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Role of Nanomaterials of Analcite, Tripoli and Silicon dioxide in Plants under Drought Conditions in Triticum aestivum L.

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Abstract. The effect of the application of nanoparticles of analcite, tripoli and silicon dioxide to gray podzolic soil at a dose of 250 mg per 100 g on drought resistance of wheat seedlings was studied under laboratory conditions. The dependence of the silicon effect on soil moisture level was also evaluated. For this, four levels of soil moisture were modeled: 20%, 40%, 60% and 80% field capacity. The positive effect has been established for all variants tested: seedlings growth criteria as well as content of photosynthetic pigments increased, while transpiration intensity decreased under water deficit. Application of nanoparticles caused accumulation of protective antioxidants. While proline, malondialdehyde, souble sugars content decreased. The dependence of analcite, tripoli and silicon dioxide effect on soil moisture level was discussed.

Key words: analcite, tripoli, silicon dioxide, nanoparticles, drought resistance, *Triticum aestivum* L.

Introduction

Drought is a worldwide issue that impacts seriously on the security of food production. Global climate change makes this even worse. Winter wheat is almost the most important crop in the world (Gornall *et al.*, 2010; Curtis & Halford, 2014). Studies have shown that drought has many adverse impacts on crops, inhibiting growth and photosynthesis, changing morphology, decreasing production and photosynthetic pigment content, affecting ion balance in plants, etc. (HongBo *et al.*, 2005; Sofo et *al.*, 2005; Tian & Lei, 2007; Singh *et al.*, 2012; Sharma *et al.*, 2012).

Silicon has not been considered as a generally essential element for higher plants, partly because its roles are poorly understood. However, numerous studies have demonstrated that silicon plays an important role in tolerance of plants to environmental stresses, especially to drought (Gong *et al.*, 2005; Pei *et al.*, 2010; Shen *et al.*, 2010; Guntzer *et al.*, 2012; Rositska, 2012; Zaimenko *et al.*, 2014), but the defense mechanisms provided by Si are far from being understood. Silicon fertilization has the potential to mitigate environmental stresses and soil nutrient depletion and as a consequence is an alternative to the extensive use of phytosanitary and NPK fertilizers for maintaining sustainable agriculture (Guntzer *et al.*, 2012). We suggest that silicon is useful for drought tolerance improvement of plants.

The objective of this study was to evaluate experimentally the effects of silicon application under drought conditions.

Materials and Methods

Wheat leaves were isolated from plants *Triticum aestivum* L., cv. Podil'ska 90 (drought–sensitive) grown during one month under laboratory conditions at 22-24 °C. Soil humidity of gray podzolic soils was maintained at 20%, 40%, 60%, 80% of field capacity. As a source of silicon was used natural minerals (analcite and tripoli) and silicon dioxide (SiO_2) at a dose of 250 mg per 100 g of soil. The concentration of silicon compounds was selected on the basis of our previous studies (ROSITSKA, 2014).

Lipid peroxidation was measured as malondialdehyde (MDA) in the leaves and was analyzed following KABASHNYKOVA *et al.* (2007) method. Proline was extracted from freshly cut leaves by 3% sulfosalicyl acid. Its quantitative content was determined using qualitative reaction with ninhydrin on spectrophotometer "Specord 200" (STATSENKO & BUTYLKIN, 1999). Catalase activity was determined by the method of PLESHKOV (1985).

The content of pigments was extracted from leaves by acetone. The solution mixture was analyzed for Chlorophyll-a, Chlorophyll-b and carotenoids content on spectrophotometer "Specord 200" (POCHINOK, 1976).

Soluble sugars were determined based on the method of Bertrand (KOLUSHEVA & MARTINOVA, 2011). The transpiration rate was determined by registering changes in weight of cut transpirating leaves for short time intervals (TRETYAKOV, 1990).

The results presented in the tables are the means of four replications. The data were statistically analyzed using the least significant difference (LSD) test ($p<0.05$). The effect of analcite dose, soil type, moisture level, and species of tested plants on their adaption to soil drought was assessed using analysis of variance (ANOVA) and correlation analysis with the help of Statistica 6.0 software (STATSOFT INC., 2001).

Results and Discussion

Plant growth and development

Water stress depressed the growth of shoot and root. There is a general agreement on the positive effect of Si application on the biomass yield under water stress (ENEJI *et al.*, 2008). Indeed, increases of biomass and grain yields have been observed on a large set of crops (ENEJI *et al.*, 2008; SHEN *et al.*, 2010; PEI *et al.*, 2010). We report the results of the study of natural minerals' effects on wheat leaves growth rate in Fig. 1 and 2. It appears that the values of the parameters in experimental plants exceed the control ones. After adding silicon in different forms to the substrate, we observed a significant increase in growth rate in comparison to control plants. Particularly, the growth rate of aboveground part of wheat seedling in experimental plants exceeded growth rate in the control plants at the level 34−73% (analcite), 10−94% (tripoli) and 30−77% (silicon dioxide) depending on the soil humidity. The growth rate of underground part of wheat seedling was increased by 12−66% (analcite), by 7−91% (tripoli) and by 5−66% (silicon dioxide) in comparison to control.

The content of chlorophyll and carotenoids

Along with determining the growth rate of wheat leaves, we studied distribution of photosynthetic pigments in leaves under different moisture conditions (Table 1). It is well established that drought inhibits photosynthesis, induces changes in chlorophyll content and composition, and also damages the photosynthetic apparatus (NAYYAR & GUPTA, 2006). SAIRAM *et al.* (1997/98) reported an increase in lipid peroxidation and a decrease in the level of total chlorophyll and carotenoids. Increased MDA accumulation has been correlated with a reduction in the relative water content and photosynthetic pigment content of leaves subjected to prolonged water deficit (JIANG & HUANG, 2001). Furthermore, dehydration of tissue inhibits photochemical activities and brings about a reduction in the activity of Calvin–Benson–Basshamn cycle enzymes (MONAKHOVA & CHERNYADEV, 2002). GONG *et al.* (2005) observed that Si increased antioxidant defenses and therefore maintained physiological processes such as photosynthesis.

The detailed analysis reveals increasing of the total amount of chlorophyll. That is to say the chlorophyll content from 20 to 60% soil humidity in experimental plants increased by 1.4–2.7 times (analcite), by 1.6–2.8 times (tripoli) and by 1.8–3.3 times (silicon dioxide). But at 80% humidity, these figures, in contrast, decreased, except for analcite. Our experiments showed a positive effect of silicon compounds in the biosynthesis of carotenoids.

Soluble sugars

The concentration of soluble sugars in general increases or at least remains constant under water deficit stress. Recent studies report the accumulation of simple sugars such as glucose and fructose following an increase in the invertase activity in the leaves of the drought challenged plants (PINHEIRO *et al.*, 2001; TROUVERIE *et al.*, 2003).

Analysis of the results showed (Table 2) reduction of soluble sugars in all treatments of the experiment compared to the control plants. The most significant decrease fixed in 20% soil humidity. In particular, analcite reduced concentration of mono- and disaccharides by 1.2–2.1 times, tripoli and silicon dioxide – by 1.1–2.7 and by 1.1–1.5 times respectively.

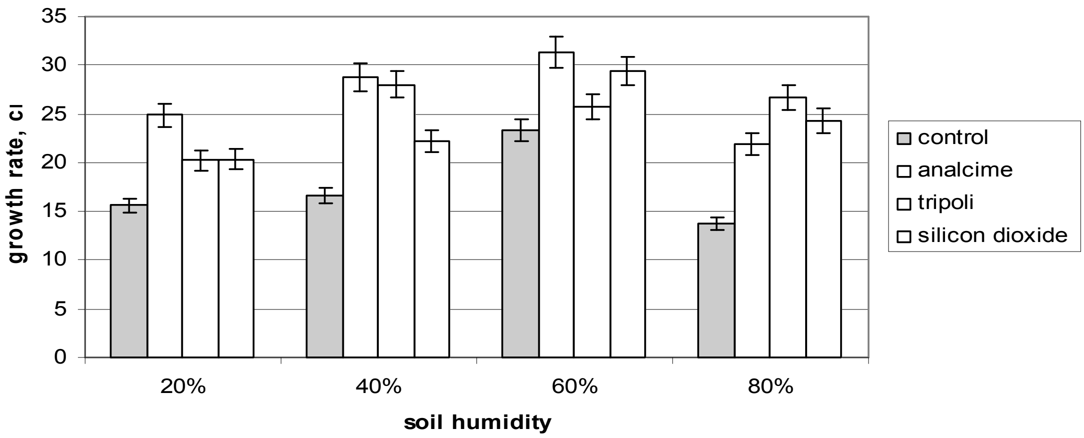

Fig. 1. The impact of different forms of silicon on the growth rate of aboveground part of wheat seedling. Vertical bars – LSD (least significant difference).

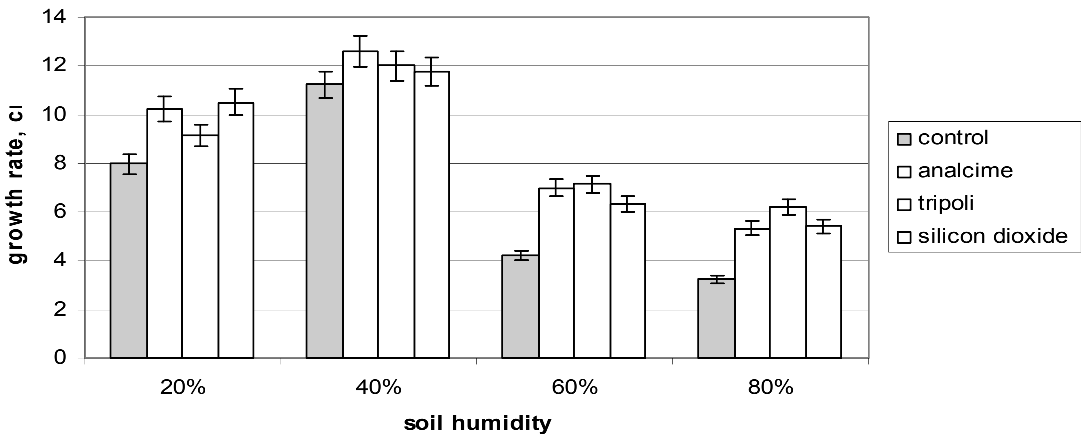

Fig. 2. The impact of different forms of silicon on the growth rate of underground part of wheat seedling. Vertical bars – LSD (least significant difference).

Table 1. The impact of silicon on photosynthetic pigments content in wheat leaves.

Soil humidity	Chlorophyll, mg/g fresh weight		Carotenoids, mg/g fresh weight
	a	b	
	Control		
20%	0.20	0.13	0.06
40%	0.33	0.14	0.06
60%	0.19	0.08	0.05
80%	0.58	0.23	0.11
	Analcite		
20%	0.63	0.25	0.14
40%	0.45	0.20	0.09
60%	0.43	0.18	0.09
80%	0.60	0.25	0.13
	Tripoli		
20%	0.54	0.20	0.10
40%	0.55	0.23	0.11
60%	0.54	0.23	0.10
80%	0.50	0.20	0.09
	Silicon dioxide		
20%	0.77	0.32	0.14
40%	0.72	0.28	0.13
60%	0.35	0.15	0.08
80%	0.56	0.23	0.10
LSD	*0.021*	*0.007*	*0.004*

Table 2. The impact of silicon on soluble sugars content in wheat leaves, %.

Soil humidity	Control	Analcite	Tripoli	Silicon dioxide
		Monosaccharides		
20%	0.57	0.27	0.21	0.40
40%	0.21	0.15	0.10	0.15
60%	0.17	0.10	0.10	0.16
80%	0.10	0.10	0.10	0.04
		Disaccharides		
20%	0.89	0.74	0.77	0.59
40%	0.95	0.87	0.88	0.82
60%	0.94	0.90	0.88	0.78
80%	0.89	0.79	0.79	0.83
LSD	*0.02*	*0.02*	*0.02*	*0.02*

Transpiration intensity

Wheat plants subjected to drought and treated with Si maintained higher stomatal conductance, relative water content, and water potential than non-treated plants. Besides, leaves were larger and thicker, thereby limiting the loss of water through transpiration (GONG *et al.*, 2003; HATTORI *et al.*, 2005) and reducing water consumption (ENEJI *et al.*, 2005). It was established decreasing in the intensity of transpiration after adding analcite by 2.0–8.0 times, tripoli – by 1.5–4.0 times, and silicon dioxide – by 1.4–3.2 times compared to the control (Fig. 3).

Lipid peroxidation

It has been shown that under stress conditions, MDA accumulation takes place in plants due to membrane lipid peroxidation (HONGBO *et al.*, 2005). It is an effective means of assessing oxidative stress induced membrane damage including changes to the intrinsic properties of the membrane, such as fluidity, ion transport, loss of enzyme activity and protein cross–linking. These changes eventually result in cell death (MELONI *et al.*, 2003; SAIRAM *et al.*, 2005; SHARMA *et. al.*, 2012). It is possible that the detrimental effect of water deficit in soil is associated with levels of lipid peroxidation in tissues. SINGH *et al.* (2012) showed that instability of biological membranes, as reflected by lipid peroxidation, was greater in drought–sensitive than in drought–tolerant wheat (*Triticum aestivum* L.) genotypes.

Thus, the concentration of MDA in all variants of the experiment decreased with increasing of soil humidity (Fig. 4). In particular, analcite and tripoli reduced concentration of MDA by 1.2–2.4 times, silicon dioxide – by 1.2–3.6 times. These results suggest that stress-induced membrane lipid peroxidation could be partly alleviated by added silicon.

Proline content

Proline is one of the minor amino acids in non-stressed plants, but accumulates very strongly during adaptation to water stress. Special functions proposed for proline are the stabilization of membranes under water deficit or role as small compatible molecule (BÜSSIS & HEINEKE, 1998). Some of the crop plants for instance wheat is marked by the accumulation and mobilization of proline was found to increase tolerance towards water deficit stress (NAYYAR & WALIA, 2003). The analysis revealed that adding analcite reduced proline concentration by 1.1–1.5 times, tripoli – by 1.1–2.1 times and silicon dioxide – by 1.3–1.5 times (Fig. 5).

Catalase activity

It was reported that catalase is highly expressed enzyme, particularly in plant cell, and is thus an integral part of the plant antioxidant system. Catalase functions as one of the key enzymes in the scavenging of reactive oxygen species and affects on toxic H_2O_2 levels in a cell (SOFO *et al.*, 2005). Furthermore, SINGH *et al.* (2012) concluded that the progressive increase in MDA during plant development may have resulted from greater levels of hydrogen peroxide. Moreover, a very strong and positive correlation was reported to exist between levels of hydrogen peroxide and MDA in the leaves of wheat plants grown under irrigated and rain–fed conditions. Conversely, the correlation between H_2O_2 and MDA was positive (TIAN & LEI, 2007). The analysis of the data showed a positive effect of silicon compounds on the activity of catalase (Fig. 6). In experiments observed a direct correlation between the level of soil humidity and the activity of the enzyme.

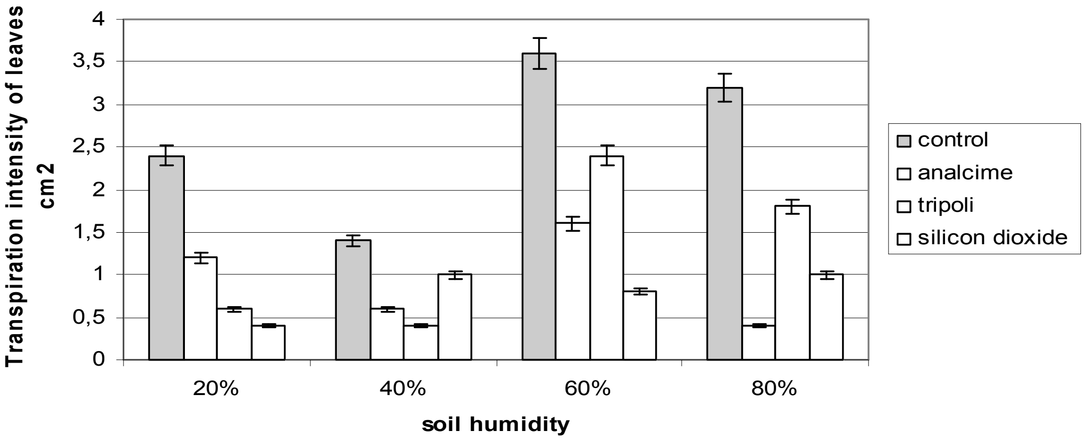

Fig. 3. Transpiration intensity of wheat seedling under different sources of silicon. Vertical bars – LSD (least significant difference).

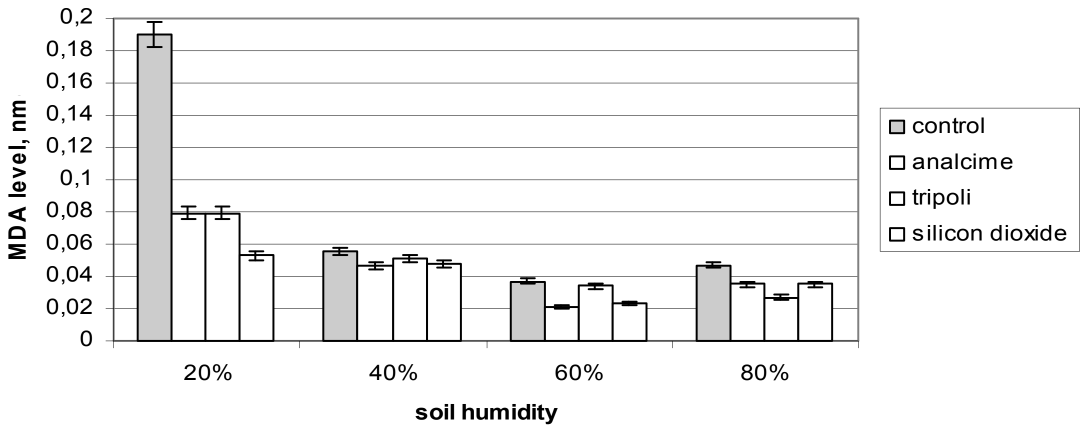

Fig.4. The impact of silicon on MDA content in wheat leaves. Vertical bars – LSD (least significant difference).

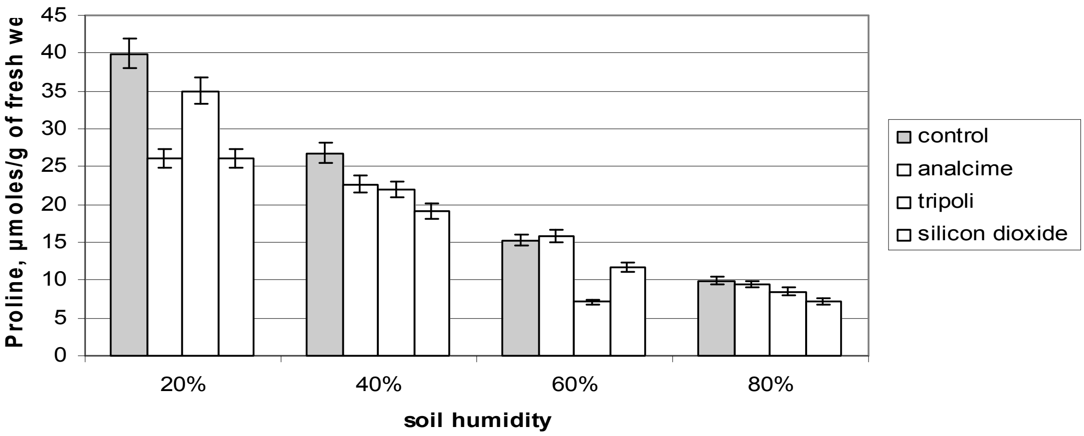

Fig.5. The impact of silicon on proline content in wheat leaves. Vertical bars – LSD (least significant difference).

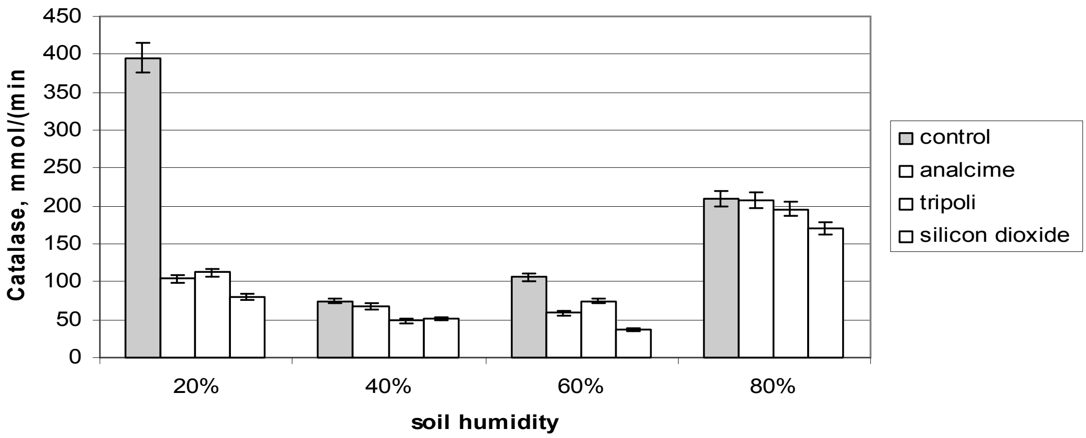

Fig. 6. The impact of silicon on catalase activity in wheat leaves. Vertical bars – LSD (least significant difference).

Conclusions

The data presented indicate that the application of nanoparticles of analcite, tripoli and silicon dioxide to gray podzolic soil contributes to the increase in the resistance of wheat plants to drought stress. The positive effect has been established for all variants tested: seedlings growth criteria as well as content of photosynthetic pigments increased, while transpiration intensity decreased under water deficit. Application of nanoparticles also caused a decrease in the content of proline, souble sugars and malondialdehyde.

Thus, the physiological and biochemical parameters measured indicated that Si could alleviate seedling damage under water stress conditions, improve mineral nutrition, increase drought resistance of plants *Triticum aestivum* L., and stimulate growth processes by enhancing antioxidant system.

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Animal Road Mortality (Aves & Mammalia) from the New Section of the Maritsa Highway (South Bulgaria)

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Abstract. A total of 197 road kill victims were recorded in the period November 2015 - May 2017 from the new section of the Maritsa Highway in South Bulgaria. The Northern White-breasted Hedgehog (*Erinaceus roumanicus* Barret-Hamilton, 1900) - 18.6%, n=118, Eurasian Badger (*Meles meles* (Linnaeus, 1758)) - 16.9%, n=118, Golden Jackal (*Canis aureus* Linnaeus, 1758) - 15.2%, n=118 and Common Buzzard (*Buteo buteo* (Linnaeus, 1758)) - 25.3%, n=79 were the most frequently observed victims. The number of victims detected at varying traffic intensities is different and increases with increasing road traffic. There are several parts of the highway where road kill victims are concentrated.

Key words: road kills, Maritsa Highway, South Bulgaria, Aves, Mammalia.

Introduction

Bulgaria is situated at the crossroad of the Balkans. Some of the main roads between Western Europe and the Middle East pass through it. In Bulgaria, plans have been developed to expand the road network significantly. Transport corridors are one of the causes of habitat fragmentation (VAN DER GRIFT *et al.*, 2008). The last decade is characterized by intensive road building. The routes of two major highways ("Trakia" and "Maritsa") in Bulgaria have been completed. The Maritsa highway is a part of the Pan-European Transport Corridor IV (Bulgarian Road Infrastructure Agency).

The number of wildlife casualties on roads and railways has constantly grown worldwide as traffic and vehicle speeds have increased and infrastructure networks expanded (SEILER *et al.*, 2004). Victims of road traffic among various animal groups are relatively well studied in a number of European countries. Problem sections and regularities have been identified during the different annual seasons (see HANER *et al.*, 2002; HELL *et al.*, 2005; LODÉ, 2000; HUIJSER & BERGER, 2000; HAIGH, 2012; ROIG-MUNAR *et al.*, 2012). In Bulgaria, identified problem areas with a concentration of road traffic casualties are reported by VAN DER GRIFT *et al.* (2008), data on road casualties in two types of road network give KAMBUROVA-IVANOVA *et al.* (2012), MOLLOV *et al.* (2013) and MICHEV *et al.* (2017).

The purpose of this study is to present the results of the animal road traffic victims for a 19 months period on the new section of the Maritsa highway (South Bulgaria) since it was put into exploitation.

Materials and Methods

In the period November 2015 - May 2017 the animal victims of road traffic were reported in the 70th km section of the

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Maritsa highway. The start of the research area was put on the beginning of the highway (0 km), the end was the 71st kilometer of the road near Harmanli Town (Fig. 3). Surveys were conducted twice a month over a 2-week period. The number and species of killed birds and mammals were recorded by traveling with a car with a speed of less than 40km/h by searching the roadway in both directions. Only mammals and birds were reported across the width of the roadway. For each victim we recorded the exact location and by the accumulation of these points we established the sections of the road with concentrations of killed animals. All found animals were removed when it was possible to avoid double records in the next survey. Victims have been identified as accurately as possible using key features given by Popov & Sedefchev (2003) and Svensson & Grand (1999). When birds were in poor condition we also used feather keys according to Hansen & Synnatzschke (2015).

The intensity of road traffic was determined by counting the number of vehicles passing for one hour at 36.4 kilometers (Fig. 3). The road traffic was determined in every report. Then the traffic was classified into three categories: up to 20 vehicles – low(1); between 21 and 60 vehicles – medium(2); 61 and more vehicles – high(3).

Differences in the number of victims during different seasons, and according to different traffic intensity, were tested by the Kruskal-Wallis test. Pearson's correlation coefficient was used to determine the relationship between the number of victims and the intensity of road traffic (Fowler & Cohen, 1987). All statistical analyses were performed using PAST (Hammer *et al.*, 2001).

Results

The total number of victims in the survey period is 197. Their number during the first months after the start of the road was 16, mainly birds (Table 1).

The most frequently recorded road kill victims were the Northern White-breasted Hedgehog (*Erinaceus roumanicus* Barret-Hamilton, 1900) - 18.6%, n=118, Eurasian Badger (*Meles meles* (Linnaeus, 1758)) - 16.9%, n=118 and Golden Jackal (*Canis aureus* Linnaeus, 1758) - 15.2%, n=118 within the mammals, and Common Buzzard (*Buteo buteo* (Linnaeus, 1758)) - 25.3%, n=79 within the birds.

There are no statistically significant differences in bird deaths by months for the study period (Kruskal-Wallis Chi square = 7.887, p>0.05). However, there was a slight increase in May-June and December. Although mammalian victims are slightly more in March-April, June and December, there are no significant differences in road kills between months (Kruskal-Wallis Chi square = 22.14, p>0.05) (Fig. 1).

The number of victims detected at varying traffic intensities is different and grows when the road traffic increases (Kruskal-Wallis Chi square = 13.91, p<0.001) (Fig. 2). There is a moderate positive correlation (r=0.45, p<0.001) between the number of passing vehicles per hour and the number of reported victims.

There are several areas where the victims are concentrated: 7th – 8th km (pointer 1), 21st -22nd km (pointer 2), 26.5th - 27.5th km (pointer 3), 42.5th - 43.5th km (pointer 4), about 54th km (pointer 5), 59th km (pointer 6) and 70th kilometer (pointer 7) (Fig. 3).

Discussion

In the present study, White-breasted Hedgehog and Eurasian Badger are the predominant victims within the mammals. Our results are in agreement with those from similar surveys (Haigh, 2012). The number of dead Hedgehogs increased from April to July and gradually decreased to single individuals in November-February. Most of the losses reported in June. Some studies explain these facts by the growing of the home range of males in this period and correspondingly increasing the road casual-

Table 1. Species composition and number of road traffic victims of the Maritsa Highway for the period November 2015 - May 2017.

Species	Number of individuals (2015)	Number of individuals (2016)	Number of individuals (2017)	Total	Number per km
Mammalia					
Erinaceus roumanicus Barret-Hamilton, 1900	0	20	2	22	0.32
Lepus europaeus Pallas, 1778	0	1	3	4	0.06
Canis lupus familiaris L., *1758*	0	9	1	10	0.15
Canis aureus L., 1758	0	15	3	18	0.26
Vulpes vulpes (L., 1758)	1	5	4	10	0.15
Meles meles (L., 1758)	1	17	2	20	0.29
Mustela nivalis L., 1766	0	1	0	1	0.01
Mustela putorius L., 1758	0	5	1	6	0.09
Mustela sp.	0	4	0	4	0.06
Martes foina (Erxleben, 1777)	1	9	4	14	0.21
Felis silvestris Shreber, 1777	0	5	1	6	0.09
Felis silvestris catus L., 1758	0	3	0	3	0.04
Subtotal Mammalia	3	94	21	118	1.74
Aves					
Buteo buteo (L., 1758)	6	13	1	20	0.29
Falco tinnunculus L., 1758	0	1	0	1	0.01
Falconiformes unind.	1	0	0	1	0.01
Larus michahellis J. F. Naumann, 1840	0	3	0	3	0.04
Streptopelia turtur (L., 1758)	0	1	0	1	0.01
Tyto alba (Scopoli, 1769)	2	6	0	8	0.12
Athene noctua (Scopoli, 1769)	0	2	0	2	0.03
Merops apiaster L., 1758	0	3	2	5	0.07
Delichon urbicum (L., 1758)	0	1	0	1	0.01
Motacilla alba L., 1758	0	1	0	1	0.01
Turdus merula L., 1758	1	1	1	3	0.04
Pica pica (L., 1758)	0	2	0	2	0.03
Sturnus vulgaris L., 1758	3	0	4	7	0.10
Passer hispaniolensis (Temminck, 1820)	0	1	0	1	0.01
Passer montanus (L., 1758)	0	1	0	1	0.01
Fringilla coelebs L., 1758	0	0	6	6	0.09
Carduelis carduelis (L., 1758)	0	2	0	2	0.03
Emberiza melanocephala Scopoli, 1769	0	1	0	1	0.01
Emberiza calandra L., 1758	0	4	1	5	0.07
Passeriformes unind.	0	6	2	8	0.12
Subtotal Aves	13	49	17	79	1.16
Total	16	143	38	197	2.90

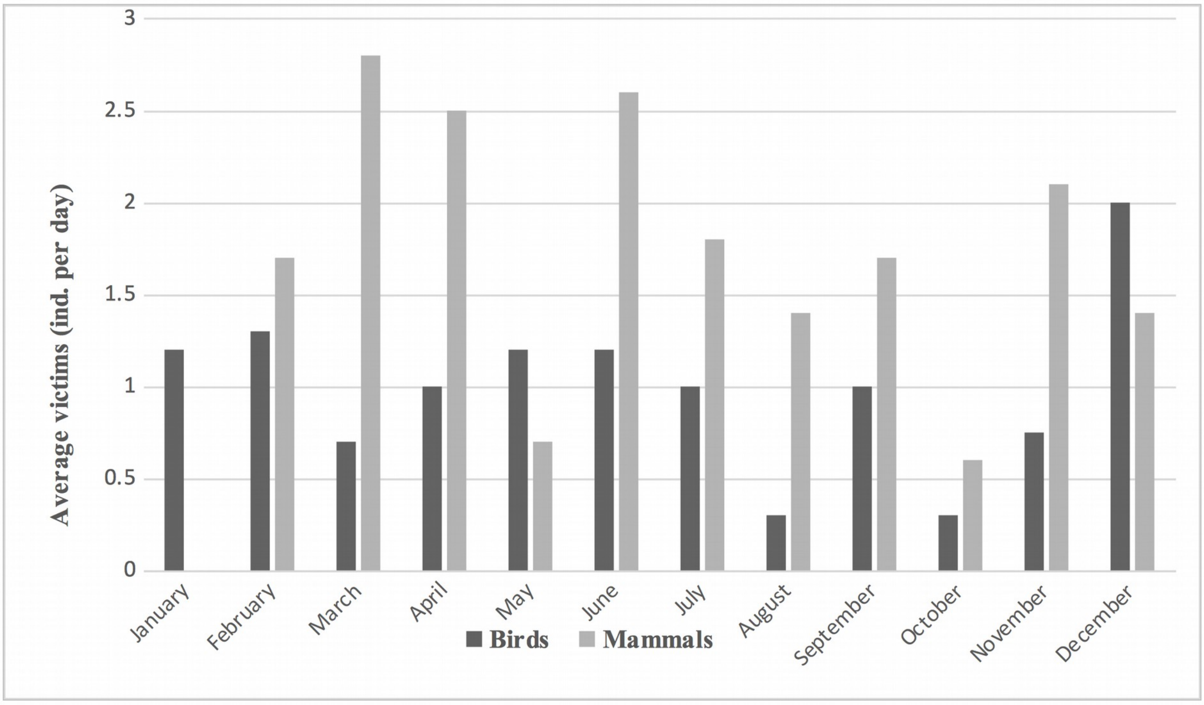

Fig. 1. Average number of victims by months for the study period.

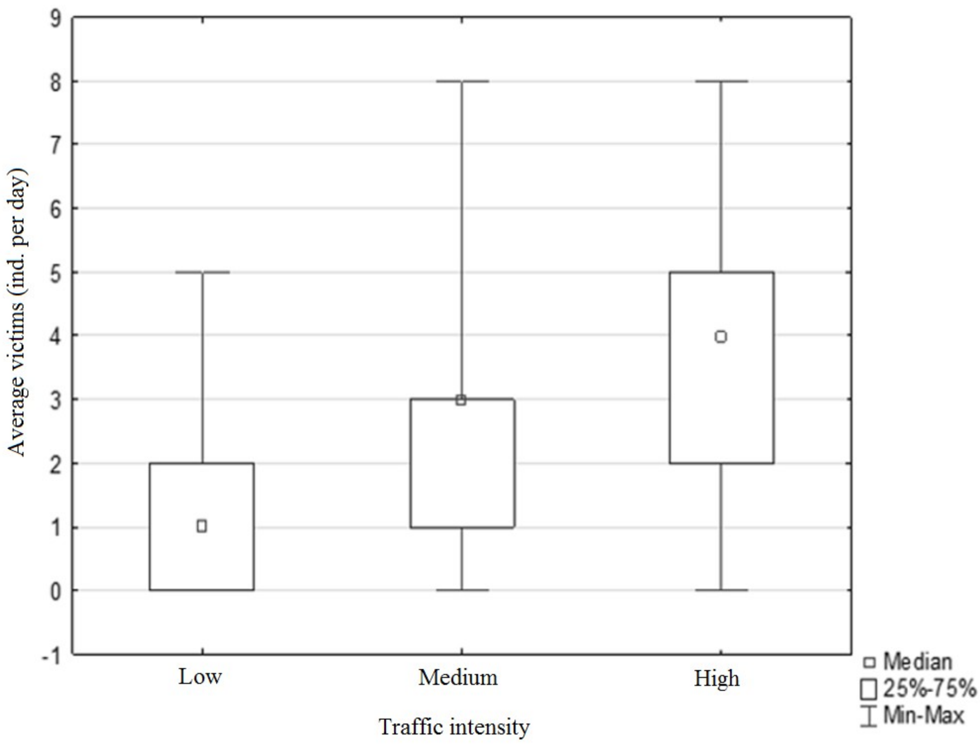

Fig.2. Box and Whiskers plot of the number of road kill victims by different traffic.

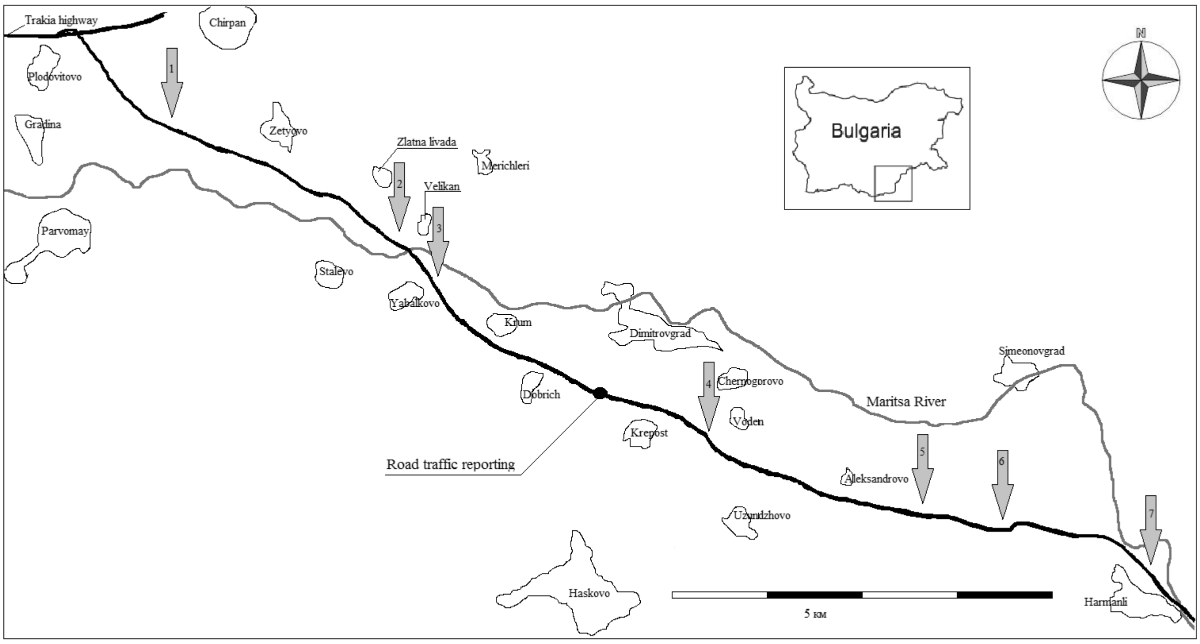

Fig.3. Study area and places with concentrations of victims in the period November 2015-May 2017.

ties of this species (Holsbeek *et al.*, 1999; Jackson, 2006; Haigh, 2012).

Badger is the second most frequently recorded victim in this study. The peak of the reported victims was observed in the spring. This species is also reported as a victim of road traffic in other studies in Britain and Ireland (Davies *et al.*, 1987; Haigh, 2012). The mortality peaks in this study can be explained by the increased activity of the badgers before the breeding season. The results are obtained consistent with those of Davies *et al.* (1987), Smiddy (2002) and Haigh (2012) and are similar to those from the latest study in Bulgaria (Kamburova-Ivanova *et al.*, 2012).

Another victim with high loss in our study is the Golden Jackal. It has a higher incidence of reported deaths compared to previous studies for Bulgaria. Kamburova-Ivanova *et al.* (2012) reported 4.3% of the number of victims in mammals, while in this study, the road killed Golden Jackals were 15.2% of the mammalian losses. We assume that this is due to the likely higher density of the jackals in the studied area. In Bulgaria, it is estimated to occupy about 72% of the territory. Our research region falls into one of the highest density areas for this species (about 15 ind./10km^2) of the species according to Stoyanov (2012). Our data does not allow the definition of the peaks of the loss of jackals, although in November 2016 we recorded the biggest number of dead individuals (6 ind.). Probable cause for losses in this case is due to the fact that jackals have gone out on the highway to feed with other dead animals. The Golden Jackal is an opportunist and moves easy to affordable food, the breadth of the food niche depends mostly on the supply in the area (Stoyanov, 2012).

From the bird victims the most common is the Common Buzzard. Our results confirm similarities in previous studies in Bulgaria (Kamburova-Ivanova *et al.*, 2012). We have seen a concentration of victims of this species in November and December and their gradual reduction from January to March. These results are also in agreement with Meunier *et al.* (2000) and Kamburova-Ivanova *et al.* (2012). According to these authors, Common buzzards use roads for hunting mainly during the winter and this is one of the reasons for the increased number of casualties in this period.

This study does not reveal significant differences in the number of victims by month. Similar results have been observed in other studies (HAIGH, 2012). Peaks in the number of road traffic victims were reported in a number of studies (GRYZ & KRAUZE 2008; KAMBUROVA-IVANOVA *et al.*, 2012). The absence of such peaks in this study is due to the relatively small number of dead animals. A small number of victims and their relatively even distribution throughout the survey period do not allow a clear distinction between seasons with maximum and minimum casualties. A disadvantage of this study is the reporting of victims by vehicle. Although the vehicle is moving slowly, some of the victims may be unreported. The analysis of data showed that the manner of surveying (car, bicycle or foot) significantly affected the number of casualties found (ERITZOE *et al.*, 2003). In Bulgaria there are restrictions on the movement on the highways by bicycle or on foot. It is also possible that some victims are not counted because other predatory mammals and birds have removed them before the report.

The growing number of losses with the increasing of road traffic is logical, because it changes the probability of crashes (ERRITZOE *et al.*, 2003). However, there are various results on the number of victims at different traffic intensities (see also BRUUN-SCHMIDT, 1994; FUELLHAAS *et al.*, 1989). Our results are consistent with those of GOŁAWSKI & GOŁAWSKA (2002) and GLISTA *et al.* (2008), according to whom the number of victims is increased as traffic increases. The reduction in road deaths while traffic increases is explained by other authors with a decrease in the density of the victims' populations (HANSEN, 1982) or the so-called "learning affects" (ERRITZOE *et al.*, 2003). The new highways may have a higher rate of road deaths than the old ones, but similar results could also be due to a reduction in the size of the species populations. (HAVLIN, 1987; ERRITZOE *et al.*, 2003). Our results show fewer victims per kilometer than the number of victims of Trakia highway (KAMBUROVA-IVANOVA *et al.*, 2012). We do not have data on the size of the victims' populations in the area around the Maritsa highway. Therefore, we cannot claim that the small number of established victims is due to the low density of their populations.

A total of seven points with the accumulation of casualties were found in this study (Fig. 3). Two of them are near to wetlands (pointer 3 and 7), one is in a section with deciduous forest – pointer 5, and this is the only point with a concentration of casualties where the highway is below ground level. The last place with a concentration of victims (pointer 7) is due to the discovered dead individuals of the Common Starling (*Sturnus vulgaris* Linnaeus, 1758) and Common Chaffinch (*Fringilla coelebs* Linnaeus, 1758) on 02.01.2017. In winter, flocks of Common Starling, Common Chaffinch and Eurasian Jackdaw (*Corvus monedula* Linnaeus, 1758) around the 70th kilometer which landed on the roadway were observed. In several studies, habitats around the road are mentioned as an important factor for the victims (ERRITZOE *et al.*, 2003). Traffic victims do increase at places situated near forests, wetlands or hedges (BROWN *et al.*, 1986; BOSH, 1989; JOHNSON, 1989). If the road is at the ground level around it or slightly higher, the number of victims increases (BRUUN-SHMIDT, 1994). This explains to a great extent our results with regard to the places where victims are concentrated.

Conclusions

This study identified the Northern White-breasted Hedgehog (*Erinaceus roumanicus* Barret-Hamilton, 1900), Eurasian Badger (*Meles meles* (Linnaeus, 1758)), Golden Jackal (*Canis aureus* Linnaeus, 1758), and Common Buzzard (*Buteo buteo* (Linnaeus, 1758)) as the main victims of road traffic on the Maritsa Highway. There are no differences in the number of casualties by months, but they grow up with the increasing of the road traffic. There are seven sections with a concentration of losses and it is probably related to the type of habitats and the situation of the road to the surrounding environment. There are several sectors with nets to prevent collisions with birds and this may be one of the reasons for the less number of casualties than other similar surveys in Bulgaria.

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Diet of the Kotschy's Gecko Mediodactylus kotschyi rumelicus (Müller, 1940) (Reptilia: Gekkonidae) from the City of Plovdiv (Bulgaria)

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Abstract. The seasonal and habitat variation of the trophic spectrum of Kotschy's gecko (*Mediodactylus kotschyi rumelicus*), as well as the species' trophic niche breadth was studied, based on feces analysis. The material was collected in spring and autumn of 2007 and 2008. The diet of *M. kotschyi* consists mainly of insects (59.51%), and from the non-insect food we registered Aranea (3.46%) and Acari (4.50%). From the insects, largest share have the ants - Hymenoptera, Formicidae (41.18%), followed by Coleoptera (11.77%), represented mainly by the Curculionidae family and Heteroptera (3.46%). In most of the samples (93.41%) were identified a significant amount of plant residues (32.52% of the total food). The taxonomic contents of the gecko's diet remains almost unchanged in the spring and in the autumn. The index of dominance of Berger-Parker (d) showed a moderate value of 0.41 and the trophic niche breadth, calculated with Simpson's diversity index (1/Simpson) was 3.53, which is relatively low value.

Key words: feeding ecology, Kotschy's gecko, diet, trophic niche, Plovdiv.

Introduction

Studying the feeding ecology of a species provides important information for the used food resources, the most common and the proportion of each food component, as well as the role of the species in the interspecific and intraspecific relationships (BONFIGLIO *et al.*, 2006). A broad food niche is common for species with high ecological plasticity, which in combination with appropriate environmental conditions, provides a sound basis for the successful occupation of new territories (RÖDDER *et al.*, 2008). From this perspective, the study of the ecological role of the species in the natural and for survival and existence in anthropogenically transformed landscapes is a necessary part of the overall ecological characteristics of the species (SEMENOV & SHENBROT, 1988).

Widely popular opinion among herpetologists is that lizards are opportunistic predators and the major variations in their food spectrum are due to the size of their body and differences in the availability of food (AVERY, 1966; ARNOLD, 1987). True specialists, regarding food among lizards are rare, and most species use a variety of different organisms as food (GREENE, 1982). Moreover, the trophic

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spectrum of lizards may vary in different habitats and in different seasons (JAMES, 1991).

Most of the lizards, occurring in Bulgaria, feed exclusively on invertebrates. With the exception of some representatives of the Lacertidae family (PETERS, 1963; ANGELOV *et al.*, 1966; 1972a; b; c; KABISCH & ENGELMANN, 1969; 1970; TOMOV, 1990; MITOV, 1995; DONEV, 1984a; b; DONEV *et al.*, 2005; MOLLOV *et al.*, 2012; MOLLOV & PETROVA, 2013), the diet of the rest of the lizard species in Bulgaria remains unstudied (MOLLOV *et al.*, 2012). From the other lizard families occurring in Bulgaria, regarding the trophic spectrum, the only studied species is the slow worm (*Anguis fragilis*) by ANGELOV *et al.* (1966) and MOLLOV (2010).

The Kotschy's gecko (*Mediodactylus kotschyi*) is the only representative of the Gekkonidae family in Bulgaria (BESHKOV & NANEV, 2002; BISERKOV *et al.*, 2007), which is represented with three subspecies in the country (*M. k. bibroni* (Beutler and Gruber, 1977) - distributed in the southern part of Struma Valley (Petrich Town, Sandanski Valley and north to Kresna Gorge), also occurring in the cities of Gotse Delchev and Blagoevgrad; *M. k. danilewskii* (Strauch, 1887) - occurs in the Eastern Rhodopes Mts., the Maritsa River Valley and Tundzha River Valley, north to Nova Zagora City and Yambol City and along the Black Sea coast north of Balchik Town, isolated habitats are located in Rousse City and the Orlova Chuka Cave and *M. k. rumelicus* (Müller, 1940) - recorded in the western parts of the Upper Thracian Plain (the cities of Pazardzhik, Plovdiv and Asenovgrad and Kuklen Village). This is a primarily synanthropic species and in Bulgaria there are only few known populations from natural habitats (BESHKOV & NANEV, 2002). So far there is only partial and incomplete data in the literature on the composition of the diet and feeding ecology of this species and there are no comprehensive studies on this subject, conducted in Bulgaria. The aim of this study was to research the qualitative and quantitative composition of the trophic spectrum, trophic niche breadth and trophic specialization of the Kotschy's gecko (*Mediodactylus kotschyi rumelicus*) in Plovdiv City (Bulgaria).

Material and Methods

For the purposes of the current study we collected a total of 91 samples of Kotschy's Gecko (*Mediodactylus kotschyi rumelicus*) feces from three protected areas (three of the Plovdiv Hills - Nature Monument "Mladeshki halm" Hill, NM "Halm na osvoboditelite" Hill and NM "Danov halm" Hill) and part of one protected zone (NATURA2000 site "Maritsa River" - BG0002087) in Plovdiv City (Fig. 1). The material was collected in the spring and the autumn of 2007 and 2008 and is deposited in the herpetological collection of the Department "Ecology and Environmental Conservation" at the University of Plovdiv.

The samples were collected from typical habitats inhabited by geckos, namely supporting walls along the alleys of the hills of Plovdiv and the supporting wall of Maritsa River in the central part of the city (see MOLLOV & VELCHEVA, 2010). We chose precisely these areas, since they are few of the only available places, where we have access to the geckos and their habitats, from where the feces were collected, unlike the rest of the city, which is densely built with residential buildings and houses, where there is no free access.

Analysis of feces can provide some information about the composition of the diet of the geckos, although faeces do not provide a complete assessment of the dietary spectrum of a given species, because the soft body parts of the prey tend to be less well preserved in remains (see HÒDAR & PLEGUEZUELOS, 1999; LUISELLI & AMORI, 2016). Geckos seek refuge during the day in crevices of the supporting walls, and at night they move around on the ground a few meters from the walls (MOLLOV, 2005). During each visit we collected all feces found at the sites. In the studied areas there are no other gecko species and the feces of other lizards are clearly distinguishable from the geckos' (HÒDAR & PLEGUEZUELOS, 1999). Since sampling took place in the same areas around the supporting walls and we collected all visible feces on each visit, we assume that there is equal probability that feces from every living gecko in the particular micro-habitat had been sampled. of course this method does not allow for the identification of the individual who has deposited the single scat (LUISELLI & AMORI, 2016). The feces were collected dry in plastic bags with zip and labeled. Later in the laboratory the samples were soaked in water and examined under a microscope. The food remains were separated and sorted and identified to the lowest taxonomic level possible, depending on the level of decomposition, using available guides (IVANOV *et al.*, 1981). The taxonomy follows Fauna Europaea (DE JONG *et al.*, 2014).

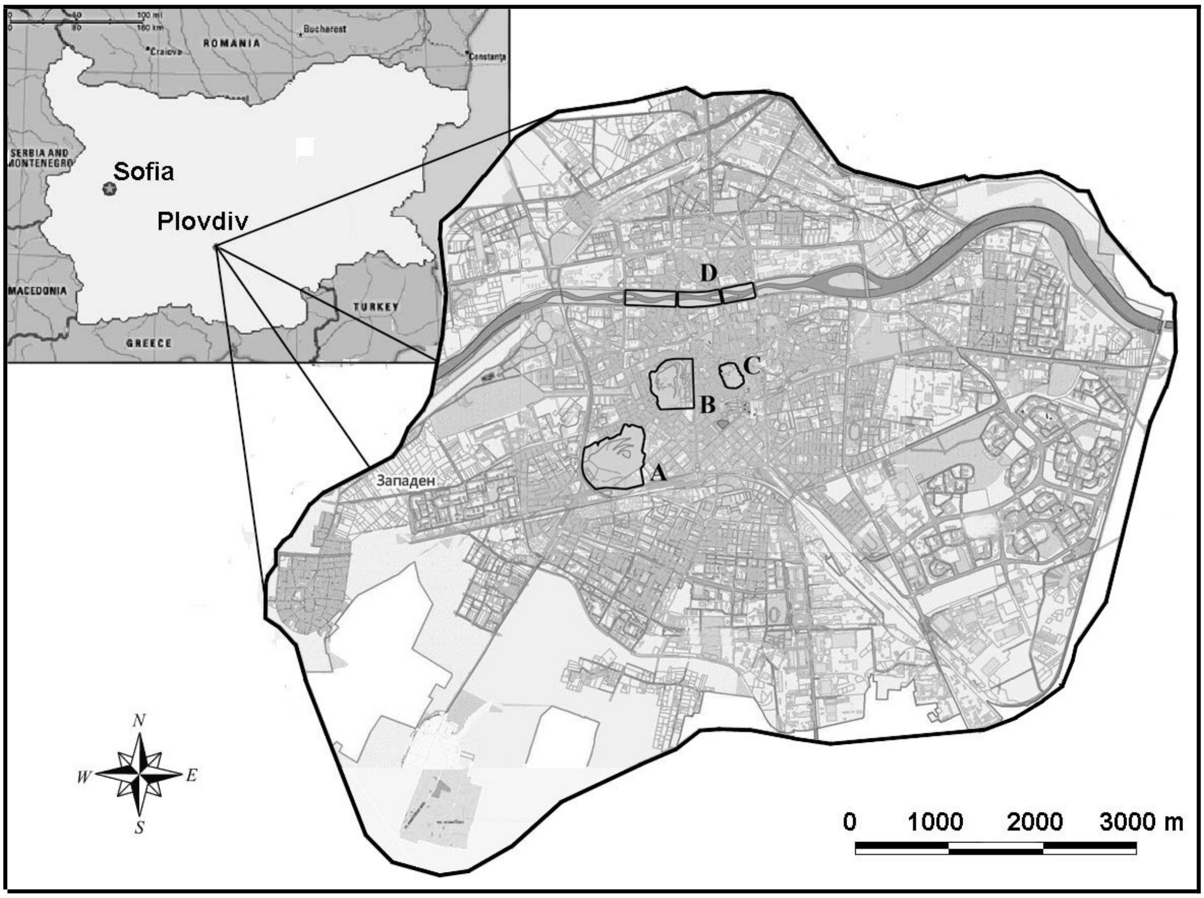

Fig. 1. Indicative map of the city of Plovdiv and the studied sites. Legend: A - NM "Mladezhki halm" Hill, B - NM "Halm na osvoboditelite" Hill, C - NM "Danov halm" Hill, D - part of Maritsa River.

For each recorded taxa in the trophic spectrum are given the number of prey categories, the number of prey items and percentage proportion, the number of samples that contained the taxon and frequency of occurrence (the ratio between the number of samples that contain a certain taxon prey and the total of analyzed samples, the obtained value being expressed in percentages).

Sampling adequacy was determined using Lehner`s formula (LEHNER, 1996):

$$Q = 1 - \frac{N_1}{I},$$

rising from 0 to 1, where N_1 is the number of the food components occurring only once, and I is the total number of the food components.

The diversity of the diet (niche breadth) was calculated, using the reciprocal value of the Simpson's diversity index (MAGURRAN, 1988):

$$S = \frac{1}{\sum p_i^2},$$

where: S - trophic niche breadth; P_i - proportion of food component i.

To determine the level of trophic specialization of we used the index of dominance of Berger-Parker (d), calculated by the following formula (MAGURRAN, 1988):

$$d = \frac{n_i \max}{N},$$

where: N - the number of all recorded food components (taxa); $n_i$max - the number of the specimens form taxon i (the most numerous taxon in the diet). The Berger-Parker index (d) varies between 1/N and 1. A value closer to 1 means a higher specialization in the choice of food; a value closer to 1/N is typical for a species that is a general feeder (polyphage).

The results were statistically processed using descriptive statistics and cluster analysis (Paired Group Linkage, Hamming Similarity Measure), for determining the similarity of the trophic spectrum of the geckos from the different localities (FOWLER *et al.*, 1998). For the descriptive statistical processing of the data and 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

For the whole period of study we recorded totally 289 prey items in the trophic spectrum of the Kotschy's Gecko in the studied sites in the city of Plovdiv, which were determined in 17 taxa (food categories) (Table 1).

The qualitative and quantitative composition of the trophic spectrum of the Kotschy's Gecko (*Mediodactylus kotschyi rumelicus*) in the city of Plovdiv, for the entire period of study is presented in Table 2. The adequacy of the samples, calculated by the Lehner's formula is 0.76 for all samples and even higher by localities (see Table 4). This value is considered sufficient for this kind of research. The data from the table shows that the food of *M. kotschyi* consists mainly of insects (59.51%), and from the non-insect food we registered Aranea (3.46%) and Acari (4.50%). From the insects, largest share have the ants - Hymenoptera, Formicidae (41.18%), followed by Coleoptera (11.77%), represented mainly by the Curculionidae family and Heteroptera (3.46%), to which belongs to only determined to species level taxon - *Neides tipularius*.

In most of the samples (93.41%) were identified a significant amount of plant residues (32.52% of the total food). The plant remains consisted of seeds in sizes from 0.8 to 4.8 mm; leaves and parts of leaves with a length of 5 to 20 mm, and a stem 7 mm long as well as other plant remains.

The index of dominance of Berger-Parker (d) showed e moderate value of 0.41 and the trophic niche breadth, calculated with Simpson's diversity index (1/Simpson) was 3.53, which is relatively low value.

The analysis of the seasonal distribution of the food showed that, the taxonomic contents of the gecko's diet remains almost unchanged in the spring and in the autumn (Table 3). In both season predominant are the ants (38.54% - in the spring and 42.49% - in the autumn), followed by Coleoptera (7.29% - in the spring and 14.00% - in the autumn), while in the spring samples we recorded only undetermined to family level coleopterans and representatives of the Curculionidae family, and the other families from the Coleoptera order, were registered only in the autumn samples. From the non-insect invertebrates in the spring predominant are Acari (mainly Oribatida) - 6.25% (3.63% in the autumn) and Aranea (5.21% - in spring and 2.59% - in authumn). The third predominating prey taxa in the autumn was Heteroptera with 4.15% from the trophic spectrum, while their quantity in the spring samples was significantly lower (2.08%). Plant residues are present in significant quantities as in spring (37.50% in all samples), as well as in autmn (30.05% in about 88% of the samples).

Table 4 shows the trophic spectrum of the 4 different populations of the Kotschy's gecko in Plovdiv. The most diverse is the diet of the geckos from "Mladezhki halm" Hill. Aside the plant remains, the predominant taxon is Hymenoptera, Formicidae (35.43%), followed by Coleoptera (13.14%) and Acari (5.14%). In the samples from "Halm na osvoboditelite" Hill most numerous are again Formicidae (68.18%), aside from plant residues that occupy second place, followed again by Coleoptera (10.61%) and Aranea (3.03%). In the samples from "Danov Halm" Hill the most numerous are the plant remains. From the animal prey taxa most numerous are again Hymenoptera, Formicidae (27.78%), followed by Acari (16.67%) and Aranea (11.11%). In the samples from the Maritsa River again the most numerous are the plant remains and in the food of animal origin predominate Formicidae (23.33%), followed by Coleoptera (13.33%) and Aranea and Trichoptera (Hydropsychidae) with 6.67%. We recorded representatives from the Hydropsychidae family, which are aquatic organisms inhabiting the shores of the various aquatic basins, only in the samples from Maritsa river.

To determine the similarity between the samples from the four localities, we used cluster analysis, based on quantitative data (Fig. 2). From the dendrogram it is visible that with similarity of around 4% the samples from "Mladezhki halm" Hill separate in independent cluster. From the remaining samples, with 60% similarity Maritsa River is separated in second cluster. And the samples from "Halm na osvoboditelite" Hill and "Danov halm" Hill are grouped in a third cluster with about 65% similarity.

Discussion

The diet on the Kotschy's gecko (*M. kotschyi rumelicus*) consists mainly of arthropods, as pointed out by the partial data of other authors for this species in Bulgaria (Kovachev, 1912; Milenkov, 1962; Zhivkov & Dobrev, 2001; Beshkov & Nanev, 2002; Profirov, 2003). The main food source for the Kotschy's Gecko in Plovdiv are the ants, which occupy the highest percentage and frequency in both studied seasons and in all populations. The representatives of the order Coleoptera also play an important role in the species' diet. From the non-insect invertebrates the most important prey items are Araneae and Acari. The beetles and ants are basic food most probably due to the abundance of this preys and the wide range of habitats where they can be found (Mollov, 2008). Despite the predominance of these two taxa it appears that the Kotschy's gecko shows no specialization in its food and consumes animals with low and high nutritional value.

Table 1. Descriptive statistics of the samples by seasons and localities.

	Number of food categories	Number of prey items	Mean	Standard deviation (SD)
Spring	17	96	5.65	11.76
Autumn		193	11.35	22.83
NM "Mladezhki Halm"		175	10.29	19.89
NM "Halm na osvoboditelite"		66	3.88	10.99
NM "Danov Halm"		18	1.06	1.89
Maritsa River		30	1.76	3.68
Total		289	17.00	34.41

Table 2. Composition of the trophic spectrum of the Kotschy's Gecko (*Mediodactylus kotschyi rumelicus*) in the city of Plovdiv, for the entire period of study. *Legend:* **n** - number of prey items from the selected taxon, **n%** - percentage proportion from the total number of prey items, **s** - number of samples, the selected taxon was recorded in; **s%** - frequency of occurrence (percentage proportion of the samples, containing the selected taxon).

Prey items	n	n%	s	s%
Araneae	10	3.46	9	9.89
Acari-undet.	2	0.69	2	2.20
Acari, Oribatida	11	3.81	11	12.09
Insecta-undet.	3	1.04	3	3.30
Hymenoptera-undet.	1	0.35	1	1.10
Hymenoptera, Formicidae	119	41.18	68	74.73
Coleoptera-undet.	22	7.61	19	20.88
Coleoptera, Buprestidae	1	0.35	1	1.10
Coleoptera, Curculionidae	8	2.77	8	8.79
Coleoptera, Scarabeidae	1	0.35	1	1.10
Coleoptera, Elateridae	2	0.69	2	2.20
Homoptera, Cicadomorpha	2	0.69	2	2.20
Heteroptera	8	2.77	8	8.79
Heteroptera (*Neides tipularius*)	2	0.69	2	2.20
Trichoptera (Hydropsychidae)	2	0.69	2	2.20
Lepidoptera (larvae)	1	0.35	1	1.10
plant remains	94	32.53	85	93.41
Total	**289**	**100.00**	**91**	**-**
Lehner's index		**0.765**		
Berger-Parker		**0.412**		
1/Simpson		**3.531**		

Table 3. Seasonal composition of the trophic spectrum of the Kotschy's Gecko (*Mediodactylus kotschyi rumelicus*) in the city of Plovdiv. *Legend:* **n** - number of prey items from the selected taxon, **n%** - percentage proportion from the total number of prey items, **s** - number of samples, the selected taxon was recorded in; **s%** - frequency of occurrence (percentage proportion of the samples, containing the selected taxon).

Prey items	Spring				Autumn			
	n	n%	s	s%	n	n%	s	s%
Araneae	5	5.21	5	15.63	5	2.59	4	6.78
Acari-undet.	1	1.04	1	3.13	1	0.52	1	1.69
Acari, Oribatida	5	5.21	5	15.63	6	3.11	6	10.17
Insecta-undet.	1	1.04	1	3.13	2	1.04	2	3.39
Hymenoptera-undet.	0	0.00	0	0.00	1	0.52	1	1.69
Hymenoptera, Formicidae	37	38.54	25	78.13	82	42.49	43	72.88
Coleoptera-undet.	5	5.21	4	12.50	17	8.81	15	25.42
Coleoptera, Buprestidae	0	0.00	0	0.00	1	0.52	1	1.69
Coleoptera, Curculionidae	2	2.08	2	6.25	6	3.11	6	10.17
Coleoptera, Scarabeidae	0	0.00	0	0.00	1	0.52	1	1.69
Coleoptera, Elateridae	0	0.00	0	0.00	2	1.04	2	3.39
Homoptera, Cicadomorpha	0	0.00	0	0.00	2	1.04	2	3.39
Heteroptera	2	2.08	2	6.25	6	3.11	6	10.17
Heteroptera (*Neides tipularius*)	0	0.00	0	0.00	2	1.04	2	3.39
Trichoptera (Hydropsychidae)	2	2.08	2	6.25	0	0.00	0	0.00
Lepidoptera (larvae)	0	0.00	0	0.00	1	0.52	1	1.69
plant remains	36	37.50	32	100.00	58	30.05	52	88.14
Total	**96**	**100.00**	**32**	**-**	**193**	**100.00**	**59**	**-**

Table 4. Composition of the trophic spectrum of the Kotschy's Gecko (*Mediodactylus kotschyi rumelicus*) in the city of Plovdiv, by localities. *Legend:* **n** - number of prey items from the selected taxon, **n%** - percentage proportion from the total number of prey items, **s** - number of samples, the selected taxon was recorded in; **s%** - frequency of occurrence (percentage proportion of the samples, containing the selected taxon).

Prey items	**"Mladezhki halm" Hill**				**"Halm na osvoboditelite" Hill**				**"Danov halm" Hill**				**Maritsa River**			
	n	**n%**	**s**	**s%**	**n**	**n%**	**s**	**s%**	**n**	**n%**	**s**	**s%**	**n**	**n%**	**s**	**s%**
Araneae	4	2.29	3	5.08	2	3.03	2	12.50	2	11.11	2	33.33	2	6.67	2	20.00
Acari-undet.	2	1.14	2	3.39	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Acari, Oribatida	7	4.00	7	11.86	0	0.00	0	0.00	3	16.67	3	50.00	1	3.33	1	10.00
Insecta-undet.	2	1.14	2	3.39	0	0.00	0	0.00	1	5.56	1	16.67	0	0.00	0	0.00
Hymenoptera-undet.	1	0.57	1	1.69	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Hymenoptera, Formicidae	62	35.43	41	69.49	45	68.18	16	100.00	5	27.78	4	66.67	7	23.33	7	70.00
Coleoptera-undet.	12	6.86	10	16.95	6	9.09	6	37.50	0	0.00	0	0.00	4	13.33	3	30.00
Coleoptera, Buprestidae	1	0.57	1	1.69	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Coleoptera, Curculionidae	7	4.00	7	11.86	1	1.52	1	6.25	0	0.00	0	0.00	0	0.00	0	0.00
Coleoptera, Scarabeidae	1	0.57	1	1.69	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Coleoptera, Elateridae	2	1.14	2	3.39	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Homoptera, Cicadomorpha	2	1.14	2	3.39	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Heteroptera	6	3.43	6	10.17	1	1.52	1	6.25	1	5.56	1	16.67	0	0.00	0	0.00
Heteroptera (*Neides tipularius*)	2	1.14	2	3.39	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Trichoptera (Hydropsychidae)	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	2	6.67	2	20.00
Lepidoptera (larvae)	1	0.57	1	1.69	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
plant remains	63	36.00	59	100.00	11	16.67	11	68.75	6	33.33	6	100.00	14	46.67	9	90.00
Total	**175**	**100.00**	**59**	**-**	**66**	**100.00**	**16**	**-**	**18**	**100.00**	**6**	**-**	**30**	**100.00**	**10**	**-**
Lehner's index	**0.76**				**0.88**				**0.88**				**0.94**			

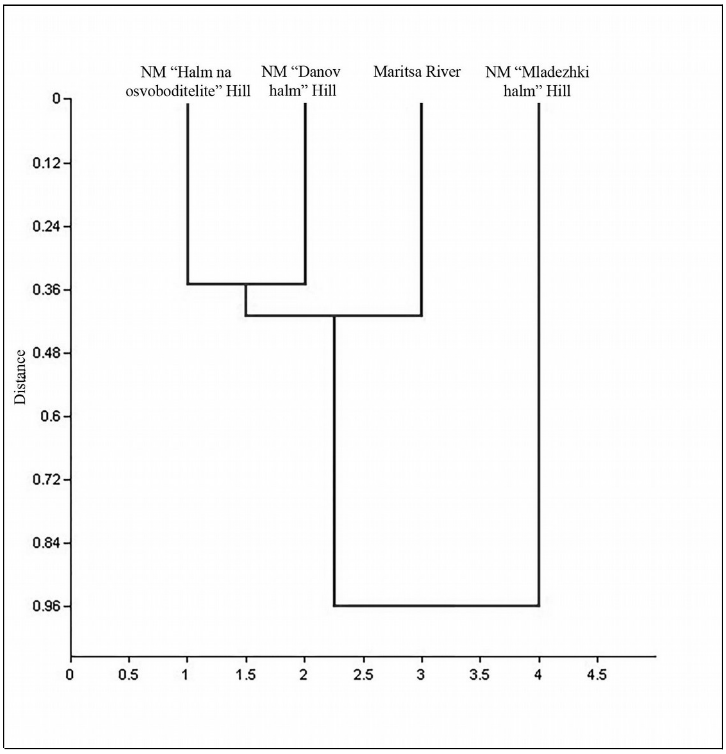

Fig. 2. Cluster analysis, based on quantitative data (Paired Group Linkage, Hamming Similarity Measure) of the trophic spectrum of the Kotschy's gecko from the four studied localities in the city of Plovdiv.

M. kotschyi can be classified as zoophagous polyphage with opportunistic feeding behavior. Depending on the season and the characteristics of the habitat the food can vary and certain taxa may prevail.

The high percentage of registered plant residues in food of the Kotschy's gecko in Plovdiv suggests that they are not accidentally ingested while catching animals, and it is possible that under certain conditions the geckos may actively include plant food in their trophic spectrum.

Predators are extremely sensitive to the complexity and structure of the ecosystems and lizards, because of their small size and opportunistic feeding behavior, usually overcome shortage of food resources, in some cases by adding plants in their diet (VAN DAMME, 1999; SCHWENK, 2000; COOPER & VITT, 2002). Many lizard species often include plant material in their trophic spectrum, however, most studies investigating these omnivorous species concluded that despite the size of the lizards in most cases they have low trophic specialization (COOPER & VITT, 2002). That is why most omnivorous lizards are considered opportunistic herbivorous species with a common morphology, as they do not show any specific morphological "adaptation" characteristic of actual herbivorous species (SCHWENK, 2000).

The trophic spectrum of *M. kotschyi* in Plovdiv does not differ significantly between the different localities in the city, but with the greatest prey variety is characterized the diet of the geckos from "Mladezhki halm" Hill. The reason for this is probably the fact that this is the largest protected area in the studied area and contains the greatest diversity of habitats which is a prerequisite for a rich food base. The food range of the populations of the other two hills ("Halm na osvoboditelite" Hill and "Danov halm" Hill) show high similarity, which can be explained by the fact that both areas are located close together in the city center and offer similar habitats and environmental conditions. The diet of the population from Maritsa River shows some differences and includes moisture-loving species that are only found near ponds and absent from the hills. We think the only reason the Kotschy's gecko can be found along the banks of Maritsa River in Plovdiv is the presence of a retaining wall along the banks of the river, where geckos find excellent hiding spots and living conditions (similar to those on the retaining walls on the alleys of the hills).

This is the first detailed study on the diet of the Kotschy's gecko in Bulgaria and in the Bulgarian herpetological literature so far there are only partial data for the qualitative composition of the food spectrum without quantification.

The first data about the food of *M. kotschyi* can be found in the work of KOVACHEV (1912) where he reports that the species gets out at night to "hunt for insects and stalking prey on the walls of houses under street lamps around the city." According to MILENKOV (1962) *M. kotschyi* begins to catch their food at night, which consists of "flies, mosquitoes, spiders, beetles and other arthropods." ZHIVKOV & DOBREV (2001) report that the diet of the Kotschy's gecko is composed of "insects, spiders, centipedes and other night-active arthropods". BESHKOV & NANEV (2002) supplement that geckos are feeding mainly on "insects, spiders and other". According to Profirov (2003) of the food geckos are "different insects, spiders, millipedes, and other", while according BISERKOV *et al.* (2007) geckos eat "night-active invertebrates".

According to SHTERBAK (1960) based on the stomach contents of geckos from Criema, the food of *M. kotschyi daniliewskii* consists essentially of Diptera (22.48%), followed by Hymenoptera (19.38%), Aranea (19.05%), Lepidoptera (12.79%) and Coleoptera (12.7%). In another study by the same author (SHTERBAK, 1966) from Crimea on the same subspecies, the largest share in the diet belonged again to Diptera (20.08%), followed by Lepidoptera (18.5%) and Aranea (14.17%). According to VALAKOS & VLACHOPANOS (1987, 1989), the main prey taxa in the composition of the diet of *M. kotschyi* from Greece are Coleoptera-larvae (63.9%), Aranea (9.17%) and Coleoptera (8.28%). Our data confirm only partially the data from the above mentioned authors. The main reason for this in our opinion, is the different method used in the studies - the diet of the geckos in Crimea and Greece was examined by studying the stomach contents, while our study is based on feces analysis, which is far more inaccurate, but invasive method. Not all taxa can leave a distinct residues in feces and probably this could explain the absence of taxa as Lepidoptera and Diptera, as well as the relatively low percentage of spiders in the trophic spectrum of *M. kotschyi* from Plovdiv.

Regarding the trophic niche breadth, our data is close to the results obtained by VALAKOS & VLACHOPANOS (1987) from Greece, where the niche breadth of *M. kotschyi,* calculated by us form their data is 2.32. We also calculated the niche breadth from the data by SHTERBAK (1960, 1966) from Crimea, which showed relatively high value - 10.88 and 11.19 respectively.

Our data showed no significant difference in the trophic spectrum of the Kotschy's gecko in Plovdiv in both sampling seasons. In autumn we recorded a greater amount of food intake, which can be partly explained by the accumulation of nutrients by the geckos before hibernation. According to VALAKOS & VLACHOPANOS (1987) for the geckos from Greece in March the food contains mainly larvae of Hemiptera (68.42%), Coleoptera-imago and larvae (respectively 13.6%); in July the larvae of Coleoptera (76.6%), Aranea (8.03%) and Coleoptera-imago (7.3%) prevail, and in November - Aranea (30.77%), Hymenoptera, Formicide and Coleoptera (respectively 11.54%). According to the same authors of another study in Greece in 1989 (VALAKOS & VLACHOPANOS, 1989) in July in the diet was consisted by larvae of Coleoptera (70%), Coleoptera-imago and Aranea (approximately 7%) and in November - Aranea (about 36%), Diptera (about 20%) and Acari (about 12%). This data once again confirms the opportunistic behavior of feeding the geckos as during different seasons they feed on organisms that are the most numerous and present in the habitat.

Another study by VALAKOS & POLYMENI (1990) shows that the insect larvae takes even bigger portion of the gecko's diet – 72%, as the second most important taxa were Tysanura and Coleoptera (12.5%) and Aranea (9.9%). The low percentage of insect larvae in our samples can be explained with used method by analyzing scat samples.

The analysis of the trophic spectrum of other closely related to *M. kotschyi* species, shows that, similar to the Kotschy's gecko, other gecko species from the genus *Mediodactylus* also exhibit opportunistic feeding behavior. According to BOGDANOV (1960) *M. russowii* of Russia, feeds mainly Hymenoptera (56.15%), of which the ants predominate (28.57%), Coleoptera (49.21%) and Heteroptera (23.82%), whose diet resembles the one of *M. kotschyi* from the city of Plovdiv. According to the same author *Tenuidactylus caspius* feeds mainly on Coleoptera (48.15%), Diptera (14.81%) and Isopoda (5.56%), while *T. fedtschenkoi* feeds on Hymenoptera (37.93%), from which ants occupy 31.03%, followed by Coleoptera (24.14%) and Hemiptera and Diptera (by 10.34%).

The two main predating strategies in lizards are "sit-and-wait" and "active search". The first strategy requires the predator to be standing still in one place and attack prey that runs around, while the second strategy requires the predator to move actively attacking any animal, with suitable size that it meets (HUEY & PIANKA, 1981; COOPER JR., 1995; PERRY & PIANKA, 1997). Although the active search for food requires more effort and burns more calories species using this feeding strategy capture more animals from the "sit-and-wait" species (PIANKA, 1986). This approach may be effective for nocturnally active animals, especially ectotherms, because as temperatures get lower at night this can significantly reduce the time to hunt and therefore the animal will need the most effective method of hunting. The benefits the "sit-and-wait" strategy is that the predator saves energy and the risk of being caught by other predators is reduced to a minimum (PIANKA, 1986). In our opinion, the Kotschy's gecko uses both strategies to catch its prey, depending on environmental conditions, season and habitat. According to SHTERBAK (1960) at low temperatures geckos can even completely stop foraging. This behavior can be explained by the fact that *M. kotschyi* is thermophilic species (MOLLOV & VELCHEVA, 2015). The species hunts mostly at night, as in the early hours of the evening before they air temperature gets colder, "active search" is used and at that time geckos can often be seen on the walls around the illuminated areas under outdoor lights that attract insects (ZHIVKOV & DOBREV, 2001), while later when the air temperature gets colder or under less favorable conditions geckos use the "sit-and-wait" technique.

Conclusions

The Kotschy's gecko (*M. kotschyi rumelicus*) in Plovdiv City feeds mainly on arthropods, of which the largest share have the representatives of the Formicidae family, orders Coleoptera and Hemiptera. In urban environment *M. kotschyi* can use plants as food - a mechanism to overcome shortage of food resources under certain conditions or habitats. The qualitative composition of the food remained almost unchanged in spring and in autumn, as in both seasons are predominating Formicidae and Coleoptera. Most diverse is the diet of the geckos Mladezhki Halm Hill. As from the other studied areas, more specific is the diet of the population from Maritsa River, while the populations from "Halm na osvoboditelite" Hill and "Danov halm" Hill show high similarity in the trophic spectrum. *M. kotschyi* can be classified as zoophague with opportunistic feeding behavior. Depending on the season and habitat characteristics the trophic spectrum and trophic niche breadth of the species can vary and certain taxa may predominate.

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State of Forest Plantations Used for Afforestation of Heavy Metals Polluted Lands Around Former Metallurgic Plant "Kremikovtsi" (Bulgaria)

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Abstract. A study was carried out on the sustainability of forest plantations established on soils contaminated with heavy metals in the vicinity of the former "Kremikovtsi" metalurgic plant. Soils and forest vegetation near the village of Buhovo (black pine *Pinus nigra* Arn.) and Lokorsko I (red oak *Quercus rubra* L.) and Lokorsko II (winter oak *Quercus sessiliflora* Salisb.) were studied. The soils of the area are geochemically enriched with lead, reaching up to 136 mg/kg for Pb under the Black Pine Plantation. The lowest levels of assimilation have the red oak leaves, as evidenced by the analysis of the heavy metals content: Zn, Cu, Pb, Cd and the biological absorption coefficient Ah, and it is recommended that for the soil reclamation to be used this tree species.

Key words: red oak, black pine, biological absorption coefficient.

Introduction

In the conditions of intense industrialization and active technogenic impact, afforestation with forest plantations on contaminated lands is proved to be a stable, lasting and cheap opportunity to rehabilitate ecosystems, to improve hygienic and sanitary conditions and microclimate of the environment. For many years, defensive or meliorative afforestations have been widely used in combating erosion, natural disasters and for providing a healthy, clean and aesthetic environment in cities and other settlements (ZAHARIEV, 1977; POPOV *et al.*, 2018). The tree vegetation is successfully applied around roads and highways to reduce soil pollution with heavy metals (CHULGIJAN & PETROV, 1985; KACHOVA & ATANASSOVA, 2014). By forest vegetation are improved the structure and properties of the soil, and by the fall and decomposition of the dead forest litter (DFL) is improved soil nutritional status (SOKOLOVSKA *et al.*, 2001). Forestry-biological restoration is successfully used and around a number of industrial enterprises (GENCHEVA & GELEVA, 1984; MALINOVA, 1998). Significant aesthetic, mitigating and meliorative effect of the forest plantations makes the forest-biological restoration particularly effective. Forest vegetation improves the hydro-thermal regime and the fertility of disturbed lands, creating more favorable growing conditions in future land uses (PROKOPIEV, 1967; ALEXANDROV *et al.*, 2001). Forest vegetation used for restoration is also considerable reservoir of carbon in the ecosystem (ZHIYANSKI *et al.*, 2014). Of particular

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importance is the choice of species for afforestation. Individual tree species thanks to their biological, physiological and morphological features are distinguished by different adaptability and resistance to contaminated lands. Less sensitive species are recommended for industrial areas: acacia (*Robinia pseudoacacia* L.), american ash (*Fraxinus americana* L.), elms (*Ulmus spp.*), sycamore (*Acer negundo* L.), simple dogwood (*Cornus mas* L.), oaks (*Quercus spp.*), silver spruce (*Picea pungens*), douglas- fir (*Pseudotsuga menziesii* (Mirb.) Franco), west tuya (*Thuja occidentalis* L.), juniper (*Juniperus spp.*) (PROKOPIEV, 1967; TSAKOV & DIMITROVA, 2002). The lands around the former "Kremikovtzi" plant are remarkable with poor quality, mainly with the presence of heavy metal pollution (HM). Questions such as: "Which are the most suitable forest plant species for afforestation around the former plant?"; "What is the adaptability of plantations to the specific pollution of the soils in the area?", remain open yet.

The aim of this study is to analyze the accumulation of heavy metals in the leaves of several tree species, used for afforestation in the area of former Kremikovtsi plant; to assess the state of the established plantations and to recommend forest species suitable for future afforestation.

Material and Methods

The sample plots (SP) are located in the pre-mountainous climatic region of the Sofia region with a typical temperate continental climate and an annual sum of precipitation ~ 600 mm. Western winds predominate (up to 2/3 of the cases).

The sample plots (SPs) in the following objects were used for study:

- *Buhovo* with altitude of 750 m, brown forest soil (Eutric Cambisols) and black pine plantation (*Pinus nigra* Arn.);
- *Lokorsko I* at altitude of 650 m, cinnamoric soils (Chromic Luvisols) and red oak plantation (*Quercus rubra* L.);
- *Lokorsko II* with altitude 650 m, cinnamonic soils (Chromic Luvisols) and a winter oak plantation (*Quercus petraea* Liebl.).

Soil samples were taken from 5 depths: 0-5, 5-10, 10-20, 20-40, 40-80 cm in 4 repetitions for each site. Laboratory analyzes were performed according to established methods (DONOV *et al.,* 1974): mechanical composition – by Kaczynski method; pH - potentiometrically with "Pracitronic MV 88"; total carbon – by Turin method. Total heavy metal content was determined on a "Perkin Elmer 370 A" atomic absorption spectrophotometer after treatment with aqua regia (HNO_3 : HCl 1: 3) (ISO 11466); mobile forms of HMs – by atomic absorption after treatment with 1N $CH3COONH_4$ at pH = 4.8.

Analyses of the elements in the leaves were made after processing with concentrated HNO_3 and was determined by AAC Method (ICP Forests, 2006). An average sample of 5 representative trees was taken.

Dendrometrical characterization of the trees from the SPs (20/50m = 0.1ha.) was carried out on the basis of measuring the diameters of the trees (with accuracy of 2 cm) and at 3 heights (0.5 m accuracy). To assess the general physiological state of the trees an entomological characteristic was made. Separately, plastid pigments: chlorophyll "a", chlorophyll "b" and carotenoids in leaves in deciduous trees and annual needles of coniferous trees were defined, through the method of MACKINNEY (1941). It was done in three repetitions for June and October.

The average diameter (cm) is obtained by the arithmetic basal area – formula:

$$DbH=SQRT(1.274*\sum G/n)*100$$

where: $G=\pi*d^2/4$ – is a basal tree area(m^2);

d - is the tree diameter(cm);

n - is number of trees;

DbH – is the average diameter (cm).

The average height (m) is calculated as a weighted average according the Lorey's formula. The average height was obtained as weighted mean from the heights of all trees in the relevant sample plots after the formula of LOREY (1878):

$$H = h_1g_1 + h_2g_2 + \ldots + h_ng_n / g_1 + g_2 + \ldots + g_n \text{ (m)}$$

where: h – is a height of each tree (1,2, ….n) (m);

g – is a basal area of each tree (m²).

Stand quality class is determined by interpolating and using the tables for determining the quality of black pine plantations according Tsakov (PORYAZOV *et al.,* 2004), of red oak plantations according Krastanov and Hristov (PORYAZOVPORYAZOV*et al.,* 2004) and of high-stemmed oak stands according Nendyalkov (KRASTANOV & RAYKOV, 2004).

Results and Discussion

Soil Analyses. When considering the condition of plantations on polluted lands, the specific characteristics of soils are of fundamental importance. Soil determines the quality of the growth environment. The results for the general characterization of soils and the content of total forms of heavy metals (HM) in SPs are given in Table 1.

The soil of Buhovo has an alkaline reaction (pH 7.85 - 8.10). The soils of the other SPs are defined as medium to strongly acid along the profile depth. According to data from the mechanical composition, the soil substrates under the wood pine plantation in Buhovo have the highest content of clay and fine clay. They are the most loamy along the profile compared to the rest. The majority of the soils in the surveyed areas are poor in organic carbon, especially those under the winter oak in Lokorsko II. This low content of organic matter shows, that the organic matter has no significant role in the sorption of HMs. The surface horizons of Buhovo are relatively richer in organic carbon. This shows a relatively higher level of buffer capacity of this soil towards toxic pollution. For the soils from the investigated area the Zn content is not high. These concentrations do not exceed the levels for precautionary concentrations and maximum permissible concentrations for industrial/production sites and also intervention concentrations for HMs adopted in the country (Regulation № 3 / 01.08.2008). This is applied for the medium sandy-loam soil from Buhovo too. In the soils of the SPs, the Cu content is within the range of this element's variation in the country. The content of Cu is within the frame of the adopted permissible concentrations (including the soils from Buhovo, where pH>6). The data for Pb show pollution with technogenic and geochemical origin along the entire depth of the soil from Buhovo. Precautionary concentrations of Pb in the soil (at the rate for sandy loam soil rate of 40 mg/kg) have been exceeded. But the maximum permissible and interventional concentrations (at the rate in pH > 7.4 of 150 mg/kg) are not exceeded. For the soils from Lokorsko I and Lokorsko II there are a technogenic contamination of the surface layers of 0-5 cm with exceeding of the precautionary values (concentrations > 40 mg/kg for sandy clay soils). The analysis of the obtained results shows presence of HM contamination in the soils from Buhovo. But these exceeding concentrations are also associated with higher pH values, which in the other hand makes these soils more resistant to pollution. The Cd values vary below the regulatory concentrations adopted in the country. There is one with exception: the surface layer 0-5 cm of the soil from Buhovo, where the concentration is equal to the precautionary value for sandy loam soils (0.6 mg/kg).

For the specific clarification of the accumulation of HMs into tree species and in particular to clarify their toxicity, the amo-acetate extractable mobile forms of HMs in the soils were determined. The results are given in Table 2.

Table 1. Characteristics of soils and total contents of Cu, Zn, Pb, Cd (mg/kg).

SP	Depth, cm	Mechanical composition (%)			pH	C g/kg	Zn mg/kg	Cu mg/kg	Pb mg/kg	Cd mg/kg
		clay	sand	fine clay						
Buhovo	0 -5	31.18	68.82	8.29	7.85	22.0	67	42	136	0.6
	5 – 10	29.22	70.78	8.29	7.95	16.8	60	38	108	0.5
	10– 20	31.36	68.64	8.31	8.00	14.9	94	34	82	0.5
	20 - 40	30.68	69.32	8.31	8.00	12.3	63	35	84	0.5
	40 - 80	36.38	63.62	8.29	7.80	9.7	59	29	110	0.6
Lokorsko I	0 -5	25.74	74.26	4.05	5.20	15.8	25	21	48	0.05
	5 – 10	21.27	78.73	4.05	4.50	6.5	22	18	23	0.1
	10– 20	20.60	79.40	8.08	4.45	5.2	17	10	16	0.06
	20 - 40	25.30	74.70	4.06	4.85	3.8	21	20	21	0.06
	40 - 80	21.25	78.74	8.10	5.10	1.9	20	19	21	0.06
Lokorsko II	0 -5	20.97	79.03	8.06	6.40	11.0	17	20	64	0.1
	5 – 10	35.35	64.15	4.03	5.40	7.1	13	14	26	0.1
	10– 20	17.12	82.88	8.06	4.70	5.8	10	10	17	0.02
	20 - 40	17.32	82.68	4.03	4.65	4.5	14	13	19	0.02
	40 - 80	25.08	74.92	8.09	4.90	1.3	20	17	29	0.07

Table 2. Mobile forms of Cu, Zn, Pb, Cd (mg/kg) and % of their total content.

SP	Cu		Zn		Pb		Cd	
	mobile	% of total	mobile	% of total	mobile	% of total	mobile	% of total
Buhovo	2	5	2.5	4	15.5	11	0.45	75
	2.5	7	1.5	3	10	9	0.4	80
	2.5	7	1.5	2	12	14	0.35	70
	3.5	10	4.5	7	13	15	0.35	70
	3	10	2	3	14	18	0.5	83
Lokorsko I	1	5	1	4	3.5	7	-	-
	2	11	0.5	2	1.5	6	-	-
	2	12	0.5	3	5	31	-	-
	2.5	12	0.5	2	4.5	21	-	-
	2	10	1	5	4	19	-	-
Lokorsko II	2	10	1	6	9.5	15	-	-
	1	7	0.7	5	5	19	-	-
	1	10	1	9	5	29	-	-
	2	15	1	7	5	8	-	-
	2	15	1.5	7	4.5	15	-	-

Table 3. Total content of heavy metals in leaves of the trees.

SP species	Age	Zn	Cu	Pb	Cd
		mg/kg			
Buhovo - black pine	1	77	38	249	1.4
	2	29	16	28	0.3
	3	20	7	28	0.3
Lokorsko I - red oak		24	4	5	0.2
Lokorsko II – winter oak		18	8	19	0.4

Data show that despite the increased total concentrations of HMs in the soil of Buhovo, due to the alkalinity of the medium (pH ~ 8) their solubility and mobility, and hence their accessibility to plants is low. The percentage of mobile forms to the total forms of Cu and Zn is below then the other more acidic soils, which is the reason for the poor suppling of this soil with plant-accessible mobile forms of these two elements. Concerning Pb where there are high total concentrations the concentration of the mobile forms slightly higher than those of the more acidic soils. The major buffering factor is again the alkalinity of the medium. In relation with Cd there is the opposite fact: the alkaline medium is not a buffering factor, and the movable forms of this element are high as a percentage of the total concentrations.

Foliar analysis is also used to determine the level of nutrition of plants, which also affects their overall general condition. The background concentration of Pb in white pine needles for Western Europe is 2.45-3.90 mg/kg, but according to our data (YOROVA & KUCHUKOV, 1996) for the Western Stara Planina Mts. it is up to 4 mg/kg. In terms of critical levels the values, indicated in the literature vary widely: Pb 3-300 mg/kg, and for Cu 2-100 mg/kg. In most cases the data reported are higher than the tolerant dose (10mg/kg for Pb). The data from the present study show too high Pb content in the black pine needles in Buhovo. Concerning Cu and Zn there are also higher contents of these elements in the black pine needles. Cd is within background content. According to the literature, the background concentrations of trace elements in oak trees are: Pb - 6mg/kg, Zn - 25mg/kg, Cu - 10 mg/kg, Cd – 0.5 mg/kg. In this sense, there is only contamination with Pb in the needles of the winter oak from Lokorsko II. Low values of HMs are found in the leaves of the red oak (*Q. rubra* L.) of Lokorsko I, especially with respect to Cu, Pb and Cd.

To elucidate the absorption of HMs in the woody vegetation, depending on their concentrations in the soil, a bio-absorption coefficient was calculated. This coefficient shows the intensity of biological uptake of HMs, and is calculated by the formula:

$$Ah = Lx / Nx,$$

where: Lx is the content of a chemical element in the ash residue of the plant;

Nx is its content in the surface layer of the soil (GIGOV *et al.*, 1998).

If: $10 > Ax > 1$ there is a high degree of biological absorption of the element; $1 > Ax > 0.1$, an average bioavailability of the element is achieved and at $Ax < 0.1$ a low bioavailability of the element is showed (Table 4).

The lowest degree of absorption of all HMs (Zn, Cu, Pb, Cd) is found in the leaves of the red oak (*Q. rubra* L.) from Lokorsko I.

In order to compare the tree species growth peculiarities in the studied plantations, some dendrometrical indicators were defined (Table 5).

The determined stand quality class of each of the studied plantations is a complex dendrometrical indicator for the quantitative productivity of the respective tree species for a given age and under the appropriate habitat conditions. The results show, that the trees of the winter oak (Lokorsko II) is the most productive, followed by the red oak (Lokorsko I) and last but not least the black pine plantation (Buhovo).

In Table 6 are shown the more important types of pest disease-causing agents, as a part of the entomological characterization of the test areas from Buhovo and Lokorsko. With the "+" sign, the following degrees of distribution of pests are differentiated: + - rarely encountered; ++ - commonly encountered; +++ - encountered very often.

A pathogenic fungus, cancer agent was discovered in Buhovo, which could cause major damage. But it was too low met, which gives grounds for assertion, that it could hardly develop. No insect pests were found in the same SP. In the Lokorsko I, in only one single case wounds were found on the stem of a single tree from the wood decomposing fungi *Stareum hyrsutum*. No pathogenic fungi and pests were found in in Lokorsko II. But part of the lower leaves of the winter oak were attacked by flour mana (fungi of the family Peronosporaceae and Pythiacea), and up to 10% of the leaves were damaged by insect pests (Torticidae, Geometridae, Noctuidae).

Quantitatively were determined plastid pigments. The results are given in Table 7.

Table 4. Biological absorption coefficient.

SP	Age	Ax			
		Cu	Zn	Pb	Cd
	1	0.9	1.2	1.8	2.3
Buhovo – black pine	2	0.4	0.4	0.2	0.6
	3	0.2	0.3	0.2	0.5
Lokorsko I – red oak		0.2	1	0.09	2
Lokorsko II – winter oak		0.4	1.05	0.3	4

Table 5. Dendrometrical indicators in studied SPs.

SP	Composition	Age	$H_{av.}$ m	$D_{av.}$ cm	Stand quality class	Number trees/ha
Buhovo	Black pine (*Pinus nigra* Arn.)	38	11.5	13.2	IV	2130
Lokorsko I	Red oak (*Quercus rubra* L.)	43	18.0	14.7	III	5150
Lokorsko II	Winter oak (*Quercus petraea* Liebl.)	43	15.5	17.5	I	2050

Table 6. Species composition and occurrence of major fungus disease.

SP	Find	Species	Importance	Meeting
Buhovo *Pinus nigra* Arn.	branches	*Gremmenilla abietina*	+++	+
Lokorsko I *Quercus robur* L.	trunk	*Stereum hyrsutum*	+	+

Table 7. Plaster pigment content.

Tree species SP	Month	Chlorophyll "a"	Chlorophyll "b"	"a"/"b"	"a"+"b"	Carotene	a+b/ Carotene
P. nigra	VI	0.980	0.471	2.08	1.451	0.280	5.18
Buhovo	X	0.817	0.361	2.26	1.178	0.242	4.86
Q. rubra L.	VI	1.125	0.407	2.77	1.532	0.320	4.78
Lokorsko I	X	0.917	0.402	2.28	1.319	0.378	3.49
Q. sessiliflora S.	VI	1.080	0.471	2.29	1.552	0.352	4.09
Lokorsko II	X	0.870	0.399	2.18	1.269	0.480	3.05

The biosynthesis of green pigments: chlorophyll "a" and chlorophyll "b" is not impaired. Their total amount decreases in June - October. This decrease is mainly due to the reduction of chlorophyll "a", which is a more labile pigment. Its lability is more intense in case of a higher degree of atmospheric contamination and acid deposition. The ratio of chlorophyll "a"/chlorophyll "b" is considered an indicator determining the degree of gas resistance of tree species. It shows lower values than those considered as optimal 3:1. In our case, the results are about a 2:1 ratio. According this indicator, the studied tree species used for afforestation around the "Kremikovtsi" metallurgic plant can are considered sustainable. The amount of yellow pigments: the carotenoids are less varied, especially in deciduous species. The increase is regarded as a protective response under unfavorable conditions. Higher quantities have the leaves of winter oak in October. The quantities in leaves of black pine are stable. The ratio of green/yellow pigments is within the optimal 4:1 range, 5:1. Analogous analyzes were made for the plantations of black pine and white pine around metallurgic plant - Plovdiv (MIRCHEVA, 1996, Sofia, pers. comm.). Greater resistance of *Pinus nigra* Arn have been determined, compared to *Pinus sylvesstris* L. in terms of atmospheric pollution.

Conclusions

The studies conducted and the results obtained are a reason to say that although winter oak trees show greater productivity, the red oak plantations are best suited for establishing on technogenically contaminated terrains, especially those polluted with lead and low-acid pH of the soil. On so studded sample plots at these altitudes, red oak plantation has a low degree of uptake of leaf toxicanta with very good growth indicators and good entomological situation. It is in dependance of the meteorological and ecological conditions of the specific habitats: altitude, exposure, moisture and others.

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Short Note

Is the Spiny-tailed Lizard Darevskia rudis (Bedriaga, 1886) Active All Year?

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Abstract. The current literature and our field observations have shown that spiny-tailed lizard may also be active in the winter season. Activity of *Darevskia rudis* in the February was recorded for the first time in the province of Giresun, Eastern Black Sea Region, Turkey. In conclusion, we assessed that the spiny-tailed lizard could be active throughout the year due to global climate changes.

Key words: hibernation, Giresun, activity pattern, Trabzon, climate change.

Introduction

Global warming and climate change generally effect on the animal species (Corn, 2005; Borenstein *et al.*, 2009; Huey *et al.*, 2012; Blaustein *et al.*, 2010; Li *et al.*, 2013) and reptiles, specifically (Hawkes *et al.*, 2009; Bickford *et al.*, 2010; Le Galliard *et al.*, 2012; Escobedo-Galván, 2013; Kurnaz *et al.*, 2016). The majority of these impacts has negative consequences for reptile populations (Winter *et al.*, 2016). First of all, the climatic changes affect the summer and winter activity of reptiles (Zug *et al.*, 2001). Hibernation, one of the behavioral responses to seasonal change in reptiles, is directly influenced by the lack of climate (Gregory, 1982; Adolph & Porter, 1993).

There is a known fact that the members of family Lacertidae are hibernating species in the Northern Hemisphere (Smith, 1946). In general, *D. rudis* hibernates between October and March in the bottom of stones and rockies (Demirsoy, 2003).

Darevskia rudis (Bedriaga, 1886) ranges from sea level to 2400 m a.s.l., inhabiting rocky areas in temperate forests, but also montane-steppes, and walls and other human structures (Akarsu *et al.*, 2009 and Kaska *et al.*, 2009).

The researchers reported winter activity for different lizard species; *Podarcis erhardi* (Buresh & Tsonkov, 1933; Beshkov, 1977), *Sceloporus jarrovi* (Tinkle & Hadley, 1973), *Podarcis muralis* (Buresh & Tsonkov, 1933; Beshkov, 1977; Beshkov & Nanev, 2002; Westerstrom, 2005; Tzankov *et al.*, 2014), *Lacerta viridis* (Vongrej *et al.*, 2008), *Zootoca vivipara* (Grenot *et al.*, 2000), *Mediodactylus kotschyi* (Mollov *et al.*, 2015) and *Darevskia derjugini* (Kurnaz *et al.*, 2016).

In the present study, we present data on the extraordinary activity of *Darevskia rudis* in Turkey.

Materials and Methods

The individuals were found during excursions in the province of Trabzon (in

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November 2016, 2017; December, 2017) and Giresun (in February, 2017) during the day. No individuals were caught to avoid the disturbing them. The sexes of the individuals were diagnosed by the secondary sex characters (e.g., dark blue spots on the margins of ventral plates and dorsal coloration of males). All individuals were photographed with a digital camera.

Results and Discussion

November

In total, 7 individuals (4 adults and 3 subadults) of *D. rudis* were found during an excursion in Mansion of Timurcular, Trabzon Province of Turkey between 12:33 and 14:40 AM on 27th November 2016. The observation site was located at the 100 m a.s.l. (41°01′21′′ N; 39°33′83′′ E). The lizards were observed on the walls, which were covered with faded leaves, of Timurcular Mansion. The air temperature in the locality was 15°C in the observation time. In addition, 12 adults and 3 subadult individuals of *D. rudis* were found during an excursion in Akoluk District, Trabzon between 15:15 and 16:00 AM on 12th November 2017. The observation site was located at the 370 m a.s.l. (40°48′77′′ N; 39°36′65′′ E). The lizards were observed on the rocks on the edge of a stabilize road. The air temperature was 17.5°C in the observation time.

December

Two subadult and 8 adult individuals of *D. rudis* were found during an excursion in Ortahisar, Trabzon at 13:45 AM on 2th December 2017. The observation site was located at the 116 m a.s.l. (40°59′45′′ N; 39°45′50′′ E). The lizards were observed on the rocks on the edge of the main road. The air temperature was 16.5°C in the observation time. Besides, 4 adult individuals of *D. rudis* were found during an excursion in Hagia Sophia Museum, Trabzon at 12:00 AM on 10th December 2017. The observation site was located at the 23 m a.s.l. (41°00′19′′ N; 39°41′76′′ E). The lizards were observed among the vines on the retaining walls around the museum. The air temperature was 16°C in the observation time. Furthermore, 5 subadult and 15 adult individuals of *D. rudis* were found during an excursion in İkisu Village, Trabzon at 14:00 AM on 10th December, 2017. The observation site was located at the 323 m a.s.l. (40°55′39′′ N; 39°48′28′′ E). The lizards were observed on the rocks near the village road. The air temperature was 16°C in the observation time.

February

Two adult individuals were observed in Tirebolu, Giresun, between 13:34 and 15:20 AM on 19th February, 2017. The observation site was located at the 17 m a.s.l. (41°04′56′′ N; 38°48′87′′ E). The individuals were found among the rocks on the roadside. The air temperature in the locality was 10°C in the observation time. Two individuals were photographed.

Several active subadult and adult individuals of *D. rudis* were photographed in November 2016 (Fig. 1-A), December 2017 (Fig. 1-B) and February 2017 (Fig. 1-C) brings to mind that this species is active during the winter months. In addition to our field observations, an unusual *D. rudis* activity was observed on December 1989 by Franzen (2000). He reported that the lizards of the *D. rudis* were regularly observed being active in his short visit at the Turkish Black Sea coast between Trabzon and Hopa. Our findings and reports of Franzen (2000) show that the Spiny-tailed lizard may be active during the winter months, except in January.

According to Mollov *et al.* (2015), the records of several subadult and adult individuals of *Mediodactylus kothcyi* in a winter season show that if the temperatures are high enough and they can maintain in certain levels especially in their habitats geckos can be active as long as temperatures allow it. So, we can evaluate that *D. rudis* can be active as long as temperatures allow it during winter, similar to *Mediodactylus kotschi* (Mollov *et al.*, 2015).

Although active periods during the winter were reported for some lizards (SHTERBACK & GOLUBEV, 1986; OKE, 1982, CAMILLONI & BARROS, 1997; VONGREJ *et al.*, 2008; MOLLOV *et al.*, 2015; KURNAZ *et al.*, 2016), this phenomenon was not previously reported for *D. rudis* except the report of FRANZEN (2000). Our data may contribute to the knowledge of the annual activity of *D. rudis*. In our opinion, the unusual activity may be a result of global warming of the world in the Northern Hemisphere.

Fig. 1. The observed individuals of *D. rudis*. **A)** A female individuals from Mansion of Timurcular, Trabzon. **B)** A female individuals from Hagia Sophia Museum, Trabzon. **C)** Two female individuals from Tirebolu, Giresun.

One of the effective factors on hibernation of the lizards is cold environment. Certain vital activities (e.g. mobility, fertility, food availability and escaping behavior) of these animals mainly depend on air temperature and lower temperatures have negative effects on these activities (ADOLPH & PORTER, 1993).

Accordingly, the air temperature during the observation time of the individuals in the present study was very low to be carried out the vital activities of *D. rudis*. Regular change of air temperature can be thought of as the reason for the early awakening from hibernation of some lizards (KURNAZ *et al.*, 2016). In conclusion, we assessed that the spiny-tailed lizard could be active throughout the year due to global climate changes.

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Short Note

First Record of the Narrow Stink Bug Mecidea lindbergi Wagner, 1954 (Hemiptera: Heteroptera: Pentatomidae: Mecideini) from Turkey

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Abstract. In this study, genus *Mecidea* is recorded from Turkey for the first time with the species *Mecidea lindbergi* Wagner, 1954. The newly recorded species is compared with the similar species of the genus *Mecidea* distributed in the Middle East.

Key words: Heteroptera, Pentatomidae, *Mecidea lindbergi*, new record, Turkey.

Introduction

Most Pentatomidae species are known to be phytophagous however, some Asopinae species are predatory, feeding on other insects. Nearly 760 genera and 4100 species of Pentatomidae are known worldwide (YAZICI *et al.*, 2014). Some faunistic studies on this family in Turkey have been done by AHMAD & ÖNDER (1990); AKTAÇ & FENT (1999); AWAD & PEHLIVAN (2001); BEYAZ & TEZCAN (2002); BOLU *et al.* (2006); GHAHARI *et al.* (2014); KIYAK *et al.* (2004); KMENT & JINDRA (2005); KÜLEKÇI *et al.* (2009); LODOS *et al.* (1978, 1987, 1998); LODOS & ONDER (1983); ÖZGEN *et al.* 2005a; b; TEZCAN & ONDER (1999); KULEKCI *et al.* (2009); FENT & AKTAÇ (2009); FENT (2010); FENT *et al.* (2010), DURSUN & FENT (2011), YAZICI *et al.* (2014) and DURSUN & FENT (2015). The genus *Mecidea* (Hemiptera: Pentatomidae) comprises a group of stink bugs with remarkably slender body shape. they occurs in the subtropical and adjacent temperate parts of the world (SAILER, 1952). Until present, genus *Mecidea* was not represented by any species in Turkey.

Material and Methods

The material was collected in the Siirt province of Turkey. The specimen was captured using a sweeping net during the morning period. The material is deposited at the Bioengineering Department laboratory of Fırat University, Turkey.

Results and Discussion

Mecidea lindbergi Wagner, 1954

Material examined: Siirt, Merkez, 08.09.2009, 1 female, Özgen leg., B. Çerçi det.

Genus *Mecidea* is represented by 5 species in the Palearctic region (RIDER, 2006). Only *M. lindbergi* Wagner, 1954 and *M. pallidissima* Jensen-Haarup, 1922 are known from the Middle East. The species belonging to genus *Mecidea* are similar and mostly have

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sexual dimorphism. *Mecidea lindbergi* is distinguished from *M. pallidissima* by the following characters: size between 11-12 mm (in *M. pallidissima* 8-11 mm); second antennal segment only slightly expanded (in *M. pallidissima* second antennal segment significantly expanded); second antennal segment of male 1.6-1.7 times as long as the third antennal segment (in *M. pallidissima* second antennal segment of male 0.6 times as long as the third antennal segment); body 3.6-3.8 times as long as the width of the posterior margin of the pronotum (in *M. pallidissima* body 4.1-4.2 times as long as the width of the posterior margin of the pronotum) (SAILER, 1952; HEISS, 1997). *M. pallidissima* is known from Canary Islands, Egypt and Libya in North Africa; India, Iraq, Israel, Kuwait, Saudi Arabia and Yemen in Asia (RIDER, 2006). *M. lindbergi* is known from England, France, Greece, Italy, Spain (including Canary Islands), Portugal and Romania in Europe; Algeria, Egypt, Morocco and Tunusia from North Africa; Afghanistan, India, Iraq, Israel, Jordan, Saudi Arabia, Syria, Turkmenistan and Yemen in Asia (RIDER, 2006; TRISTAN, 2016; ROCA-CUSACHS & GOULA, 2017). *Mecidea lindbergi* is recorded from Turkey for the first time in this work.

Host Plant: M. lindbergi is known to feed on different species of Poaceae, including Marram Grass *Ammophila arenaria* (CARAPEZZA, 1997).

Fig. 1. Habitus of *Mecidea lindbergi* Wagner, 1954, female.

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Synopsis

Mussels in Ecotoxicological Studies - Are They Better Indicators for Water Pollution Than Fish?

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Abstract. EU Member states are required to apply the EU Water Framework and its Daughter Directives in order to achieve Good Environmental Status (GES) for all 11 qualitative descriptors by 2015 in all water bodies for a list of priority and specific pollutants. Therefore, environmental indicators and biological-effect techniques have to be carefully selected for the management of chemicals in the aquatic environment and for developing an integrated framework. The most commonly applied biological-effect tools are measures of the biochemical and physiological state of selected organisms, such as mussels or fish. The present article provides basic information on the EU Water Directive, the essence of biomarkers, and outlines why mussels may be the better choice of indicators in toxicological research and monitoring programs in order to study the impact of contaminants in water ecosystems.

Key words: mussels, contamination, water, indicators.

Water contamination as a global issue

Water is the key resource required to sustain life on this planet and it is important to both society and ecosystems (Paul, 2017). In addition, freshwater is the most important resource for mankind, crosscutting all social, economic and environmental activities. It is a condition for life on the Earth, an enabling or limiting factor for any social and technological development, a possible source of welfare or misery, cooperation or conflict (Bouwer, 2002). As stated by Lomsadze *et al.* (2017) we depend on a reliable, clean supply of drinking water to sustain our health. We also need water for agriculture, energy production, recreation and manufacturing. However, many of these uses put pressure on water resources. Therefore, in recent years, water contamination has been considered as a global issue (Cui *et al.*, 2011; Yuan *et al.*, 2011). Studies conducted in Arabian Gulf (Naser, 2013); Argentina (Marcovecchio, 2004); Bangladesh (Rahman *et al.*, 2012); Chile (Tapia *et al.*, 2009); China (Wang *et al.*, 2005; Qiao-qiao *et al.*, 2007; Yi *et al.*, 2011; Li *et al.*, 2013; Zhang *et al.*, 2013); Croatia (Klobučar *et al.*, 2008); Egypt (Rashed, 2001a); Ethiopia (Yohannes *et al.*, 2013); France (Shinn *et al.*, 2009); India (Sankar *et al.*, 2006; Sivaperumal *et al.*, 2007; Rajeshkumar *et al.*, 2013; Arockia

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VASANTHI *et al.*, 2013); Malaysia (TAWEEL *et al.*, 2013); Norway (NÆS *et al.*, 1999; BERGE *et al.*, 2011); Pakistan (SHAHBAZ *et al.*, 2013); Serbia (SUBOTIĆ *et al.*, 2013); Slovenia (TOLLEFSEN *et al.*, 2006; MILAČIČ *et al.*, 2017); Spain (OLMEDO *et al.*, 2013); Sudan (PRAGST *et al.*, 2017); Tailand (PEEBUA *et al.*, 2006); Turkey (TÜRKMEN *et al.*, 2005; GÖRÜR *et al.*, 2012); Zimbabwe (BIRUNGI *et al.*, 2007) and even Arctic regions (SONNE *et al.*, 2009) show that contamination is an ecological problem worldwide, suggesting that it could be affecting the aquatic organisms and having a considerable impact on the health of water ecosystems. Thus, the scientific community suggests that urgent actions and strategies need to be taken to prevent and control the pollution.

One of the dangerous types of water pollution is with heavy metals (As, Cu, Cd, Hg, Pb, Zn) (MOISEENKO & KUDRYAVTSEVA, 2001; SHAH *et al.*, 2009; CARRASCO *et al.*, 2011; PAUL & SINHA, 2013; AVKOPASHVILI *et al.*, 2017); persistent organic pollutants (POPs) (HYLLAND *et al.*, 2006; 2008), pesticides (DEVAULT *et al.*, 2009; LOOS *et al.*, 2009; YADAV *et al.*, 2010); crude oil (OLSEN *et al.*, 2007; HOLTH *et al.*, 2009; HOLTH *et al.*, 2011; BALK *et al.*, 2011; VESTHEIM *et al.*, 2012) and plastics (ANDRADY, 2011; LÖHR *et al.*, 2017). Furthermore, it has been reported that in recent decades the level of foreign compounds in aquatic ecosystems such as heavy metals, pesticides and other persistent organic pollutants has increased alarmingly as a result of domestic, industrial and agricultural effluents (SEKABIRA *et al.*, 2010; LUSHCHAK, 2011; ONDARZA *et al.*, 2011; PEREIRA *et al.*, 2013; JÖRUNDSDÓTTIR *et al.*, 2014).

Heavy metals are toxic, non-biodegradable and persistent environmental contaminants (HAS-SCHÖN *et al.*, 2008). Large quantities of heavy metals have been released into aquatic systems, both fresh and marine worldwide due to a global rapid population growth and intensive domestic activities, as well as expanding industrial and agricultural production (SREBOTNJAK *et al.*, 2012; ISLAM *et al.*, 2014). Thus, they have severely deteriorated the aquatic ecosystems because of their toxicity, abundance, persistence, and subsequent bioaccumulation and biomagnification (ALI *et al.*, 2013).

POPs include many different contaminant groups such as polyaromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs), including dichlorodiphenyltrichloroethane (DDT), polybrominated diphenyl ethers (PBDEs) used as flame-retardants, and industrial organochlorines (OCs) such as polychlorinated biphenyls (PCBs) (CIESIELSKI *et al.*, 2017). Since most POPs are lipophilic they accumulate in the lipid-rich tissues of living organisms, and thereby biomagnify in the food web (LETCHER *et al.*, 2010). Under this consideration, the United States Environmental Protection Agency (USEPA) classified 16 of them as priority pollutants (QIAO *et al.*, 2006).

Pesticides have been widely applied to protect agricultural crops since the 1940s, and since then, their use has increased steadily (GRUNG *et al.*, 2015). The organochlorine pesticides (OCPs) became the dominant pesticides after the Second World War. With the publishing of "Silent Spring" by Rachel Carson in 1962, a wider audience was warned of the environmental effects of the widespread use of pesticides. Since 1970s, after evidence of their toxicity, persistence and bioaccumulation in environmental matrices OCPs production, usage and disposal have been regulated or prohibited in most of the developed countries (JAN *et al.*, 2009; MARIN-MORALES *et al.*, 2013). In this sense, organophosphorous pesticides (OPs) in many cases replaced organochlorine pesticides (OCs) in agriculture. Even though they have a short-term degradation and fewer residues, OPs unfortunately lack target specificity and can also cause severe, long lasting population effects on aquatic non-target species (FULTON & KEY, 2001; SUN *et al.*, 2011).

Crude oil consists of variety proportions of hydrocarbons such as alkanes, aromatics and PAHs, asphaltene and resins, as well as non-hydrocarbons namely sulphur, nitrogen, oxygen and traces of metals, particularly copper, iron, nickel and vanadium (Brandt, 2006; Sakthipriya *et al.*, 2015; Wilberforce, 2016). At present, the petroleum industry has entered into a period of modernization and transition. Due to this industrial development, pollution of seawater by crude oil has been increased and creating a serious issues (Bao *et al.*, 2014). As a result, each year enormous quantities of crude oil are disposed into the environment, either deliberate or accident during crude oil production and transportation. Between 1.7 and 8.8 million metric tons of crude oil are estimated being released in the water environment each year (NRC, 1985), of which >90% as deliberate waste disposal, directly related to human activities (Ndimele, 2010). According to Effendi *et al.* (2017) oil spills often bring devastated effects on the aquatic organisms and consequently ruin the aquatic ecosystem homeostasis. Lennuk *et al.* (2015) state that the impact of oil toxicity on various ecosystem elements have been increasingly reported since 1960s and the majority of studies have focused on the oil spill effects on organisms such as plankton, fish, birds and marine mammals. In addition, oil spills incidences all over the world occasionally occur and one of the most devastating ones are linked with the explosion of Deepwater Horizon (Weisberg *et al.*, 2017) and Exxon Valdez (Lindeberg *et al.*, 2017).

Last but not least, worldwide there is a growing concern about the risks and possible adverse effects of (micro) plastics because since the 1950s the amount of plastics in the marine environment has increased dramatically (Jambeck *et al.*, 2015; UNEP, 2016). Furthermore, substantial quantities of microplastics are already present in the global marine ecosystem (Andrady, 2011; Desforges *et al.*, 2014), from the tropics to the poles, including in Arctic sea ice and numerous studies have already showed the negative effects of microplastics on marine biota (Lusher *et al.*, 2013; Lusher, 2015; Romeo *et al.*, 2015).

Legislation in the field of water conservation

According to Vethaak *et al.* (2015) many maritime countries in Europe have implemented marine environmental monitoring programs, which include the measurement of chemical contaminants and related biological effects. Moreover, the European Union (EU) has, over the last twenty years, developed its water policies so that now there is significant European legislation covering marine waters, as well as the lakes and rivers that ultimately flow into our coastal ecosystems.

To maintain the water at an adequate quality for humans and to preserve natural ecosystems and biodiversity, it is necessary to sustainably use, protect and manage the water resources. In the countries of the European Union (EU), national water agencies, which follow EU policy and the requirements of the EU Water Framework and its Daughter Directives (Directive 2000/60/EC; Directive 2008/105/EC; Directive 2008/56/EC; European Communities Environmental Objectives 272/2009; European Communities Technical Report, 2010), implement regular water monitoring with the aim to control and prevent pollution (Milačič *et al.*, 2017).

Recently, the Water Framework Directive (WFD, Directive 2000/60/EC) of the European Union specified monitoring programs required to assess the achievement of good chemical and ecological status for all water bodies by 2015 for a list of specific pollutants (Sanchez & Porcher, 2009). This list currently stands at 41 pollutants (33 priority substances (PSs) and 8 other pollutants) given in Annex I of Directive 2008/105/EC; furthermore, the European Commission (EC) proposed recently to include 15 additional PSs on this existing list (Besse *et al.*, 2012).

More recently, the European Union has implemented the Marine Strategy Framework Directive (MSFD, DIRECTIVE 2008/56/EC). At its heart is the concept of "good environmental status" (GES) for all European waters and the provision of a framework for the protection and preservation of the marine environment, the prevention of its deterioration, and, where practicable, the restoration of that environment in areas where it has been adversely affected (VETHAAK *et al.*, 2015). In addition, the MSFD is a wide-ranging framework directive with the overall objective of maintaining GES in the seas of Europe by 2020 (LYONS *et al.*, 2010).

GES will be assessed on a regional basis. The Regional Sea Conventions (OSPAR Commission, Helsinki Commission (HELCOM), Barcelona Convention (In 1975, 16 Mediterranean countries and the European Community adopted the Mediterranean Action Plan (MAP), the first-ever Regional Seas Program under UNEP's umbrella) and the Black Sea Commission), which aim to protect the marine environment, were required to support the implementation of the MSFD (VETHAAK *et al.*, 2015). According to LYONS *et al.* (2010) the Joint Research Centre (JCR) and the International Council for the Exploration of the Sea (ICES) were also commissioned to facilitate the preparation of scientific bases for criteria and to propose methodological standards in relation to 8 of the GES descriptors during the course of 2009.

As stated by BESSE *et al.* (2012) the application of the WFD for the surveillance of chemical contamination of surface waters involves two main objectives: (1) to assess the chemical status of the water bodies, by determining whether contamination levels are compliant with the regulatory Environmental Quality Standards (EQSs); and, (2) to assess the temporal trends of the contamination in the different environmental compartments of aquatic ecosystems. EQSs are defined as "the concentration of a particular pollutant or group of pollutants in *water, sediment or biota,* which should not be exceeded in order to protect human health and the environment". In addition, the assessment of chemical status under WFD is undertaken in fresh, transitional and coastal waters using EQS, which are derived from toxicological information and used to set acceptable limits for individual priority contaminants (LYONS *et al.*, 2010).

Choice of bioindicator species – fish or mussels

The use of living organisms in the study of environmental quality is widely accepted (STAMENKOVIĆ *et al.*, 2013). However, according to SHERMAN (1994) selecting indicators can be challenging, given the variety of ecosystem characteristics they are intended to track. Furthermore, according to BESSE *et al.* (2012) there are currently two different strategies for chemical biomonitoring (i.e. monitoring contamination in biota) that can be adopted: *passive or active.* Passive approaches rely on indigenous organisms, while active approaches rely on transplanted (or caged) individuals from a reference site (see SCHØYEN *et al.*, 2017).

In the review of REES *et al.* (2008) it is explained the ideal indicator should be: (i) capable of conveying information that is responsive and meaningful to decision-making (directly tied to management questions and linked to thresholds for appropriate action relative to designated ecosystem goals); (ii) linked to a conceptual stressor–response framework (with the ability to communicate potential cause–effect relationships); (iii) capable of measuring change or its absence with confidence (robust to influences of confounding environmental factors); (iv) highly sensitive and anticipatory (early warning of potential problems); (v) applicable over a variety of spatial scales and conditions (to support global as well as local comparisons); (vi) desirable operationally (easy to measure, reproducible with minimum measurement error, cost-effective); (vii) integrative (serves multiple indicator purposes); (viii) non-destructive (measurement does not cause ecosystem damage); (ix) easy to understand and communicate (non-specialists need to act on findings); (x) scientifically and legally defensible (robust to peer review and wider challenge).

CAIRNS *et al.* (1993) encapsulated the issue of single vs. multiple indicators by noting that "... everything is an indicator of something but nothing is an indicator of everything" in a review of ecosystem health indicators. UNESCO identified three classes of ecosystem indicators (slightly modified): (i) ecological indicators: to characterize and monitor change in the state of various physical, chemical, and biological aspects of the environment relative to defined quality targets with thresholds for management action (see also FISHER, 2001); (ii) socio-economic indicators: to measure whether environmental quality is sufficient to maintain human health, human uses of resources, and favorable public perception (see also CAIRNS *et al.*, 1993); (iii) governance indicators: to monitor the progress and effectiveness of management and enforcement practices towards meeting environmental policy targets. As stated by REES *et al.* (2008) until recently, *ecological indicators* have received most attention.

According to WFD, fish represent one of the key elements to evaluate the rivers ecological status (SCARDI *et al.*, 2008; HERMOSO *et al.*, 2010).

BESSE *et al.* (2012) explain that fish are ideal organisms for checking compliance against biota EQSs, since the protection objectives targeted are human health and/or high-level predators. Hence, out of the 3 biota EQSs defined under Directive 2008/105/EC and the 8 biota EQSs given in the EC proposal to revise this Directive, 10 are based exclusively on a fish matrix. However, BESSE *et al.* (2012) state that if fish are the test organisms of choice for checking compliance with biota EQSs, they have several characteristics that limit their use for active biomonitoring, while macroinvertebrates represent a good compromise in terms of feasibility and fulfilling the objectives of the WFD. In addition, the authors add that fish carry disadvantages for long-term trend analysis on contamination patterns, as they are known to strongly metabolize certain contaminants, and that makes them less valuable as indicators for some PSs. For example, fish strongly metabolize PAHs, limiting in this way their relevance as reliable indicators of PAH pollution; indeed, it is only possible to estimate recent exposure to PAH indirectly by determining biliary concentrations of PAH metabolites (VAN DER OOST *et al.*, 1994). Furthermore, caging is generally unsuitable for fish species and the use of fish for active biomonitoring purposes remains difficult to achieve because in logistical terms, the size of these organisms requires large caging systems that are difficult to handle in the field; and, fish are easily stressed, especially in caging-experiment conditions (which limit their mobility), which risk introducing bias in the responses obtained (OIKARI, 2006).

YANCHEVA *et al.* (2015, 2016a) describe in reviews why in ecotoxicology fish have become the major vertebrate model. Indeed, a tremendous body of information has been accumulated (STEINBERG *et al.*, 1995; BRAUNBECK *et al.*, 1998; MOISEENKO, 2005; RAISUDDIN & LEE, 2008; MURTHY *et al.*, 2013; CZÉDLI *et al.*, 2014). Fish are among the group of aquatic organisms, which represent the largest and most diverse group of vertebrates. They are present virtually in all environments and many species have been found to be susceptible to environmental pollutants (VAN DER OOST *et al.*, 2003). A number of characteristics make fish excellent experimental models for toxicological research, especially for the contaminants which are likely to exert their impact on aquatic systems (LAW, 2003; DE LA TORRE *et al.*, 2010). According to DE LA TORRE *et al.* (2005) monitoring sentinel fish species is widely used to assess the degree of accumulation of pollutants and the effects on health status. In addition, fish have been found to be good indicators of water contamination in aquatic systems because they occupy different trophic levels; they are of different sizes and ages and in comparison with invertebrates, are also more sensitive to many toxicants (DALLINGER *et al.*, 1987; POWERS, 1989; BARAK & MASON, 1990; WESTER *et al.*, 1991; BURGER *et al.*, 2002) Fish respond to environmental toxic changes with adapting of their metabolite functions (MISHRA & SHUKLA, 2003). They are also preferred in toxicological research because of their well-developed osmoregulatory, endocrine, nervous, and immune systems compared to invertebrates (SONG *et al.*, 2012). In addition, the studies of PÉREZ CID *et al.* (2001); FISK *et al.* (2001); RASHED (2001b); MONDON *et al.* (2001); MANSOUR & SIDKY (2002); USERO *et al.* (2004); MENDIL & ULUÖZLÜ (2007); ÖZTÜRK *et al.* (2009); SOUNDERAJAN *et al.* (2010); ROWAN (2013) show that fish may absorb toxicants directly from the surrounding water and sediments (waterborne exposure), or ingest them through contaminated food in the food chain (dietary exposure), enabling the assessment of pollutant transfer through the tropic web.

The pollutants are accumulated through different organs of the fish because of the affinity between them. In this process, many of them are concentrated at different levels in different organs of the fish body (RAO & PADMAJA, 2000). Therefore, in teleost fish, the gills, liver, kidney and muscles are the tissues most frequently utilized in toxicological studies because they are metabolically active tissues and accumulate toxicants at higher levels (HEIER *et al.*, 2009; JOVIČIĆ *et al.*, 2014).

In their review BESSE *et al.* (2012) present information about different national and international project involving fish as a test organism. The authors provide data about the French ''Plan National PCB'', which is a public health-oriented program deployed between 2008–2010. It was designed to step up the monitoring of contamination in aquatic environment and fish products, in order to adopt appropriate risk-management measures. Dioxins, furans, dioxin-like PCBs and indicator PCBs, as well as total Hg were investigated within this program. The project framework included sampling schemes on several fish species across more than 300 sites (around 100 sites a year). The fish species were selected for their PCB bioaccumulation profiles, their national geographical distribution profiles, and their history of use as human food. Another program is the ''Flemish Eel Pollutant Monitoring Network'', which was launched in 1994 in Belgium by the Research Institute for Nature and Forest (Inbo). This monitoring network, public-health oriented, aimed at monitoring the dispersion of pollutants in Flanders for continental waters using European eel (*Anguilla anguilla*). Over a 10-year period, around 360 sites (streams, rivers, canals, ponds and lakes) across Belgium were sampled for environmental analysis of PCB, OCPs and heavy-metal pollution.

To monitor the health of water ecosystems, other sentinel organisms such as mussels (bivalves) have been identified as suitable candidates to indicate the levels of contaminants and their effects in the aquatic environment and as such, they have been proposed to be suitable "biomonitors" of pollution (NAIMO, 1995; BESADA *et al.*, 2011).

Their filtering habits, low metabolism and ability to bioaccumulate pollutants make them an excellent choice to assess their bioavailability and effects (BAYNE, 1989; FOSSATO *et al.*, 1989; WIDDOWS & DONKIN, 1992). Mussels are also sessile, sedentary, long-lived and easy to collect, have a reasonable size for chemical analyses, they are worldwide distributed and present across a very wide geographical areas (basically the entire range of North Atlantic, Baltic, and Mediterranean coastal areas, see THAIN *et al.* (2008)), particularly abundant in coastal and estuarine waters and often found in large amounts (FARRINGTON *et al.*, 1983; GOLDBERG, 1980; 1986; BOLOGNESI *et al.*, 2006; BELLOTTO & MIEKELEY, 2007; CHANDURVELAN *et al.*, 2015).

The U.S. Environmental Protection Agency (EPA) classifies mussels as biomonitors because they react to changes in the surrounding environment. Additionally, since mussels are filter feeders, they bioaccumulate heavy metals and POPs and other contaminants in their bodies and shells (RAINBOW, 1993; CAPPELLO *et al.*, 2013). In comparison to fish and crustaceans, bivalves have a very low level of activity of enzymatic systems capable of metabolizing POPs. Therefore, bivalves are widely used as bioindicators of organic pollution in freshwater, marine and estuarine ecosystems because they are known to provide a time integrated indication of environmental contamination, as well as reliable information on the potential impact of seafood consumption on human health (TURJA *et al.*, 2013).

To monitor the nature and extent of coastal pollution, a Mussel Watch Programme (MWP) was developed by GOLDBERG (1975; 1986) in an attempt to quantify the levels of pollutants in coastal systems. BESSE *et al.* (2012) describe in their review that in marine waters, the NOAA "Mussel Watch" represents the oldest, continuous running contaminant-monitoring program in US coastal and Great Lakes waters. This project has been running since 1986, but the original "US Mussel Watch" program was initiated in 1975 as shown above. The program covers 300 coastal sites and monitors over 100 organic and inorganic contaminants (PAHs, PCBs, DDTs, TBT, OCPs and toxic trace elements) in sediment and biota tissue. It is based on yearly collection and analysis of oysters and mussels. Since there is no species of mussel or oyster common to all coastal regions, a variety of species are collected (*Mytilus* species, *Crassostrea virginica* and *Dreissena* species) in order to gain a national perspective (BESSE *et al.*, 2012).

As stated by BESADA *et al.* (2011) the use of mussels to monitor coastal pollution is now widely accepted and supported by many international organizations (see APETI *et al.*, 2010; MARUYA *et al.*, 2014; MELWANI *et al.*, 2014; SCHÖNE & KRAUSE JR., 2016; BEYER *et al.*, 2017). According to SCHØYEN *et al. (2017)* most commonly the mussel watch studies involve collection of samples from natural blue mussel populations, but the adoption of an active biomonitoring alternative by using transplanted blue mussels has gained considerable popularity in ecotoxicology research and monitoring.

Mytilus sp. have been widely used since the 90s, and have been shown to be one of the most successful model organisms for time-integrated responses to complex mixtures of pollutants (UNEP/RAMOGE, 1999). Furthermore, the blue mussels such as *Mytilus edulis* and *Mytilus galloprovincialis* are widely used as sentinels in coastal pollution monitoring (mussel watch) programs, mainly because their biological characteristics make them very suitable as bioindicators for assessing the quality status of coastal waters (ZATTA *et al.*, 1992; HAGGER *et al.*, 2008; WEPENER & DEGGER, 2012; SPARKS *et al.*, 2014; ROUANE-HACENE *et al.*, 2015; FARRINGTON *et al.*, 2016).

In this sense, in France, Ifremer (French research institute for exploration of the sea) has been running the "RNO" network (National observatory network on marine environment quality) since 1974 and since re-named "ROCCH" – National chemical contamination network. This approach is based on monitoring natural populations of three bivalve mollusks – one oyster species (*Crassostrea gigas*) and two mussel species (*Mytilus edulis* and *Mytilus galloprovincialis*) in order to ensure national-scale coverage. About 50 chemical contaminants (PAHs, PCBs, OPs and metals) are currently being monitored at 80 sampling sites around the French coastline. Organisms that have spent at least 6 months seeded in the zone are sampled semi-annually for metal pollutants and annually for organic pollutants (BESSE *et al.*, 2012). In addition, in 1996, Ifremer developed RINBIO ("Re´seau Inte´grateurs Biologiques") as a methodology built around caging marine mussels (*Mytilus galloprovincialis*). Indeed, using transplanted organisms made it possible to compensate for the scarcity of wild-mussel colonies in certain sections of the French Mediterranean coastline as explained by BESSE *et al.* (2012). The authors also mention the recent "Projet Mytilos" initiative, based on the same structural principle as RINBIO, was developed to study heavy-metal contamination (Cd, Hg, Ni and Pb) in caged mussels (*Mitilus galloprovincialis*) at over 120 sites spanning the entire Mediterranean Basin. Another monitoring program in which mollusks were used is the National Water-Quality Assessment (NAWQA) program in the USA, which was launched in 1993 by the United States Geological Survey (USGS) to monitor spatial and temporal trends of contamination for a selection of micropollutants (PAHs, OPs, PCBs, dioxins and metals). This monitoring program encompasses several species: (1) a bivalve mollusk (*Corbicula fluminea*); (2) three insect genus (*Trichoptera, Chironomidae, Plecoptera*); (3) six fish species; and, (4) vascular plants (*Potamogeton sp.* and *Elodea sp.*). Chemical analysis in resident biota was integrated as part of the first cycle of assessments (1993–2001), but was dropped from the second cycle due to the high costs involved and to data-interpretation difficulties (see BESSE *et al.*, 2012).

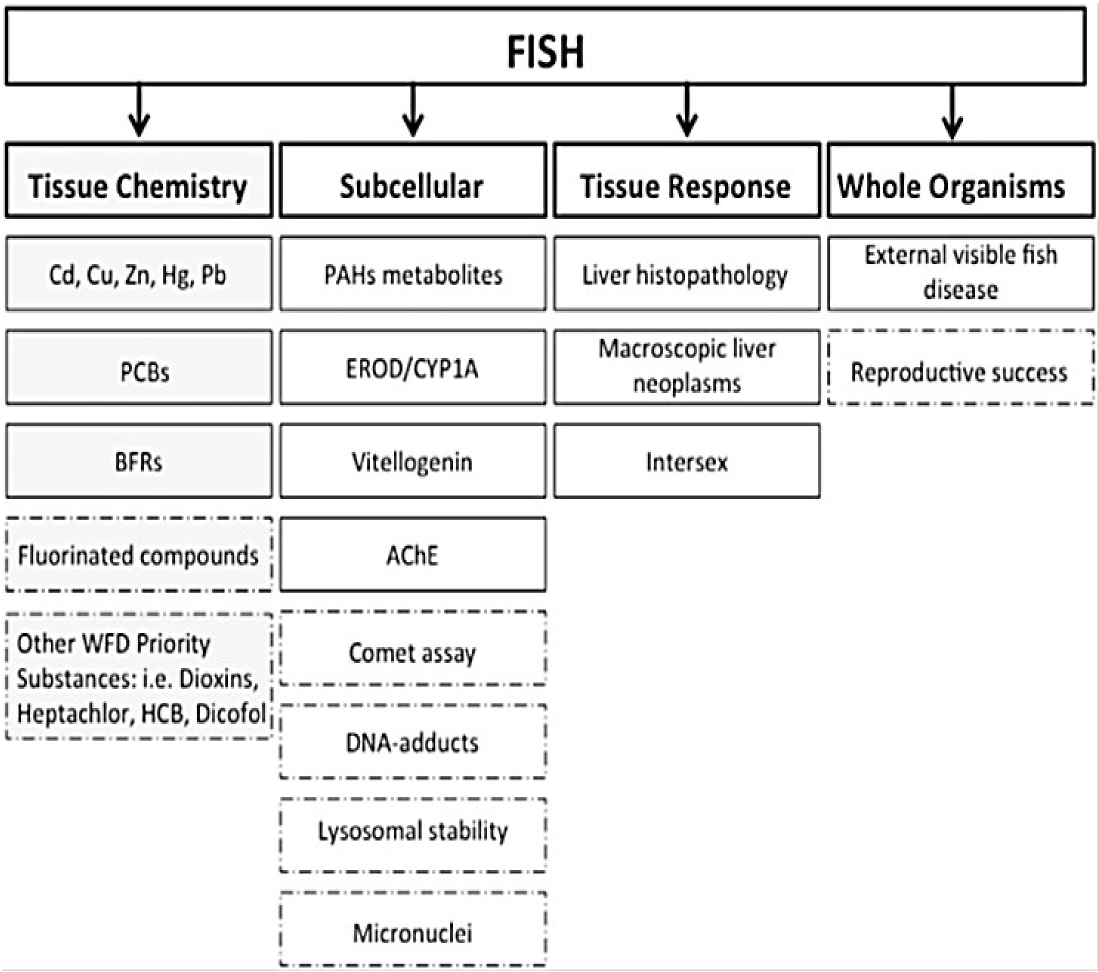

Fig. 1. Determinants and measurements included in the fish component of the ICES/OSPAR integrated monitoring framework. *Legend:* solid lines - core methods; broken lines - additional methods; PCBs - polychlorinated biphenyls; BFRs - brominated flame retardants; AChE - acetylcholinesterase; WFD - Water Framework Directive, WFD - priority substances are required in biota under DIRECTIVE 39/2013/EU (see THAIN *et al.* (2008) and VETHAAK *et al.* (2015)).

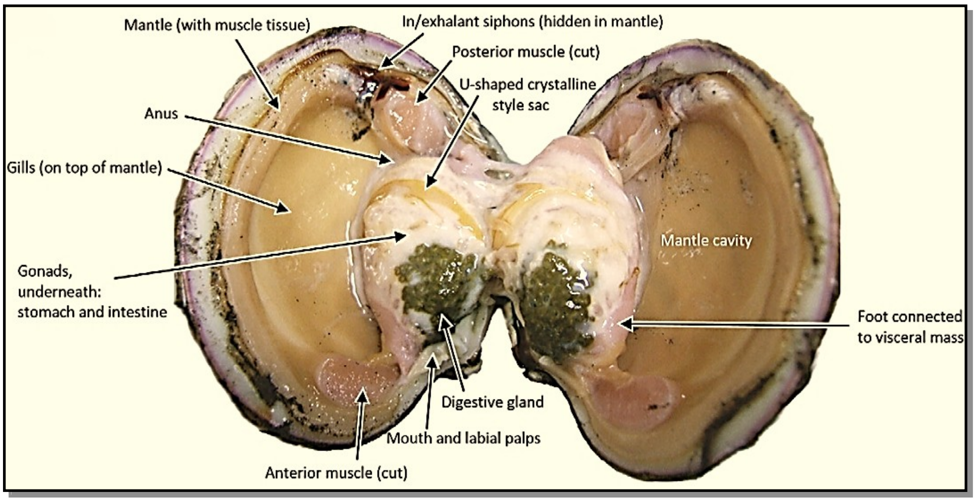

Fig. 2. Overall bivalve anatomy (after SCHÖNE & KRAUSE JR., 2016).

However, other freshwater bivalves such as zebra mussel (*Dreissena polymorpha*) can be used as a freshwater substitute of *Mytilus* sp. (LEPOM *et al.*, 2012) and has already been successfully used in ecotoxicological studies and monitoring programs (CAMUSSO *et al.*, 1994; JOHNS & TIMMERMAN, 1998; DE LAFONTAINE *et al.*, 2000; GUERLET *et al.*, 2007; BINELLI *et al.*, 2010; FARIA *et al.*, 2010; YANCHEVA *et al.*, 2016c; d; 2017).

It is important to outline that the organisms used to cover all three main trophic levels in monitoring programs are – bryophytes, macroinvertebrates, and fish. Furthermore, the most commonly applied species are *Fontinalis antipyretica* for bryophyte species, invasive bivalves *Dreissena polymorpha* and *Corbicula fluminea* for invertebrate species, and *Cyprinus carpio for* fish species (see BESSE *et al.*, 2012).

According to BINELLI *et al.* (2015) one of the fundamentals in the ecotoxicological studies is the need of data comparison. In their study the authors showed the advantages of *Dreissena polymorpha* application in biomonitoring, as well as for the evaluation of adverse effects induced by several pollutants, using both *in vitro* and *in vivo* experiments. Since Directive 2013/39/EU establishes EQS for biota because it has been demonstrated that pollutants bioaccumulate in aquatic organisms, BENITO *et al.* (2017) evaluated bioaccumulation of inorganic elements in the soft tissues of zebra mussels in order to assess the usefulness of this species as a bioindicator of contaminant presence in superficial waters along the Ebro River Basin (Spain). The authors concluded that *Dreissena polymorpha* not only supplies information about current water quality but also acts as a witness of past water quality by bioconcentrating toxic elements present in the environment and providing relevant results about historical water contamination. BESSE *et al.* (2012) explain that in Switzerland, the International Commission for the Protection of the Waters of Lake Geneva (CIPEL), established in 1962, has monitored trends of contamination in biota, notably fish, for 30 years, with the dual purpose of public health and the environment. For example, in 2004, organic micro-pollutants (mercury, PCBs and organotins) and trace metals in aquatic ecosystems and biota (fish and mussels) of Lake Geneva were investigated. Metals and organotins were monitored in *Dreissena polymorpha*, as were mercury and PCBs in several fish species (*Perca fluviatilis, Lota lota, Coregonus sp*. and *Salvelinus alpinus*).

Although, zebra mussels is a harmful and among the top 100 most dangerous invasive species in aquatic habitats, its pervasiveness means that it can be also used as a bioindicator to assess current and past presence of elements in water. Moreover, according to BESSE *et al.* (2012) for active strategies, the use of invasive species should be avoided, meaning that the use of zebra mussels and Asian clams (*Anodonta*) (which are among the most widely-used species) should be limited to sites that these species have already colonized. However, MERSCH *et al.* (1996) and BOURGEAULT *et al.* (2010) successfully used zebra mussels in caging experiments dealing with the effects of different pollutants.

Lastly, the most commonly used bivalve tissues in toxicological studies are gills and digestive gland (Fig. 2) because they play major part in the process of bioaccumulation of toxicants. Thus, they are used in biomarker analyses to the study the subsequent negative effects.

Choice of biomarkers in integrated monitoring

Biomarkers are defined as responses to any exposure evidenced in histological, physiological, biochemical, genetic and behavioral modification (FOSSI & MARSILI, 1997; FOSSI, 1998). Furthermore, as stated by VIARENGO & CANESI (1991) the effects of pollutants on living organisms can be evaluated at different levels of organization (molecular, cellular, individual, population and community.

More recent, VAN DER OOST *et al*. (2003) defined biomarkers as biological indicators from an exposure to a stressor responding in various ways. PICADO *et al.* (2007) add that biomarkers, which act as early warning signals of the presence of potentially toxic xenobiotics, are useful tools for assessing either exposure to, or the effects of these compounds providing information about the toxicant bioavailability.

A range of techniques (Fig. 1 and 3) has been developed to quantify or indicate the effects of pollutants on aquatic organisms from cellular to community levels (ICES, 2004). In addition, THAIN *et al.* (2008) explains that the most widely used biological-effect tools are measures of the biochemical and/or physiological state of selected organisms, such as mussels or fish.

According to HYLLAND *et al.* (2006) over the past decade there has been evidence of effects at low exposure levels. The authors state that many chemicals are metabolized or cause effects at very low levels (e.g. endocrine disrupting substances, PAHs and OPs). Therefore, descriptor 8 in MSFD defines that "concentrations of contaminants are at levels not giving rise to pollution effects" (Table 1).

According to MARIA *et al.* (2009) connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects and determining the extent and severity of such contamination only by the result of water chemical analysis is insufficient and often overestimates the proportion and duration of exposure to the toxic agent. Therefore, integration of chemical analyses with biomarker responses in organisms has been recommended for monitoring anthropogenic activities (HYLLAND *et al.*, 2008). According to BAYNE (1986) good interpretation of the data can be obtained by studying the effects of pollutants in individuals, with the aim of understanding and eventually predicting the possible consequences at higher levels.

In the past 25 years, numerous biomarkers have been developed with the objective to apply them for environmental biomonitoring and risk assessment programs (NRC, 1987; HINTON *et al.*, 1992; HINTON, 1994; SCHMITT *et al.*, 2007; PANDEY *et al.*, 2008; ARDESHIR *et al.*, 2017). PINTO *et al.* (2009) suggest that the biomonitoring process should include analyses at different levels of biological organization, from sub-cellular and cellular analysis of tissues and organs, to the of population and community levels. Therefore, studies using biomarkers are essential to complement environmental monitoring in order to control pollution effects on the animals that inhabit the water bodies (Au, 2004).

The usefulness of any biological-effect method will depend on how well it is able to separate anthropogenic stressors from the influence of environmental or host-related processes (Lyons *et al.*, 2010). According to Hylland *et al.* (1998) it is well established that there are seasonal variations, most commonly coordinated with reproduction, for most biomarkers in temperate organisms. Furthermore, according to Hylland *et al.* (2006) the methods must be able to separate between effects from contaminants and natural variation or the effects from other environmental factors. For example, Kammann *et al.* (2005) found that certain biomarkers such as hepatic EROD activity in fish are influenced by a number of non-contaminant related factors including ambient water temperature and stage of sexual development. As explained by Hylland *et al.* (2006) European flounder (*Platichthys flesus*), which is a common test species in monitoring programs in Europe and numerous toxicology studies, shows natural variability through a year (Hylland *et al.*, 1998); response to individual contaminants (Beyer *et al.*, 1996); combination of contaminants (Sandvik *et al.*, 1997) or response in caging experiments (Beyer *et al.*, 1996). On the other hand, there is enough evidence for already established cause-and-effect relationships between the presence of contaminants and biological-effect responses in the organisms. A good example here would be tributyltin (TBT)-induced imposex in dogwhelk as Thain *et al.* (2008) explain. Once population effects that could be directly related to concentrations of organotins in the marine environment were observed (Gibbs & Bryan, 1996), management actions were taken to reduce TBT emissions and introduce international policies through the EU and International Maritime Organisation (IMO), which has resulted in a decrease in the prevalence and severity of imposex (Birchenough *et al.*, 2002).

WHO (1993) state that biomarkers have been classified into three separate categories that correspond to three major parameters necessary to conduct ecological risk assessments. To perform such an accurate ecological risk assessment, ecological effects, as well as exposure and susceptibility to contaminants must be well-characterized following identification or formulation of a problem. In each of these processes, well-defined biological indicators can be used in certain cases to help make inexpensive predictions regarding the bioavailability (exposure), mechanism of action (effect) and uncertainty of response (susceptibility) elicited by various anthropogenic substances (Schlenk, 1999). According to Vethaak *et al.* (2015) the concept of a background level of response (residual noise of the measurement found from responses of animals in relatively clean waters) is applicable to all effects measurements. Assessment criteria analogous to the Environmental Assessment Criteria (EAC) (i.e. representing levels of response below which unacceptable responses at higher, e.g. organism or population, levels of biological organization would not be expected) are applicable for some biological effects measurements, and these have been termed *"biomarkers of effect"*. In other cases, the link to higher level effects is less clear, and these measurements have been termed *"biomarkers of exposure"*, in that they indicate that exposure to hazardous substances has occurred.

Biological effects measurements and chemical methods have been selected for the *biota matrix (separated as fish, mussels and gastropods)* using different criteria – general designs for integrated monitoring of fish are presented in Fig. 1 and of mussels in Fig. 3.

Table 1. Qualitative descriptors for determining Good Environmental Status as defined in Annex I of the MSFD (see LYONS *et al.*, 2010).

1. Biological diversity is maintained. The quality and occurrence of habitats and the distribution and abundance of species are in line with prevailing physiographic, geographic and climatic conditions.
2. Non-indigenous species introduced by human activities are at levels that do not adversely alter ecosystems.
3. Populations of all commercially exploited fish and shellfish are within safe biological limits exhibiting a population age and size distribution that is indicative of a healthy stock.
4. All elements of the marine food webs, to the extent that they are known, occur at normal abundance and diversity and levels capable of ensuring the long-term abundance of the species and the retention of their full reproductive capacity.
5. Human-induced eutrophication is minimized, especially adverse effects, such as losses in biodiversity, ecosystem degradation, harmful algae blooms and oxygen deficiency in bottom waters.
6. Sea-floor integrity is at a level that ensures that the structure and functions of the ecosystem are safeguarded and benthic ecosystems, in particular, are not adversely affected.
7. Permanent alteration of hydrographical conditions does not adversely affect marine ecosystems.
8. Concentrations of contaminants are at levels not giving rise to pollution effects.
9. Contaminants in fish and other seafood for human consumption do not exceed levels established by Community legislation or other relevant standards.
10. Properties and quantities of marine litter do not cause harm to the coastal and marine environment.
11. Introduction of energy, including underwater noise, is at levels that do not adversely affect the marine environment.

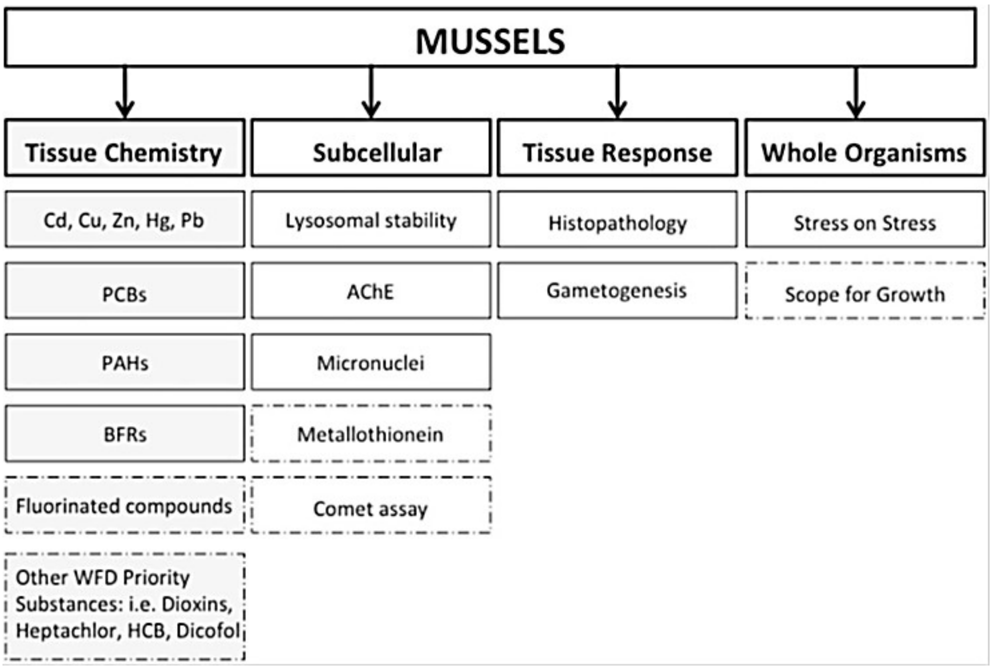

Fig. 3. Determinants and measurements included in the mussel component of the ICES/ OSPAR integrated monitoring framework. *Legend:* solid lines - core methods; broken lines - additional methods; PCBs - polychlorinated biphenyls; PAH - polycyclic aromatic hydrocarbon; BFRs - brominated flame retardants; AChE - acetylcholinesterase. WFD - Water Framework Directive. WFD priority substances are required in biota under Directive 39/2013/EU (see THAIN *et al.* (2008); VETHAAK *et al.* (2015)).

Table 2. OSPAR status of biological-effect techniques for invertebrates and fish (JAMP) (see THAIN *et al.*, 2008).

Method	In JAMP	CEMP category	Rec. by WGBEC	QC
Mussels				
Whole sediment bioassays	Yes	II	Yes	B
Sediment pore water bioassays	Yes	II	Yes	B
Sediment seawater elutriates	Yes	II	-	-
Water bioassays	Yes	II	Yes	B
In vivo bioassays	No	-	Yes	B (some)
In vitro bioassays	No	-	Yes	-
Lysosomal stability	No	-	Yes	B-a
Multidrug resistance (MXR/MDR)	No	-	Yes	-
Scope for growth	No	-	Yes	B-a
AChE	No	-	Yes	-
Metallothionein (MT)	No	-	Yes	B-a
Histopathology	No	-	Yes	-
Imposex/Intersex in gastropods	Yes	I (M)	Yes	Q
Benthic community analysis	Yes	-	Yes	B
Fish				
AChE in muscle	No	-	Yes	-
Lysosomal stability	Yes	II	Yes	B-a
Externally visible diseases	Yes	I (V)	Yes	B-a
Reproductive success (eelpout)	Yes	II	Yes	B-a
Metallothionein (MT)	Yes	II	Yes	B-a
ALA-D	Yes	II	Yes	B-a
Oxidative stress	Yes	II	-	-
CYP1A-EROD	Yes	II	Yes	Yes
DNA adducts	Yes	II	Yes	B-a
PAH metabolites	Yes	II	Yes	Q
Liver neoplasia/hyperplasia	Yes	I (V)	Yes	B
Liver nodules	Yes	I (V)	Yes	B
Liver pathology	Yes	I (V)	Yes	B
Vitellogenin in cod	No	-	Yes	Yes
Vitellogenin in flounder	No	-	Yes	-
Intersex in male flounder	No	-	Yes	B-a

CEMP category: II, method suitable for marine monitoring purposes; I, method suitable and analytical-quality control (AQC) is available; M, mandatory method in place, with AQC and assessment criteria established. Quality control: V, voluntary method in place, with AQC but conducted voluntarily. Recommendations for inclusion by WGBEC (ICES, 2007a) and information on existence of quality control [QC: B, Biological Effects Quality Assurance in Monitoring Programmes; B-a, available online (BEQUALM, 2009); Q, Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME, 1992).

WGBEC (ICES, 2007a) has reviewed the status of biological-effect techniques regularly and recommended in its reports those techniques for fish and invertebrates that are in the research phase, look promising, and require development and analytical-quality control, or are available for use and take-up in national and international monitoring programs. Some of the recommended methods have been included in OSPAR guidelines for contaminant-specific or general monitoring (JAMP, 1998a; b) and have, after a process of quality assurance, been included in CEMP (JAMP/CEMP, Joint Assessment Monitoring Programme/Co-ordinated Environmental Monitoring Programme). The updated list (Table 2) includes information on the current position of each technique relative to these guidelines.

Conclusions

In summary, in 2006 Working Group on Biological Effects of Contaminants (WGBEC; ICES, 2006) made a preliminary proposal, endorsed by Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open-Sea Areas (WKIMON) for combining methods in a program of integrated chemical- and biological-effect monitoring that contains three ecosystem components: water, sediment, and biota, restricted so far to *fish, bivalves, and gastropods* (THAIN *et al.*, 2008). Moreover. according to Directive 2008/105/EC Member States are now required to check compliance with EQSs and to monitor the contamination trends for PSs, and they are strongly recommended to use integrating matrices, e.g., *biota* in order to meet these objectives for bioaccumulative substances (BESSE *et al.*, 2012). Overall, we can conclude that it is especially important to choose fish as a test species when the research concerns human health, but mussels are also commercial products in terms of aquaculture and are consumed extensively in some areas of the world. From our case studies (GEORGIEVA *et al.*, 2014a; b; 2015; 2016; YANCHEVA *et al.*, 2014a; b; 2016b; 2017) we can confirm that mussels are easier to collect on the field compared to fish, but fish are particularly sensitive to contamination, which is essential in the field of ecotoxicology. Therefore, we would recommend that both, fish and mussels are used in monitoring programs on water contamination, along with combined biomarkers which can be applied on vertebrates and invertebrates for better results.

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The standard order of sections should be: Abstract, Key words, Introduction, Material and Methods, Results, Discussion (or Results and Discussion), Conclusions (optional), Acknowledgements (optional) and References.

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In the *Acknowledgements* section all persons and organizations that helped during the study in various ways, as well as the organization that financed the study must be listed.

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Example:

Table 1. Shannon-Wiener indexes in the burned ($\mathbf{H}_{burned}$) and control ($\mathbf{H}_{control}$) territory for the total duration of the study (2004–2006).

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Literature citations in the text should indicate the author's surname in SMALL CAPITALS (for more information on SMALL CAPITALS and how to make them in Word) with the year of publication in parentheses, e.g. CARLIN (1992); BROOKS & CARLIN (1992); SHAPIRO *et al.* (1968). Citations in brackets should be divided with semicolons and the author's name and the year of publication with comma (*example:* CARLIN, 1992; BROOKS & CARLIN, 1992; SHAPIRO *et al.*, 1968). If there are more than two authors, only the first should be named, followed by "*et al.*" in *italic*. References at the end of the paper should be listed in alphabetical order by the first author's family name and chronologically. If there is more than one work by the same author or team of authors in the same year, a, b, etc. is added to the year both in the text and in the list of references. Each citation in the text must be accompanied by a full reference in the list of references and vice versa. **Please provide DOI numbers for all electronic resources (journal articles, online articles, books), if they have DOI, when cited in the references list!**

Examples:

A journal article:

AUTHOR A. 1990. Title of the article. - *Full title of the journal*, 56(3): 35-105.

AUTHOR A., B. AUTHOR. 1990. Title of the article. - *Full title of the journal*, 56(2): 35-105.

AUTHOR A., B. AUTHOR, C. AUTHOR. 1990. Title of the article. - *Full title of the journal*, 56(1): 35-105.

A book:

AUTHOR A. 2000. *Title of the book.* Vol. I. Place of publication. Publishing house.

Proceedings or book chapter:

AUTHOR A., B. AUTHOR 1990. Title of the contribution. - In: Author A. (Ed.): *Title of the book or proceedings.* Place of publication. Publishing house, pp. 235-265.

Software:

STATSOFT INC. 2004. *STATISTICA (Data analysis software system)*, Vers. 7. Computer software. Available at: [http://www.statsoft.com].

Website:

FAUNA EUROPAEA. 2007. *Invertebrates. Fauna Europaea.* Vers. 1.1. Available at: [http://www.faunaeur.org]. Accessed: 12.10.2009.

In case of papers written in other than Latin letters, if there is an English (or German, or French) title in the summary, it is recommended to be used. If there is not such a summary, the author's names must be transcribed and the title of the paper must be translated into English and put in square brackets. If the name of the journal is also not in Latin letters it also should be transcribed (not translated). This should be noted in round brackets at the end of the paragraph, for instance: (In Bulgarian, English summary).

Example:

ANGELOV P. 1960. Communications entomologiques. I. Recherches sur la nourriture de certaines espèces de grenouilles. – *Godishnik na muzeite v grad Plovdiv*, 3: 333-337. (In Bulgarian, Russian and French summary).

KOROVIN V. 2004. [Golden Eagle (*Aquila heliaca*). Birds in agricultural landscapes of the Ural]. Ekaterinburg, Published by Ural University, 57 p. (In Russian).

Names of persons who provided unpublished information should be cited as follows: "(ANDERSSON, 2005, Stockholm, pers. comm.)".

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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 nonsignificance. 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 detail as precisely 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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