlite (NPK:14-16-18), and sand in proportion 2:1 (v/v), the larger ones alone, and the rosette clusters together with the initial leaf, partially exhausted during the soilless cultivation. They were additionally grown in a room phytotron under controlled conditions (day-and-night temperature and air humidity fluctuations: 24 ± 4 °C, 55 ± 15%) and finally in an unheated greenhouse. Final number of survival rosettes was counted 13 months after the beginning of the first experiments. Well-shaped rosettes with diameter over 3 cm were defined as large, while the minor ones, often forming clusters of individuals not well distinguished from one another were called small.

Statistical analysis. Statistical analyses were done using Excel "Data analysis tool – Anova: Two Factor Without Replication". All percentage data were transformed using Excel formula: ASIN(SQRT()) to normalize error distribution prior variance analysis.

Results and Discussion

Leaf rooting and formation of rosettes took about 6 months on both vertical and horizontal aero-hydroponic systems (Fig. 1a, 1b); however, some differences were noticed.

Leaf rooting and rosette formation on the vertical aero-hydroponic system

Both substrate and IBA influenced the success of the overall process of rosette formation on Green Diamond vertical system (Table 1). In most variants the percentages of leaf rooting and survival were relatively high: 60.0 to 86.7%. Non-rooted leaves turned brown and died.

Concerning the percentage of leaf rooting, no significant differences were noticed neither between variants differing by their substrate, nor between IBA-treated and control non-treated leaves (Table 1-A). However, some variants differed regarding the root quality and the time of their formation. Thus, best results were obtained in variant P-AP-IBA where all survived leaves developed vigorous root system for 12 weeks (Fig. 1c). Rooting in the other variants took 8 to 12 weeks more. Only the organic peat substrate proved inappropriate and roots in variants PC-AP-C and PC-AP-IBA remained short and weak, many leaves dropped out and only few rosettes formed; therefore, these variants were further ignored.

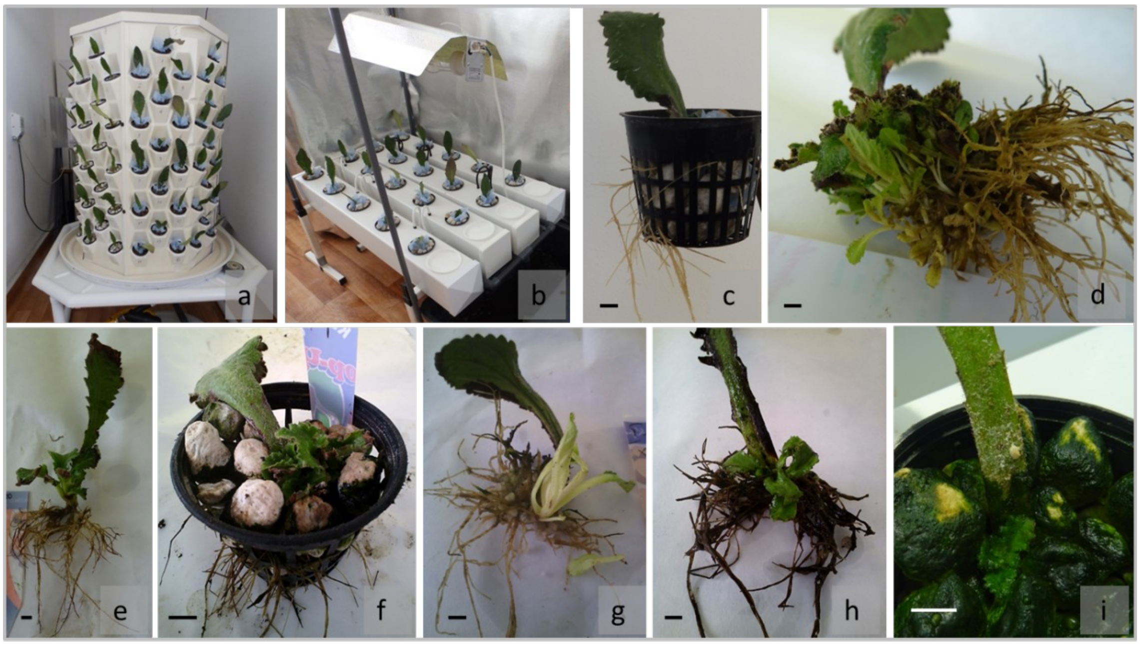

Fig. 1. *H. rhodopensis* rosettes formation on aero-hydroponic systems: a) Vertical Green Diamond system; b) Horizontal Aeroflo-20 system; c) Roots on variant P-AP-IBA, 12 weeks old; d) Numerous rosettes formed at the leaf base in variant MW-AP-IBA; e) A single rosette formed in variant MW-AP-C; f) Rosettes formed on Aeroflo-20 in variant AP-6m-L; g) Etiolated rosette formed under the agrolava pebbles; h) Partial putrefaction of the root system; i) Algae development on the substrata. Scale bars = 10 mm.

Table 1. Leaf rooting and rosettes formation on the vertical aero-hydroponic system, for 6 months. Effect of substrate and IBA-treatment on leaf rooting (A), rosette formation (B), and number of large rosettes (C), estimated with ANOVA Two-factor without replication. Legend: * - plus additional small rosettes; PC – peat cubes; AP – agrolava pebbles; IBA – IBA-treated leaves; C – control non-treated leaves; MW – mineral wool; P – perlite; KP – keramzite pellets.

Variant	Rooted leaves (%)	Leaves with rosettes (%)	Number of large rosettes	Large rosettes per leaf
PC-AP-C	53.3	13.3	2	1.0
PC-AP-IBA	46.7	6.7	1	1.0
MW-AP-C	73.3	33.3	6	1.2
MW-AP-IBA	66.7	46.7	17*	2.4
P-AP-C	60.0	40.0	8	1.3
P-AP-IBA	86.7	46.7	20*	2.9
P-KP-C	66.7	26.7	10	2.5
P-KP-IBA	53.3	40.0	7*	1.2

(A)

Source of variation	Df	MS	F	p-value
Substrate type	3	0.026007676	1.247809922	0.42995825
IBA Treatment	1	0.000162102	0.007777413	0.935283122
Error	3	0.020842658		

(B)

Source of variation	Df	MS	F	p-value
Substrate type	2	0.005684031	6.592599167	0.131707203
IBA Treatment	1	0.020022653	23.22319022	0.040464754
Error	2	0.000862184		

(C)

Source of variation	Df	MS	F	p-value
Substrate type	1	6.25	25.0	0.126
IBA Treatment	1	132.25	529.0	0.028
Error	1	0.25		

The choice of the relevant substrate is considered as very important for the success of the soilless cultivation. It should depend on the plant species requirements as well as on the way the constant wetting is realized (Giurgiu et al., 2014). Optimal conditions are determined mostly for vegetables usually grown on mineral wool that keeps 80% of the nutrient solution and contains 15% air while its fibers represent only 5% of the substrate. The proportion between the solution and the air inside is important for the root growth and over-wetting could cause their putrefaction. Giurgiu et al. (2014) recommended fraction size between 2 and 7 mm of diameter like perlite. Among the substrata used in the present experiment perlite has better perviousness compared to mineral wool and peat cubes, and both agrolava and keramzite were of larger fraction sizes than perlite. In nature, *H. rhodopensis* plants are adapted to very specific conditions presenting combination of humid ground and steep slopes and escarps allowing water drainage; therefore, we tested several substrata differing in fraction size and capacity to retain water.

Single rosettes of *H. rhodopensis* began to appear after 3 months of leaf cultivation. For the 6-month duration of the experiment, half of the survived leaves gave rise of rosettes (38 from 76 rooted leaves). Unexpectedly, the formation of rosettes was significantly enhanced by IBA-treatment ($p < 0.05$, Table 1-B). No significant difference was noticed between the three substrata regarding the percentage of the leaves forming rosettes (Table 1-B).

The total number of well-shaped rosettes with 4-6 leaves each, and diameter between 4 and 8 cm obtained in all variants was 71, and 63.4% of them formed in the variants stimulated with IBA (Table 1). In these variants additional numerous small rosettes were observed whose number was impossible to count (Fig. 1d). The highest numbers of large rosettes were noticed in variants P-AP-IBA and MW-AP-IBA: 20 and 17, respectively, while in the corresponding control variants P-AP-C and MW-AP-C their numbers were much lower (Fig. 1e) ($p < 0.05$, Table 1-C). The average numbers of rosettes per leaf in these variants were twice higher when leaves were IBA-treated, the highest one being 2.9, recorded in variant P-AP-IBA (Table 1). All these parameters, along with the fastest leaf rooting, distinguished variant P-AP-IBA as the best one on the vertical system.

Leaf rooting and rosette formation on the horizontal aero-hydroponic system

On the Aeroflo-20 horizontal system the trials took different time due to the differences in the leaf age and status which proved to be of crucial importance (Table 2). Thus, the previous leaf rooting in variant AP-6m-L facilitated the formation of rosettes: they were numerous and well-shaped, grew faster and reached the size suitable for potting in soil mixture for only one and a half month (Fig. 1f). Eighty-five percent of the leaves in this variant formed rosettes. In variant AP-1m-L, with the same substrate, leaf stalks had callus tissue at the beginning of the treatment, and most of them developed roots, but only 25% of the leaves formed rosettes and half of them represented clusters of very small rosettes. The worst variant was that with freshly gathered leaves put in perlite surrounded by agrolava pebbles (P-AP-FL) due to the rooting difficulties.

Comparison between the two aero-hydroponic systems showed important differences related to the way of the substrate wetting. On the horizontal system the excessive solution could not strain off from the dense perlite particles thus causing over-wetting, whereas the drainage on the vertical system was easy. The larger agrolava fraction applied alone was most appropriate for the horizontal system, avoiding the substrate over-wetting. However, the space between agrolava pebbles allowed formation of some rosettes below the substrate surface, which caused their etiolation (Fig. 1g). Young roots appearing on agrolava pebbles in variant AP-1m-L were vulnerable to the excessive solution, which caused partial root putrefaction (Fig. 1h). Although 16 leaves rooted (80%) only 6 of them survived and finally 5 leaves formed either one well-shaped rosette or a cluster consisting of 4 to 7 small rosettes (Table 2).

Also, algae multiplication was observed after about 2 or 3 months from the beginning of the cultivation. Microscopic Zygnemophyta green algae that are largely spread easily contaminated the solutions used in the soilless cultivation. Their fast growing and reproduction led to formation of compact algae layer on the substrate surface and hampered the development of *H. rhodopensis* rosettes (Fig. 1i).

Acclimation of rosettes to soil and growth rate comparison

Seven months after the transfer to soil mixture, the number of rosettes formed on the vertical Green Diamond system decreased almost twice (47.9% survival rosettes) and the effect of IBA was lost in a long-term period because most of the smaller rosettes died. New rosettes formed rarely after the transfer to the soil. At this stage the survived rosettes in the greenhouse were with diameter more than 10 cm and height of about 7 cm, with several well-shaped leaves (Fig. 2a). At the same, the rosettes obtained during the second trial on the Aeroflo-20 horizontal system (variant AP-6m-L) and transferred to soil mixture 2 months later, were about 7 cm in diameter (Fig. 2b). Young rosettes in the natural populations have also 4-6 leaves, and numerous mature plants are growing close to each other, the length of the leaves depending on the environmental conditions (Fig. 2c). A total of 47 rosettes (34 obtained on the vertical and 13 on the horizontal aero-hydroponic systems) were further grown in the greenhouse.

The control leaves under glace cover in the flower box with wet perlite needed about 2 months to develop roots. Their survival was high as 86.1% of the leaves rooted and were transferred to pots with light soil mixture and agrolava pebbles in proportion 2:1. At the end of the 13th month, 13.9% of the leaves had rosettes and the total number of rosettes was 11, but they were much smaller compared to those obtained on the aero-hydroponic systems (Fig. 2d). Even the 5-month old rosettes from the last test on the horizontal system were larger (Fig. 2e).

Formation of daughter rosettes on *H. rhodopensis* leaves under *ex situ* conditions was previously reported as possible but not effective, even if only ten leaves were used for the test (Bogacheva-Milkoteva et al., 2013). Authors used light soil for leaf rooting and observed rosettes on three of the leaves but they were extremely small and unable to grow and develop. Taking in consideration the slow rate of *H. rhodopensis* plant growth in nature, our results with soilless production of rosettes on leaves are promising as the largest rosettes reached autonomy for only one year.

Trials for propagation by seeds

Seeds were abundant and too small (Fig. 3a), therefore they were sown on the peat cubes without counting, most probably several hundred on each cube. First seeds germinated two weeks after their setting on the Cutting Board hydroponic system. At the end of the first month, numerous seeds germinated and formed small green cotyledons, on 23 of all 27 pots, running into several dozens of seeds per peat cube (Fig. 3b, 3c). The growth of the seedlings was very low; rosettes with diameter about 5 mm, consisting of 4 to 6 leaves, formed at the end of the third month. Several rosettes at the periphery of the pots grew faster, reaching 10 mm for the same time, probably because they had more space (Fig. 3d). Meantime, mosses developed on the peat cube surface and the substrate was infested by insects and fungi, causing putrefaction of part of the rosettes (Fig. 3e). Plantlets were treated with bio fungicide and bio insecticide but the infection was heavy and the effect of the treatments was temporary. At the end of the 5th month, the largest 30 rosettes were transferred to a tray with soil mixture and perlite in proportion 1:1, but 2 months later only one rosette survived. After 6 more months it reached diameter 4 cm (Fig. 3f) but its growth was extremely slow.

Table 2. Rosette formation on the Aeroflo-20 horizontal aero-hydroponic system. Legend: P – perlite; AP – agrolava pebbles; FL – fresh leaves; L – leaves stored in wet perlite: 6m – for 6 months, 1m – for one month.

Variant	Time	Leaves with rosettes (%)	Number of well-shaped rosettes	Number of clusters with small rosettes
P-AP-FL	6 months	10.0	1	1
AP-6m L	1.5 months	85.0	38	0
AP-1m L	2 months	25.0	2	3

Fig. 2. Potted rosettes and native plants: a) Rosettes formed on the vertical aero-hydroponic system, 7 months after the transfer to soil mixture; b) Rosette formed on the horizontal aero-hydroponic system, 5 months after the transfer to soil mixture; c) Native mature plants in their habitat; d) Rosettes formed at the base of a control leaf in wet perlite in flower box, 13 months after the start; e) Rosette cluster at the base of a leaf from the last trial on the horizontal system, 5 months after the experiment start. Scale bars = 10 mm.

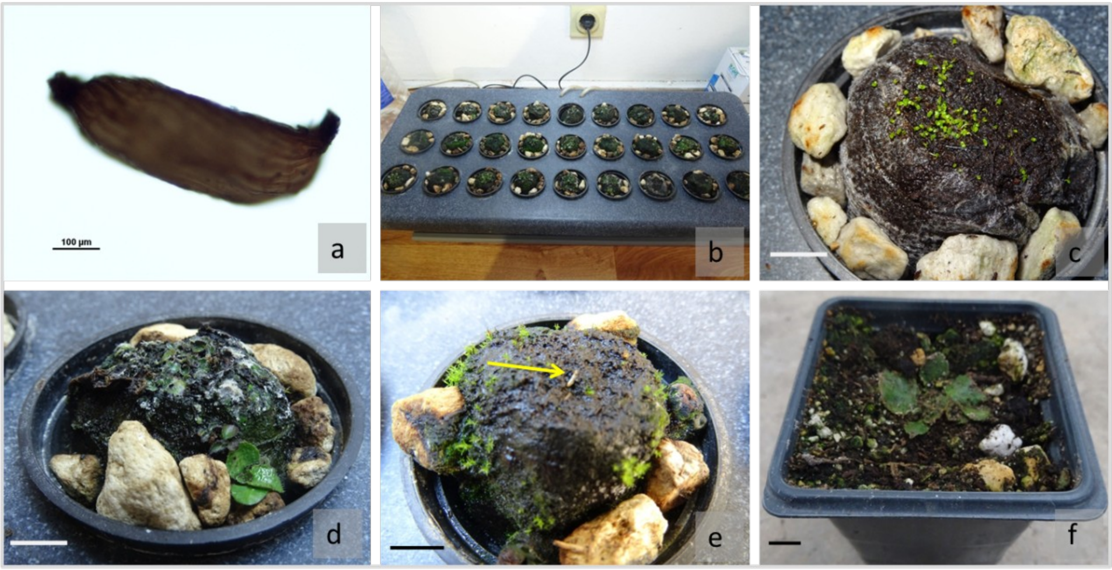

Fig. 3. Seeds derived rosettes: a) Seed; b) Seed germination on Cutting Board hydroponic system; c) One month old seedlings; d) Rosette with diameter 10 mm; e) Infected substrate with insects (the arrow indicates insect larvae); f) Rosette with diameter 3 cm in the greenhouse, 13 months after seed germination. Scale bars = 10 mm.

Coco-fiber cubes seemed less appropriate as few seeds germinated on this substrate, and some of them necrotized 2 weeks later, while the rest remained several months at cotyledon phase and finally died.

Obviously, there was no problem with seed fertility and germination, which corresponds to the results reported about the high rate of pollination of the native plants from several populations (Bogacheva-Milkoteva et al. 2013). The same authors reported easy seed germination in laboratory conditions on wet filter paper in petri dishes, but extremely slow growth (seedlings at cotyledon stage with length less than 4 mm for 5 months), and only 1% of survival rosettes.

Seeds of *H. rhodopensis* were germinated also *in vitro* after a drastic surface disinfection procedure to eliminate microbial contamination (Djilianov et al., 2005). Authors obtained plantlets after long germination and succeeded first in finding out the appropriate medium for this species, and then in multiplying rosettes by direct organogenesis, using pieces of *in vitro* leaves as explants. They obtained clusters of very small plantlets, and finally fully developed *in vitro* plantlets, for about 7 months. *Ex vitro* adapted plantlets were designed to serve as uniform and initial plant material of high quality for further research. Other authors tried to build a strategy for conservation of *H. rhodopensis* including *in vitro* propagation as a tool for production of numerous plants intended to be used for reinforcement of the natural populations (Daskalova et al., 2011a, 2011b). Authors claimed to have created *in vitro* gene bank of the species with plants from many populations; however, their later work was focused on research such as genetic diversity and chloroplast genome, and there was no more information about plant propagation, most probably because of the difficult *ex vitro* adaptation of the plants and their very slow growth.

Conclusions

First trials on soilless propagation of *H. rhodopensis* using leaves as initial material are promising, as plant production, growth and development have been enhanced; seeds are not recommended. Among the two tested aero-hydroponic systems, the variant with perlite/agrolava substrate was the best one on the Green Diamond vertical system, while the horizontal Aeroflo was more appropriate in combination with agrolava pebbles alone as substrate. However, fraction with smaller pebbles in horizontal system should be used to avoid rosette development below the surface. Rooting of IBA-treated leaves in wet perlite for two months should be applied prior to the soilless cultivation in order to synchronize the process of rosette formation and to ensure fast growth of the rosettes, thus allowing their transfer to pots with soil mixture at the end of the second month. Use of lower concentration of IBA would be better, ensuring rooting but avoiding excessive formation of miniscule rosettes, thus increasing rosette survival. Shortening of the soilless cultivation stage will contribute to avoidance of algae contamination as well. The whole optimized procedure including rosette strengthening in greenhouse and development to 10 cm in diameter could be performed in 9 months. Plants are designed for further grown in the *ex situ* collection of IBER-BAS with consideration of their specific requirements to the environmental conditions.

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Wind Regime and Wave Fetch as Factors for Seagrass Habitat Distribution: A Case Study form Bulgarian Black Sea Coast

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Abstract. The paper presents a study on the relative importance of wind regime and wave fetch for limitation of seagrass spatial distribution in Burgas Bay, the Black Sea. In Burgas Bay the relative importance of both factors is obviously changing from northern to southern coasts. The seagrass meadows facing north-northeast direction have two times smaller integral fetch and are three times more often wave impacted than those facing south-southeast. Along the southern coast the fetch (maximum and number of azimuths) is more important to allow seagrass presence, while in front of the northern coast – it is the wind speed recurrence. In the first scenario the habitat is expected to be less frequently but more strongly affected by the wave action, contrary to the second scenario, where the reversed effect is supposed.

Key words: Seagrass, wave exposure, wind regime, wave fetch.

Introduction

Seagrass communities inhabit the shallow coastal zone of oceans and seas where they are subjected to the impact of the environmental factors such as temperature, salinity, wave action, bottom substrate, riverine and suspended matter inflow, which dynamics varies significantly in time and space. One of the main natural ecological factors which determine the presence of seagrasses habitats is the wave action (Koch, 2001). In the shallow coastal zone of a micro-tidal sea like the Black Sea (Poulos, 2020) the wind waves are a key limiting factor for seagrass spatial distribution.

Thus along coasts predominantly exposed to the prevailing wave action such as the Bulgarian Black sea coast, the habitats suitable for seagrass growth are limited by wind waves. In such areas, it is a matter of scientific and practical interest to study their effect on seagrass meadows distribution, as a critical step for a complex shallow water ecosystem model design.

In temperate, mixomesohaline, mesotrophic water bodies like the surface layer of the Black Sea the diversity of seagrasses is low. It is a habitat of only wave sensitive species: *Zostera noltei*, Hornemann, *Z. marina* L., *Zannichellia palustris* L., *Stuchenia pectinata* (L.) Borner, *Ruppia cirrhosa* (Petagna) Grande (Milchakova, 2011).

Along Bulgarian coast the present natural seagrass meadows are mostly concentrated in the Burgas Bay (Berov et al., 2015). Most of the meadows are formed in

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areas protected by rocky capes which act as a wave shelter. The known shallow-water meadows are located along coastlines facing north-northeast or south-southeast directions.

The aim of this study is to explore the importance of the wind regime, fetch and coastline orientation as a precondition for seagrasses distribution along Bulgarian coast.

Due to the specific wind regime and coastline orientation, it is interesting to analyze how the wave exposure changes along the cost and what are the consequences for the seagrass meadows distribution. The working hypotheses are: 1) seagrass meadows along differently oriented coastline are under different exposure pressure and 2) the relative importance of the fetch and wind regime changes depending on the coastline orientation and thus affects presence/absence of seagrass meadows.

Material and Methods

The present study covers the area of several bays with well-established, perennial seagrass meadows, open towards different azimuth directions: south-southeast and north-north-east (Fig. 1).

Nesebar Bay is a part of Burgas Bay, enclosed between c. Emine and Nesebar Peninsula. It is widely open towards south, southeast and east. Sveti Vlas and Elenite meadows are located along its north coast, as well as the small marina of Elenite resort. (Fig. 2).

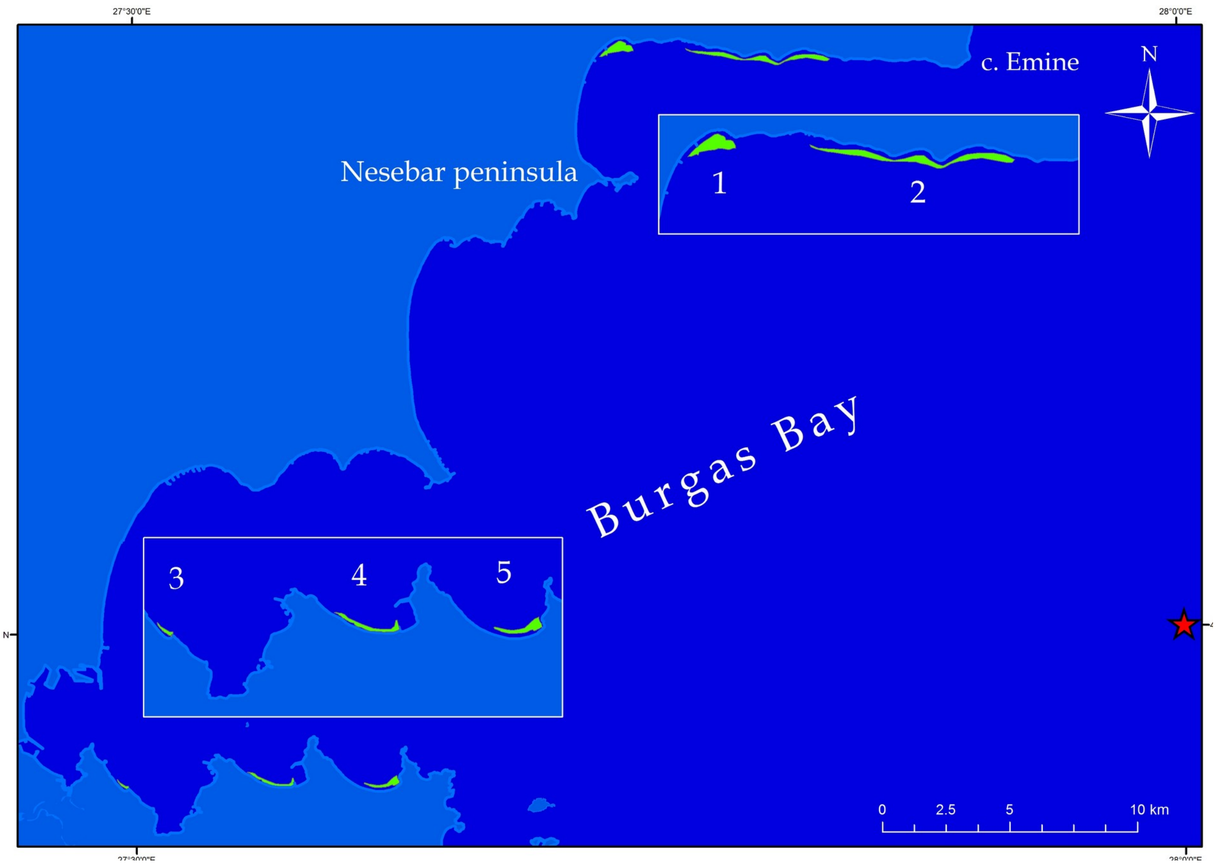


Fig. 1. Study area in Burgas Bay, Black sea: Sveti Vlas (1), Elenite (2) meadows facing south-east; Kraimorie (3), Atia(4) and Vromos Bay (5) facing north, north-east. All meadows are in light green. Red star shows location of wind data collection. Scale line = 10 km.

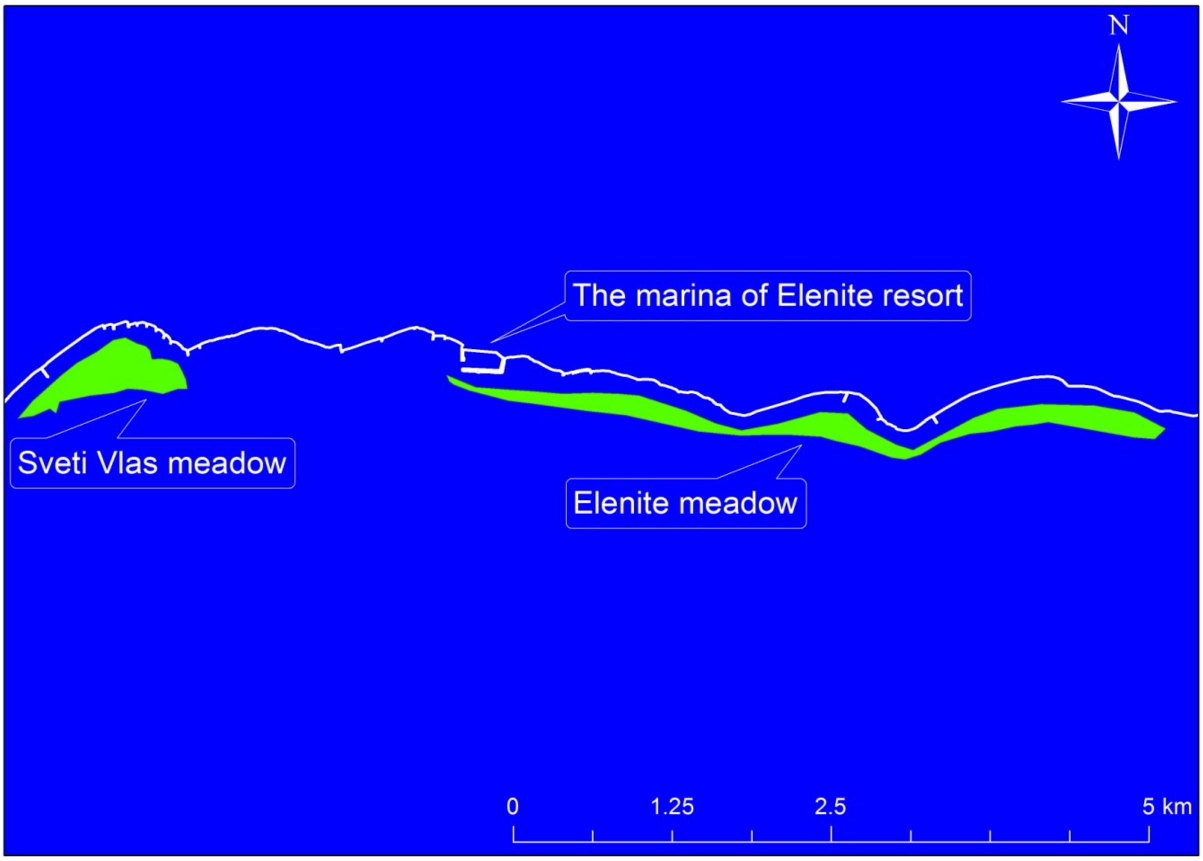


Fig. 2. Sveti Vlas and Elenite meadows and the marina of Elenite resort. Scale line = 5 km.

Vromos Bay (between c. Atia and c. Akra), Kraimorie and Atia meadows are open towards north, northeast (Fig. 1).

The methodology of wind regime, wave exposure and wave parameters calculation as well as statistical modelling and seagrass distribution in Nesebar Bay are presented in detail in Hineva (in press). Modelled data of wind speed and azimuth (available for 6 hours period) in front of Burgas Bay for a 5 year period (MHI-RAS, 2020) were used for wind regime study. The wind data, wave fetch GIS tool (Rohweder et al., 2012) and CMS v. 2.5 (University of Cantabria) modeling software were used for wave parameters calculation in "deep water" and "shallow water" conditions, respectively. The bottom orbital velocity was calculated according to Hunt's method (Soulsby, 2006). In order to find the azimuths of limiting waves a generalized linear modelling (Zaionts, 2019) between the seagrass upper boundary and the bottom orbital velocity was used. It is assumed that if the model for a given direction was well-fitted, with large area under the ROC curve (AUC) and high percent of correct forecasts (Zaionts, 2019, Schubert et al., 2015), waves coming from that direction limit the seagrasses, while the opposite is true when the model showed a bad discriminative ability.

The seagrass meadows boundaries were outlined in two ways. In Nessebar Bay they were traced out in situ with an echosounder and GPS (September, 2018); in Vromos Bay, Kraimorie and Atia areas the upper boundaries were delineated after a Google Earth image taken on 19.9.2013 had been georeferenced in ArcGIS. The *in-situ* validation of the image was done by SCUBA diving (September, 2018). The spatial data were transformed into binary presence-absence data sets for further statistical treatment. "Presence" of a seagrass meadow means an outstanding, significant part of bottom area covered by seagrass (>20 %) perennially, continuously (i.e. not fragmented to separate patches) and more or less homogenously. Where the patches themselves irrespectively of their own percent cover, occupy less area than the unoccupied surrounding area, these were not considered as a "meadow".

The integral exposure was defined as the area between fetch curve (the maximum fetch along an azimuth) and the x axis for each studied seagrass area only for seaward directions (azimuth from 10 to 180 degrees, see Fig. 5). Calculation was done by the Image J software. Based on the "deep water" wind wave generation theory (Smirnov, 1987) two scenarios for seagrass limitation were considered: 1) small recurrence of winds able to generate highly energetic waves combined with big exposure and 2) high recurrence of moderate to fresh breeze combined with a small "window of exposure".

The observed pattern corresponds to the typical picture in front of Bulgarian coast (Valchev et al., 2014).

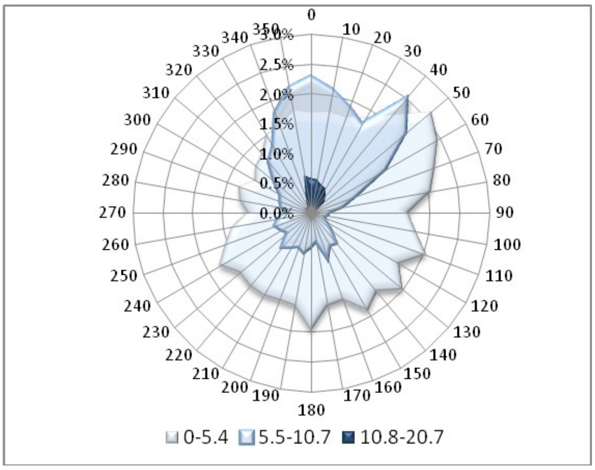


Fig. 3. Wind rose for the open sea in front of Burgs Bay: "calm weather to gentle breeze", "moderate to fresh breeze" and "strong wind to gale". The wind speeds are given in m/s, azimuth- in degrees.

Results

Wind regime

The wind regime characteristics are presented in detail in Hineva E. (in press). Wind rose (Fig. 3) shows that there is significantly inhomogeneous distribution of the relative frequency of the wind blowing from various directions within each category.

The highest recurrence had winds within "calm weather to gentle breeze" (0-5.4 m/s) category. Their distribution by directions was relatively even and illustrated the approximately equal probability of winds, to blow from each direction.

"Moderate to fresh breeze" winds had significantly uneven distribution, with most frequent winds blowing from north (0°) and northeast (40°).

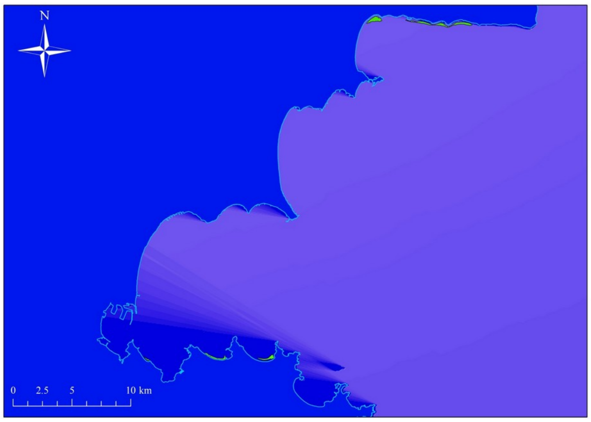
Winds in the category "strong breeze to gale" were the least frequent and with highly uneven distribution, prevailing from north, northwest and northeast directions. North-northeast winds have occurred under 1 % of all cases.

Wave fetch

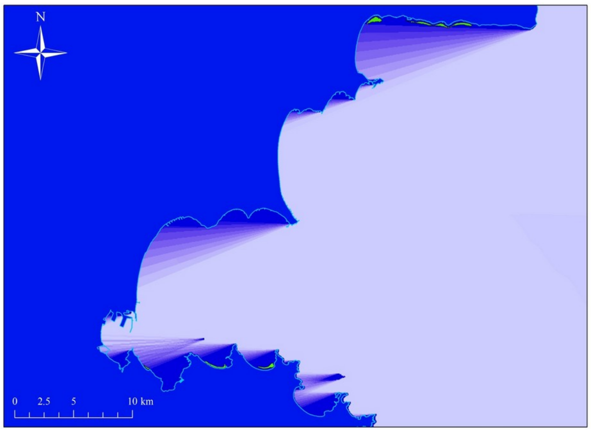
The coastline of Burgas Bay changes its orientation from east-west in its northern part, to north-south in its middle part and again to east-west and northwest-southeast direction in its south part (Fig. 1). The coastline indentation also differs between the regions. In the northern part of the bay the coast is almost a straight line (low coefficient of indentation) while in the remaining part it is a sequence of smaller or bigger bays and rocky capes (Sailing Directions for the Black Sea, 1956). As a result some parts of the shallow coastal zone of Burgas Bay are to a different extent exposed to waves approaching from north to south (Fig. 4). This creates a variable picture of habitats for marine macrophytes, where the impact of the wave factor is changing along the coastline.

The northern coast of Burgas Bay is mostly exposed to waves approaching from east-southeast and south direction. The maximum wave fetch in this region is at c. Emine (580 867 m from azimuth 110 °, Fig.

4). The seagrass meadows Sveti Vlas and Elenite are located along its northern coast (Fig. 1 and 2).



A



B

Fig. 4. Effective wave fetch along azimuth 110^0 (A) and 80^0 (B). Colour codes: light colour – exposed areas, dark colour – shadowed areas. For simplicity labels are not shown. Scale line = 10 km.

The southern coasts are predominantly exposed towards north, northeast and partially to east (Fig. 4). The maximum wave fetch here is 827 028 m from 70° azimuth. Kraimorie, Atia and Vromos Bay meadows are located in this area (Fig. 1).

The maximum wave fetch in Burgas Bay is at the western coast (1 019 211 m from azimuth 80^0 (Fig. 4)).The maximum wave fetch where seagrass meadow can persist in Burgas Bay is at the southern coast (597 961 m) located in Kraimorie (Fig.4). The same one at the northern coast is 464 811 m at Sveti Vlas meadow.

Depending on the coastline orientation the meadows are to a different extent exposed to the approaching waves (Fig. 5).

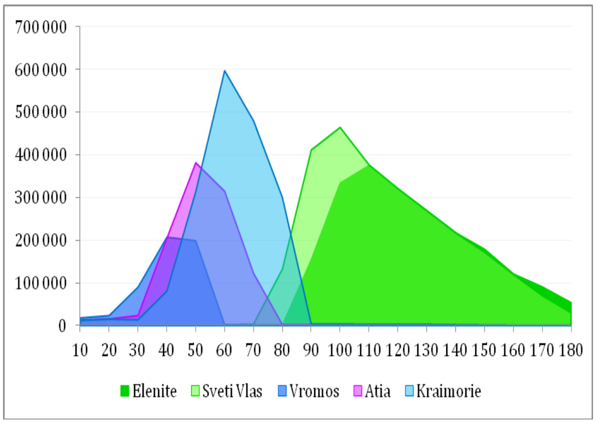


Fig. 5. The value of the maximum wave fetch from different azimuths for two groups of fields: Elenite and Sveti Vlas – open towards south-southeast, Vromos, Atia, Kraimorie – open towards north-northeast. Along x axis are azimuths and along y axis is the fetch, m.

While the meadows facing north-northeast are exposed to lower number of azimuths (smaller window of exposure), with higher maximum fetches, the meadows opened towards south and southeast are facing more azimuths and have shorter maximum fetches. The total fetch of each vegetated area (the integral of maximum fetches along each seaward azimuth) shows that meadows facing north-northeast directions have smaller total fetch (77 730 ± 11 772 conventional units) than those exposed towards south-south east directions (168 462 ± 1 105 conventional units) (Fig. 5 and 8).

The results of the statistical analysis has shown poor, satisfactory, very good and excellent discriminative ability of the constructed models (Table 1).

Results for Nesebar Bay meadows presented in detail in Hineva E. (in press) have shown that both meadows are significantly limited by the wave action, as indicated by the mostly excellent and very good discriminating ability of the logit functions (Hineva E., in press). Stveti Vlas meadow, where statistical tests has shown poor discriminating ability if waves approach from 170 and 180 degrees, is better protected than Elenite meadow due to the wave shadow of Nesebar Peninsula (Table 1, Fig. 6).

The results from statistical analysis for Kraimorie, Atia and Vromos Bay are shown in Table 2.

Kraimorie and Atia meadows are protected from the north and partially from the northeast by dry land (c. Chukhalia and c. Atia, respectively). Atia, the most protected meadow facing north-northeast, is limited only by the waves coming from 70, 80 and 90 azimuth degrees. Kraimorie one is more exposed as the waves approaching from 30 and from 60 to 80 degrees can explain the observed meadow location (Table 2.).

Vromos Bay is partially open towards north, northeast from small number of azimuths (6). From the north it is protected by the Pomorie Peninsula and the short fetch (13 033 m) limits the wave growth. The waves which approach from 20 to 80 degrees can explain the observed seagrass location. The statistical models have excellent to satisfactory discriminative ability and high enough number of correct forecasts (Table 2, Fig. 7).

When the waves come from east and southeast c. Akra creates a wave shadow over the whole bay and those waves could not limit the upper meadow boundary.

The coasts open to north-northeast are facing 10 azimuths (from 0 to 90 azimuth degrees), whose total relative frequency of moderate to fresh breeze is 15.1 % (Fig. 8). This shows that the whole area has been wave impacted approximately 3 times more often than Nesebar Bay, where only 4.8 % of cases were moderate to fresh breezes and the respective waves. The small number of statistical relationships between bottom orbital velocity and seagrass upper boundary, which have excellent discriminative ability, indicates a better protection of the meadows facing north-northeast; where the wave fetch is limited by some natural features (peninsulas, capes etc.) models have bad discriminative ability, i.e., waves approaching from those directions could not limit the seagrass. Thus the smaller window of exposure decreases the overall possibility the meadows to be regularly damaged by wave action and their persistence here to be prevented.

Discussion

It is well known (e.g. Smirnov, 1987) that the main factors which determine wave parameters in deep water conditions are: 1) wind regime (speed, recurrence and period of action) and 2) the free space (fetch) over which wind can travel in a constant direction (Rohweder, 2012).

There are generally four possible combinations of both of factors and their relevant impact on wave-sensitive seagrasses (Fig. 9 A, B, C, D). In extremely sheltered areas (Fig. 9 A) seagrasses are not limited by wind waves due to the short fetches (e.g. Rubegni et al., 2013, van Djik, 1993) or due to both the short fetches and low speed winds. In this case there is not enough wind energy which can be transferred to the sea surface in order to generate the relevant waves. The short fetch indicates that a protection from the swell is also available. Usually in such areas the rooted plants are stressed due to water stagnation and stimulated by wave action (van Djik, 1993). In the situations where both fetches and wind characteristic are high (Fig. 9 D) there is no possibility of wave sensitive seagrass species to survive in the highly energetic shallow water area (Koch et al., 2006). When at least one of them has small enough value the probability to cause seagrass absence is also small but the upper boundary of the meadow is wave limited (Koch, 2006, Infantes et al., 2009, Stevens & Lacy, 2012) (Fig. 9 B and C).

Table 1. Seagrass meadows along the coastline of Burgas Bay and the corresponding directions of the limiting waves.

№	Meadow	Limiting waves approach from azimuths:	Meadow is facing:	Source:
1	Elenite	90-180	East-southeast-south	Hineva E. (in press)
2	Sveti Vlas	90-160	East-southeast	Hineva E.(in press)
3	Vromos	20-80	North-northeast	Present study
4	Kraimorie	30, 60-80	North-northeast	Present study
5	Atia	0-90	North-northeast	Present study

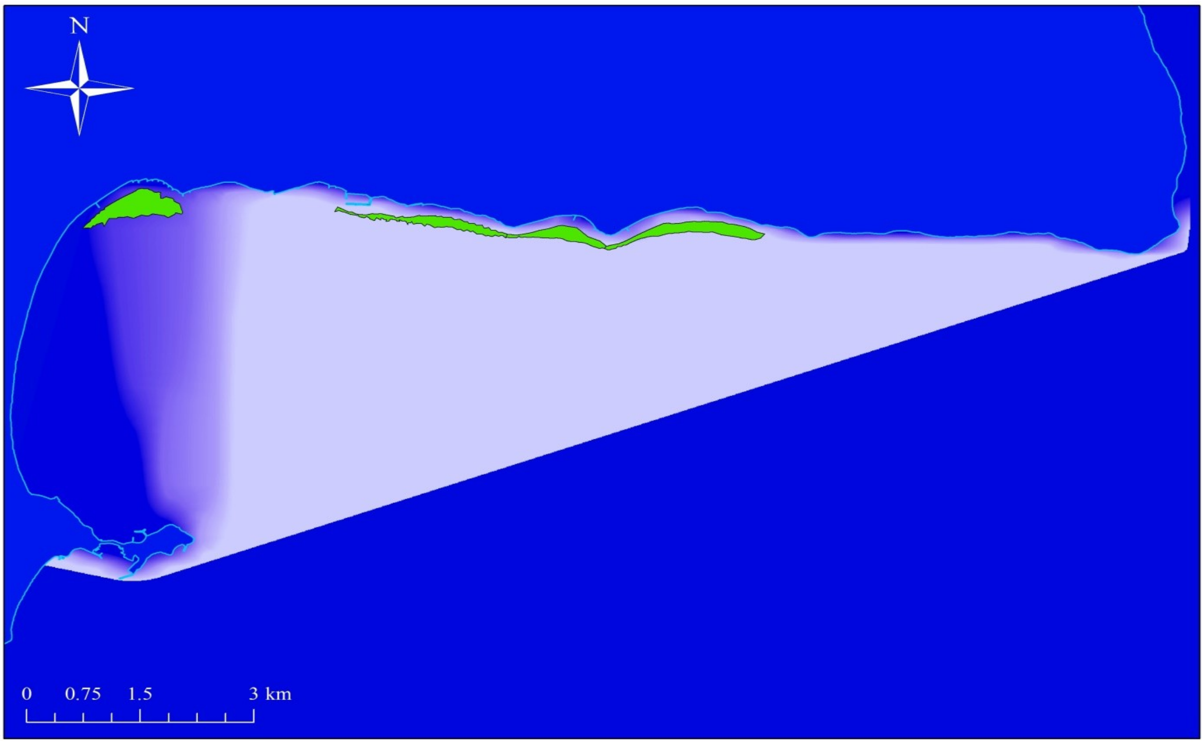


Fig. 6. Distribution of the average wave height in Nesebar Bay when approaching from azimuth 180°. "Colour codes" are the same as in Fig. 4. Scale line = 3 km.

Table 2. Parameters of the logistic model for the upper boundary of the meadows Kraimorie, Atia and Vromos Bay. Coefficient b0 and b1 – coefficients of the equations, AUC – area under the ROC curve.

№	Azimuth, degrees	b0	b1	Correct forecasts (%)	AUC	Discriminative ability (according to Hosmer & Lemeshow, 2000)
			Kraimorie meadow			
1	20	0,3	-1 278,8	51,8	0,52	poor
2	30	0,8	-39 390,8	72,7	0,72	satisfactory

3	40	0,63	-38,1	53,6	0,62	poor
4	50	0,25	-2,17	49,1	0,54	poor
5	60	0,56	-18,76	79,0	0,72	satisfactory
6	70	2,8	-22,9	73,6	0,81	very good
7	80	7,02	-58,19	84,5	0,91	excellent
8	90	3,4	-65,4	75,5	0,76	satisfactory
			Atia meadow			
1	20	1	-49,6	67,4	0,57	poor
2	30	0,8	-13,9	67,4	0,34	poor
3	40	0,4	1,5	61,8	0,64	poor
4	50	0,9	-4,4	64,0	0,47	poor
5	60	1,1	-21,0	71,9	0,53	poor
6	70	1,8	-24,7	77,5	0,71	satisfactory
7	80	2,9	-41,5	79,8	0,84	very good
8	90	2,4	-58,1	77,5	0,81	very good
			Vromos Bay			
1	20	3,12	-82,3	81	0,94	excellent
2	30	4,17	-155,5	85	0,95	excellent
3	40	4,43	-200,3	88	0,97	excellent
4	60	3,47	-175,6	85	0,96	excellent
5	70	8,35	-180,9	91	0,98	excellent
6	80	2,25	-553,6	72	0,74	satisfactory
7	90	0	0	60	0,38	poor

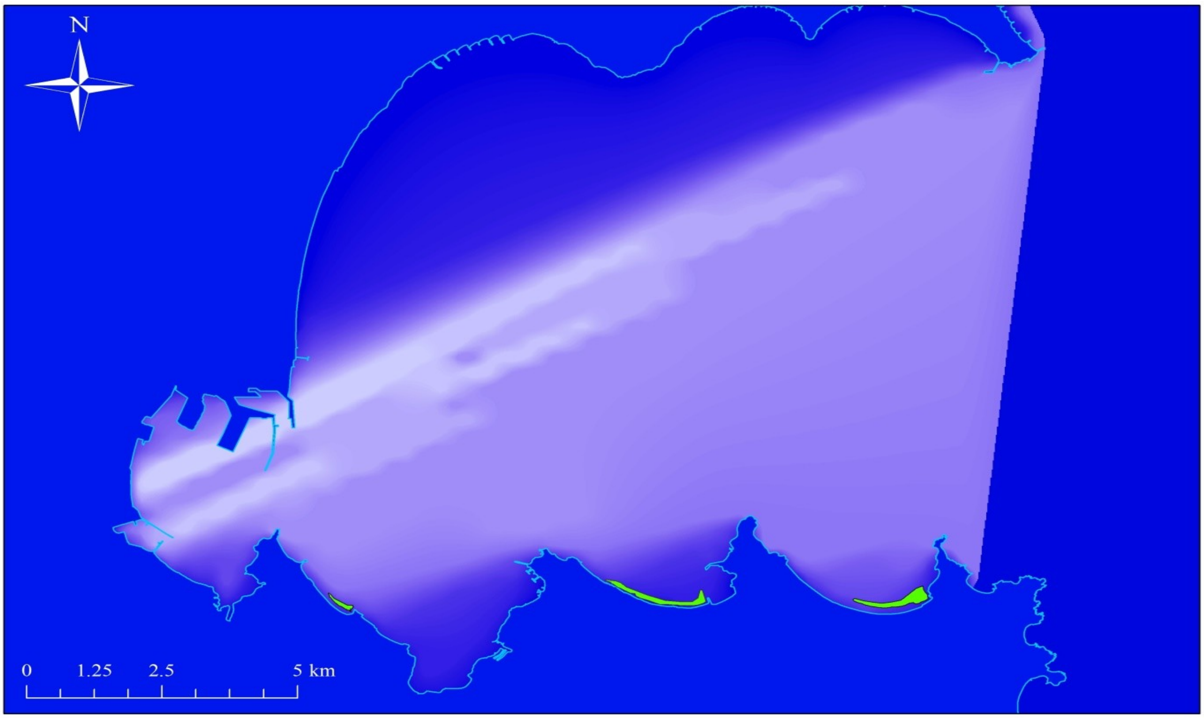


Fig. 7. Distribution of the average wave height in Kraimorie, Atia nad Vromos Bay when approaching from azimuth 70°. Port infrastructure is not assumed in modelling. "Colour codes" are the same as in Fig. 4. Scale line = 5 km.

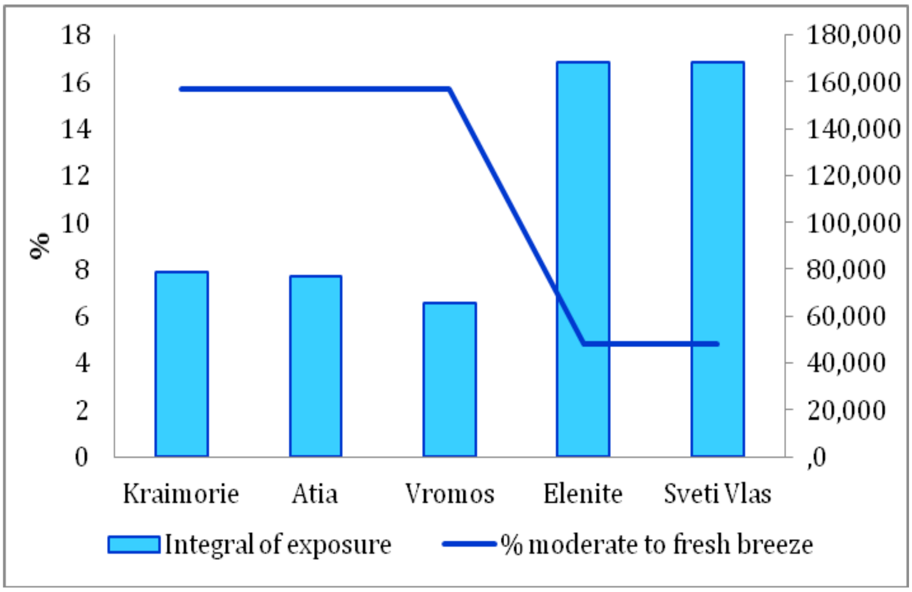


Fig. 8. Integral of exposure, percent of cases when wind has blown from north-northeast and south-southeast for the five seagrass meadows.

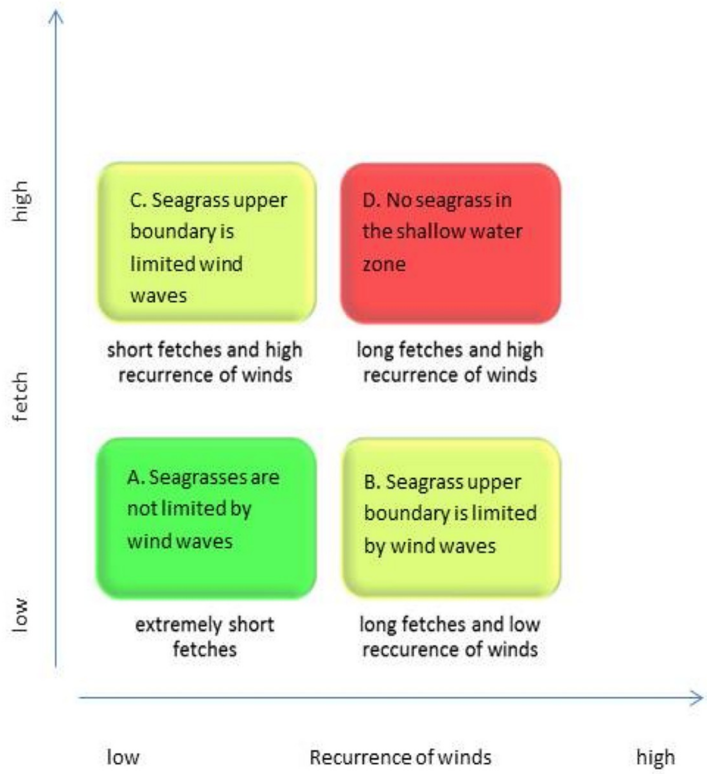


Fig. 9. Four possible scenarios for limiting wave sensitive seagrass by wind waves. “Winds” corresponds to “wind speed recurrence and duration of action” high enough to create waves able to limit seagrass presence in the shallow coastal zone.

Both types of wave generation factors have their specific features in Burgas Bay.

The wind rose for the open sea area against Burgas Bay shows that the distribution of relative frequency of wind speeds is different for each category: "gentle breeze" is relatively evenly distributed by directions, while in the other two categories the distribution is significantly uneven, as winds from north, northeast, and northwest predominate (Fig. 3).

The „gentle breeze"category obviously does not generate waves that could limit the seagrasses: the similar probability wind to blow from each direction does not explain the observed meadow distribution. Similar finding based on seasonal wind regime pattern has been reported by Keddy, 1982, who found that the measure of exposure has non-significant or poor correlation with vegetation during summer period (low wind speeds). In addition Schubert et al., 2015 have found that the "best model" which explains *Z. marina* L. distribution in the Baltic Sea is the one that considers wind speed ≥ 6 m/s (i.e. above "the gentle breeze" category). One can expect winds, within the categories "fresh to strong breeze" and "strong breeze to gale", to have a potential limiting effect. Strong hydrodynamic conditions are well known to have a destructive effect on seagrasses (e.g. Short & Willey-Echeveria, 1996, Portillio, 2014). Strong winds are the rarest along Bulgarian coast they occurred only in 0,1 % of cases from wave exposed directions (Fig. 3). Their strength creates waves with high energy, which probably have destructive effect upon seagrasses but their small probability of recurrence does not allow them to determine the perennial location of seagrass meadows. They could explain only a snap-shot picture observed immediately after a strong wind event. If for a long enough period after such an event there is no strong wind conditions, seagrasses would recolonize the previously abandoned bottom area up to the boundaries determined by less strong but more often wind waves.

The category "moderate to fresh breeze" has wide enough speed range and includes winds which have occurred often. Based on the wind rose (Fig. 3) it is expected that wave effect is strongest along coastlines open towards north and northeast and that conditions for seagrass growth there will differ from those along south-south east ones.

The maximum wave fetch along the northern parts of the coastline is significantly shorter from that along southern parts (Fig. 4). The maximum fetch alone could not explain the observed seagrass presence in Burgas Bay (poor discriminative ability of the logit function, data not shown), thus emphasizing the importance of the wind regime.

The impacts of both factors: wind regime and fetch also depends on the number of seaward azimuth directions (Fig. 5) (WFD CIS Guidance N 5). There is much higher possibility seagrasses along the coasts, open towards more seaward directions, with higher probability of "moderate to fresh breeze" to be limited by the wind wave and not to be present there. This fact is reflected in the design of simplified exposure indicators: exposure measure (Keddy, 1982), relative exposure index (Fonseca & Bell, 1998), where each term of the sum is a product of the fetch multiplied by the wind speed (average or exceedance or monthly average, etc.) and by the relevant wind percent frequency. Despite their obvious advantages and extremely wide application in aquatic ecology (e.g. Santana-Garson et al., 2010, Bekkbi et al., 2014, Mason et al., 2018), these indices focus mostly on the cumulative effect and not on the role of each type of wave generation factor for the creation of the habitat conditions.

The comparative analysis of conditions for seagrass inhabitance along the northeast and south-southeast coastlines of the study area allows for highlighting the relative importance of the wind regime versus the integral fetch. Examples of the four combinations of wave generation factors (Fig. 9) can be found in Burgas Bay.

The marina of Elenite resort along the north coast of Nesebar Bay limits the fetch and prevents the possibility of wind waves and swell to enter its aquatory (Fig. 2). It is representative of situation where both the short fetches and low wind recurrence create stagnate conditions (Fig.9A). The soft bottom macrophyte community there consists of three wave sensitive rooted aquatic plants: *Z. marina* L., *S. pectinata* (L.) Borner *Z. palustris* L. The scenario D (Fig. 9 D) is typical of the unvegetated area neighboring the meadows which lies outside the wave shadow of the adjacent capes. Examples are available both in northern and southern stretches of the coastline: the area adjacent to c. Emine (Nesebar Bay), areas neighboring the meadows of Kraimorie, Vromos etc. (Fig. 1). The situation on Fig. 9 B is represented by the Elenite and Sveti Vlas meadows opened towards a great number of directions (8-10 azimuths). The meadows are limited by wave action but can survive here having a deeper upper meadow boundary (> 2.5 m depth). These meadows are the most limited by wind waves: the statistical models have predominantly excellent to very good discriminative ability (Table 1). Regardless the higher most probable and average monthly maxima wind speeds from south-southeast; the low frequency of occurrence of those winds allows a fully open bay like Nesebar to accommodate seagrasses. In such areas the recurrence of wind is a key factor in the seagrasses presence because it creates more calm conditions, suitable for their inhabitance. The situation on Fig. 9 C is represented by the coasts exposed towards less number of azimuths (3 - 6 azimuths) with higher recurrence of "moderate to fresh breeze". The integral of all fetches in the most exposed southern coasts (open towards north-northeast) where seagrasses live is 2 times smaller than the integral of maximum fetches for most exposed northern coast (opened towards south-southeast) (Fig. 5). The wind rose (Fig. 3) shows that if a "moderate to fresh breeze" is observed the probability that it comes from north and northeast is much higher than from south-southeast. This indicates that when moderate to fresh breeze winds frequently occur, the fetch (integral) can significantly impact the seagrass distribution. The high recurrence of those winds has to be "compensated" to some extent by the smaller number of fetches of the limiting waves in order perennial seagrass meadows to be present (Tables 1 and 2). Therefore in bays open towards north, northeast the wave fetch has significant importance for presence or absence of seagrasses.

Conclusion

The combination of predominating winds and available free space for generation and growth of waves through the wave climate determines the probability of shallow water seagrass presence along Bulgarian Black Sea coast. In the regions where integral exposure is high seagrass could survive only if the wind regime creates milder wave climate corresponding to low frequency of moderate to fresh breeze. In regions where the moderate and fresh breeze occurrences are frequent, the smaller integral fetch creates suitable conditions for seagrass habitat. In the first situation the habitat is expected to be less frequently but more strongly impacted by the wave action, while in the second situation the opposite is supposed. Whether this has any consequences for the adaptation of seagrass communities to local hydrodynamic conditions is yet to be studied in future experiments.

For the Bulgarian coast the relative importance of the factors: wind regime and fetch is obviously changing from northeast to southeast coasts. Along the first ones the fetch (maximum and number of azimuths) is more important, while in front of the second, the wind speed recurrence is more important to allow for seagrass presence.

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Turtle Dove (Streptopelia turtur Linnaeus, 1758) Distribution Dependence of Habitat Variables in Central South Bulgaria

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Abstract. In the period 2016–2019, we monitored the presence of Turtle Doves in 153 census points within a study plot in Central South Bulgaria. The presence of singing males varied slightly throughout the study period and there was no significant difference between years. The riparian and oak forests are characterised by a greater presence of Turtle Doves than other habitat types. The Multiple regression model showed a relation between the presence index, the height of trees and the distance to water resources.

Key words: breeding habitats, habitat preference, presence index, tree height, water sources.

Introduction

Avian populations are one of the important indicators of biodiversity applied worldwide (Burchard et al., 2010; Gregory et al., 2005; Gamero et al., 2017). Agricultural intensification is considered to be one of the main reasons for the decline of farmland birds (Donald et al., 2006; Emmerson et al., 2016; Rief & Vermouzek, 2018; Traba & Morales, 2019). One of the species affected by the Pan-European agricultural practices is the Turtle Dove (*Streptopelia turtur*). Its breeding population is declining which is why it falls under the category of vulnerable species (IUCN, 2019). Threats in Europe include fragmentation and reduction of nesting habitats (Browne et al., 2004; Dunn & Morris, 2012; Kleeman & Quillfeld, 2014), as well as changes in agricultural practices leading to a decrease in food availability (Browne & Aebisher, 2003, 2004; Baptista et al., 2015). Other factors that contribute to the Turtle Dove's decline are associated with wintering grounds and migration routes (Browne & Aebisher, 2001).

Although the main food resources for the Turtle Dove are seeds in open areas (Browne & Aebisher, 2001, 2003), breeding habitats are forests close to the feeding areas (Browne & Aebisher, 2003; Browne et al., 2004). Within nesting sites, Turtle Doves are influenced by forest type, forest characteristics, and the type of adjacent open areas offering food resources (Bakaloudis et al., 2009; Dias & Fontoura, 1996; Dias et al., 2013; Rocha & Hidalgo, 2002). This defines the complex interrelationships between the environmental factors that determine the spread and the preferences of the Turtle Doves, which are sometimes difficult to understand. There are few studies on breeding habitat variables and the Turtle

Dove's presence (Bakaloudis et al., 2009; Dias et al., 2013), and differences in landscape structure in various nesting habitats that determine different preferences for nesting habitats.

The purpose of this study is to determine the preferences of the Turtle Dove to certain breeding habitats in the study area. It also analyses the impact of some habitat characteristics affecting the distribution of breeding birds.

Material and Methods

Study area. The study area falls in Central Southern Bulgaria and occupies parts of the Sakar Mountain and the Upper Thracian Plain (Fig. 1).

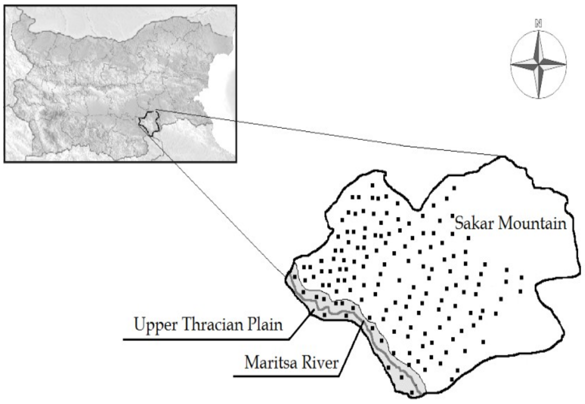

Fig. 1. Study area and distribution of points.

The study area covers an area of 1019 km^2 and includes several Natura zones: Sakar (BG 0000212; BG 0002021), Radinchevo (BG 0002020) and Maritsa River (BG 000578).

The forest flora is represented by *Quercus fraineto* (Ten.), *Quercus pubescens* (Willd.) and *Quercus virgiliana* ((Ten.) Ten.)). The main species at the shrub floor are: *Crataegus monogina* (Jacq.), *Rosa canina* (L.), *Rosa gallica* (L.), *Paliurus spina-christi* (Mill.), *Prunus*, *Cornus* etc. In wetlands, other groves can also be seen in patches: *Salix alba* (L.), *Salix fragilis* (L.), *Populus,Fraxinus Acer*, etc. (Bondev, 1991). Part of the territory is occupied by coniferous cultures: *Pinus nigra* (Arnold) and *Cedrus*. Open habitats are cultivated lands – mainly wheat and rapeseed. These are divided by narrow strips of deciduous trees and shrubs.

Field methods. In the period 2016–2019, we monitored the presence of Turtle Doves in 153 census points in the studied area. The number of singing male birds within 100 meters of the centre of each point was recorded from May 1 to July 20 two times per year. The data were collected in clear and quiet weather, with no rainfall, between 5:00a.m. and 8:30a.m.. The number of singing Turtle-Doves was determined from duration of 10 minutes, after a two-minute wait on the part of the observer before the onset of the measurement of each point. Several types of nesting habitats were distinguished in the study area and points were distributed to all of them (Table 1).

Table 1. Number of points count by habitat type.

Habitat type	Number of points
Coniferous cultures	24
Oak forest (*Quercus pubescens, Q. fraineto, Q. virgiliana*)	22
Deciduous forests dominated by Oaks	22
Riparian forests	22
Shrubs predominated by *Paliurus spina-christi*	24
Strips of trees and shrubs amid vineyards	19
Strips of trees and shrubs amid arable land	20

Coniferous cultures are composed mainly of Black Pine (*Pinus nigra* Arnold) and there is no shrubby floor. Single spots consist of *Cedrus*. The Oak forests are represented by Hungarian Oak (*Querqus frainetto* Tenn.), Austrian oak (*Querqus cerris* L.), and Downy oak (*Querqus pubescens* Willd.). They have an average height of 10 meters and no shrubby floor. The Deciduous forests have a mixed composition of Narrow- leafed ash (*Fraxinus ornus* L.), Oriental hornbeam (*Carpinus orientalis* Mill.) and Downy oak (*Quercus pubescens* Willd.). They are characterized by a shrub floor of Common hawthorn (*Crataegus monogina* Jacq.), Dog rose (*Rosa canina* L.), Provence rose (*Rosa gallica* L.), Jerusalem thorn (*Paliurus spina-christi* Mill.), Cornelian cherry (*Cornus mas* L.) and average height of 8meters. The riparian forests are representing of poplars, willows and ash trees. They are up to 20 meters in height and single shrubs. Jerusalem thorn communities are the lowest (3.2 m height). Among the shrubs there are single pears and oaks. The stripes of trees and shrub amid vineyard and arable lands are represented by oaks, pears and ash trees. They have a shrub floor of Blackthorn (*Prunus spinosa* L.), Hawthorn (*Crataegus*) and Jerusalem thorn with an average height of 4 meters.

We calculated the Turtle Dove's abundance index as the average number of singing birds in each point by year (total number of singing birds in each point/number of observations per year). In addition, we used data from the study of the Turtle Dove in 2014–2016 at MG 14 (UTM) (Gruychev & Mihaylov, 2019). In 2014–2016, data was used only for reports in the period May 1 –July 20, so that there are no time distortions.

Flora characteristics were determined at each point within the 100-metre radius around it: 1) cover of a dominant tree species (%); cover of a dominant shrub species (%); tree density (number of trees in a spot of ten by ten metres) (number); average vegetation coverage (%); grass height (metres); tree height (metres); shrub height (metres); deciduous coverage (%); total shrub coverage (%); grass coverage (%) (Bibby et al., 1992). Next, we characterised the flora in each separate type of habitat by averaging the values measured at each point. This allowed us to compare the Turtle Dove's abundance in each separate type of nesting habitat with the characteristics of the vegetation therein. We measured the shortest distances from each point to open areas and to water sources. The distances were measured with QGIS (QGIS, 2019). These variables were included in a model to look up the relations between the Turtle Dove's presence and the habitat variables.

Statistical methods. We used one-way ANOVA to compare the abundance of Turtle Doves in different years and habitats and ANOVA main effect to compare the abundance of birds in different crops in the neighbourhood. In this case, the dependent variable was the Turtle Dove's presence and the independent variable was habitat type and crops in the neighbourhood. A multiple regression model was used to determinate the relations between habitat variables and the Turtle Dove's presence. The presence of Turtle Doves was a dependent variable and the habitat characteristic was an independent variable (Dytham, 2011).

The habitat selection by Turtle Doves was assessed comparing the bird's abundance index in each habitat to its availability in the whole study area by using the Jacobs' index of selection J (Jacobs, 1974):

$$J = (H1/H2-A1/A2) / (H1/H2+A1/A2),$$

where: H1 – Turtle Doves abundance in habitat 1; H2 – Turtle Doves abundance in all habitats; A1 – area of all study plots in habitat 1 (coniferous, oak etc.); A2 – area of all study plots in all habitats (Jacobs, 1974).

All statistical analyses were performed using Statistica 8.0 StatSoft, Inc (Hill & Lewicki, 2006).

Results

The presence of singing males varied slightly throughout the study period (Fig. 2), and there was no significant difference between years (F = 1,47; p = 0,199; df = 290).

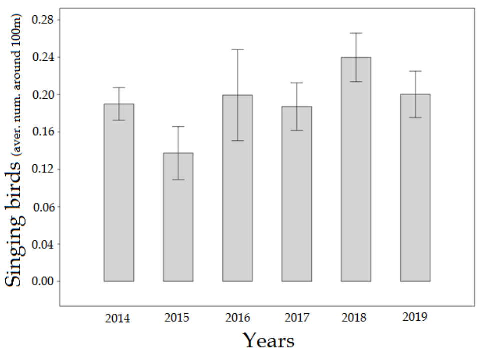

Fig. 2. Average number of singing Turtle Doves (*Streptopelia turtur*) by year around 100 meters (average, min.-max., st. err.).

The riparian and oak forests are characterised by a greater presence of Turtle Doves than other habitat types (F = 4.43; p < 0.0001; df = 6), (Fig. 3).

The Jacobs' index has the highest positive values for riparian and oak forests and negative ones for coniferous cultures and strips of trees and shrubs amid vineyards and arable land (Fig. 4).

The Multiple regression model showed a relation between the Turtle Dove's presence and height of trees and distance to water sources, but the general model is not significant (Table 2).

Although the presence of Turtle Doves is higher (Fig. 5) in the habitats with meadows our model does not recognise any significant differences (Table 3). The ANOVA main effect model is significant but does not show any differences in Turtle Dove's presence in addition by crop neighbourhood (Table 3).

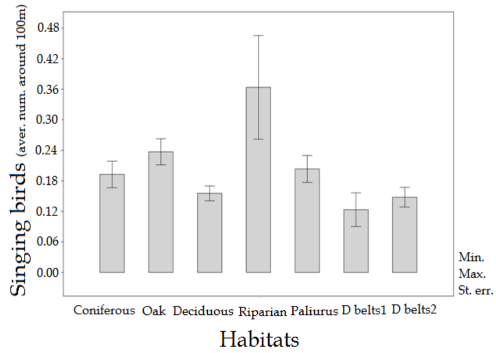

Fig. 3. Number of singing birds around 100 meters in different habitats of study area. (D belts 1 - strips of trees and shrubs amid vineyards; D belts 2 - strips of trees and shrubs amid arable land).

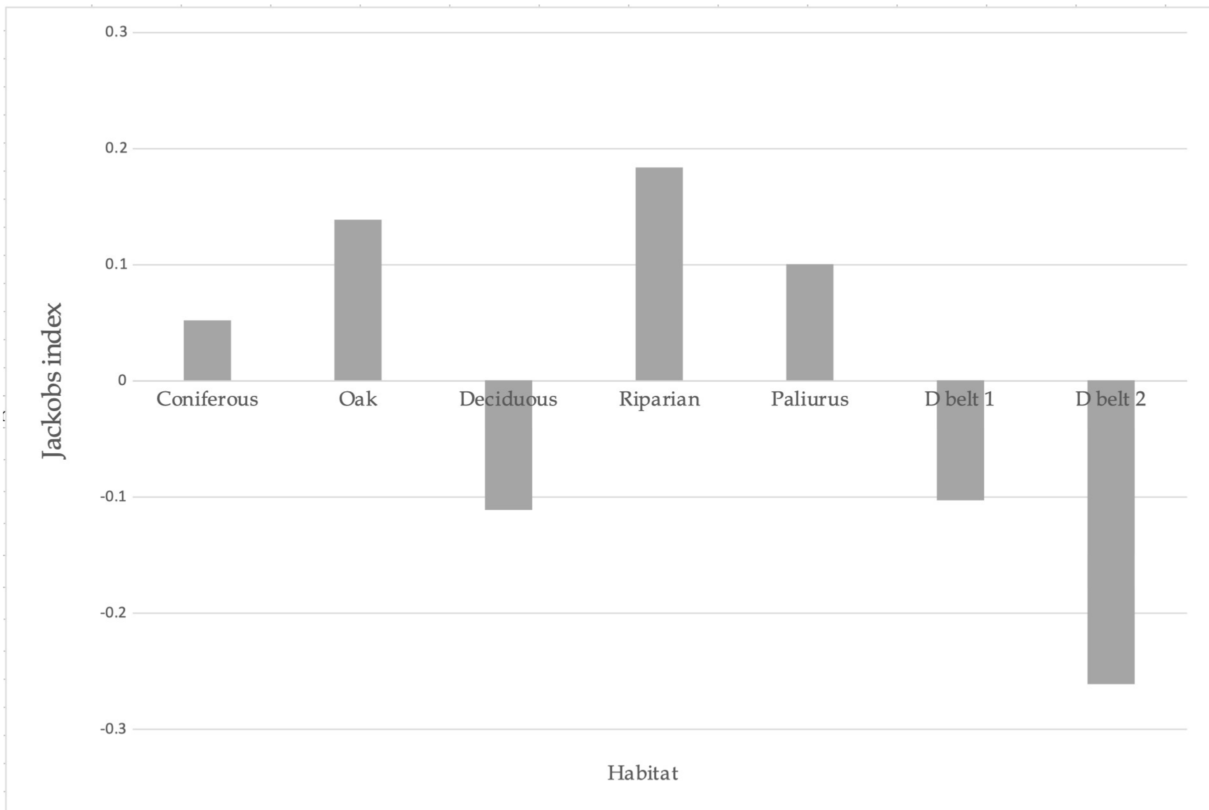

Fig. 4. Jacob's index by habitats. (D belts 1 - strips of trees and shrubs amid vineyards; D belts 2 - strips of trees and shrubs amid arable land).

Table 2. Results from multiple regression model for Turtle Dove's presence and habitat variables. Legend: in bold font are the results with p<0.05.

Regression Summary for Dependent Variable: Turtle Dove presence R= ,23344816 RI= ,05449804 Adjusted RI= ,03211930 F(12,507)=2,4353 p<,00438 Std.Error of estimate: ,32657						
	b*	Std.Err.	b	Std.Err.	t(507)	p-value
Intercept			-0.331731	1.391095	-0.23847	0.811615
Cover of a dominant tree species	-0.36656	1.111859	-0.003900	0.011830	-0.32968	0.741777
Cover of a dominant shrub species	0.20078	0.416050	0.005220	0.010816	0.48258	0.629601
Tree density	-1.42350	1.289121	-0.012616	0.011425	-1.10424	0.270012
Average vegetation cover	-0.44411	1.207920	-0.003838	0.010438	-0.36767	0.713273
Grass height	1.82990	1.480129	0.158592	0.128278	1.23631	0.216916
Tree height	**0.36096**	**0.161182**	**0.035462**	**0.015835**	**2.23943**	**0.025561**
Shrub height	-0.03551	0.212853	-0.000499	0.002991	-0.16681	0.867587
Deciduous coverage	1.07495	1.036068	0.010317	0.009944	1.03753	0.299985
Total shrub coverage	-0.18301	0.232824	-0.005093	0.006479	-0.78605	0.432206
Grass coverage	0.55540	0.537269	0.006874	0.006650	1.03374	0.301751
Distance to open areas	0.10052	0.055043	0.000672	0.000368	1.82623	0.068404
Distance to water areas	**-0.10891**	**0.050475**	**-0.000102**	**0.000047**	**-2.15775**	**0.031416**

Discussion

We did not find significant differences in the Turtle Dove's presence indices over the years. In a previous study, differences in species density by year (Gruychev & Myhailov, 2019) and a decrease in breeding density over previous periods (Simeonov & Petrov, 1978) were found in a part of the Sakar Mountains.

The riparian and oak forests are characterised by a higher abundance index than other breeding habitats. These results are confirmed for similar but smaller areas of Sakar Mountain (Gruychev & Myhailov, 2019) and other parts of Bulgaria (Nankinov, 1994; Simeonov et al., 1990;). Appropriate forest habitats can maintain 6.5 times higher density than open areas (Browne et al., 2004). The riparian forests are preferred by Turtle Doves in other parts of its range (Saenz de Buruaga et al., 2012). The Jacobs' index has the highest values for the last two habitat types. There are various studies on the Turtle Doves' breeding range in Europe which, to some extent, highlight dependencies between density and different habitats. In some parts of the Iberian Peninsula, the species prefers forested and agricultural landscapes with single trees (Dias et al., 2013). The Jacobs' negative index of trees and shrubs among arable land and vineyards in this study is probably due to a mechanized spraying in these areas, often with aviation creating anxiety during the breeding season. The Jacobs' negative index for deciduous forests remained interesting. The probable reason is the shrub floor in these habitats and reduced visibility, which is reported as a negative factor by other similar studies (Bakaloudis et al., 2009; Camprodon & Brotonos, 2006; Dias et al., 2013; Santos et al., 2002; Saenz de Buruaga et al., 2012). Coniferous cultures and shrub communities also have a positive Jacobs' index. In a number of studies, such habitat types have been reported to be positively related to breeding density of Turtle Doves

(Bakaloudis et al., 2009; Browne & Aebisher, 2004; Dias et al., 2013; Dunn & Morris, 2012). In the study area, the last two habitat types have lower presence index. A probable reason might be the preference of the Turtle Dove to riparian and oak forests.

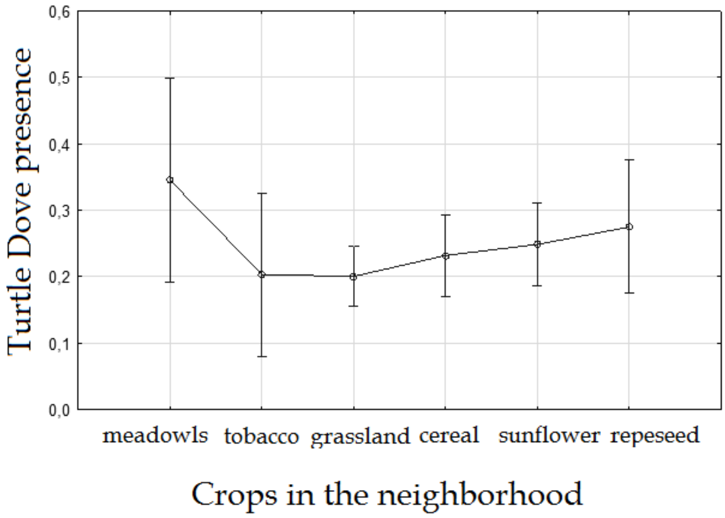

Fig. 5. Turtle Dove (*Streptopelia turtur*) presence by different crop in neighbourhood. (current effect F (5,519)=0.988; p=0.42, effective hypothesis decomposition, vertical bars denote 0.95 confidence intervals)

Table 3. The result of ANOVA main effect model by habitat type and crop neighborhood. Legend: in bold font are the results with significance.

	SS	Degree of freedom	MS	F	p
Intercept	**14.32005**	**1**	**14.32005**	**134.4404**	**0.00000**
Crop neighborhood	0.52608	5	0.10522	0.9878	0.424508
Habitat	**2.14193**	**5**	**0.42839**	**4.0218**	**0.001371**
Error	55.28181	519	0.10652		

The Multiple regression model showed a relation between the presence index, the height of trees and the distance to water resources, but the overall model is not significant. However, a similar relation has been established for another part of the Turtle Dove's range (Kafi et al., 2015). Recent authors have found that the number of hatchlings increases with the height of the trees up to about 1.6 m and then sharply decreases. In this study, we have a height restriction on trees, because we do not have many counting points in habitats with tree heights over 20 meters. Therefore, we do not know what height is optimal. There is a slight negative correlation between the presence index and the distance to water resources. The presence decreases gradually with the increasing distance to water resources. The presence of permanent water sources can largely determine the distribution of some more sedentary bird species in dry areas, such as some Galliformes (Borralho et al., 1998; Larsen et al., 2010; Lee et al., 2003). But Turtle Doves are quite mobile species that can travel considerable distances and water should not be a limiting factor. There are studies which do not identify the impact of water resources on breeding density (Gutierrez-Galan et al., 2018; Kleeman & Quillfeld, 2012) and those that claim a relation (Dunn & Morris, 2012; Saenz de Buruaga et al., 2012). The mean distance to permanent water sources in this study is 486 ± 354 (27–1970 m) and although very close to other similar studies (Gutierez-Galan et al., 2018), there is a slight correlation here with the presence of the Turtle Dove. The probable reasons are the points with greater distances to the water. In addition, the habitats in the study area where we reported the highest presence indices are located near different water sources. Our results did not find a significant relationship between Turtle Dove's presence and other habitat characteristics. The analysis of variance did not consider a significant relationship between the presence of the Turtle Doves and the type of arable land in the neighbourhood. However, the presence is highest when there is a combination of forest habitats with meadows, followed by crops such as rapeseed, sunflower and cereals. The Turtle Dove's feeding areas consist of areas occupied by low grassland and arable land (Browne & Aebisher, 2003). Thus, wild and cultivated seeds are the main nutritional components for Turtle Doves (Dias & Fontoura, 1996; Gutierrez-Galan et al., 2018; Jimenez et al., 1994;). In the study area, the meadow probably offers the best food for the species in May and June. At the same time, they have lower degree of chemisation than the other feeding sites. Positive effects of grassland places have also been reported in other studies (Kleeman & Quillfeld, 2014). Although cereals and oilseeds are the preferred food for the species, they are only available in the study area in July and August. The presence of Turtle Doves is greater in habitats close to cereals and such offering wild seeds included in the local diet of the species (Gutierez-Galan et al., 2018; Mansouri et al., 2019). The reason for the lack of relationship between the Turtle Dove's presence and crops in neighbourhood within the present study area is likely due to its heterogeneity. It is a combination of many different habitats close to each other, and the mosaic of agricultural lands ensures a good food supply in the whole area. The study area includes habitats similar to those that are known to be preferred by Turtle Doves in other parts of the breeding range. The results confirm similar outcomes from other studies of the species (Barbaro et al., 2007; Browne & Aebisher, 2004; Dias et al., 2013).

Conclusions

In this study, no difference was found in the presence of Turtle Doves during the study period. We found that the riparian and oak forests have a higher presence index and a positive Jacobs' index. Of the habitat characteristics, the height of the trees and the distance to water resources are important for the presence of the species during the breeding season. These results must be taken into account when planning activities in the habitats concerned. Often, forestry activities, such as logging during the breeding season, can lead to significant losses of broods. The nesting habitats of the Turtle Doves must be managed through a comprehension of the breeding season of the species in order to avoid loss of biodiversity.

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Fire Dangerous Properties of the Most Common Plants of Grass Ecosystems in Ukraine

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Abstract. The impact of grass fires on environment and grass ecosystems is mainly negative. The fire hazard of herbaceous plants causes the occurrence and spread of fires in herbaceous ecosystems. Various indicators, in particular the humidity of the combustible material, the self-ignition temperature, etc., can estimate it. These indicators depend on the type of plants and the natural conditions that determine the properties of the combustible materials. The goal of the research is to determine the fire hazard indicators of five the most widespread herbaceous plants in Ukrainian ecosystems (*Festuca arundinacea*, *Festuca pratensis*, *Elymus repens*, *Phleum pretense* and *Trifolium arvense*) and to substantiate these indicators due to results of thermogravimetric analysis and values of absolute humidity and self-ignition temperature. Within 5 days, the absolute humidity of the samples as well as the self-ignition temperature were determined by weight method and using the OTP testing device. Complex thermal analysis of samples was also perform using a Q-1500D derivatograph. The results of the research show that absolute humidity and self-ignition temperature of certain types of plants specify differences in their fire-fighting properties. According to the results of complex thermal analysis for each plant species, three stages occur at different temperatures: evaporation of free and bound water, self-ignition of samples and combustion of carbonized residue. The maximum exothermic effect for each plant was characterized by different value of temperature, as well as ash residue, which impedes the combustion process.

Key words: Herbaceous ecosystem, fire hazard, absolute humidity, self-ignition temperature, thermal analysis, ash content.

Introduction

Grass fires make the impact on environment and ecosystems. The soil properties are change after fires. The biodiversity of grass ecosystems is also changes. The most negative impact of fires is on fauna. However, sometimes fires make a positive impact which uses for grassland management (Pereira et al., 2017). There are several types of grass ecosystems in Europe. According to the MAES classification, they are pastures and natural grasslands (Maes et al., 2013). In East Europe there are many abandoned agricultural lands with grass cover which also belong to grass ecosystems with the same fire impact on them (Khanina et al., 2018). In general the processes of occurrence and spread of grass fires in all types of grass ecosystems are very similar and are determined by fuels characteristics. Grassy ecosystems under favorable weather and herbaceous plants'

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conditions are fire hazardous: they may effect on fire, spread of fire and cause the fires of various objects. In 2016 in Ukraine grass and shrub fires covered an area of more than 36,000 hectares, which amounts 48.3% of the total area of natural ecosystems' fires (Center of fire statistics of CTIF, 2018). The fire hazard of herbaceous plants, like other combustible materials of vegetable origin, depends most on their quantity, moisture content, specific combustion heat, surface to volume ratio, the part of minerals (ash) in their composition, as well as the bulk density (Simpson et al., 2016). Moisture content is the most important fire hazard property (Yebra et al., 2018). However, plants on a site may have different states during a given period: long-withered, recently withered, withering and alive (Cruz et al., 2016). These features are due not only to the climatic and weather conditions, but also to the biological features that cause not only the spatial structure but also the flammability of the grasses (Harris et al., 2016).

Vegetable combustible materials were studied in (Keane, 2015). There are well-known studies of the fire risk of individual plant species in the United States (Havill et al., 2015) and Mediterranean countries (Simpson et al., 2016). A review of the literature that deals with the plant flammability and the definition of flame retardant plants are provided in (Bethke et al., 2016). The classification and fire-hazard characteristics of combustible materials of vegetable origin, including herbs, is given in Amer Mehmood et al. (2017). In Ukraine the fire-hazardous properties of herbaceous plants in forest ecosystems have been studied (Kuzyk, 2019). However, by now still there was no research on the fire hazard of herbaceous plants of meadow ecosystems of Ukraine, which necessitates their carrying out.

The ignition characteristics of herbaceous plants in the field conditions are determined mainly by experimental methods, in particular, laboratory study of their fragments in terms of absolute humidity, ignition and self-ignition temperatures (Thakur et al., 2017). Thermogravimetric analysis (Thakur et al., 2017; Hlavsova et al., 2016) is one of the most promising and fast methods of investigating the combustible properties of plants and their fragments, which makes it possible to quickly investigate the processes of mass loss of combustible material upon the application of heat using derivatograph. The output of pyrolysis products depends on the biochemical composition, secondary reactions and the content and composition of the ash (Hlavsova et al., 2016), causing differences in the ability of different types of combustible materials to ignition.

To analyze the fire hazard of grassy ecosystems and to predict the fire spread, it is necessary to evaluate the fire risk of plants of individual species. This can be done using traditional fire safety methods and derivatographic method, which will not only establish the fire risk of plants of meadow ecosystems, but also provide a comparative analysis of the results obtained by different methods.

The article is dedicated to establishment of fire hazard characteristics of the most widespread representatives of Ukrainian grasslands and their justification by the values of absolute humidity, self-ignition temperature and the results of thermogravimetric analysis.

Materials and Methods

The absolute humidity of the samples was determined by weight method. The self-ignition temperature was determined using an OTP device according to the standard (DSTU 8829:2019, 2019) for solid materials. The reaction chamber inside OTP device vas preheated up to 500 Celsius degrees. Chapped and formed like tablets plant samples was putted inside chamber. The self-ignition temperature was identified by chromel-alumel thermocouple putted inside testing sample at the moment of flame apperance. The thermal analysis of the samples was performed on a Q-1500D derivatograph of the "Paulik-Paulik-Erdey" system using a computer to record an analytical signal of mass loss and thermal effects. The studies were performed dynamically with a heating rate of 5°C per minute in air. Thermal stability of the samples was studied in the temperature range of 20-1000°C. The reference substance was aluminium oxide.

The studies were conducted in May 2018. Fragments of the aerial parts of herbaceous plants were selected as the test material on May 16 on the grasslands near Lviv, Ukraine (49°53'42.2"N 24°03'52.5"E). We chose the most widespread representatives of Ukrainian grasslands in Western Ukraine (Baistruk-Hlodan & Khomiak, 2016), Western Forest-steppe (Yarmoliuk et al., 2010), Forest-steppe (Kirian et al., 2018) and widely uses for forage producing (Bugayov et al., 2008) such as: *Festuca arundinacea* Schreb., *Festuca pratensis* Huds., *Elymus repens* (L.) Could, *Phleum pretense* L. and *Trifolium arvense* L. After delivery to the laboratory, part of the material was left for further drying at a temperature of 20±2 Celsius degrees (the samples were formed and weighed) and the other part was used to determine the self-ignition temperature. In total, 18 samples with a weight of 0.05 kg were formed for each of plant species. In the next 4 days, the temperature of self-ignition was determined for the partially dried samples. Self-ignition temperature was determined by conducting 3 experiments for each type every day. Drying lasted 4 days, and on the 5th day the samples were placed in the moisture-testing oven, kept for 3 hours at 105°C and weighed. 3 samples of each plant species were tested. The masses of the samples in an absolutely dry state were used to determine their absolute humidity.

Results and Discussion

Elymus repens had the highest initial humidity and *Festuca pratensis* had the lowest initial humidity. However, on the 5th day of research, the lowest humidity was observed for *Festuca arundinacea* and *Festuca pratensis*, the highest humidity – for *Trifolium arvense* (Table 1).

The self-ignition temperature of samples is given in Table 2. It was determined every day during the drying process as well as for a completely dry condition. The samples were placed to the cylindrical furnace of the OTP device heated to 550°C, and the self-ignition temperature was determined by the thermocouples disposed inside the samples.

On the first day of the studies, the temperature of self-ignition was the highest for all plants and ranged from 432 to 493°C, and further decreased daily. *Elymus repens* had the highest, and *Festuca pratensis* had the lowest self-ignition temperature. On the 5th day of research, the temperature of self-ignition decreased for all species by approximately 100°C and remained the lowest for *Festuca pratensis*, while *Phleum pretense* had the highest self-ignition temperature. For a completely dry condition, the *Trifolium arvense* samples had the lowest self-ignition temperature (265°C), while the *Festuca pratensis* samples and the *Trifolium arvense* samples had the highest auto-ignition temperatures (296 and 298°C).

As can be seen from the results of studies, the fire hazard characteristics of different plants vary differently during drying, which indicates their dependence on the species. To determine the causes of such differences, a complex thermal analysis of the samples was conducted, the results of which are presented on thermograms (Fig. 1). The average weight of the samples was 100 mg.

Table 1. Absolute humidity (per cent) of plant samples during their drying.

Herbaceous plant	Date of test				
	16.05	**17.05**	**18.05**	**19.05**	**20.05**
Festuca arundinacea	307.46	174.21	108.58	26.71	8.03
Festuca pratensis	382.55	192.09	90.79	16.78	9.00
Elymus repens	630.57	275.51	89.44	45.81	12.37
Phleum pretense	277.56	225.01	134.29	53.41	17.39
Trifolium arvense	471.56	308.97	202.28	76.47	19.59

Table 2. Self-ignition temperatures (°C) of plant samples in the process of drying them.

Herbaceous plant	Date of test 16.05	17.05	18.05	19.05	20.05	Absolutely dry state
Festuca arundinacea	451	440	419	395	342	273
Festuca pratensis	432	408	362	346	326	296
Elymus repens	493	481	465	414	374	283
Phleum pretense	457	446	439	421	380	298
Trifolium arvense	490	473	443	414	358	265

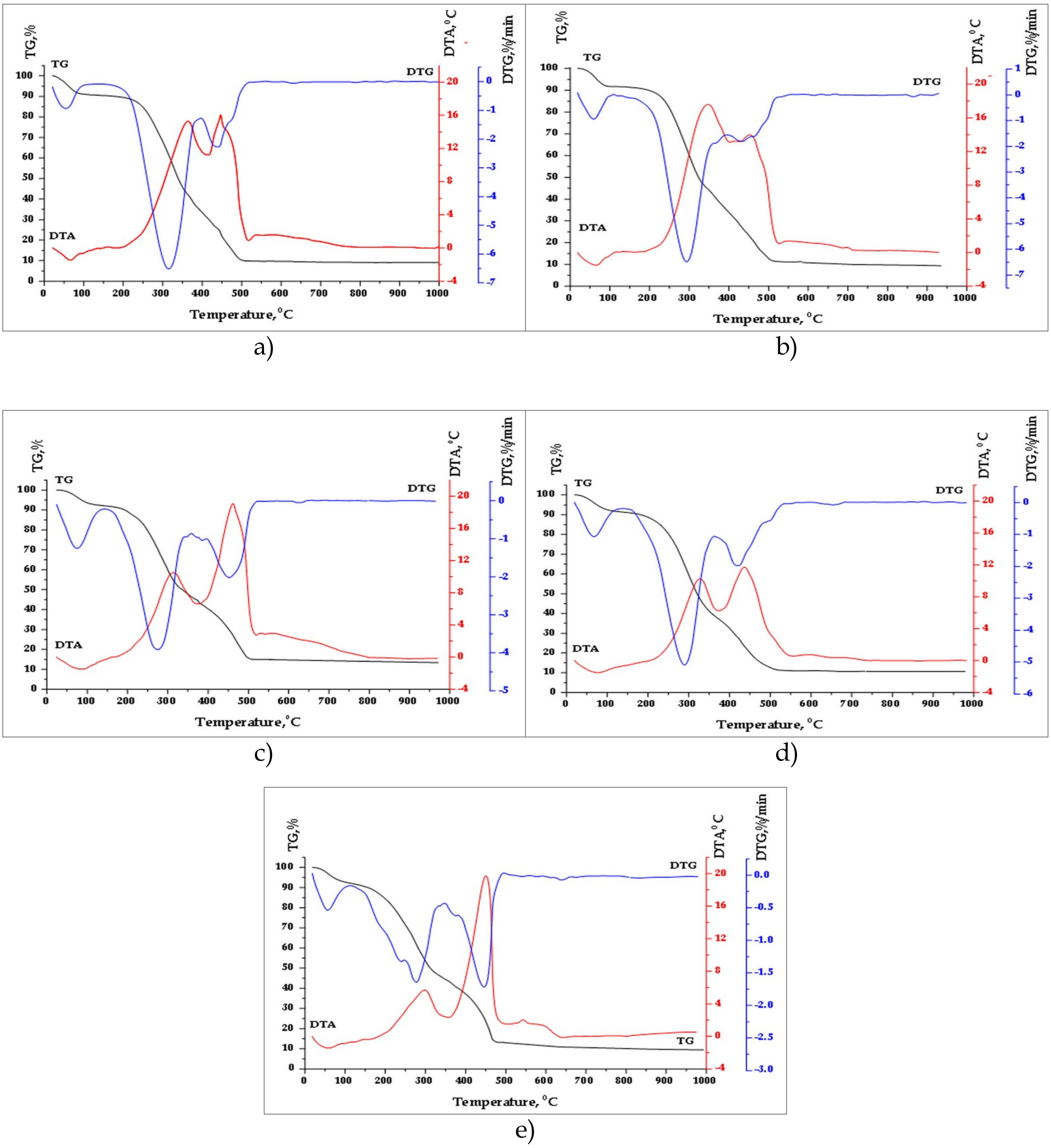
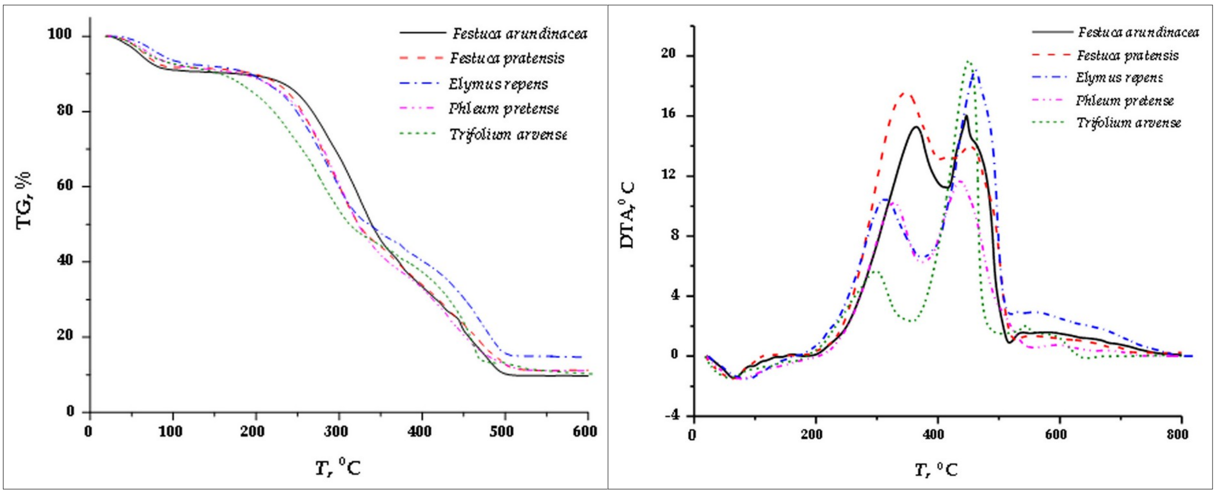


Fig. 1. Thermograms of herbaceous plants of meadow ecosystems: a) *Festuca arundinacea*; b) *Festuca pratensis*; c) *Elymus repens*; d) *Phleum pretense*; e) *Trifolium arvense*.

Thermogravimetric (TG) curves illustrate the weight loss of the samples during the heating process. Differential thermal analysis (DTA) curves indicate the sign and magnitude of the thermal effect of the process. And the curves of differential thermogravimetric analysis (DTG) are the result of differentiation of the TG curves and correspond to the weight loss velocity at the appropriate temperature. In Fig. 2 TG and DTA curves of the samples are shown for comparison.

In the table 3 the results of thermogravimetric analysis of the samples and the maximum temperature of the first exothermic effect (*Tmax*,°C) corresponding to the flame combustion of the samples, are shown. For each of plant species three stages characterizing the corresponding pyrological properties are identified. Their temperature intervals are different for each plant species, which indicates unequal fire hazard characteristics.

In all the tested samples, the endothermic processes occur in the temperature range 20–245°C (stage I). These processes are accompanied by the weight loss of the samples. In this temperature range, the evaporation of unbound water and the release of constitutional water that is part of the plant material has place. It should be noted that the *Trifolium arvense samples* had the highest content of unbound and constitutional water (15.01%), which was lost in the temperature range of 20–197°C.



a) b)

Fig. 2. Thermogravimetric curves of herbaceous plants of grassy ecosystems: a) TG curves of the samples; b) DTA curves of the samples.

Table 3. Results of thermogravimetric and differential thermal analyzes of the samples.

Herbaceous plant	Stage	Temperature range, °C	Mass loss, %	Maximum temperature of the first exothermic effect Tmax, °C
Festuca arundinacea	I	20 – 183	10.08	364
	II	183 – 397	55.64	
	III	397 – 1000	25.12	
Festuca pratensis	I	20 – 184	10.10	347
	II	184 – 400	56.24	
	III	400 – 1000	26.03	

Elymus repens	I	20 – 195	10.21	316
	II	195 – 366	44.45	
	III	366 – 1000	32.46	
Phleum pretense	I	20 – 211	12.29	333
	II	211 – 373	49.94	
	III	373 – 1000	25.62	
Trifolium arvense	I	20 – 197	15.01	300
	II	197 – 349	40.27	
	III	349 – 1000	35.54	

The samples of *Festuca arundinacea* contain the smallest amount of water (10.08%), which is lost in the temperature range of 20–183°C. Such results are in good agreement with the data on the humidity of the samples obtained during their drying.

Intensive destruction of the samples in the second stage, which is illustrated by rapid weight loss, begins at temperatures higher than 183–211°C. The points of extremum on the DTA and DTG curves of the samples differ significantly, indicating differences in their chemical composition.

In the temperature range of 183–400°C, there are a number of complex processes: along with the endothermic processes of pyrolysis of the samples, exothermic oxidation processes take the place, leading to the combustion of the decomposition products. The DTA curves exhibit clear exothermic effects in this temperature range.

It should be noted that the *Trifolium arvense* samples are characterized by the lowest thermal resistance. The thermo-oxidation processes in this sample flow at lower temperatures (Fig. 1), the maximum of the exothermic effect (300°C), which corresponds to the flame combustion of the decomposition products of the sample, is displaced to the area of lower temperatures in comparison with other samples (Fig. 2).

Samples of *Festuca arundinacea* and *Festuca pratensis* exhibit the highest thermal resistance. The temperature interval of intense mass loss, which corresponds to the combustion of the decomposition products, is displaced to the area of higher temperatures (Fig. 1). The maximums of the exothermic effect of these samples are manifested on the DTA curves at the highest temperatures as compared to other samples (Fig. 2). For the sample of *Festuca arundinacea*, it is observed at a temperature of 364°C, and for the sample of *Festuca pratensis* – at a temperature of 347°C. Samples of *Elymus repens* and *Phleum pretense* demonstrate moderate thermal resistance. The maximums of the exothermic effects of these samples correspond to temperatures of 316°C and 33°C.

The results of thermal analysis are satisfactorily consistent with the data obtained in studies on the determination of self-ignition temperatures of samples.

In the temperature range 349-1000°C (stage III), the carbonized residue formed during the flame combustion of the samples is combusted. On the DTA curves of the samples there is a pronounced exothermic effect, the points of extremum of which, as in stage II, also differs from each other and indicate the difference in chemical composition. After completion of the combustion a small quantity of residue (ash) remains. For samples of *Elymus repens* and *Phleum pretense* this quantity is the largest.

Studies have only been conducted on the 5 most common herbaceous plants since we cannot describe every part of a grassland that can sometimes number more than 150 species. However, the focus of ignition occurs on a small part of the site and therefore is determined by the properties of the dominant plants. In addition, single plants do not significantly affect the spread of fire.

Conclusions

The fire hazard of herbal plants is caused by their physical and chemical properties. It depends significantly on their species and conditions and causes differences in the processes of ignition and spread of fires. Differences in fire hazard of different plants can affect the occurrence of fires, as well as the rate of fire spread in grassy ecosystems. As a result of laboratory thermogravimetric studies, it is established that the highest thermal resistance due to the exothermic effect is peculiar to *Festuca arundinacea* and *Festuca pratensis*. The largest is the ash residue for *Elymus repens* and *Phleum pretense*, which indicates that they have less ability to ignite in comparison with other plant species. Fire hazard by values of self-ignition temperature depends on the humidity of the plants, which decreases in the process of drying, and at high humidity may be lower for some types of plants, in particular the *Elymus repens* and *Trifolium arvense*, and at low humidity – for others types, in particular the *Elymus repens* and *Phleum pretense*.

To evaluate the fire hazard of a particular ecosystem, it is necessary to explore the plants that are part of it. It is advisable to take into account the difference between the fire hazard properties of herbaceous plants in the analysis of the fire danger of the grassy areas, prediction the spread of fires and their extinguishing. Plants with lower fire hazard characteristics should be planted near high-risk objects. For the territories of nature reserves, taking into account the fire-fighting properties of the most common herbs will minimize the risk of fires and their spread to forests. Because it is not possible to change the species composition in grasslands, which has a high fire risk, we have to use grass mowing before the end of a vegetation period where it possible and additionally introduce restrictive measures for mitigate the fire hazard.

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Habitat Selection of "Mad Cocks" of the Western Capercaillies Tetrao urogallus (Galliformes: Phasianidae) from the Fringe of the Range: A Case Study from Rila Mts. (Bulgaria)

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Abstract. We investigated the habitat selection of "mad" Western Capercaillies males from the isolated and threatened Rila-Rhodope population. These "mad" Capercaillies are highly territorially aggressive individuals, that approach and attack people; this abnormal behavior, rarely occurs beyond single individuals in one lek and no studies that we know of have been published on such cases. In 2014–2015, we tagged with GPS transmitters three adult "mad" males associated with one lek. We evaluated the temporal variation in the Capercaillie habitat selection based on three periods ("summer", "winter", and annual). Based on Manly's selection ratios (design III), at the home range scale, males used measured habitat variables non-randomly. Birds selected forests dominated by Scots pine (annually) and Macedonian pine (summer). Males used forest stands in the age class "81 to 120" years more than the availability. They highly avoided stands dominated by Norway spruce, bare rocks and ski slopes, as well as forest stands less than 81 years old (summer and annually). The birds demonstrated significant avoidance of flat and highly slopped terrains as well as those with northern and northwestern exposure. Notwithstanding the abnormal aggressive behavior of Capercaillie males and their affiliation with an edge population, the habitat selection of "mad cocks" in the Rila Mts. is consistent with the principal habitat preference of the species.

Key words: Capercaillie, "mad cocks", GPS devices, habitat utilization, resource selection.

Introduction

The organization of animals in space and time and the resource selection patterns are central questions of ecology. Habitat selection refers to a hierarchical process of behavioral responses that may result in the disproportionate use of habitats to influence survival and fitness of individuals (Block & Brennan, 1993; Jones, 2001). Understanding how animals establish their home ranges and how they select and use the resources within the home range is crucial for conservation and wildlife management efforts (Rechetelo et al., 2016).

The Western Capercaillie *Tetrao urogallus* L. (hereafter Capercaillie) is the

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largest and most dimorphic grouse of the boreal and montane forests of Eurasia (Rolstad et al., 1988; Storch, 2002). In the late winter and spring, males establish permanent ranges clumped around lek centers, where they display and interact with females in early spring, when the latter visit the lek to mate (Wegge & Larsen, 1987; Storch, 1997). After the display activity, in early summer, Capercaillie males move to distinct summer ranges and return to their leks in autumn and winter (Rolstad et al., 1988; Storch, 1995; Hjeljord et al., 2000). The Capercaillie requires extensive areas and is regarded as an important indicator of intactness and high structural diverse of mountain forest ecosystems (Grimm & Storch, 2000). The species is habitat specialist with affinity to old conifer forest (Storch, 2002). Due to its broad spatial and specific habitat requirements, the Capercaillie is a popular model species for the analysis of species–habitats interrelationships (Braunisch & Suchant, 2007). The Capercaillie is an anthropophobic species (Boev et al., 2007). However, throughout its range, some male birds might exhibit an abnormal aggressive behavior reaction, resulting in approach and attack of humans during the lekking period. These aggressive Capercaillie males are referred to as "mad cocks" (Storch, 2013). The aggressive abnormal behavior can be regarded as stress-coping responses (Jansen, 1986; Storch, 2013) but there are no indications if it is related to changes in habitat selection of birds.

The Capercaillie is a priority conservation species because much of its population is endangered and protecting Capercaillie habitat will benefit a host of other species of conservation concern (Suter et al., 2002; Pakkala et al., 2003; Storch, 2007; Mikoláš et al., 2016). As other endangered populations, Europe's southernmost Capercaillie's meta-population in the Rila-Rhodope Massif, Bulgaria and Greece, is threatened due to its low population size (709–1185 displaying males in Bulgaria; 350–500 individuals in Greece), isolation, habitat degradation, and decreasing distribution area (Storch, 2007; Boev & Nikolov, 2015; Plachiyski et al., 2018). While the habitat preferences of the Capercaillie are well recognized in Scandinavia (e.g. Rolstad et al., 1988; Rolstad & Wegge, 1989; Gjerde, 1991a, 1991b; Finne et al., 2000; Hjeljord et al., 2000), Scotland (e.g. Moss et al., 1979; Picozzi et al., 1996; Summers et al., 2004), Central Europe (e.g. Storch, 1993a, 1993b, 1995; Saniga, 2002, 2004; Thiel et al., 2007), and the Iberian Peninsula (e.g. Ménoni, 1991; Quevedo et al., 2006; Blanco-Fontao et al., 2010; González et al., 2012), the knowledge of the Rila-Rhodope population is based only on direct observations on the habitat use of the species. Because populations at the edge of a species' distribution use ecologically marginal habitats, observations made in one part of the range are not always applicable at the edge of a species' distribution (Quevedo et al., 2006).

We had the rare chance to identify and to equip with GPS transmitters three "mad" adult Capercaillie males associated with one lek. We used GPS telemetry to understand their habitat selection at home range scale. The main questions asked were: (a) What is the habitat selection in adult Capercaillie males with abnormally aggressive behavior?; (b) Did the habitat selection of adult Capercaillie males with abnormally aggressive behavior differ from the known for the species in general? In addition, the results obtained will indicate potential differences in habitat selection between birds from the southernmost edge and the others populations that will inform conservation-oriented management of the threatened subspecies.

Material and Methods

Study area

The field study was conducted in 2014–2015 in the northeastern part of Rila Mts., southwestern Bulgaria (Fig. 1). The study area (defined precisely as a result of the obtained telemetry data) encompassed 2162 ha of forests and glades from 1430 to 2330 m a.s.l. between the upper wellsprings valleys of the rivers Beli Iskar and Maritsa. The climate is a montane variant of the transitional, with mean temperatures of 0 to 14 °C in July and -7 to -4° C in January, with annual precipitation of 700–900 mm. The ground is usually covered with snow from mid-November to late April or May (depending on altitude and aspect) (Kopralev, 2002). The forest communities are represented by old (mean age 103 yrs. ± 35 SD; authors' unpubl. data), open, mixed and unmixed coniferous communities dominated by Scots pine *Pinus sylvestris* L., Macedonian pine *Pinus peuce* Griseb, Norway spruce *Picea abies* (L.) Karsten, dwarf Mountain pine *Pinus mugo* Turra and European silver fir *Abies alba* Mill (Fig. 2).

The study area was situated predominantly (1491.76 ha) within Rila National Park (IUCN category II), managed with priority on ecosystems diversity maintenance and wildlife protection. Hunting is banned. The remaining territory (670.36 ha) was managed by the ski zone concessionaire and local State Forestry. Forestry activities included predominantly harvesting, afforestation, protection against erosion and floods, and hunting. In the western part of the study area were the infrastructure facilities (ski slopes, lifts, etc.) of the "Borovets" Ski Resort. The resort is located between 1300 and 2560 m a.s.l. The skiing infrastructure included three ski centers with 58 km marked ski trails as well as ski roads, lifts (11.13 km), and roads between the ski centers. In addition, 20 km of mountain bike tracks were marked and used predominantly during the summer (Fig. 1).

Study of individuals and telemetry

In May and August 2014, three displaying "mad" males ("Birds 1–3") associated with one lek were captured at the lek using large fisherman's landing nets and by hand. According to beak depths (Moss et al., 1979; Wegge & Larsen, 1987), all birds were more than three years old. Two young males displaying at the lek's periphery were identified but not captured.

Each of the three adults was equipped on site with a "Bird 2A" backpack GPS tags (e-obs Digital Telemetry, Grünwald, Germany) and released. The devices were fitted to the bird's backs using a 5 mm Teflon ribbon and a 3 mm thick neoprene pad glued to the bottom of the device. The tags weighted 88 g (2.5–2.75% of bird's body mass), provided positional accuracy of about ± 10 m, and could save about 10000 GPS fixes. Timestamped readings were obtained every 1 hour (in the displaying season) and 2 hours otherwise. Data were downloaded wirelessly, with a maximum range of 200–500 m in dense forest, 15 km hilltop to hilltop, and 10 km by use of small plane.

Based on 18241 GPS fixes for the three individuals (for "Bird 1" – 6638 GPS fixes in 428 days; for "Bird 2" – 5678 fixes in 357 days; "Bird 3" – 5925 fixes in 362 days), we obtained the Minimum Convex Polygons utilized by each individual: "Bird 1" - Annual – 735.8 ha; Summer – 604.12 ha; Winter – 110.33 ha; "Bird 2" - Annual – 276.49 ha; Summer – 187.96 ha; Winter – 84.47 ha; "Bird3" - Annual – 1138.95 ha; Summer – 1117.51 ha; Winter – 136.61 ha.

Ethics statement

Strict protocols to minimize stress and potential injury to the birds was followed. Handling was minimal and all procedures were carried on site by a veterinarian. The scientific permit was issued by the Rila National Park Directorate (№ РД–СР–25/10.05.2014). At the end of the study, the tags were removed and the birds were released in good health.

Data analyses

To determine resource selection, we used Manly's selection ratios (w) for habitat selection design III, where individual animals are identified and both utilized and available resources (resource units) are measured at the scale of the individual (Thomas & Taylor, 1990; Manly et al., 2002).

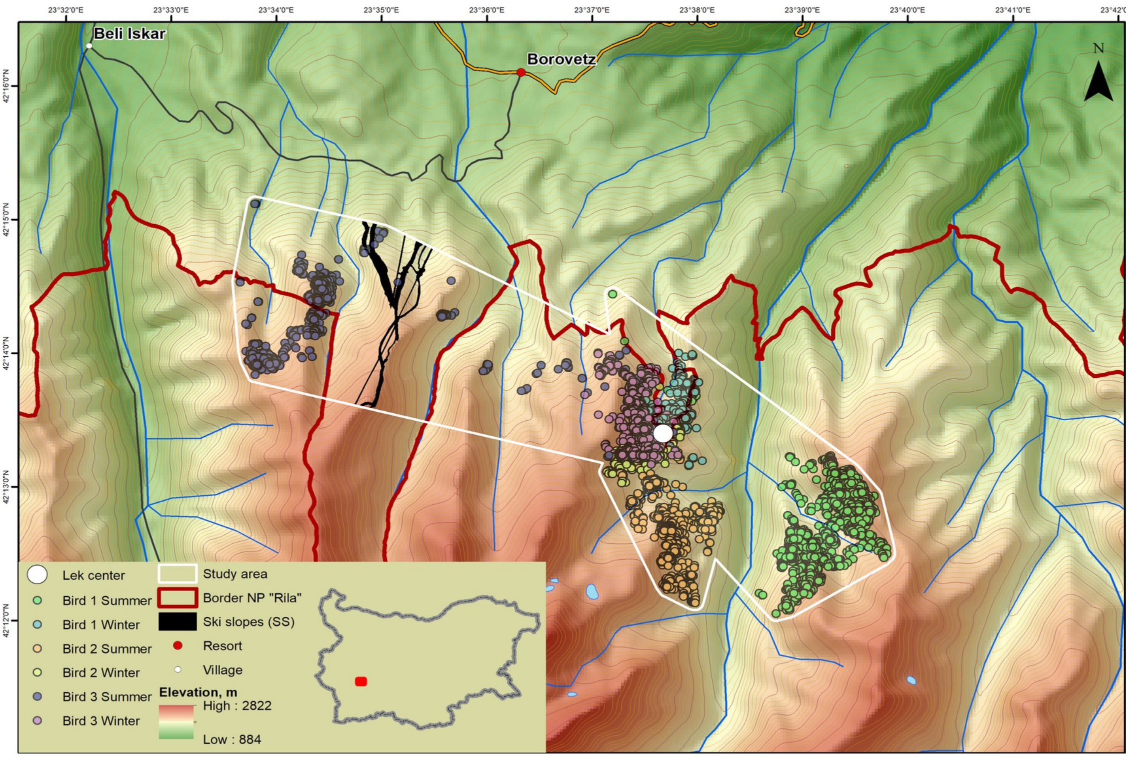

Fig. 1. Study area in Rila Mts., Bulgaria,
with temporal distribution of the positions of the three "mad" Capercaillie males.

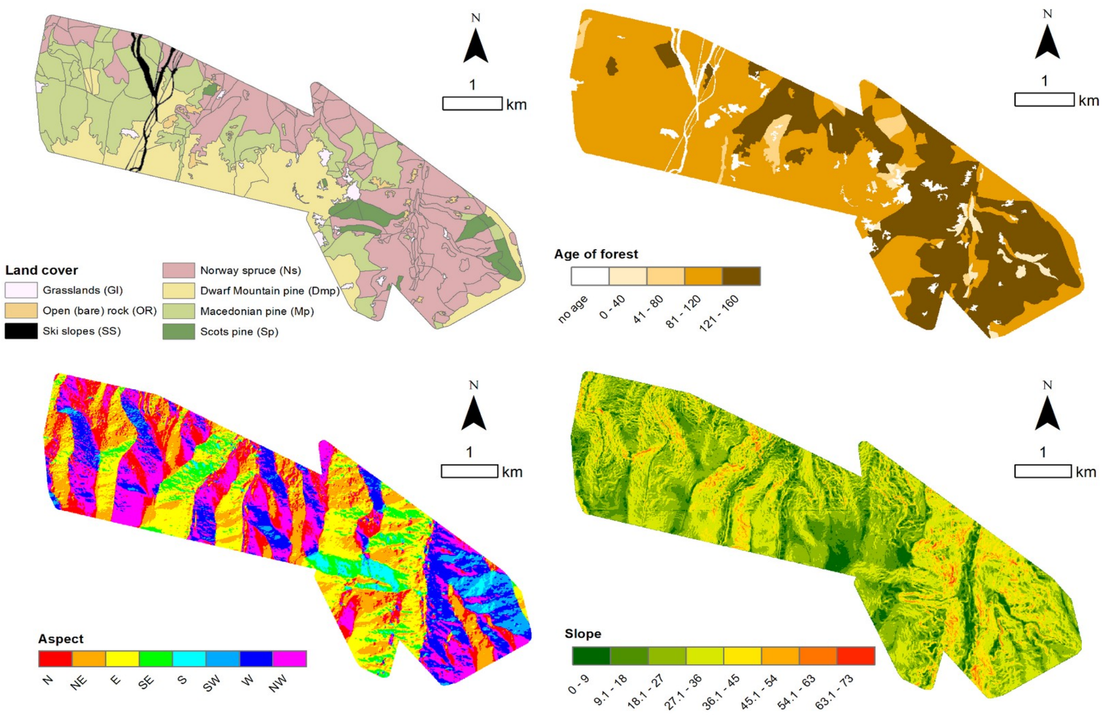

Fig. 2. Distribution of Land cover type, Age of Forest, Aspect and Slope within the study area.

The preference for a given habitat *j*, given by its selection ratio *wj*, is the number of times a Resource Unit (RU) within habitat *j* was used, divided by the available number of RUs for that given habitat. For our study, all the RUs (8 m pixels) within an individual's MCP were defined as available. According to the hypothesis of no particular selection or avoidance, *wj* should approach one, which means that the habitat *j* is used in proportion to its availability (Bengtsson et al., 2014). Habitat selection was defined as the difference in recorded use (distribution of GPS locations) to expected availability of RUs using a log-likelihood chi-square test (Khi2L) for overall habitat selection (Calenge, 2019; Ramesh et al., 2016). Capercaillie habitat preference was computed using Manly's habitat selection ratios combined with 90% Bonferroni simultaneous confidence intervals (Manly et al., 2002). We considered significant habitat preference if the lower *CI* limit was above 1 and significant avoidance if the upper *CI* limit was below 1. Habitat preference analyses were conducted by means of "adehabitatHS" package in R (Calenge, 2007) using a log-likelihood non-random statistic, setting α = 0.05. We used software ArcGIS ver. 10.2.1 (ESRI, 2014), in combination with R version 3.5.1. (R Development Core Team, 2013) and R Studio version 1.1.463-2009-2018 (RStudio Team, 2015).

Environment definitions

Dominant tree species and forest stand succession (Table 1, Fig. 2) were based on a digital layer from the Forest Data Base (FDB) (Executive Forest Agency, 2014). Non-forested habitats were added based on CORINE Land Cover level 3 (2012) and by hand-delineating habitats using current high-resolution aerial and satellite imagery (Google Maps Hybrid, Google, 2019; World Imagery Map, ESRI, 2019). The slope and aspect (Table 1, Fig. 2) were derived from a digital elevation model (DEM) with 8-m resolution. The layers were rasterized into 8-m pixels using the "feature to raster" conversion tool and "cubic convulsion" resampling in ArcGIS. All the environment variables were turned into categorical and reclassified either as equal size partitions or using "Jenk's natural breaks" function in ArcGIS. To calculate individual habitat, use and habitat selection, for each GPS location we determined in ArcGIS the respective habitat and surface characteristics.

To study the temporal variation in habitat selection, the data were partitioned conditionally into groups according to distinct movements of tracked birds for occupation of seasonal (winter–spring and summer–autumn) habitats. A distinct movement was defined as a directional movement of 1 km or more within a 5-day period (Rolstad et al., 1988). For each individual, we defined the pre-displaying and displaying period when the male birds inhabit permanently the lek and the territories around it and do not make distinct movements more than 1.5 km from the lek center, as "winter" (usually from December–February to the first week of June). The conditional "summer" (non-breeding) period is then the remainder of the year (usually second week of June to November–January). The annual period then combines the winter and summer.

Results

At the home range scale (using MCP), "mad" males used vegetation cover non-randomly (annual: Khi2L = 5738.89, df = 14, $p < 0.001$; winter: Khi2L = 3773.28, df = 13, $p < 0.001$; summer: Khi2L = 3646.32, df = 14, $p < 0.001$). They significantly selected forests dominated by Scots pine (annually) and Macedonian pine (in summer) (Table 2, Fig. 3). Habitats dominated by Dwarf Mountain pine were used in proportion to its availability, during summer and annually. Forests dominated by Norway spruce, open (bare) rocks and ski slopes were highly avoided (Table 2, Fig. 3). Ski slopes were only present in the summer home range of "Bird 3".

Roosters used forests of different stages non-randomly (annual: Khi2L = 3492.57, df = 8,

$p < 0.001$; winter: Khi2L = 2075.18, df = 8, $p < 0.001$; summer: Khi2L = 1670.1, df = 6, $p < 0.001$). They clearly avoided forest stands less than 81 years old in the summer and annually. Males used forest stands in the age class "81–120" years more than the availability but the selection was not significant (Table 2, Fig. 4).

Birds did not use slopes within the class "63.1–73.0" (Table 2). In the summer, males significantly preferred slopes within the class "27.1–36.0" and avoided "0–9", "9.1–18.0" and "54.1–63.0". The birds also demonstrated significant avoidance of flat terrains ("0–9.0") annually (Fig. 5, annual: Khi2L = 608.24, df = 17, $p < 0.001$; winter: Khi2L = 1148.37, df = 16, $p < 0.001$; summer: Khi2L = 906.54, df = 17, $p < 0.001$).

The roosters demonstrated significant preference for southeastern exposure during the winter and annually. They showed significant avoidance of northern exposure overall, as well as avoidance of north-eastern aspect during the winter and southern aspect during the summer (Fig. 6, annual: Khi2L = 4671.87, df = 18, $p < 0.001$; winter: Khi2L = 3909.04, df = 16, $p < 0.001$; summer: hi2L = 3095.84, df = 18, $p < 0.001$). Despite the high availability of northwestern exposures (19.2%, Table 1), the birds did not use them during the year (Table 2).

The large *CI*s for selection of variables indicate individual variation in the preference among the tagged birds.

Table 1. Availability of environmental variables within the study area. pix (n) = the number of the pixels (8×8 m) within the corresponding variable class. ha = variable class area in hectares. % = percent of pix (n) per variable.

Variable	Variable class	Available resource units		
		pix (n)	ha	%
Slope (Steepness, °)	0.0–9.0	16633	106.45	4.9
	9.1–18.0	59498	380.79	17.6
	18.1–27.0	108095	691.81	32.0
	27.1–36.0	102046	653.09	30.2
	36.1–45.0	38819	248.44	11.5
	45.1–54.0	10756	68.84	3.2
	54.1–63.0	1811	11.59	0.5
	63.1–73.0	173	1.11	0.1
Aspect (Exposure)	N	46607	298.28	13.8
	NE	63798	408.31	18.9
	E	65909	421.82	19.5
	SE	22878	146.42	6.8
	S	9144	58.52	2.7
	SW	15263	97.68	4.5
	W	49521	316.93	14.7
	NW	64711	414.15	19.2
Forest stand succession (Age, years)	0–40	6217	39.79	1.9
	41–80	9044	57.88	2.8
	81–120	187246	1198.37	58.1
	121–160	119750	766.4	37.2
Land cover: Dominant tree species	Norway spruce (Ns)	134312	859.6	39.8
	Macedonian pine (Mp)	95349	610.23	28.2
	Dwarf Mountain pine (Dmp)	78345	501.41	23.2
	Scots pine (Sp)	14251	91.21	4.2
Non-forest cover	Grasslands (Gl)	6215	39.78	1.8
	Ski slopes (SS)	4896	31.33	1.5
	Open (bare) rock (OR)	4463	28.56	1.3

Table 2. Average selection ratios (*Wi*) at the home range scale for three Capercaillie adult males. *Legend:* Ns = Norway spruce, Mp = Macedonian pine, Dmp = Dwarf Mountain pine, Sp – Scots pine, Gl – Grasslands, Ski slopes (SS), OR – Open (bare) rock, SE = standard error, ± 90% *CI* = confidence interval with lower and upper limits, Use % = habitat use, as % of Capercaillie locations within variable class from total number of locations per variable.

Variable	Variable class	Annual					Winter					Summer				
		Wi	SE	± 90% *CI*		Use %	*Wi*	SE	± 90% *CI*		Use %	*Wi*	SE	± 90% *CI*		Use %
Slope (Steepness, °)	0–9.0	0.45	0.15	0.10	0.81	2.6	0.39	0.27	-0.28	1.06	3.8	0.35	0.11	0.08	0.63	1.8
	9.1–18.0	0.93	0.05	0.80	1.06	16.7	0.95	0.10	0.70	1.20	27.5	0.54	0.11	0.27	0.81	9.4
	18.1–27.0	1.00	0.05	0.88	1.11	32.2	0.81	0.18	0.38	1.24	29.8	1.05	0.08	0.84	1.25	33.9
	27.1–36.0	1.14	0.11	0.88	1.40	33.4	1.44	0.35	0.58	2.29	26.2	1.27	0.07	1.10	1.43	38.3
	36.1–45.0	1.03	0.24	0.45	1.61	11.4	1.65	0.45	0.54	2.76	8.1	1.21	0.49	0.02	2.40	13.6
	45.1–54.0	1.06	0.24	0.48	1.64	3.3	3.81	1.99	-1.06	8.69	4.1	0.86	0.34	0.04	1.68	2.8
	54.1–63.0	0.73	0.25	0.13	1.33	0.4	3.08	1.20	0.14	6.01	0.5	0.52	0.06	0.38	0.65	0.3
	63.1–73.0	NA	NA	NA	NA	0.0	NA	NA	NA	NA	0.0	NA	NA	NA	NA	0.0
Aspect (Exposure)	N	0.48	0.2	0.1	0.9	7.1	0.32	0.3	-0.4	1.0	4.4	0.59	0.1	0.44	0.73	8.9
	NE	0.84	0.4	-0.3	1.9	20.6	0.33	0.2	-0.2	0.9	6.0	1.26	0.6	-0.09	2.61	30.5
	E	0.89	0.3	0.2	1.5	21.9	0.65	0.4	-0.2	1.5	19.8	0.96	0.4	0.07	1.85	23.3
	SE	2.04	0.2	1.6	2.5	22.3	2.45	0.5	1.3	3.6	44.6	0.72	0.3	0.07	1.36	7.2
	S	1.46	0.7	-0.2	3.1	9.5	2.06	1.2	-1.0	5.1	19.9	0.51	0.1	0.27	0.75	2.4
	SW	1.82	0.6	0.3	3.4	8.9	0.40	0.3	-0.4	1.2	0.9	2.58	0.8	0.72	4.44	14.3
	W	0.72	0.5	-0.4	1.9	9.8	0.59	0.4	-0.3	1.5	4.3	0.84	0.6	-0.51	2.20	13.4
	NW	NA	NA	NA	NA	0.0	NA	NA	NA	NA	0.0	NA	NA	NA	NA	0.0
Forest stand succession (Age, years)	0–40	0.41	0.21	-0.06	0.89	1.1	0.78	0.53	-0.42	1.97	1.9	0.26	0.06	0.14	0.39	0.6
	41–80	0.37	0.21	-0.11	0.84	1.1	1.28	0.68	-0.24	2.81	2.5	0.05	0.05	-0.05	0.16	0.1
	81–120	1.26	0.37	0.43	2.09	56.7	1.29	0.53	0.11	2.47	57.2	1.16	0.22	0.66	1.66	56.3
	121–160	0.83	0.38	-0.01	1.68	41.2	0.75	0.45	-0.27	1.77	38.5	0.92	0.33	0.18	1.66	43.0
Land cover:	Norway spruce (Ns)	0.38	0.09	0.16	0.60	16.2	0.21	0.11	-0.06	0.48	5.4	0.54	0.14	0.19	0.89	23.5
Dominant tree species	Macedonian pine (Mp)	1.75	0.40	0.77	2.73	44.9	1.44	0.63	-0.06	2.94	34.6	1.92	0.24	1.34	2.51	51.9
	Dwarf Mountain pine (Dmp)	0.97	0.30	0.23	1.72	16.7	1.06	0.16	0.67	1.45	25.9	0.58	0.31	-0.17	1.32	10.5
	Scots pine (Sp)	2.27	0.31	1.51	3.03	20.1	2.21	1.36	-1.04	5.46	29.4	1.92	1.36	-1.42	5.26	13.8
Non-forest cover	Grasslands (Gl)	0.58	0.51	-0.67	1.83	2.0	0.39	0.32	-0.38	1.15	4.5	0.13	0.07	-0.04	0.29	0.3
	Ski slopes (SS)	0.03	0.00	0.03	0.03	0.0	NA	NA	NA	NA	0.0	0.04	0.00	0.04	0.04	0.0
	Open (bare) rock (OR)	0.08	0.06	-0.08	0.24	0.1	0.19	0.01	0.17	0.21	0.2	0.07	0.05	-0.05	0.18	0.1

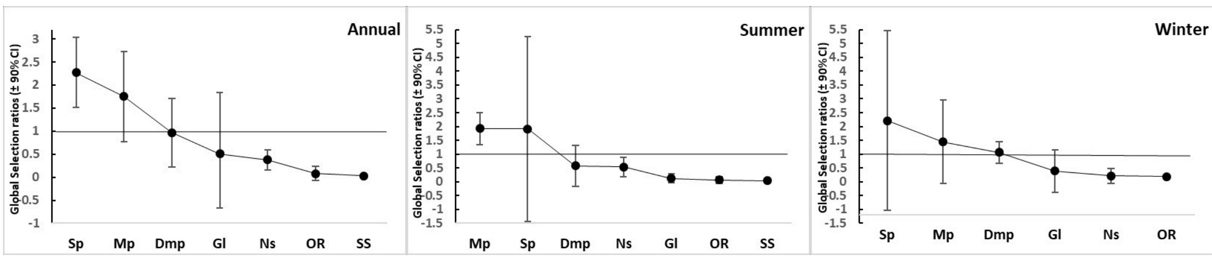

Fig. 3. Vegetation cover selection by Capercaillie males at the home range scale in Rila Mts., Bulgaria. Ns – Norway spruce; Mp – Macedonian pine; Dmp – Dwarf Mountain pine; Sp – Scots pine; Gl – Grasslands; SS – Ski slopes; OR – Open (bare) rock. Circles are Manley's Global Selection ratios' mean selectivity rates; vertical bars are Confidence Intervals. Global Selection ratios values > 1 denote habitats considered positively selected by the birds, while those in the interval 0–1 are considered to be avoided.

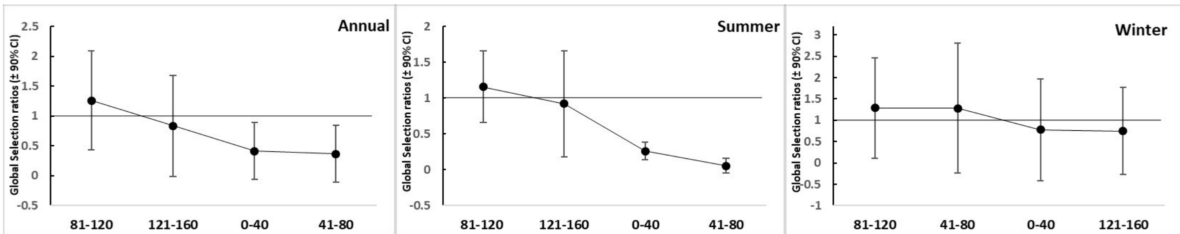

Fig. 4. Forest stage age selection by Capercaillie males at the home range scale in Rila Mts., Bulgaria. See Fig. 3 caption.

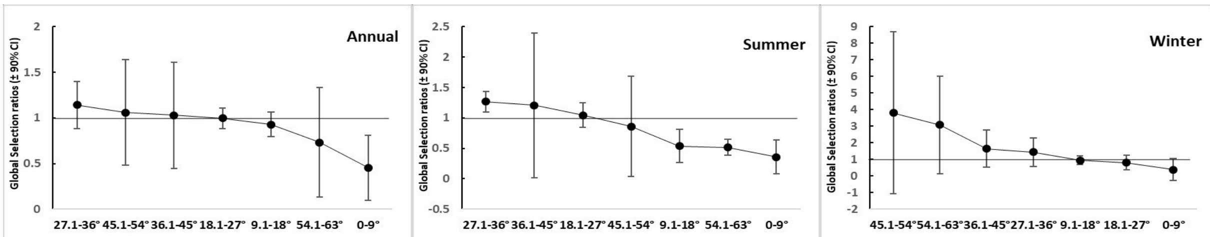

Fig. 5. Slope selection by Capercaillie males at the home range scale in Rila Mts., Bulgaria. See Fig. 3 caption.

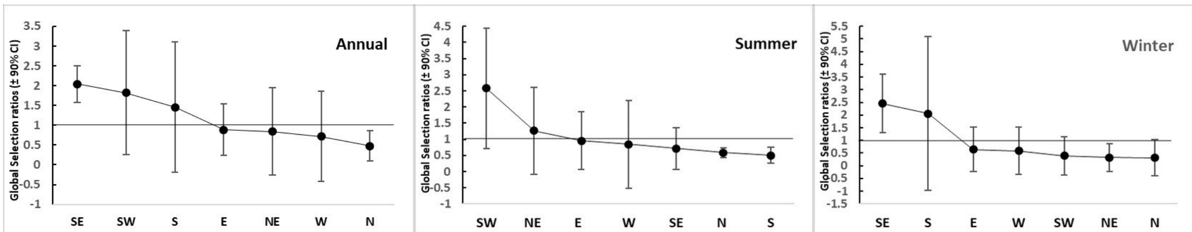

Fig. 6. Aspect selection by Capercaillie males at the home range scale in Rila Mts., Bulgaria. See Fig. 3 caption.

Discussion

Our results are the first to evaluate the habitat selection of "mad" Capercaillie males and the first to report on the Capercaillie habitat selection in the Rila–Rhodope meta-population.

Land cover selection, forest age, and the importance of old pine forests

The Capercaillie is adapted to climax forests, with leks reportedly confined to such mature, largely undisturbed habitats (Wegge & Rolstad, 1986). Males prefer open-spaced old forests throughout the year (Rolstad et al., 1988; Gjerde & Wegge, 1989; Rolstad & Wegge, 1987; Storch, 1993a, 1993b, 1997; Picozzi et al., 1996; Saniga, 2002) and avoid young forests (Rolstad et al., 1988; Gjerde & Wegge, 1989; Storch, 1993a). Our results agree with the studies from Scandinavia, central Europe and Scotland indicating general preference of old forests (81–120 yrs. old), as well as clear avoidance of forests ≤ 80 yrs. old, both annually and during the summer. The positive selection of forests 41–80 yrs. old and the decreased avoidance of 0–40 yrs. old forests in the winter could be attributed to the territorial behavior of roosters during displaying and the resulting spatial distribution. We suppose that the avoidance of forest patches 121–160 yrs. old is predominantly due to them being dominated by the highly avoided Norway spruce (represented by 65.67% within the age class). The association with old forest fulfils basic needs for food and movement

(Summers et al., 2004) as well as anti-predator behaviors (Finne et al., 2000).

Our results that Capercaillie adult "mad" males habitat selection in winter is principally determined by the strong preference of old and pine-dominated forests agree with previous studies (Wegge & Rolstad, 1986; Rolstad & Wegge, 1987; Gjerde & Wegge, 1989; Summers et al., 2004), whereas continuous, homogenous spruce-dominated stands (Gjerde & Wegge, 1989) as well as open spaces (Finne et al., 2000; Quevedo et al., 2006) are avoided. We confirm the statements of several authors on the habitat use of the species in Bulgaria based on direct observations (Simeonov et al., 1990; Ninov et al., 1994; Botev et al., 1998; Boev et al., 2007).

Due to harsh weather conditions, resource limitation and predators' pressure, the winter habitat selection of the Capercaillie should be evaluated in terms of predator avoidance as well as in an energetic context (Storch, 1993b; Gjerde, 1991a, 1991b). The Scots pine forest are the most common winter habitat for species because the combined availability of staple food and shelter enables the birds to minimize the duration of their activity periods (Lindén, 1981, cited in Thiel et al., 2007), and thereby to minimize heat loss and predation risk (Gjerde & Wegge, 1987; Storch, 1993b). Mature trees' needles have a higher energy content than those of younger (Lindén, 1984). Old forests, such as the Scots and Macedonian pine communities selected by the "mad" males in this study, are distinguished by their open structure (Storch, 1993a). Unlike the avoided spruce trees, old pine forests provide many single trees with broad less dense crowns, with large stout horizontal branches. These trees offer enough space to fly in and are more convenient for displaying, arboreal (day and night) roosting and winter feeding (Moss et al., 1979; Picozzi et al., 1996; Summers et al., 2004). The depredation avoidance strategies of Capercaillie males come down to '*detect predator → escape*' or '*detect predator → self-defense*' (Rolstad et al., 1988). Open forests and open tree structures (typical of old forests) are advantageous to detect predators early on and to assess if defense or escape is the appropriate strategy, as well as facilitate detection of females during the mating period (Moss et al., 1979; Botev et al., 1980; Summers et al., 2004; Finne et al., 2000; Thiel et al., 2007).

Predator avoidance patterns apparently change between day and night (Thiel et al., 2007). The major Capercaillie predators – martens and foxes (Schroth, 1991; Finne et al., 2000; Jahren et al., 2016), hunt mainly during the night. Martens willingly climb trees and move by jumping between tree crowns; thus, the Capercaillie preferred roosting sites, solitary trees or trees in open old stands, are usually isolated enough to prevent this means of access (Thiel et al., 2007). Within the study area the main tree edifiers (Scots pine and Macedonian pine) had a low projective coverage in the mature forests where they dominate; this formed complex communities with the participation of a well-developed Dwarf mountain pine layer and shrub phytocenoses dominated by Common juniper *Juniperus communis* L. During the day, when Capercaillie males spend more time moving on the ground for foraging, courtship and territory defense, this dense understory of Dwarf Mountain pine and Common juniper provides secure shelter against detection by predators.

Habitat utilization in the summer

Capercaillie males face two main problems in the summer – to get enough food to cope with the nutritional cost of the molt, and to avoid predation (Rolstad et al., 1988). In our case, as summer habitats Capercaillie "mad" males strongly selected old (81–120 yrs.) forests dominated by Macedonian and Scots pines, and avoided all other vegetation cover classes. Selected pine communities were characterized by low stocking density, determining availability of well-developed field layer with high abundance of Bilberry *Vaccinium myrtillus* L., Lingonberry *V. vitis-idaea* L., Wild strawberry *Fragaria vesca* L., Common juniper *Juniperus communis* L., Raspberry *Rubus idaeus* L., R. *hirtus* Walds & Kit. (Rusakova, 2015; Dimitrov & Rusakova, 2015) and other plant species, taking significant part in the Capercaillie diet (Cramp, 1985; Simeonov et al., 1990; Storch, 1993a). The presence of a well-developed ground layer provides shelter for birds when they feed and rest on the ground. Due to the dense canopy cover within much of the spruce forests, there is almost no underbrush and, in some cases, even a grass layer (Rusakova & Dimitrov, 2015). In parallel, spruce wood and mixed forests dominated by spruces are the preferred habitat by major Capercaillie predators such as the Pine marten *Martes martes* L. (Sidorovich, 2011; Spassov & Spiridonov, 2015). Therefore, the lack of a rich trophic basis and an increased depredation risk, possibly determine the avoidance of this class of forest stands by Capercaillie males.

Avoidance of open habitats

Avoidance of the open habitat types (bare rocks, grasslands and ski slopes) by the studied “mad cocks” could be considered a predator escape strategy or disturbance avoidance behavior. The Capercaillie is not a permanent inhabitant in stands near centers of human activities (frequented tourist paths, ski slopes, etc.), although these habitats also may fulfill its habitat requirements (Saniga, 2002). The Capercaillie probably use skiing areas only when undisturbed refuges are also available within their home ranges (Thiel et al., 2008). Taking into account the clear avoidance of ski slopes and the negligible presence of “Bird 3” in suitable habitats adjacent to the ski slopes during the summer (Fig. 3), we could speculate that there was no available undisturbed refuge within the ski zone in our study area. The openings within the forest are associated with habitat fragmentation and edge effect. The increase of grass and deciduous shrubs areas trigger higher predator pressure on ground nesting birds such as the Capercaillie (Rolstad & Wegge, 1989). Forest edges and openings are preferred hunting sites of the Northern goshawk *Accipiter gentilis* L. (Storch, 1993a), Pine marten and Red fox *Vulpes vulpes* L. (Clevenger, 1994; Sidorovich, 2011), all important predators of the Capercaillie (Botev et al., 1980; Gjerde & Wegge, 1989; Tornberg, 2001; Wegge & Kastdalen, 2007).

Topographical features

Some authors described preference for upper slopes with NE, E, and SE exposures, due to higher proportions of Bilberry on east-exposed slopes (Botev et al., 1993; Storch, 1993b). According to our results, at the home range scale, Capercaillie males most clearly avoided northern exposed slopes, but excluding the significant selection of SE slopes on annual basis; all other results are difficult to interpret. We consider the avoidance of northern exposures as a consequence of the distribution of the spruce-dominated community, associated with convex relief forms on northern exposures (Rusakova & Dimitrov, 2015). Regarding the exposure of the occupied terrains there is no regularity in the distribution of the *V. myrtillus* associations and it varies according to the area occupied and the habitats specificity (Vitkova & Rusakova, 2015). Therefore, our results are in accordance with the conclusions that the use of the different categories exposure is predestined by the aspect of the hills/valley axis as well as according to the local weather conditions (Saniga, 2002) and Capercaillie select habitats independently of the exposure (Rolstad & Wegge, 1987; Storch, 1993b).

Like Central European and Scandinavian studies, our survey indicates that hills and ridges are attractive Capercaillie display grounds (see Saniga, 2002) but are also preferred terrain features in the summer. Investigations from the Teisenberg Mtn. identified general preference for gentle slopes and avoidance of steep terrains in winter, spring and autumn that were less pronounced in summer (Storch, 1993a, 1993b). In contrast, our results indicate clear avoidance of flat terrains ("0–9.0"), selection of gentle slopes ("27.1–45.0") during summer, as well as preference of wider range ("27.1–63.0") of more steep slopes during winter. However, in general, gentle slopes ("27.1–36") seemed to be preferred, most strongly pronounced in the summer. The flat terrains within the study area were dominated by grasslands and dense Dwarf Mountain pine communities, avoided by Capercaillie males, which could explain why the birds were not utilizing them.

Implications for management

Habitat management for the Capercaillie should aim to provide forests dominated by (or with significant presence of) pines in the late successional stages with open structure, low stocking density, and well-developed understory with shrub and grass communities for foraging and shelter. Extensive cuttings, construction of firebreaks and development of other forest openings within potential Capercaillie habitats should be avoided.

Conclusions

Notwithstanding the abnormal aggressive behaviour of Capercaillie males and their affiliation with an edge population, the habitat selection of "mad cocks" in the Rila Mts. is consistent with the principal habitat preference of the species - old pine dominated forest associations distributed on hills and ridges. The selection of this principal habitat is similar in winter and summer. Thus, future studies can pool results of "mad" and normal individuals, and conservation initiatives should not separate between the two types.

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Modeling and Forecasting of Air Pollution with Particulate Matter PM2.5 Depending on Weather Conditions in Urban Areas - A Case Study from Plovdiv, Bulgaria

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Abstract. One of the main global environmental issues with direct impact on public health is preserving clean air in urban areas. The latest report by the European Environment Agency details the progress made towards meeting the air quality standards comparing it against the official requirements set out in EU directives. The objective of this scientific study is to model air pollution in the city of Plovdiv with fine particulate matter smaller than 2,5 micrograms per cubic meter (PM2.5). A CART method is used and based on best results obtained forecasts are made for future particulate matter pollution for 2 days ahead. Data from actual measurements taken between 1 February 2014 and 30 June 2019 and data from meteorological measurements are used. This study is an alternative to the official reports by the Regional Inspectorate of Environment and Water for the city of Plovdiv, which enables forecasting future pollution and its prevention.

Key words: Particulate matter PM2.5, air pollution, pollution forecast, Classification and regression trees (CART) method.

Introduction

Air pollution is a serious issue relevant to many European countries. According to the latest report by the European Environment Agency (EEA, 2019), progress has been made in achieving legal requirements on air quality. The most harmful air pollutants are fine particulate matter up to 10 microns in diameter (PM10) and up to 2,5 microns (PM2.5). The two types of pollutants cause severe respiratory diseases, lung damage, allergies, etc. Smaller particulate matter PM2.5 make their way directly to lung alveoli and cause diseases such as cancer, heart disease, etc. The main source of harmful particles are the burning of solid fuels by households and large factories, gases emitted by motor vehicles, especially diesel engines, etc. It has been proven through epidemiological and toxicological studies that particulate matter are harmful to human health. For this reason, it is necessary to monitor concentration levels and to take the necessary measures to meet EU requirements and to eliminate any violations.

There are numerous scientific studies about the impact of harmful emission on human health, as well as studies on mortality related to these problematic pollutants (Herman et al., 2020; Maji et al., 2017; Cox et al., 2013).

The environmental issue of air pollution is subject to studies around the world. The goals of the researchers include finding

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pollution sources, namely (Saraga et al., 2019; Ehsanzadeh et al., 2016). The Box-Jenkins method has been used to build stochastic models (Jian et al., 2012), which consider the influence of meteorological factors on PM2.5 and PM10 emissions. Various types of methods are used to study air quality through modeling using artificial neural networks, CART, fuzzy sets and others (Ivanov & Gocheva-Ilieva, 2013; Prakash et al., 2011).

In the past decade, urban air pollution in Bulgaria has been a serious problem. Constant control and monitoring of harmful emissions is performed by the National Environment Agency. Plovdiv and Sofia are among the cities with relatively high levels of PM10 pollution in Europe in recent years (WHO, 2015). In Bulgaria, there is relatively little scientific literature to analyze the condition of atmospheric air in urban environments (Gocheva-Ilieva & Ivanov, 2019; Gocheva-Ilieva et al., 2019; Veleva & Zheleva, 2018).

The main objective of this paper is to study the dependence of PM2.5 concentration on meteorological conditions by building CART models to be used as the basis of short-term air pollution forecasts. The proposed approach can be an independent corrective to aid local governments and communities in warning about exceeded permissible limits for fine particulate matter.

Material and Methods

Study area. The city of Plovdiv is the second largest city in Bulgaria after the capital Sofia. Its population is around 350,000 people. It is situated along the two banks of the Maritza river. The climate in the city is transitional-continental with an average annual temperature of 12,3 °C. The average annual relative air humidity is 73%, which is highest in December. During the cold months there are often fogs. Plovdiv is a city with weak winds and low altitude.

Data and initial statistical processing. The analysis performed on the state of air in the city of Plovdiv is based on actual measurements by an automated measuring station "Kamenitza" located in the city center. The station is located in a predominantly residential area with moderate vehicle traffic. The data used for the scientific study are average daily measurements of the PM2.5 pollutant over a period of about 5 and a half years – from 1 February 2014 to 30 June 2019. This paper also utilizes measurement data for 8 meteorological indicators; these are: minimum average daily temperature (minT, °C), maximum average daily temperature (maxT, °C), wind speed (wind_speed, km/h), wind direction (wind_direction, rad(WDI)), precipitation (precip, %), air humidity (humidity, %), atmospheric pressure (pressure, mb) and cloud cover (cloud_cover, %). In order to account for the periodic nature of the variable wind_direction it is transformed using the formula for WDI:

$$WDI = 1 + \sin\left(wind\_direction + \pi / (k-1) \right),$$

where k is the number of different wind directions and in this case k=16.

The autoregression variables of PM2.5 , minT, maxT and wind_speed are used to build the models. They are denoted respectively by PM2.5 <1> - these are the fine particulate matter concentrations measured on the previous day, minT<1>, minT<2> - values of the minimum temperature measured on the previous day and two days before, maxT<1> - maximum average daily temperature on the previous day and wind_speed<1> - wind speed on the previous day. Since the dependent variable is a time series, variables taking into account time are also dded – month and month_day.

CART Method. CART (Classification And Regression Trees) method was developed in 1984 in a monograph (Breiman et al., 1984). The method is actively used in almost all scientific fields for the classification and investigation of dependencies. With a quantitative type variable y and independent predictors $X = (X_1, X_2, ..., X_p)$ the CART algorithm builds a binary tree structure for the observations by splitting the multivariate case space into non-intersecting regions. Starting from the root of the tree, which contains all cases, at each step, the cases are split into two upon satisfying a preset rule of the type $x_k \le \theta_{k,j}$. If the rule is met, the cases are classified in a left child node of the current node, and the rest – into the right child node. The process of growing the tree is ceased according to criteria specified by the researcher, for example minimum number of cases in a parent node (m_1) and in a child node (m_2), depth of the tree, etc (Izenman, 2008; Steinberg & Colla, 1995).

The model $\hat{y}$ can be written down as:

$$\hat{y}(\mathbf{X}) = \sum_{l=1}^{m} \hat{y}(\tau_l) I_{[X \in \tau_l]}, \; \hat{y}(\tau_l) = \overline{y}_l, \; X \in \tau_l,$$

where τ_l, $l = 1, 2, ..., m$ are terminal nodes of the tree, m – their number, $I_{[X \in \tau_l]}$ is the function, which traces the route from the root to the terminal node, $\overline{y}_l$ is the mean value of cases, classified in the terminal node τ_l, which is also the predicted value.

The quality of the models is assessed using the Root Mean Square Error (RMSE) and the coefficient of determination according to formulas:

$$RMSE = \sqrt{\frac{1}{N}\sum_{t=1}^{N}(y_t - \hat{y}_t)^2}, \quad R^2 = \frac{\sum_{t=1}^{N}(\hat{y}_t - \overline{y})^2}{\sum_{t=1}^{N}(y_t - \overline{y})^2}, \quad t = \overline{1, N},$$

where N is the number of cases, $\hat{y}_t$ is the predicted value at any given moment t, $\overline{y}$ is the mean value of y.

When selecting the best model, the guiding principle is to achieve the least root mean square error and the highest value for the coefficient of determination R^2, i.e. the degree of approximation of the model to actual data.

Modeling is performed using Salford Predictive Modeler suite (SPM, 2016) and IBM SPSS (IBM Corp, 2013).

Results

Table 1 presents the results of the initial processing of the data. The total number of observations for the pollutant PM2.5 is N=1948, and the missing data are 27, which represents about 1,37%. There are no missing values in the data about meteorological measurements.

Table 1. Descriptive statistics of the initial data on PM2.5 concentrations in the city of Plovdiv.

Statistics	PM2.5 $\mu g/m^3$	minT (°C)	maxT (°C)	wind_ speed (*km/h*)	WDI	precip (%)	humi dity (%)	pres sure (mb)	cloud_ cover (%)
N	1948	1975	1975	1975	1975	1975	1975	1975	1975
N miss	27	-	-	-	-	-	-	-	-
Mean	25.53	6.69	17.07	6.46	2.88	1.04	0.677	1017	0.30
Minimum	2.3	-17	-10	2	0.9	0	0.31	991	0
Maximum	253	22	40	26	11.6	47.6	0.99	1039	1

During the examined period, a maximum of 253 $\mu g/m^3$ was reached for PM2.5, while the legal requirements do not permit exceedance of more than 25 $\mu g/m^3$ during the year. (EC, 2008; 2015). The permitted values for PM2.5 concentrations in the air are effective as of 1 January 2015 with a maximum average annual limit of 25 $\mu g / m^3$, without any exceedance throughout the year. As of 1 January 2020, average annual limits of 20 $\mu g / m^3$ are in force. For the years between 2014 and 2019, the average values are respectively 28,79; 29,35; 27,44; 27,32; 19,17; 17,55 $\mu g / m^3$. The analysis indicates a systemic exceedance of the permissible limit.

Fig. 1 presents a graph of the observed average annual data over the examined period. The horizontal line in the graph corresponds to the average permissible limit of 25 $\mu g / m^3$. Numerous exceedances of the specified legal requirement are observed. The main reasons for the high levels of harmful emissions given in the regional reports are the use of solid fuels for heating during the winter period and the poor quality of fuels.

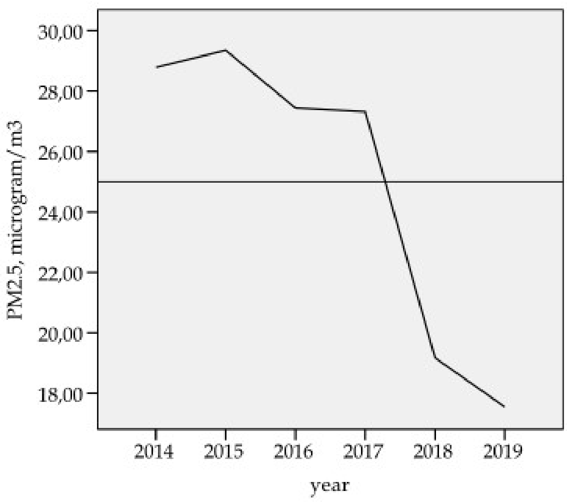

Fig. 1. Graph of measured average yearly data on PM2.5 concentrations in the city of Plovdiv. The horizontal line indicates the permissible limit of 25 $\mu g / m^3$

Construction of CART models and their analysis. When building CART models, the main goal is to find the dependence of the high levels of the PM2.5 air pollutant on meteorological conditions. Limitations are set on the minimum number of cases in the parent node (m_1) and the child node (m_2). Following numerous preliminary analyses, the values of 10 are preset for m_1 and 5 for m_2. The obtained models are denoted by $M(m_1, m_2)$.

Out of a large number of models built during the study, 3 optimal models are selected that match the requirements for best fit. Table 2 presents their basic characteristics, which indicate the extent to which the selected models approximate the actual data, the number of end nodes, and the errors reported during their build. According to the above conditions for best model, Table 2 shows the conclusion that M3 provides the best results. It describes over 82% of actual data and has the lowest RMSE=10,519.

Table 2. Summary of the obtained optimal CART models for PM2.5.

Varia ble	Model	(m1, m2)	Number of Terminal Nodes	R2 Lea rn	RMSE
PM2.5	M1	(10.5)	34	0.794	11.297
PM2.5	M2	(10.5)	47	0.814	10.727
PM2.5	**M3**	(10.5)	51	**0.821**	**10.519**

Table 3 shows the percentage ratio of meteorological variables, included in the build of the three optimal models selected. The influence of the most important indicator assumes a weight of 100%. Some of the meteorological conditions are excluded when building the models since during the analyses it was found that these have no influence. The excluded meteorological data are precipitation quantity, atmospheric pressure and cloud cover. It is obvious that for all models, the measured PM2.5 concentrations on the previous day have the greatest weight and the next most stable factor in terms of influence is the minimum average daily air temperature two days before. It can be concluded that the selected models demonstrate stability in their structure. For the best model, it was found that the greatest influence (100%) on harmful emissions is that of PM2.5 concentrations measured on the previous day, followed by the measured minimum temperature two days before, with the third being minimum temperature on the previous day, etc.

Fig. 2 shows the general structure of the obtained regression tree using model M3, which includes 51 end nodes as per the CART tree build rules. Terminal node 51 classifies the cases with the highest values of PM2.5. The rules to reach this node starting from the tree root are as follows: PM2.5<1> >48,78 $\mu g/m^3$; PM2.5<1> >128,14 $\mu g/m^3$; PM2.5<1> >184,31 $\mu g/m^3$. The predicted value of each terminal node is the arithmetic mean of cases in it.

Table 3. Variable importance of the predictors for obtained models.

Predictors	Scores		
	M1	**M2**	**M3**
PM2.5<1>	100	100	100
minT<2>	19.96	20.54	20.85
minT<1>	19.44	20.22	20.27
humidity	12.91	12.85	13.09
weather	9.90	11.09	10.70
wind_speed<1>	9.80	9.74	9.91
minT	5.55	6.51	6.62
month	5.25	-	5.34
maxT	3.18	-	-
wind_speed	2.95	-	-
month_day	-	7.93	-
maxT<1>	-	5.63	5.33
wind_speed<2>	-	-	6.84

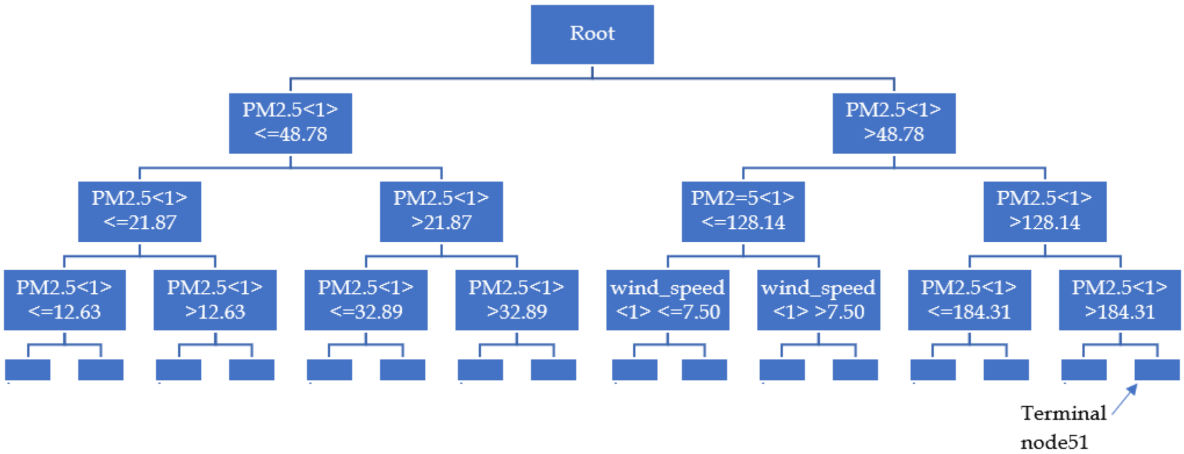

Fig. 2. Upper part of topology of the binary regression CART tree of the model M3.

Application of the models for forecasting future concentrations. Fig 3 shows a graphic of the predicted PM2.5 concentrations for 2 days ahead (1 and 2 July 2019), using data models up to 30 June 2019. The forecasts for the two days are made using actual measured data, which are not included when building the CART model but these are compared against the values predicted by the selected model. The graphic shows that the selected model approximates actual measured values of the air pollutant.

Discussion

The study uses actual measurements as data for air pollution with PM2.5 in the city of Plovdiv. The data are for the period from 1 February 2014 to 30 June 2019. The analysis shows that during the studied period there are many exceedances of the limits for healthy air quality. Modeling and forecasting the atmospheric pollutant are performed using the classification and regression trees (CART) method. Three optimal CART models of the level of fine particulate matter smaller than 2,5 $\mu g / m^3$ depending on meteorological conditions are built and analyzed. The graphic forecast of the model compared against actual measured PM2.5 concentration show very good approximation. It is found that the selected model displays very good qualities for short-term forecasts of particulate matter air pollution for 2 days ahead.

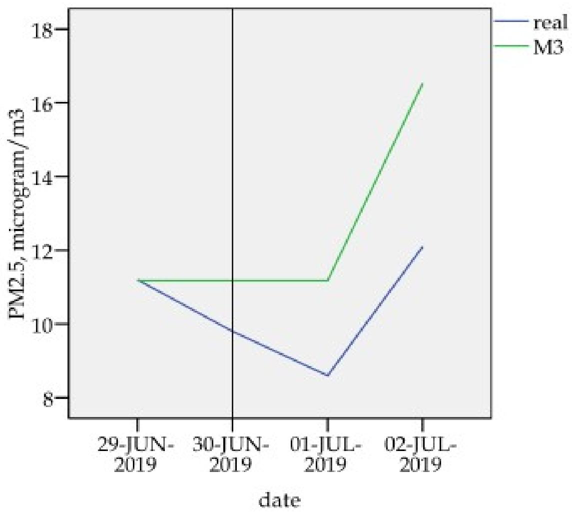

Fig. 3. Comparison of measured and predicted PM2.5.

According to the reports by the Regional Inspectorate of Environment and Water in Plovdiv on air quality (RIOSV Plovdiv, 2019), significant influence on the high levels of particulate matter in the city is due to temperature inversions, large number of days without any wind and the fogs. These factors cause accumulation and longer duration of suspension of pollutants in the air. Vehicle traffic emissions also have a significant negative impact on air quality.

Conclusions

This study shows the results of a statistical analysis of air quality in the city of Plovdiv. The obtained results show that legal limits that guarantee public health are exceeded. The proposed approach demonstrates the influence of meteorological conditions on air pollution. The selected method is suitable for forecasting these in order to prevent and control harmful emissions in the air within urban areas.

The presented study shows that the selected approach is suitable for predicting future pollution and its prevention.

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Metal Pollution Assessment in Sediments of the Bulgarian Black Sea Coastal Zone

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Abstract. The present study was conducted to assess the pollution status of the coastal sediments of the Bulgarian Black Sea coastal zone using sediment pollution indices. Eleven sites and twenty-two stations from monitoring grid were selected for the collection of samples. The heavy metal concentrations were used to calculate: geoaccumulation index (Igeo); enrichment factor (EF); contamination factor (CF); modified degree of contamination (mCd); pollution loading index (PLI). In addition, ecotoxicological level of trace metals in sediments was also assessed by comparing with the numerical sediment quality guidelines (SQGs). Mean ERM quotient (mERM-Q) and mean PEL (m-PEL–Q) were applied for assessing the potential effects of multiple heavy metal contamination in sediments. The pollution level assessed by indices related to background level of the studied sites ranged from unpolluted to moderate and only in one case considerably polluted due to heavy metals, mainly Pb and Zn. Ecotoxicological risk was assessed as low and moderately low due to the presence of Pb, Cu, Ni. The northern Danube influenced area of the Bulgarian coastal zone was categorized according to the applied indices as slightly polluted with Zn and Pb. The sediments from the "hot spot" points were with higher accumulation of Ni, Cu and Pb.

Key words: Black Sea, metal pollution, coastal sediments, sediment quality indices, sediment quality guidelines.

Introduction

Black Sea ecosystem has been heavily impacted as a result of anthropogenic activities in coastal zones and watersheds. The Black Sea is a unique semi-enclosed basin that receives a great river inflow and has a single connection with the Mediterranean Sea through the narrow Bosphorus Strait. The Black Sea area is 461 000 km^2 and its drainage area extends over more than 2 200 000 km^2. Almost one-third of the entire land area of the continental Europe drains into it which has a detrimental effect on its health (Mee, 1992). Due to the slow rate of water renewal, the Black Sea is particularly vulnerable to pollution.

Sediments may act as sinks and sources of metals to the overlying water column and biota. Contaminated sediments can decrease water quality and ecological diversity, ecological functioning, and aesthetic properties of waterbodies (Davis & Fox, 2009). Sediments play a significant role as sensitive indicators for monitoring metal contamination in aquatic systems (Ariman & Bakan, 2008). Very limited

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information exists concerning trace metals in the sediments of the Bulgarian Black Sea coastal zone.

The main objective of this investigation was to assess the pollution status of the coastal sediments in the Western Bulgarian Black Sea part based on pollution indices.

Material and Methods

Sampling was carried out on 11 stations according to EU Water Framework Directive (EC, 2000) monitoring programme at 11 sites within the 1 nautical mile zone of the Black Sea western part during surveys in 2005 and 2016. The sediments were collected on the same network of monitoring stations during both surveys, but with slightly changed positions for some stations in 2016 (Fig. 1). Depths of the stations varied between 13 and 37 meters and the distance of the stations to the shore was in the range of 1 nautical mile.

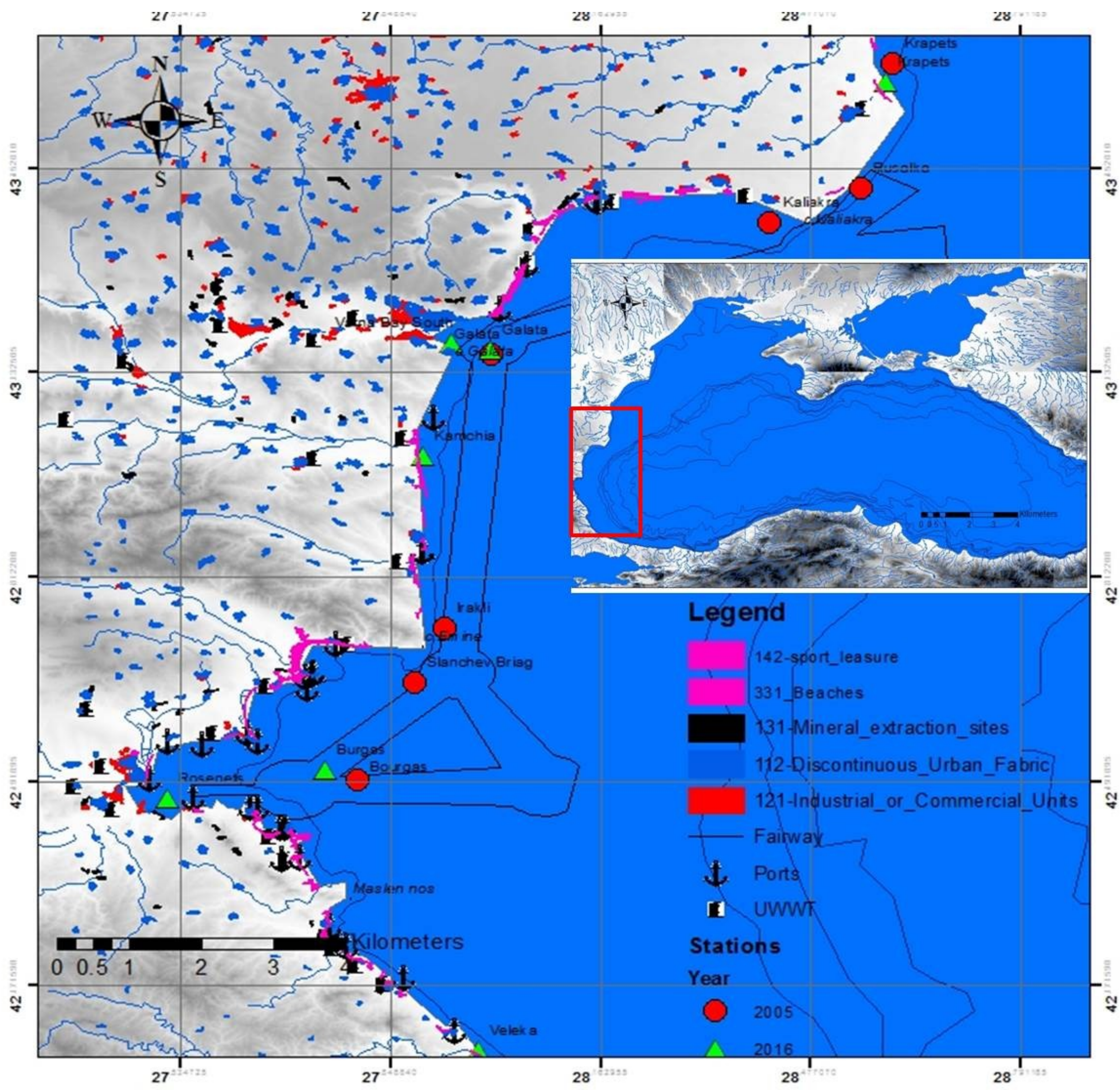

Fig. 1. Area of investigation with sampling stations (2005 and 2016) and anthropogenic pressures as Corine Land Cover (CLC) zones: Discontinuous Urban Fabric, Industrial or Commercial Units, Mineral extraction sites, Beaches, dunes, sand, Sport and Leisure facilities ("CLC Bulgaria", 2012); ports, fairway and UWWT.

The stations are in an area under the impact of different pressures. In the northern part (stations Krapets, Rusalka, Kaliakra) the influence of the Danube freshwater input along the Bulgarian Black Sea coast is the strongest. The local pressure comes from currents from the north, pollution from the waste treatment and maritime transport.

Station Kamchia is in front of the mouth of the biggest Bulgarian river Kamchia. Station Slanchev Bryag is in front of one of the biggest resorts but is in the protected sand bank "Cocketrice", an area with the highest biological diversity (Konsulova et al., 2010). Varna (st. Varna, st. Galata) and Burgas (st. Burgas, st. Rosenets) bays, and the Kamchia river mouth are exposed to direct or indirect influence of industrial and municipal discharges, port operations, tourism development and also inputs from diffuse sources.

Surface sediment samples (0-5 cm) were collected using a 0.1 m^2 Van Veen grab, placed in polyethylene bags, refrigerated and transported to the laboratory. The investigated parameters were Zn, Pb, Ni, Hg, Cu, Cd and Al. Data for 2005 were provided by the Black Sea Basin Directorate - Varna. The samples collected by IO - BAS in 2016 were analyzed in an accredited laboratories in Hungary (Biokor & Wessling) using ICP - OES technique.

The measured element concentrations were used to calculate the pollution indices.

To estimate enrichment of metal concentrations above background concentrations, the index of geoaccumulation (Igeo) as proposed by Müller (1969) was used:

$$Igeo = \log_2 Cn / 1.5 Bn,$$

where *Cn* is the measured concentration of the element *n* in the samples and *Bn* is the geochemical background value of the element *n* in the average crust. Background values and legal norms for the Bulgarian Black Sea sediments do not exist therefore the average crust value (Taylor, 1964) were used.

This index is basically a single metal approach to quantify metal pollution in sediments when the concentration of toxic heavy metals is 1.5 times greater than their lithogenic background values. The factor 1.5 was used to compensate possible variations of the background values which were attributed to lithologic variations in the sediment (Abrahim & Parker, 2008; Stoffers et al., 1986).

To identify anomalous metal concentration, geochemical normalization of the heavy metals data to conservative elements, such as Al, Fe, and Si was employed (Din, 1992; Windom et al., 1989). In this study, Al was used as a reference element to differentiate natural from anthropogenic components. The enrichment factor (EF) value of metal was defined as follows:

$$EF = \frac{(C_X / C_{Al})\,sample}{(C_X / C_{Al})\,background} \;,$$

where $(C_x/C_{Al})_{sample}$ is the ratio of sediment sample concentrations of the heavy metal and Al concentrations; $(C_x/C_{Al})_{background}$ is the ratio of backgrounds of heavy metal and Al concentrations. The average crust concentration of Al (Taylor, 1964) was taken as background. Evaluation of pollution impact (2016) using enrichment factor regarding to Pb, Cu and Ni was done.

The pollution load index (PLI) and contamination degree based on computed contamination factors were used as a tool for assessment of the degree of overall contamination in surface sediment layer. The overall contamination of sediments was assessed based on contamination factor (CF), (Hakanson, 1980). The contamination factor (CF) was calculated according to the formula:

$$CF = \frac{measured\ concentration}{background\ concentration} \;.$$

Taylor's (1964) crustal abundance was used as background concentration.

Pollution load index (PLI) was evaluated following the method proposed by Tomilson et al. (1980). PLI could be calculated by the following equation:

$$PLI = (CF1xCF2x.....CFn)^{1/n},$$

where n is the number of metals and CF is the contamination factor.

Degree of contamination (Cd) given by Hakanson (1980) was based on seven metals and one organic contaminant. The modification of the method (mCd) to avoid these limitations was proposed by Abrahim & Parker (2008) who took into consideration all the CFs for a given set of pollutants divided by the number of the analysed pollutants, e.g. heavy metals - n (Abrahim & Parker, 2002):

$$mCd = \frac{\sum_{i=1}^{i=n} CF^i}{n}.$$

To evaluate sediment contamination and potential ecotoxicological effects associated with the observed contaminant concentrations, two sets of SQGs developed by National Oceanic and Atmospheric Administration (NOAA) for marine and estuarine ecosystems (Long & MacDonald, 1998; MacDonald et al., 2000) were used. The chemical concentrations corresponding to the 10th and 50th percentiles of adverse biological effects were called the effects-range-low (ERL) and ERM guidelines, respectively (Long et al., 1995). Another sediment quality guideline used to assess the ecotoxicology of sediments was the TEL and PEL approach. This approach is based on the relation between measured concentrations of metals and observed biological effects, such as mortality, growth or reproduction of living organisms. Threshold effect level (TEL) refers to the concentration below which adverse effects are expected to occur only rarely and probable effect level (PEL) indicates the concentration above which adverse effects are expected frequently to occur (Saleem et al., 2013).

The ratio of concentration to background gives a base to evaluate sediment quality but it provides little insight into the potential ecological impact of contaminants (Gao, 2012). Based on the fact that heavy metals always occur in sediments as complex mixtures, the mean PEL and ERM quotient method was applied to determine the possible biological effect of combined toxicant groups by calculating mean quotients for a large range of contaminants using the following equations:

$$m\text{-}ERM\text{-}Q = \frac{\sum_{i=1}^{n} Ci/ERMi}{n},$$

$$m\text{-}PEL\text{-}Q = \frac{\sum_{i=1}^{n} Ci/PELi}{n},$$

where Ci is the concentration of element i in sediments, ERMi and PELi are the guideline values for the element i and n the number of metals.

Results and Discussion

The mean concentrations of elements at different sites, background concentration in the continental crust (Taylor, 1964) and threshold effect sediment quality guidelines for metals (Long et al., 1995) are given in Table 1.

The Igeo method was applied to calculate the metal contamination levels. Müller (1979) defined seven classes of the geo-accumulation index: class 0 (Igeo ≤ 0; uncontaminated), class 1 (Igeo = 0-1; uncontaminated to moderately contaminated), class 2 (Igeo = 1-2; moderately contaminated), class 3 (Igeo = 2-3; moderately to strongly contaminated), class 4 (Igeo = 3-4; strongly contaminated), class 5 (Igeo = 4-5; strongly to extremely contaminated) and class 6 (Igeo > 5; extremely contaminated).

Based on Igeo, it was found that the studied samples could be considered uncontaminated with Ni and Cu. Among the five metals studied, Pb had the highest Igeo values. 54% of the stations were unpolluted and 46% were moderately to strongly contaminated. As the Igeo class of Pb at st. Krapets (2005) and st. Kaliakra (2005) was „moderately to strongly contaminated" and for st. Rusalka (2005), st. Galata (2005), st. Rosenets (2016), st. Bourgas (2016) was „moderately contaminated".

Sediments from only one st. Kaliakra showed moderately polluted status with respect to Zn.

Sediment quality classes were evaluated using the EF value. The EF values were interpreted as suggested by Birch (2003) where EF < 1 indicated no enrichment, EF < 3 was minor; 3 ≤ EF ≤ 5 was moderate; 5 ≤ EF ≤ 10 was moderately severe; 10 ≤ EF ≤ 25 was severe; 25 ≤ EF ≤ 50 was very severe; and EF > 50 - extremely severe.

According to the EF, contamination was assessed as generally "moderate" regarding Pb and "minor" for the other elements. The lowest EF values were determined between 0.6 and 2.3 for the most of analytes. The results range of EF values was between 2.8 - 7.5 for Pb, considered as a moderate severe enrichment. Maximum enrichment Pb level was found at Varna Bay station and minimum - at Veleka station.

According to Hakanson (1980) CF < 1 indicates low contamination; 1 < CF < 3 - moderate contamination; 3 < CF < 6 - considerable contamination; and CF > 6 - very high contamination.

In the present study, the contamination factors for Zn were low except st. Kaliakra where contamination was in the moderate category.

The contamination factor for Zn was in the low category at all sites in both sampling sessions (2005 and 2016), except st. Kaliakra where contamination was in the moderate category. In the present study, the contamination factors for Pb were low for 57% of the stations. Five stations (Rusalka, Galata, Varna Bay, Rosenets and Burgas Bay) showed moderate contamination and one (Kaliakra) – considerable contamination.

PLI < 1 indicates no pollution due to anthropogenic activities and PLI > 1 indicates pollution (Harikumar et al., 2009). In the present study, the pollution load index for all studied sites showed no human associated pollution with PLI < 1.

The classification of the sediments according to the modified degree of contamination (mCd) is the following: mCd < 1.5 - zero to very low degree of contamination; 1.5 < mCd < 2 - low degree of contamination; 2 < mCd < 4 - moderate degree of contamination; 4 < mCd < 8 - high degree of contamination; 8 < mCd < 16 - very high degree of contamination; 16 < mCd < 32 - extremely high degree of contamination; mCd ≥ 32 – ultra high degree of contamination.

mCd can make an integrated assessment of the overall contamination with metallic pollutants. It was found that the mCd values ranged from 0.3 to 1.9. The minimum values were observed at stations Krapets (2016) and Veleka and the maximum at Varna Bay. Four out of 14 observations had mCd values below 1.5, indicating 'nil to very low degree of contamination'. The rest of the stations indicated 'low degree of contamination' with mCd values from 1.03 to 1.5.

The sediment quality guideline (MacDonald et al., 2000) was used to categorize element concentrations in sediments based on the toxicity to benthic organisms.

When comparing the results of the present study with ERL and ERM values, it was observed that Cd, Hg, and Zn at 100% of sampling stations were below the ERL values (1.2, 0.15 and 150 mg/kg, respectively) which indicated that those metals were not likely to have adverse effects on animals that live in the sediment. One station (Krapets) had Pb concentration higher than ERL which indicated that Pb at this station was likely to have effects adverse on 50% of animal populations that live in that sediment. On the other hand, all the rest of the studied stations had Pb concentration below the ERL value which indicated that Pb in the studied area was not likely to have adverse effects on animals that live in the sediments, except st. Krapets. Ni at two sampling stations (Burgas and Rosenets) had a value over the ERL value. St. Rosenets had concentration of Cu above the ERL value.

When compared to the TEL-PEL SQGs, the concentrations of Cd, Hg, and Zn were lower than the TEL value at 100% of the sampling stations. Pb showed values higher than the TEL value at the sampling stations in the northern part – Krapets, Rusalka and Kaliakra. In the case of Cu, 36% of the samples fell in the range between TEL and PEL at Rusalka, Kaliakra, Galata, Rosenets and Burgas indicating that associated adverse biological effects might occasionally occur. Ni concentrations were over TEL value at two stations - Rosenets and Burgas.

Only four stations (29%) had ERM-Q > 0.1 and were categorized as non-toxic and the rest of the stations were categorized as medium low toxic and might have 30% probability of being toxic. The m- ERM-Q calculated for the rest sampling sites indicated low toxicity with 12% probability of being toxic.

Fifty percent of the stations (Krapets, Rusalka, Kaliakra, Galata, Rosenets and Burgas) had m-PEL–Q within the range 0.11 – 1.5 with 25% likelihood of toxicity or categorized as slightly toxic (medium low). The rest of the stations were with m-PEL–Q below 0.1 representing non-toxic level. The m- ERM-Q calculated for the sampling sites (based on metals Cd, Cr, Cu, Ni, Pb, Hg and Zn) ranged from 0.01 to 0.34 (mean value of 0.15), indicating that the combination of Cd, Cr, Cu, Ni, Pb and Zn might have 30% probability of being toxic.

The pollution status assessed by applying different indices with respect to the investigated metals is shown in Table 2. Stations at which no pollution is detected are not included.

Metal concentrations in costal marine sediments varied considerably. The highest variation exhibited Cu (Table 3) - the difference between minimum and maximum concentrations was about 60 times. The highest concentrations of Cu and Ni were measured at st. Rosenets located in Burgas Bay near a past mining activities area. Burgas Bay and Varna Bay were the “hot spots” along the Bulgarian Black Sea coast. The concentration of Zn ranged from 2 to 96 indicating 40 fold difference among the studied stations. The maximum concentration was found in the sediments at st. Kaliakra. Maximum Pb concentration was at st. Krapets. The minimum concentrations of all metals were established at st. Slanchev Bryag, situated in the protected sand bank "Cocketrice" zone (2005).

As shown in Table 1, the average concentrations from nearly all the stations were lower than the average continental crust values. The exceptions were for Zn at st. Kaliakra and Cu at st. Rosenets which were both higher than the average continental crust value. This ratio measured concentrations / background value reflected in indices Igeo, CF, EF (Table 2). All single metal indices as Igeo, EF, CF indicated moderate contamination with Pb and Zn in the northern Danube influenced area of the Bulgarian coastal zone (stations Krapets, Rusalka and Kaliakra) and “hot spot” stations: Galata (close to Varna Bay), Rosenets and Burgas (in Burgas Bay).

The determined concentrations of Cd, Cu, and Pb at the sampling locations within the Bourgas Bay were significantly higher than those determined in the sampling sites outside the bay as a result of industrial and former mining activities in the area (Slaveykova et al., 2009). The highest Cu concentration was observed in macrophytes in Rosenets area (Strezov & Nonova, 2005).

Indices PLI and mCd indicated null overall pollution except st. Varna with low degree of contamination.

According to the low range values (ERL or TEL), adverse effects on sediment dwelling organisms were likely to occur regarding Pb, Ni and Cu concentrations at the stations in the northern part (Krapets, Rusalka, Kaliakra) and the "hot spot" stations Galata, Rosenets and Burgas. Concentrations higher than ERM or PEL were not established at the other stations indicating that adverse effects on benthic fauna were not expected to appear.

The classification of sediments according to ERMQ and PELQ showed that there was 30% or medium low probability of sediments to present toxicity in amphipod survival bioassays at the stations Kaliakra, Galata, Burgas and Rosenets and 25% probability in the northern part, the Danube current affected stations (Krapets, Rusalka and Kaliakra) and the "hot spots" (Galata, Burgas and Rosenets).

Upper range values (ERM or PEL) represent concentrations above which adverse effects are expected to appear.

Although some studies were carried out in the Bulgarian Black Sea coastal zone to assess the spatial variation of metal levels in sediments mainly in the past Andreev & Simeonov (1988; 1990); Jordanova et al. (1999); Simeonov & Andreev (1989); Simeonov et al. (2000); Stoyanov & Dimitrov (1991); none of those had assessed the status of metal pollution and the effect of pollution on benthic organisms especially in the recent period using pollution indicators.

The values were compared with other parts of the Black sea region (Table 3). Metal levels in the Bulgarian coastal zone are generally lower than those in the other Black sea regions. The maximum copper concentration at Rosenets station is comparable with the intense copper mining areas of Turkey – Rize. Average Ni and Pb concentrations are higher than the measured concentration in "hot spot" sediments from the Bulgarian Black Sea coastal zone (Simeonov et al., 2000).

Table 1. Average concentrations of metals in surface sediments from the Bulgarian Black Sea coastal zone, background concentration in the continental crust and Sediment Quality Guideline – ERL, ERM, TEL, PEL (mg/kg dry weight).

Station	Zn (mg/kg)	Pb (mg/kg)	Ni (mg/kg)	Hg (mg/kg)	Cu (mg/kg)	Cd (mg/kg)	Al (mg/kg)
Krapets	44.20	31.25	9.50	0.05	9.50	0.20	8799
Rusalka	67.00	30.40			21.50	0.14	
Kaliakra	96.10	45.80			40.30	0.14	
Varna Bay		11.00	8.00	0.05	9.60	0.25	9721
Galata	65.10	21.35	31.10	0.05	21.25	0.20	26210
Kamchia		6.20	10.70	0.05	8.70	0.25	13410
Irakli	14.30	7.60	10.60	0.01	6.70	0.08	
Slanchev Bryag	2.29	2.43	2.27	0.01	1.09	0.23	
Rosenets		29.90	42.20	0.05	66.90	0.25	43930
Bourgas	23.00	16.50	28.40	0.03	19.85	0.19	47800
Veleka		5.00	8.90	0.05	9.40	0.25	11620
Min-Max	2.29÷96.1	2.43÷57.5	2.27÷42.2	0.05÷0.02	1.09÷66.9	0.08÷0.25	8799÷47800
All Grps	45	20	18	0.04	19	0.20	23070
Continental crust (Taylor, 1964)	70	12.5	75	0.008	55	0.2	82300
ERL	150	46.7	20.9	0.15	34	1.2	
ERM	410	218	51.6	0.71	270	9.6	
TEL	124	30.2	15.9	0.13	18.7	0.68	
PEL	271	112	42.8	0.7	108	4.21	

Table 2. Pollution status of Bulgarian Black Sea coastal zone stations, established by applying individual metal indices.

Station	Igeo	EF	CF	ERL	TEL
Krapets	Pb moderately to strongly contaminated	Pb moderate	Pb considerable	Pb medium low	Pb medium low
Rusalka	Pb moderately contaminated		Pb moderate		Pb, Cu medium low
Kaliakra	Zn moderately contaminated, Pb moderately to strongly contaminated		Zn moderate, Pb considerable	Cu medium low	Pb, Cu medium low
Varna Bay		Pb significant			
Galata	Pb moderately contaminated	Pb moderate	Pb moderate		Cu medium low
Kamchia		Pb moderate			
Slanchev Bryag			Cd moderate		
Rosenets	Pb moderately contaminated	Pb moderate	Pb, Cu moderate	Ni, Cu medium low	Ni, Cu medium low
Bourgas	Pb moderately contaminated	Pb moderate	Pb moderate	Ni medium low	Ni, Cu medium low

Table 3. Comparison of sediments concentrations of heavy metals (mg/kg dry weight) with the data from literature sources.

Region/Authors/Period	Cd	Cu	Ni	Pb	Zn
West Black Sea (Unsal et al., 1998)	0.001÷0.225	2÷47		5÷143	12÷238
Whole Black Sea 1989 (Kiratli & Ergin, 1996)		29÷68	38÷130	14÷35	50÷108
Whole Black Sea 1997-1998 (Topcuoglu, 2000)	0.6÷0.9	23÷75	38÷130	14÷35	57÷127
West Black Sea 2008 (Ozkan & Buyukisik, 2012)	0.18÷0.53	40÷72	57÷93	20÷38	83÷184
South-Western (Bulgarian) Black Sea (Andreev & Simeonov, 1988)		22÷46	31÷38	23÷36	76÷105
Bulgaria Hot Spots (Simeonov et al., 2000)	0.01÷4.29	3÷786	1÷28	1.55÷118.8	14.77÷265.54
Romania 2006-2011 (Alexandrov et al., 2012)	0.01÷9.63	0.53÷147.84	0.40÷211.73	0.10÷300.78	
Midia Port (Catianis et al., 2016)	0÷2	5÷145	12÷44	2÷92	21÷222
Odessa (Dyatlov, 2015)	0.2÷17	0.1÷65.2	12.6÷79		
Range of metals in this study	0.08÷0.25	1.09÷66.9	2.27÷42.2	2.43÷57.5	2.29÷96.1
Romania 2006-2011 (Alexandrov et al., 2012)	1.03	27.54	36.54	26.71	
Rize (Alkan et al., 2015)	0.3÷0.5	33÷54	7÷9	14÷19	78÷96
Rize (Topcuoglu et al., 2003)	<0.02	67.8		67.8	483.1
Eastern Black Sea (Baltas et al., 2017)		576.31		97.33	357.2
Romania (Secrieru & Secrieru, 2002)	0.75	32.2	66.4	15	64.6
Ukraine (Wilson et al., 2008)	2.1	117		614	114
Georgia (Wilson et al., 2008)	2.7				3.35
Bulgaria Hot Spots (Simeonov et al., 2000)	0.81	80	12.96	15.96	58.97
Mean concentration in this study	0.20	18.96	18.01	19.75	44.57

Factor analysis was applied to summarize the spatial pattern of metal distribution. The only one extracted factor explains 94% of the total variance and eigenvalue of 2.9 with statistically significant loadings of Ni, Pb and Cu. The highest scores of this factor are found in the “hot spot” stations Galata, Burgas and Rosenets. Therefore, Factor 1 is attributed to anthropogenic pollution.

Conclusions

Several environmental indices were used for assessment of heavy metal contamination levels in the sediments of the studied area. The level of the most trace metals was not extremely high in the surface sediments of the Bulgarian Black Sea coastal zone and did not present a serious threat to the local fauna and flora. The northern Danube influenced area of the Bulgarian coastal zone was categorized according to the applied indices as slightly polluted with Zn and Pb. The sediments from the “hot spot” stations were with higher accumulation of Ni, Cu and Pb.

Although the results were preliminary, they did not reveal high pollution levels in the Bulgarian coastal zone.

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Short note

A Wall lizard on a Danube Island - Podarcis muralis (Reptilia) in Moldova Veche Island, Iron Gates Natural Park, Romania

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Abstract. *Podarcis muralis* was identified on Moldova Veche Island (Romania), although naturally the island lacks the species` appropriate habitats. *P. muralis* occurs in artificial habitats represented by stony piers. Probably, some individuals were brought by chance to the island together with the stones used for the pier. *P. muralis* is limited to this artificial habitat, without any possibility of further distribution.

Key words: artificial habitats, human impact, introduction, lizards.

The wall lizard, *Podarcis muralis* (Laurenti, 1768), is distributed from northern Spain to Turkey Anatolia; Romania is a part of its north-eastern distribution limit (e.g. Sillero et al., 2014). In Romania it occurs especially in the southern and central part of the Carpathian Mountains, and in Dobruja (e.g. Fuhn & Vancea, 1961; Cogălniceanu et al., 2013). It is a xerotermophylous species, widespread in rocky areas (Fuhn & Vancea, 1961). *P. muralis* was recently mentioned in Romania in anthropogenically disturbed habitats (Covaciu-Marcov et al., 2006, 2009a; Gherghel et al., 2009; Strugariu et al., 2008; Sas-Kovács & Sas-Kovács, 2014). It was frequently reported in south-western Romania, in the Danube Gorge area, where it is well represented (Covaciu-Marcov et al., 2005, 2009b; Cogălniceanu et al., 2013). Although those records refer to the Danube`s banks and surrounding slopes (Covaciu-Marcov et al., 2005, 2009b), the species was also recorded on one of the islands from the Danube Gorge, namely Ada Kaleh (Fuhn, 1975). After the construction of the Iron Gates I Dam Lake, the population disappeared as the island was flooded (Fuhn, 1975). This paper mentions the wall lizard on another island from the Danube Gorge, namely Moldova Veche Island, providing some possible explanations for its colonization.

Moldova Veche Island is situated in the Iron Gates Natural Park - IGNP. Before the dam lake the island was much larger (Ujvári, 1972). Nowadays, only some higher regions survived, covered by wetlands and some grassland. The island was a step away from becoming a tailings deposit, when the copper mine from Moldova Nouă was still functioning (Radomir & Simion, 2016; Burlacu et al., 2017). For this purpose, the island`s landscape was modified by the introduction of stones, used for propping a periisland pier and for a bridge construction (abandoned in a useless condition), which linked the island to the left Danube bank (Radomir & Simion, 2016). The stony piers, of some meters width, surround the island.

P. muralis was observed on the island by different occasions in the last years. In the autumn of 2019, during a day field study, we observed tens of individuals on the stony piers in the island`s western part (Fig. 1). From what we know, *P. muralis* was not previously recorded on the island (see Fuhn & Vancea, 1961). The species probably did not inhabit naturally the island, due to the anterior landscape, which did not fit its requirements, even if it was recorded in the surroundings (Covaciu-Marcov et al., 2005, 2009b; Tomović et al., 2014). The island landscaping with large quantity of stones has created new habitats, which were occupied by the wall lizard. Presently, the wall lizard population from the island inhabits only these stony piers. The invasive potential of this species is well known (e.g. Ulrich et al., 2012), reported from Romania (Covaciu-Marcov et al., 2006; Gherghel et al., 2009; Sas-Kovács & Sas-Kovács, 2014; Strugariu et al., 2008), Europe (e.g. Heym et al., 2013; Schulte et al., 2008; Wirga & Majtyka, 2013) or other continents (e.g. Deichsel & Gist, 2001; Deichsel & Schweiger, 2004; Hedeen & Hedeen, 1999). Thus, if the wall lizard could colonize areas on other continents, why couldn't it be established on an island from its native distribution range, with optimal climate conditions? Moreover, it was introduced in similar habitats from seaport areas situated outside its distribution range (Santos et al., 2019). The only unenlightened fact in this case is the way the species arrived at this island.

The distribution ways of the wall lizard in the new habitats over the Danube could be multiple. Most likely *P. muralis* was brought with the loads of stone used for pier propping, coming from the quarry near Moldova Nouă (information from the IGNP staff). This possibility becomes more plausible because *P. muralis* was recorded in Moldova Nouă locality (Fuhn & Vancea, 1961; Covaciu-Marcov et al., 2005) and it inhabits stone quarries (e.g. Fuhn & Vancea, 1961; Wirga & Majtyka, 2013). Similar situations were described even in areas outside its distribution range (Iftime, 2005; Sas-Kovács & Sas-Kovács, 2014). River embankments with stones are used in other regions too (Kühnis & Schmocker, 2008). The presence of *P. muralis* on Ada Kaleh Island was also considered a consequence of unintentional introduction by humans (Fuhn, 1970). On the other hand, *P. muralis* is a good climber (Avery et al., 1993; Brown et al., 1995; Žagar et al., 2017), fact suggested by its preference for steep slopes. There is a possibility that the lizard climbed the concrete pillars of the bridge and crossed to the island. This option has low chances due to the copper tailings deposit situated near the bridge pillars from the left bank of the river. Another way of distribution could be the intentional introducing by people, fact also mentioned by some authors (Deichsel & Gist, 2001; Deichsel & Schweiger, 2004; Schulte et al., 2008). In this context even its introduction by chance through boat transport must be considered.

Fig. 1. *Podarcis muralis* individual from Moldova Veche Island.

Beyond these uncertainties, *P. muralis* is a protected species (O.U.G. 57/2007). Nowadays there is sustainable population on the island, which can only be protected. The negative effects of this colonization are hard to estimate, but the anthropic changes on the island are much more serious than any consequence of the lizard's presence. Besides *P. muralis* we observed reptile species as *Lacerta viridis*, *Natrix natrix* and *N. tessellata*. In other areas of Romania even railroads are a distribution way of *P. muralis*, but its potential impact is considered limited (see in: Gherghel & Tedrow, 2019). This is similar with the case of this island, where most habitats are not favorable for this species. Thus, *P. muralis* is currently situated, and in the future will remain, captive on the artificial stony pier habitats, which permitted its establishment on the island.

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Short note

Amphibian and Reptile Diversity in Protected Site "Reka Veselina" – Current State and Prospects for Future Conservation

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Abstract. "Reka Veselina" is a protected site in the Veliko Tarnovo region and although it was declared in 2012, until now there were no specific data on the state of animal diversity within the area. Herpetological field surveys were conducted for a total of 10 days in the period April-May 2019. A total of 12 amphibian and reptile species were registered. All species except one are protected by national law and three (*Emys orbicularis, Triturus ivanbureschi, Bombina variegata*) are also included in the NATURA 2000 network. Species distribution was uneven along the river, highlighting the importance of including diverse habitats even in protected areas of small size. Results indicate that the protected site offers optimal conditions for most of the registered species and could serve as a blueprint for successful management of similar-sized wetlands.

Key words: habitats, herpetofauna, management, species, wetland.

Establishment of protected areas is a key strategy in biodiversity conservation (Vale et al., 2018), but at the same time, it is primarily driven by available opportunities rather than scientific knowledge (Baldi et al., 2017). In this regard, it is very important to study biodiversity on a local scale in order to provide the best possible management options.

Currently there are 569 protected sites in Bulgaria, most of which are of medium or small size (ExEA, 2020). Faunistic data for most of them is lacking, which hinders the establishment of adequate management plans. In particular, there is a distinct lack of published studies on the herpetological fauna for the territory of "Reka Veselina" protected site and the surrounding region. A single published record could be found in Annex 3 of a review paper on the distribution and ecological requirements of native species of freshwater turtles (Kornilev et al., 2017). The authors indicate exact geographical coordinates for the presence of the species European pond turtle (*Emys orbicularis*) in the Zlatarishka River, within the town of Zlataritsa, situated 5km northeast of "Reka Veselina" protected site.

The aim of this study was to establish a comprehensive list of amphibian and reptile species that occur within the protected site, and relate species abundance to habitat diversity, so results could be used as guidelines in future research and management plans for wetland protected areas.

The protected site of "Reka Veselina", named after the eponymous river, is located near the villages of Kapinovo and Mindia, in the region of Veliko Tarnovo. It is established in 2012 for the protection of a number of plant and animal species and has a total area of 986 ha, with elevation varying between 100 and 120 m. a.s.l. The average tidal current of the river is 2.4 m^3/s, with clearly defined spring floods from March to June and summer and autumn low water levels from July to October. Along the river there are one town (Zlataritsa) and nine villages.

Because of the lack of published data on amphibians and reptiles, the publicly available data from the SmartBirds mobile application system for wildlife mapping (SmartBirds, 2019) was reviewed using the geolocation search option, with a point in the centre of "Reka Veselina" and a 5km radius. In this way, the following species of amphibians and reptiles were identified in a 10x10km square: Marsh frog (*Pelophylax ridibundus*), Agile frog (*Rana dalmatina*), tree frog (*Hyla arborea* complex), green toad (*Bufotes viridis* complex), common toad (*Bufo bufo*), common spadefoot (*Pelobates fuscus*), European green lizard (*Lacerta viridis*), Eastern slowworm (*Anguis colchica*), Grass snake (*Natrix natrix*), Aesculapian snake (*Zamenis longissimus*), Common wall lizard (*Podarcis muralis*). Although none of the points with exact geographical coordinates for these species fall within the boundaries of the studied protected site, the presence of some of them is possible due to the existence of suitable habitats within the area and/or in its immediate vicinity. When interpreting the data from SmartBirds, it should be noted that after the recent taxonomic changes of the species *Hyla arborea*, the new species Oriental tree frog (*Hyla orientalis*) was established based on genetic traits (Dufresnes et al., 2015), and is widespread in most of the country, incl. in the study site. As there is still no evidence of morphological differences between the two species and it is virtually impossible to recognize them from each other by external features, they are not separated in the mobile application.

Field surveys were carried for a total of ten days in April and May 2019. The distribution of amphibians and reptiles was estimated on the basis of presence-absence, using exact geographical coordinates for each registered individual. The relative density of amphibians was recorded by transects with direct observations of animals and by means of non-lethal funnel traps (Sutherland, 2000). Fifteen traps (size 25x25x40 cm) were set up in the evening in preselected suitable locations and checked the next morning. After species identification, all captured individuals were released immediately at the site of capture. Reptile density was recorded by 500 m long and 2.5 m wide transects (Sutherland, 2000).

During the field surveys, a total of 80 separate observations of amphibians and reptiles were registered, and four species of amphibians and six species of reptiles were established. Additional two amphibian species were established in close proximity (<2 km) to the borders of the protected site, increasing the total number of identified species in the field surveys to 12 (Table 1).

The most abundant species by far was the Marsh frog, with a total of 303 registered adult individuals (235 in April and 68 in May), followed by the European pond turtle with 38 (37 in April and 1 in May). While the Marsh frog was uniformly distributed throughout the site, the European pond turtles were concentrated in a mid-sized marsh in the western part. The marsh had an approximate surface area of 1.28 acres and was overgrown with bulrush (*Typha* sp.); it was not connected to the river but was still a permanent water body, presumably fed by underground sources. In April, the marsh also yielded a significant number of Smooth newts (15 females, 16 males; average of 2.07 newts per trap), as well as 5 Buresch's crested newts (2 females, 3 males; average of 0.33 newts per trap). Both Smooth (5 females, 6 males) and Buresch's newts (a single male) were also registered at the eastern part of the protected site, in a shallow flooded meadow near the river. The European green lizard was registered on 28 occasions (20 in April, 8 in May) near grassy strips and shrubland along the river. The other species were registered more rarely near the river or in puddles on dirt roads: Dice snake – 3 (2 in April, 1 in May), Grass snake – 2 (1 in April, 1 in May), Eastern slowworm – 2 (both in May), Oriental tree frog (1 calling adult in April, 20 larvae in May), Common wall lizard 1 (in May). A single larva of the Fire salamander and an adult Yellow-bellied toad were observed in small streams leading to the river, to the South and West, respectively.

Table 1. Registered species during field surveys and their conservation status. * - registered outside of the protected site; BA – Biodiversity Act (2002), 92/43 – EC Habitats Directive (1992), BC – Bern convention on the conservation of European wildlife and natural habitats (1982), IUCN - International Union for Conservation of Nature (2020).

Species	**BA**	**92/43**	**BC**	**IUCN**
*Fire salamander *Salamandra salamandra* (Linnaeus, 1758)	III	-	III	LC
Smooth newt *Lissotriton vulgaris* (Linnaeus, 1758)	III	-	III	LC
Buresch's crested newt *Triturus ivanbureschi* Arntzen & Wielstra, 2013	II, III	II, IV	II	N/A
* Yellow-bellied toad *Bombina variegata* (Linnaeus, 1758)	II, III	II, IV	II	LC
Oriental tree frog *Hyla orientalis* Bedriaga, 1890	III	IV	II	N/A
Marsh frog *Pelophylax ridibundus* (Pallas, 1771)	IV	V	III	LC
European pond turtle *Emys orbicularis* (Linnaeus, 1758)	II, III	II, IV	II	NT
Eastern slowworm *Anguis colchica* (Nordmann, 1840)	III	-	III	N/A
European green lizard *Lacerta viridis* (Laurenti, 1768)	III	IV	II	LC
Common wall lizard *Podarcis muralis* (Laurenti, 1768)	III	IV	II	LC
Grass snake *Natrix natrix* (Linnaeus, 1758)		-	III	LC
Dice snake *Natrix tessellata* (Laurenti, 1768)	III	IV	II	LC

Most of the identified species are protected under Annex III of the Bulgarian Biodiversity Act (10 species) and/or under Annex IV of the EU Habitats Directive (7 species). All species are protected under the Bern Convention (7 of them are listed in Annex II, i.e., strictly protected). Species that fall simultaneously in Annexes II and III of the Bulgarian Biodiversity Act, Annexes II and IV of the Habitats Directive, and Annex II of the Bern Convention, could be accepted as species with the highest conservation status. These include the Buresch's crested newt (*T. ivanbureschi*), the Yellow-bellied toad (*B. variegata*) and the European pond turtle (*E. orbicularis*), which is also Near-threatened under IUCN. All of the above-mentioned species were established for the first time in the area with exact geographical coordinates. The species Fire salamander (*S. salamandra*), Smooth newt (*L. vulgaris*), Buresch's crested newt (*T. ivanbureschi*) and Dice snake (*N. tessellata*) were established for the first time in the region.

Most of the territory of "Reka Veselina" was covered during the field surveys, and visited habitats were in very good condition. The only recorded negative anthropogenic impact was low-level household waste pollution, mainly along the borders of the protected site; however, the degree of pollution was too low to present a threat to amphibian and reptile populations.

Joppa & Pfaff (2009) highlight the need of a more context-specific approach when planning the location and the size of protected areas. In recent decades, most protected areas have been created on the basis of consistent scientific data, rather than historic reasons, and are usually planned with regards to high biodiversity and presence of species of high conservation importance within their borders.

Results obtained during the field surveys highlight the benefit of including marshes, floodplains and other types of wetlands in protected areas specifically established alongside rivers. The large marsh in the western part of the site offers optimal habitat conditions for two species of high conservation importance, which would not have been present otherwise (or would be present in much lower numbers) – the European pond turtle and the Buresch's crested newt. The European pond turtle occurs in rivers and ponds across Bulgaria up to around 1100 m a.s.l., inhabiting a large variety of water bodies, but with the highest numbers always associated with stagnant ponds overgrown with reed and bulrush (Stojanov et al., 2011). The Buresch's crested newt inhabits stagnant water bodies and their surroundings, and is absent from ponds with predatory fish (Stojanov et al., 2011) – in this regard, conditions provided by the habitats in the western and eastern parts of the "Reka Veselina" protected site present an excellent environment for the development of this species. Although the third species of conservation importance – the Yellow bellied toad – was not registered in the protected site, the diverse habitats within its borders (flowing and standing water, puddles, flooded areas) provide suitable habitats. These diverse habitats also ensured the presence of the other registered amphibian and reptile species, all of which contribute towards a healthy ecosystem.

Some modelling studies on the planning and management of protected areas (e.g., Loyola et al., 2013) have pointed out that these areas will become less effective in maintaining species representation under changing climatic conditions. In addition, areas created for the protection of specific environments or species generally tend to be small (Marinaro et al., 2012). For these reasons, when a new protected area is declared, it is important to include as many and as diverse habitats as possible, even if the size of the area is relatively small. When planning future conservation activities, a careful integrative species-specific approach should be adopted, as sometimes seemingly beneficial activities could lead to habitat deterioration (e.g., raising water level to benefit a protected fish species from the main river could result in predatory fish appearing in the adjacent marshes, driving the newts away).

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Synopsis

Feeding Ecology of Anurans (Amphibia: Anura) in Bulgaria - A Review

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Abstract. A contemporary review of the studies on the trophic spectrum and feeding ecology of the Bulgarian frogs and toads is presented. The analysis of the Bulgarian herpetological literature showed that currently, there are 18 specialized studies on the diet of the Bulgarian anuran species. Of all 16 anuran species, occurring in Bulgaria, 9 (56.25%) have been studied in connection with their trophic spectrum. For four species (25.00%), there is very little data, and for 7 (43.75%), such studies have not yet been done. All Bulgarian species of amphibians are zoophagous, mainly insectivorous. All studied species are general feeders (polyphages), except *Bombina variegata*, which shows a slight preference to Coleoptera and *Bufo bufo* and *Bufotes viridis*, which show slight preference to Formicidae and Coleoptera. Cannibalism was recorded only in *Pelophylax ridibundus*. The highest values of the trophic niches breadths are recorded in *P. ridibundus, R. graeca, R. temporaria,* and *R. dalmatina.* The trophic niches breadths of the other anuran species found in Bulgaria have significantly lower values, the lowest being in *Hyla orientalis.*

Key words: Anura, frogs, toads, diet, trophic spectrum, synopsis, Bulgaria.

Introduction

The first step towards understanding the ecology of amphibians (or most animals, for that matter) is to collect information about their feeding ecology (Hódar, 1997). Furthermore, the determination of their feeding habits helps scientists understand how animals use the food resources available in their immediate environment (Bellocq *et al.,* 2000). Therefore their relationship with populations of other species living in the same habitat can be clearly understood by establishing where the species stands within the food chain (Duellman & Trueb, 1986). Feeding ecology is one of the most critical features of a species' natural history, as it affects survival and can provide valuable information to take conservation and management decisions regarding endangered and rare species (Watson et al., 2017). According to Çiçek & Mermer (2007), different studies suggest that food is an essential factor that can explain the structure of anuran communities in different parts of the world.

Amphibians are known to be important components in terrestrial and aquatic ecosystems (Halliday, 2008), represented with a significant number of species. As a major part of this group, anurans can be opportunistic in their feeding behavior and presumably eat any prey they find and can swallow, located in the environment (Caldart et al., 2012).

Most of the studies on the diet of the anurans in Bulgaria were conducted in the 1960s, 1970s, and 1980s. Most of them are researching the trophic spectrum for how much of the caught prey are pests in agriculture, forestry, and fisheries. Some authors analyze the seasonal variation in the trophic spectrum of the studied species, but overall most of the studies just give a qualitative and quantitative description of the diet.

Studies on the trophic niche breadth, niches overlap between the sexes and species, the position of frogs and toads in the food chains in the ecosystems, and using more advanced and contemporary research methods, were made at a much later stage. In 2006, Mollov et al. (2006) published the first country-wide synopsis of all up-to-date research on the diet of all amphibian species in Bulgaria. Three more studies on the diet of anurans in the country were published since then (Mollov, 2008; Mollov & Boyadzhiev, 2009; Mollov et al., 2010).

The current synopsis aims to complement the existing knowledge of the studies on the feeding ecology of the anurans, conducted in Bulgaria and compare and analyze the data concerning the qualitative and quantitive composition of the diet, the food specialization between the species.

Material and Methods

All available literary data on the diet of the Bulgarian frog and toad species were used for the current synopsis. From the available literature, we have summarized the data about the extend of studies on the trophic spectrum of all anuran amphibian species, that occur in Bulgaria (taxonomy of the amphibians follows Speybroeck et al. (2020), the taxonomy of the invertebrates follows Fauna Europaea, de Jong et al. (2014); the number of the studied specimens in each study, period and region of study, as well as the percentage of the three predominant taxa from the trophic spectrum of each species.

Based on the available data, the trophic niche breadth (B) is calculated for each species, using the reciprocal value of the Simpson's diversity index (Magurran, 1988):

$$B = \frac{1}{\sum p_i^2},$$

where:

B – trophic niche breadth;

P_i - proportion of taxa i.

To determine the species' trophic specialization, we used the Berger-Parker dominance index (d) calculated by the following formula (Magurran, 1988):

$$d = \frac{n_i \max}{N},$$

where: d – Berger-Parker dominance index;

N – number of individuals from all taxa;

$n_i max$ – number of individuals from the most-abundant taxon.

The Berger-Parker index (d) ranges from 0 to 1. Values close to 1 indicate a narrow trophic specialization, typical for mono- and oligophages; values close to 0 are typical for species with broad trophic specialization (polyphages).

The results were statistically processed using descriptive statistics (trophic niche breadth) and cluster analysis (Unweighted Paired Group Average Linkage, Jaccard Similarity Index), for determining the similarity of the trophic spectrum of all anurans for which quantitative data is available (Fowler et al., 1998). For the descriptive statistical processing of the data, we used MS Excel. For the cluster analysis, as well as the calculations of Simpson's diversity index and the Berger-Parker index, we used the computer software "PAST" (Hammer et al., 2001).

Results and Discussion

Currently there are 18 specialized studies conducted on the trophic spectrum of the Bulgarian anurans (Angelov, 1960; Beshkov, 1961; 1970; Hristova, 1962; Bachvarov, 1965; 1967; Angelow & Batchwarow, 1972; Angelov & Batschwarov, 1972; Donev, 1984a; 1984b; 1986; Tomov, 1989; 1990; 1991; Mollov, 2008; Mollov et al., 2006; 2010; Mollov & Boyadzhiev, 2009).

Of all 16 species of frogs found in Bulgaria, 9 (56.25%) have been studied in connection with their trophic spectrum so far. For four species (25.00%), there is very little data, and for 7 (43.75%), such studies have not yet been done.

The summarized results of the conducted studies, arranged by species, are presented in Table 1. The species for which the respective authors have conducted specialized scientific studies are marked with "+"; "?" indicates the species for which there is only partial data and "-" - no studies have been conducted.

The table shows that, of all the representatives of the Bulgarian anuran amphibians, specialized studies on their diet were conducted for only nine species, and by the number of studies they are: *Pelophylax ridibundus* - 11; *Bufotes viridis* - 7; *Bombina variegata* - 6; *Bombina bombina* and *Hyla orientalis* (*H. arborea* is mentioned in the articles, but judging by the places, where these studies were made and the new taxonomy of the species in the genus *Hyla*, it is more likely to refer to *H. orientalis*) - 3; *Bufo bufo* - 2, *Rana temporaria*, *Rana graeca*, and *Rana dalmatina* – 1 each. For the remaining seven species (30.77%) (*Pelophylax bedriagae; Pelophylax* kl. *esculentus*; *Pelobates fuscus; P. syriacus, P. syriacus*), which occur in Bulgaria has only partial data related to their food ecology and trophic spectrum or there are no such studies.

Summary of the studies of the trophic spectrum by species is as follows:

European Fire-bellied Toad (*Bombina bombina*). Data on the diet of this species in Bulgaria are presented in the works of Angelov & Batschwarov (1972); Tomov (1991) and Mollov et al. (2006). The first study conducted in 1962-1965 was based on the stomach contents of 7 individuals from Southern Bulgaria (the surroundings of Plovdiv, Pazardzhik, and Burgas). The authors found that the predominant taxa in the food of the Fire-Bellied Toad are Hymenoptera, Formicidae (84.85%), Coleoptera (9.09%), Gastropoda (0.30%) and Isopoda (0.30%). Tomov (1991) analyzed the stomach contents of 248 individuals from the surroundings of the town of Lom (Northern Bulgaria) and found that it consisted mainly of Coleoptera (26.95%), Hymenoptera, Formicidae (19.43%), Myriapoda (9.14%). Data from the study by Mollov et al. (2006), based on 46 individuals, captured in from Plovdiv District in 1974, show that the most numerous taxa in the diet of the species are Coleoptera (31.0%) and Diptera (31.0%), and Hymenoptera, Formicidae is in third place with 8.3%.

The width of the trophic niche shows significant differences due to the large difference in the number of studied individuals in the three studies - from 1.37 (Angelov & Batschwarov, 1972), 7.54 (Tomov, 1991) to 10.23 (Mollov et al., 2006).

Yellow-bellied Toad (*Bombina variegata*). The trophic spectrum of this species has been very well studied (Angelov, 1960; Bachvarov, 1965; Batchvarov, 1967; Angelov & Batschwarov, 1972; Donev, 1984b; Mollov et al., 2006). The data available at this time indicate that the Yellow-bellied Toad shows some preference for the Coleoptera order, as it is the predominant taxon in all studies except Donev (1984b), which indicates the Diptera order as the most preferred prey. According to the data provided by these authors, the trophic niche of the *Bombina variegata* varies from 2.95 to 7.22.

Common Toad (*Bufo bufo*). The available data on the diet of this species is quite scarce. Angelov & Batschwarov (1972) studied the stomach contents of only six specimens of this species from the surroundings of Plovdiv, Pazardzhik, and Burgas in the period 1962-1965. The data show that the most preferred taxon is Coleoptera (36.36%), Hymenoptera (27.27%), and Arachnida (10.60%). The low food specialization (d=0.36) creates an impression that this species maybe prefers ants, which may be due to the small number of specimens studied,

Species / Author(s)	Angelov (1960)	Beshkov (1961)	Hristova (1962)	Bachvarov (1965)	Batchvarov (1967)	Beschkov (1970)	Angelow & Batchwarow (1972)	Angelov & Batschwarov (1972)	Donev (1984a)	Donev (1984b)	Donev (1986)	Tomov (1989)	Tomov (1990)	Tomov (1991)	Mollov et al. (2006)	Mollov (2008)	Mollov & Boyadzhiev (2009)	Mollov et al. (2010)
Fire-belied Toad *Bombina bombina* (Linnaeus, 1761)	-	?	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-
Yellow-belied Toad *Bombina variegata* (Linnaeus, 1758)	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	-	-	-
Common Spadefoot Toad *Pelobates fuscus* (Laurenti, 1768)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eastern Spadefoot Toad *Pelobates syriacus* Boettger, 1889	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Balkan Spadefoot Toad *Pelobates balkanicus* Karaman, 1928	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Common Toad *Bufo bufo* (Linnaeus, 1758)	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
Green Toad *Bufotes viridis* (Laurenti, 1768)	+	+	-	+	+	-	-	+	-	-	-	-	+	-	+	-	-	-
Eastern Tree Frog *Hyla orientalis* Bedriaga, 1890	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
Common Tree Frog *Hyla arborea* (Linnaeus, 1758)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Marsh Frog *Pelophylax ridibundus* (Pallas, 1771)	+	?	+	+	+	-	+	-	+	-	+	+	-	-	+	+	-	+
Edible Frog *Pelophylax kl. esculentus* (Linnaeus,1758)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Levant Green Frog *Pelophylax bedriagae* (Camerano, 1882)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pool Frog *Pelophylax lessonae* (Camerano, 1882)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Greek Stream Frog *Rana graeca* Boulenger, 1891	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Common Frog *Rana temporaria* Linnaeus, 1758	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Agile Frog *Rana dalmatina* Fitzinger in Bonaparte, 1838	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-

Table 1. Summary of the conducted studies on the trophic spectrum on the Bulgarian anuran species (explanations are in the text).

but given the high value of the trophic niche breadth (9.13), it is possible that this species is polyphagous. The study of Mollov & Boyadzhiev (2009), based on eight individuals caught in 1967 in the area of the Rowing Canal of Plovdiv City, shows a much lower value for the trophic niche breadth - 1.96 and a very high percentage of Hymenoptera, Formicidae in the diet - 70.20%, which suggests that perhaps depending on the season, there is a certain preference for ants as a food source.

More research is needed over the different seasons to determine variations in diet, trophic specialization of the species, and the trophic niche breadth.

Green Toad (Bufotes viridis). The trophic spectrum of the Green Toad is quite well studied in Bulgaria (Angelov, 1960; Beshkov, 1961; Bachvarov, 1965; Batchvarov, 1967; Angelov & Batschwarov, 1972; Tomov, 1990; Mollov et al., 2006).

The first study of the Green Toad's diet in Bulgaria was conducted by Angelov (1960). The study aims to clarify issues related to the role of frogs as predators of pest insects. The material was collected in 1954 and 1955 from April to September, and 20 individuals were collected from the vicinity of Plovdiv City. The diet consists almost entirely of insects, only 0.8% of the food is non-insect. Predominating taxa consist of the order Hymenoptera, of which the species of the family Formicidae are 95.1%, and only 4.9% are others.

From the study of Beshkov (1961), based on the stomach content of 180 individuals from Sofia City, shows the following composition of food: Hymenoptera, Formicidae (46.89%), Aphidoidea (18.97%), Diptera and Diptera - larvae (11.90%), Coleoptera, Carabidae (4.71%), Arachnida (1.77%), Oligochaeta, Lumbricidae (1.73%), Lepidoptera and Lepidoptera - larvae (1.56%), Hymenoptera (1.25%), Coleoptera Curculionidae (1.11%), Coleoptera, Chrysomelidae (1.03 %), Hemiptera (1%), Gastropoda (0.69%), Arthropoda, Myriapoda (0.66%), Isopoda, Oniscoidea (0.55%). The remaining 6.19% is distributed among the different Coleoptera families - Dermaptera, Orthoptera, etc., as none of the listed groups exceeds 1%.

Bachvarov (1965) studied the food of the Green Toad caught from the Kardzhali Region, as part of a parasitological study. The toads were collected in 1962 and 1964. The stomach contents of 11 specimens were represented by several taxa with a more significant number of Coleoptera, families Carabidae and Cerambycidae, Orthoptera, Gryllidae, Gastropoda, Clausiliidae, and Diptera, without any quantification, given by the author. A similar study by the same author (Batchvarov, 1967) was conducted in May 1965 along the Tundzha River from the towns of Kazanlak to Elhovo. Twenty seven individuals were caught, and their gastric contents show that the most numerous taxa consumed by the Green Toad are Coleoptera, Hymenoptera, Gastropoda (Helicidae, Zonitidae), Oligochaeta, Lumbricidae, Myriapoda, etc.

Angelov & Batschwarov (1972) studied the diet of *B. viridis* in 1962 and 1965 in the regions of Plovdiv, Pazardzhik, and Burgas. The data show that the most numerous taxa in the stomachs are Coleoptera, Carabidae (46.38%), Hymenoptera, Formicidae (18.52%), and Hemiptera (16.91%).

According to Tomov (1990) data on the trophic spectrum of *Bufotes viridis* from the town of Lom for the period 1980-1982, the following results were obtained. The author studied a large sample of 394 individuals, and the material is distributed by seasons as follows: spring - 158 ind. (March - 30, April - 38, May - 90); summer - 151 ind. (June - 13, July - 36, August - 102); autumn 85 ind. (September - 58, October - 31). The diet of the Green Toad in the study area was composed of representatives of 6 classes of invertebrates. The largest are the insects (Insecta); in addition to being the most numerous among the ingested animals - 12,579 preys (96.73%), they were found in the largest number of examined stomachs - in 384 stomachs (97.46%). The second place takes the crustaceans - Crustacea (terrestrial Isopoda only), and in third place, with almost equal participation, are Oligochaeta (exclusively Lumbricidae), as well as Arachnida (mainly Aranea and single Pseudoscorpiones, Opiliones and Acarina). Third place is taken by the centipedes - Myriapoda (mainly Chiliopada and only a few Diplopoda). The average number of specimens found in one stomach is 33, which shows both the very high trophic activity of the toads and the presence of rich and various prey in the study area.

The seasonal feeding activity of the Green Toad in the study area is well illustrated by the average number of invertebrates found in one stomach, as for spring, this value is 30.61, for summer - 36.56 and in autumn - 31.13. It is visible that the trophic activity of the toads is highest in the summer; the lowest values in the spring are probably due to the breeding season in which the toads do not feed actively. In this regard, it should be noted the presence of empty stomachs only in the spring season, while in summer and autumn, there are none. The most numerous and with the highest percentage of occurrence in the spring are Hymenoptera, Formicidae, Coleoptera, Carabidae, and Diptera. During the summer, Hymenoptera, Formicidae, and Coleoptera take the first and second place, and Heteroptera is in third place, while the number of Lepidoptera - larvae are significantly increasing. In the autumn, ants and carabids are again the most numerous, but significant increase in various species of small Hymenoptera have been observed; the number of Diptera, Isopoda, and Hemiptera also remains relatively large.

According to Mollov et al. (2006), the most numerous taxon in the food spectrum of the Green Toad from Plovdiv City is Coleoptera (52.2%), followed by Hymenoptera, Formicidae (23.9%) and Crustacea (11.0%). According to their data, the trophic niche breadth is 4.98.

The majority of studies on the feeding ecology of *Bufotes viridis* have shown that this species consumes predominantly Hymenoptera, Formicidae, which has the highest value in the data given by Tomov (1990). The second most preferred taxon, which is presented as the first in some studies (Bachvarov, 1965; Batchvarov, 1967; Angelov & Batschwarov, 1972; Mollov et al., 2006) is the order Coleoptera. Based on the available data, the width of the trophic niche of the green toad varies from 1.72 to 8.38.

Marsh Frog (Pelophylax ridibundus). This is the best-studied species in terms of its trophic spectrum in Bulgaria (Angelov, 1960; Beshkov, 1961; Hristova, 1962; Bachvarov, 1965; Batchvarov, 1967; Angelow & Batschwarow, 1972; Donev, 1984a; 1986; Tomov, 1989; Mollov, 2008; Mollov et al., 2006; 2010). The studies conducted in Bulgaria on the food spectrum of *P. ridibundus* can be conditionally divided into two periods. The first period covers all studies conducted in 1954-1985, most of which were conducted in southern Bulgaria (Plovdiv, Pazardzhik, Burgas, Sofia, Kardzhali Region, the Tundzha River Valley and in various places in southwestern Bulgaria). Only one study was conducted in the vicinity of the town of Lom in northern Bulgaria (Tomov, 1989). The second period covers articles published in the period 2006-2010, but they are also based on material collected in 1974-1995 from localities in Plovdiv and Pleven.

The first study on the diet of the Marsh Frog was done by Angelov (1960). The author has researched the diet of some species of frogs, intending to provide some new data on their role in combating pests. From his data, it is clear that the trophic spectrum of the species consists mainly of invertebrates, and insects predominate (Coleoptera – 47,2%; Hymenoptera – 16,8%; Diptera – 13,6 %).

The most extensive and detailed study of the trophic spectrum of *P. ridibundus* was conducted by Hristova (1962), who was the first author to establish that the Marsh frog causes some damage (catches a large number of fish) in fish farms. This is the only study so far on the food spectrum of the larvae in Bulgaria. The author reports that vegetation plays a significant role in the life of frogs, which largely directly determines the food available in the habitat, as frogs feed mainly on insects, and they are closely dependent on plants. The higher the species richness of the vegetation, the more abundant the insect fauna in this place. Thus, vegetation has indirect and direct importance for the feeding of frogs, as the larvae of *P. ridibundus* are mainly herbivores.

In her research on 162 larvae of the Marsh Frog in the Chelopechene Fish Farm (Sofia Region), Hristova (1962) found that all the larvae ingested plants - 33,1%, animals - 11,1% and detritus – 45,5%, during the study period. Along with these nutrients in the diet are absorbed plant particles 7.7%, as well as sand and silt (8.6%). In the analysis of the change in food depending on the growth of larvae, the author found that until the start of the metamorphosis, they feed almost exclusively on algae and detritus (up to body size of 4.5 cm). In the stage with a single pair of limbs (body size 4.5 to 7.5 cm), plants are also predominant, but tiny invertebrates are also found in the diet. In the stage with two pairs of limbs (over 7.5 cm), more animal food predominates.

According to the same author, the food of the larvae of other species of anurans (*Bufotes viridis, Pelobates fuscus*, and *Hyla orientalis*), which inhabit the fish farms examined by her, does not differ in quality from the diet of the larvae of the Marsh Frog. There are some small differences, expressed in the fact that in some of the pools, a more considerable amount of a particular type of food is found in than in others. In none of these species, can we talk about food selectivity.

Prof. Georgi Bachvarov, in few studies (Bachvarov, 1965; Batchvarov, 1967; 1968), examines the food spectrum of the Marsh Frog from the point of view of possible ways of infecting frogs with helminths. He also found that the three most essential taxa in the diet are Coleoptera, Hymenoptera, Diptera, but did not give any quantitative data (Bachvarov, 1965; Batchvarov, 1967) and that in fish farms, the species also catches a certain amount of fish and specimens of the same species (Bachvarov, 1968), again without any quantitive data. Cannibalism has also been reported in almost every study of the species' food spectrum (Mollov et al., 2010).

It is known from the literature that the feeding of the frogs depends on the temperature of the environment. At low temperatures, frogs stop feeding. This fact was also established in the study of Batchvarov (1968). In late autumn and early spring (immediately after waking from hibernation), only frogs with empty stomachs were caught. In April, as the weather warms and the frequency of insect activity increases, frogs begin to feed and lead an active lifestyle. In the stomachs of the studied frogs from the spring, remains of Coleoptera were found. During the summer months, the food is abundant and consists of remains of Coleoptera, Oligochaeta, larvae of *P. ridibundus*, and even small frogs (Bachvarov, 1965, Batchvarov, 1967).

Donev (1984a, 1986) is the second author to study the Marsh Frog's trophic spectrum in the Plovdiv State Fishery. He also confirmed Hristova's (1962) study that the species can cause severe damage in fish farms. According to the second study from 1986, fish is the third most important food component. In many cases, the author also establishes cannibalism.

Tomov (1989) reports new data regarding the trophic spectrum of *P. ridibundus* from Northern Bulgaria, from where so far, in this respect, there is no data from this part of the country. The study was conducted in the vicinity of the town of Lom, and the author examines both the qualitative and quantitative composition of food and its seasonal dynamics. The author's data show that the diet of the Marsh Frog in the studied area is diverse - it includes representatives of 9 classes of animals (7 classes of invertebrates and 2 classes of vertebrates). Insects are vibrant and constitute an essential component in the food. The seasonal feeding activity of frogs in the study area is highest in summer and significantly lower in autumn and spring.

At a much, later stage in 2006 in a review article by Mollov et al. (2006) published unpublished data on the food of the Marsh Frog and reported that the diet consists predominantly of Coleoptera (36,8%), Diptera (13,0%), and Hymenoptera (12,2%).

Mollov (2008) was the first author in the country to study the sex-based differences in the diet of *P. ridibundus*. According to the author, the trophic niche breadth has a much higher value in males (22.70) than in females (17.45). There is also an overlap of the trophic niches between the sexes, which is 63.8%. Morphology appears to influence the feeding behavior of *Pelophylax ridibundus* significantly, and differences in the diet between sexes can be explained by the fact that males are smaller than females. The author also found that the caught prey was mostly terrestrial, but given the frequency of encounters and the size of aquatic organisms, he concluded that feeding primarily occurs on land, but frogs also hunt in the water. This suggests that the Marsh Frog has an essential position in food chains located in the ecotone area between aquatic and terrestrial ecosystems.

The last article on the Marsh Frog's trophic spectrum was published in 2010 by Mollov et al. (2010). The authors found that in the diet was predominating Coleoptera, Diptera, and Hymenoptera and that the share of aquatic organisms was significantly smaller than terrestrial ones, with the authors again recording a case of cannibalism and the presence of vertebrates in the stomach contents. The variations of the trophic spectrum by seasons are also studied. Feeding is particularly intense in the spring, especially before the breeding season and decreases in the fall with falling air temperatures and changes in climatic conditions.

Most of the publications show Coleoptera as the most numerous taxon in the diet of the Marsh frog and has the highest value in the study of Hristova (1962), which is also confirmed by the results of Mollov et al. (2006; 2010). In the publication of Donev (1986), Collembola is mentioned as the most preferred taxon. Tomov (1989) mentions Hymenoptera as the most numerous taxon, which is confirmed in Mollov's (2008) study. From the above, we can conclude that *P. ridibundus* has no particular preference for certain prey and can undoubtedly be classified as polyphagous. This is confirmed by the high values of the trophic niche breadth, which, based on the data from the above studies, it varies from 3.65 to 19.21. This is the only anuran in our country that exhibits cannibalism.

Greek Stream Frog (Rana graeca). Only one study by Beschkov (1970) was conducted on the diet of this species in Bulgaria. However, since the study is based on a significant number of specimens (n=155) and done in a relatively large area (South-eastern Bulgaria), we can draw some conclusions about the food ecology of this species. The author's data show that the food of the Greek Stream Frog consists of 97% of terrestrial taxa, with Coleoptera (15.73%) predominating, followed by Diptera (13.48%) and Arachnida (9.81%) and showing no preference for a particular taxon (d=0.16) and can, therefore, be classified as a polyphagous. The breadth of the trophic niche, calculated according to the author's data, also has quite high value - 12.85, which confirms this statement.

The author also examines the food of *Rana graeca* by seasons and finds that in the spring, the percentage of empty stomachs is highest, which is the lowest in the fall. In all three seasons, Coleoptera predominates as the most preferred food. In the spring, a large percentage of Plecoptera (imago + larvae) is observed, which shows that the Greek Stream Frog inhabits mainly clean mountain springs and rivers. In summer, in addition to Coleoptera, Araneae and Diptera predominate in the diet, while in autumn, in addition to the beetles, Gastropoda, Homoptera and Collembola predominate. The author also notes that *Rana graeca* does not search its food underwater, but hunts entirely on land. Also, the Greek Stream Frog feeds both during the day and at night.

Comparing the diet of young and adult frogs, Beschkov (1970) found that characteristic components of the diet of juveniles are Collembola, Aphidodea, Plecoptera, Cicadellidae, Formicidae and some tiny organisms, such as pseudoscorpions, small ticks and leaf fleas, which are absent in the stomachs of the adults. The number of Gastropoda and Orthoptera is very small, which are represented by a much higher percentage in adult individuals. The author also notes that the main food of *Rana graeca* - Coleoptera and Diptera are consumed equally by the juveniles and adults.

Common Frog (Rana temporaria). The trophic spectrum of this species has been studied only by Angelov & Batschwarov (1972). The data given by the two authors, based on a study of 25 individuals from the surroundings of Plovdiv, Pazardzhik, and Burgas, show that the three predominant taxa are Arachnida (14.23%), Hymenoptera, Formicidae (9.61%), Diptera (8.18%). The trophic spectrum of the species to some extend is similar to that of *Rana graeca*, which can be explained by the fact that the two species inhabit similar habitats - mountain springs, streams, and rivers. The Common Frog does not show particular preferences in food (d=0.14) and has high values of the trophic niche breadth - 16.01, which leads us to conclude that it is probably polyphagous.

Agile Frog (Rana dalmatina). Only one study by Angelov & Batschwarov (1972) was conducted for this species. The authors' data based on a small sample (n = 12) show that the Agile Frog feeds mainly on Coleoptera (56%), Gastropoda (16%), Myriapoda (8%) and has a high value of the trophic niche breadth. - 8.11. Further research on the species' diet based on larger samples is needed.

Eastern Tree Frog (Hyla orientalis). So far, three studies have been conducted on the trophic spectrum of this species in Bulgaria (Angelov, 1960; Batchvarov, 1967; Angelov & Batschwarov, 1972). Unfortunately, the three studies were conducted on small samples and give very contradictory results: according to Angelov (1960) the predominant taxa in the diet of *Hyla orientalis* are Coleoptera (67.1%), Hymenoptera (14.6%) and Diptera (7.3%); Batchvarov (1967) mentions only Coleoptera, without any quantification and according to Angelov & Batschwarov (1972), the predominating taxes are: Lepidoptera (larvae) - 30.59%, Arachnida - 23.53%, Diptera (larvae) - 16.47%). Due to this fact, we cannot draw any specific conclusions. *Hyla orientalis* is probably polyphagous, and this is partly confirmed by the values of the width of the niche of the species, which varies from 2.11 to 5.68.

Currently, in Bulgaria, there are no studies on the trophic spectrum of the following species: *Pelobates fuscus, P. syriacus, P. balkanicus, Hyla arborea, Pelophylax* kl. *esculentus, P. bedriagae,* and *P. lessonae*.

Figure 1 presents the average values and the standard deviation of the trophic niche breadths, calculated according to the data from all available studies. The chart shows that the highest values are presented in the trophic niches of the aquatic frogs - *P. ridibundus, R. graeca, R. temporaria, R. dalmatina*. The trophic niches breadths of the other anuran species found in Bulgaria have significantly lower values, the lowest being in *Hyla orientalis*.

From the analysis of the summarized data, we can conclude that all anuran species in Bulgaria are zoophagous, mainly insectophages, because insects occupy a significant part of their trophic spectrum. Summarized data from the Berger-Parker index from all studies show that all species show broad trophic specialization (Table 2) and can be classified as polyphagous, except for *Bombina variegata*, which has narrower limits in the index and shows a preference for the order Coleoptera. Some authors report plant and inorganic components (pebbles, sand, etc.) in the food spectrum of the studied species, which are considered to be accidentally ingested.

Figure 2 shows a dendrogram showing the similarity between the trophic spectrum of all studied Bulgarian anuran species, calculated based on quantitative data for each species, from the available studies. The figure shows that the studied species in terms of their diet are grouped into three clusters. At 5% similarity, *Rana dalmatina* was isolated in a separate cluster, but this may also be due to the fact that only one study on this species was conducted on a very small sample. The other species are divided into two clusters, the first, which is separated at about 7% similarity, consists of two species - *Bufotes viridis* and *Pelophylax ridibundus*. The reason for the grouping of the two species, in our opinion, is first of all the fact that most of the studies were done on them. On the other hand, the food of the two species is similar, as they hunt mainly on land, and the main taxa they catch are Coleoptera, Hymenoptera, etc. The beetles and ants are basic food most probably due to the abundance of this preys and the wide range of habitats they occupy.

The third cluster is further divided into two sub-clusters. The first one is isolated at about 25% similarity and includes *H. orientalis, R. temporaria*, and *R. graeca*. We have already commented that the trophic spectrum of *R. temporaria* and *R. graeca* is similar mainly because the two species inhabit very similar habitats - clear, mountain streams, rivers, lakes, and rarely swamps. *H. orientalis* appears to show higher similarity to the diet of *R. temporaria* with approximately 43% similarity. The second sub-cluster is separated at about 35% similarity and includes *Bufo bufo, Bombina bombina, B. variegata*, as the last two species have a higher similarity in their trophic spectrum with about 40%.

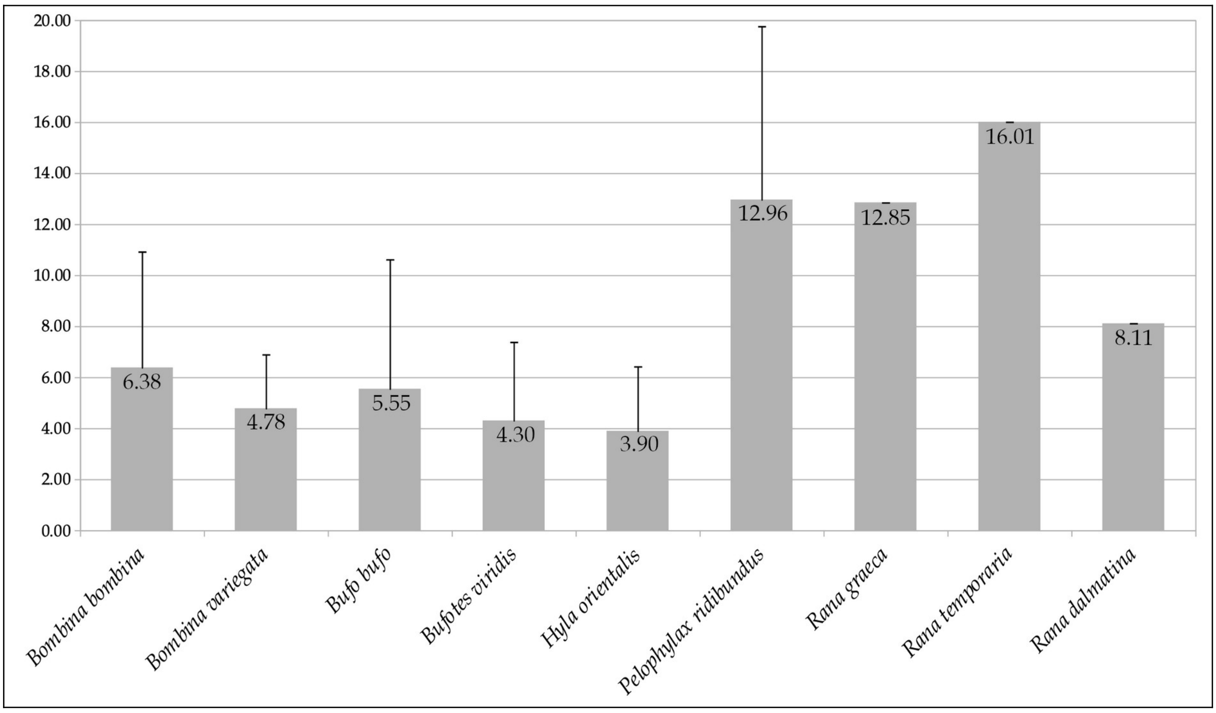

Fig. 1. Average values and standard deviation of the trophic niche breadth of all studied species, calculated based on data from all available studies, conducted in Bulgaria.

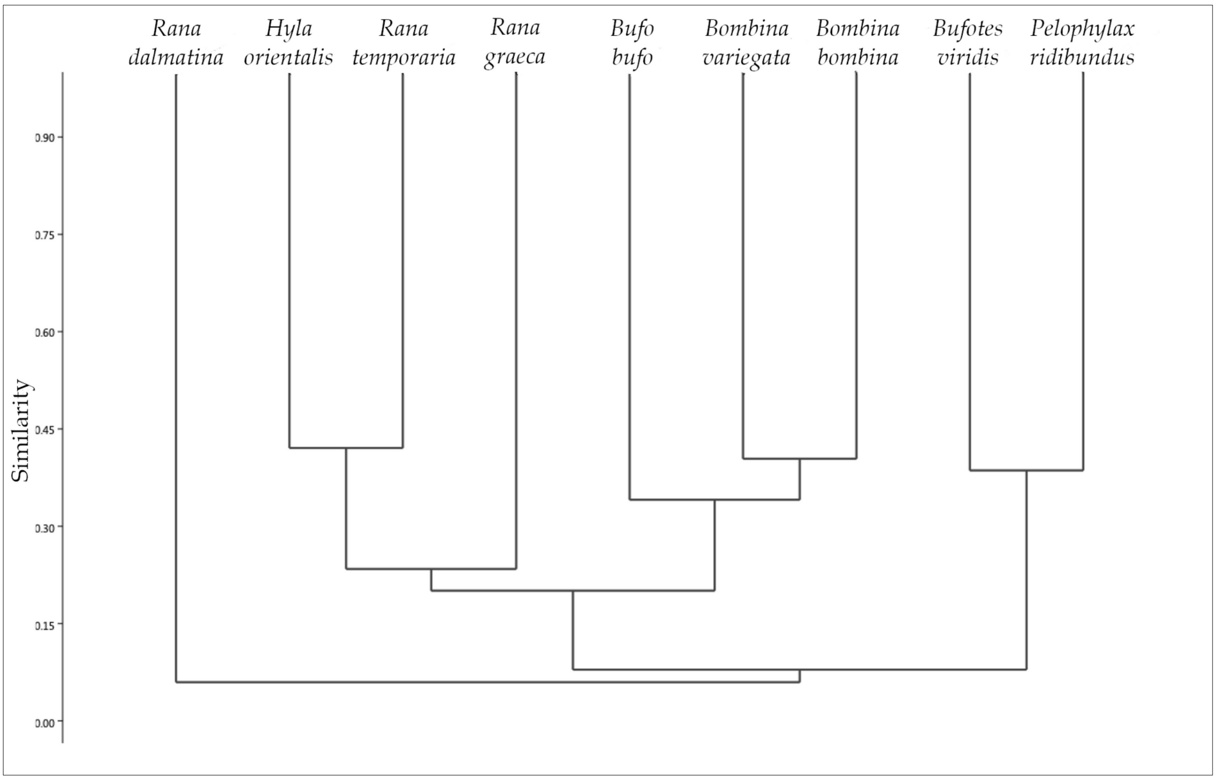

Fig. 2. Similarity of the trophic spectrums of all studied Bulgarian anurans (cluster analysis, Jaccard similarity index, Unweighted Pair Group Average), calculated based on the quantitative data from all available studies, conducted in Bulgaria.

Table 2. Trophic specialization of the Bulgarian anuran species, based on the Berger-Parker index values, calculated from the available studies in Bulgaria.

Species	Berger-Parker index	Trophic specialization
Fire-belied Toad (*Bombina bombina*)	0.27-0.85	polyphage?
Yellow-belied Toad (*Bombina variegata*)	0.44-0.62	oligophage?
Common Toad (*Bufo bufo*)	0.36-0.70	polyphage?
Green Toad (*Bufotes viridis*)	0.46-0.76	polyphage?
Eastern Tree Frog (*Hyla orientalis*)	0.31-0.67	polyphage
Marsh Frog (*Pelophylax ridibundus*)	0.17-0.58	polyphage
Greek Stream Frog (*Rana graeca*)	0.16	polyphage
Common Frog (*Rana temporaria*)	0.14	polyphage
Agile Frog (*Rana dalmatina*)	0.56	polyphage?

All studies conducted in Bulgaria until 1991 are characterized by research only of the qualitative and quantitative composition of the diet of the Bulgarian anurans and an analysis of what part of the prey are pests in agriculture, forestry, and fisheries. Some authors analyze the seasonal variations in the trophic spectrum of the studied species. Studies on the trophic niche breadth, the overlap of niches between the sexes, and the role of anuran amphibians in food chains in biocenoses are done by Mollov (2008);

Mollov et al. (2006; 2010). There is still no study on the overlap of trophic niches between species that occur together, as well as a studies of the diet regarding its volume.

More studies need to be conducted on the qualitative and quantitative composition of the diet anurans in Bulgaria (especially the species that have not yet been studied in this regard), as well as more detailed studies of their feeding ecology.

Conclusions

Currently, there are 18 specialized studies on the diet of the Bulgarian anuran species. Of all 16 anuran species, occurring in Bulgaria, about half have been studied in connection with their trophic spectrum so far. All Bulgarian species of amphibians are zoophagous, mainly insectivorous, and can be classified as general feeders (polyphages), except *Bombina variegata*, which shows a slight preference to Coleoptera and *Bufo bufo* and *Bufotes viridis*, which show slight preference to Formicidae and Coleoptera. Cannibalism was recorded only in *Pelophylax ridibundus*. *P. ridibundus, R. graeca, R. temporaria,* and *R. dalmatina* has the highest values of the trophic niches breadths. In contrast, the other anuran species found in Bulgaria have significantly lower values, the lowest being in *Hyla orientalis*.

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

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

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

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