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Variations in the Antioxidant Defense System of the Black Sea Mussel, Mytilus galloprovincialis (Lamarck, 1819)

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Abstract. The study aimed at assessing changes in the pro-antioxidant balance in gills, foot and digestive gland, of the mussels, *Mytilus galloprovincialis* Lam., gathered in June and September in two successive years (2017 – 2018) from 11 different habitats with various environmental conditions of the Bulgarian Black Sea coastal area. The glutathione (GSH) concentration and enzyme activity of catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-S-transferase (GST), superoxide dismutase (SOD), together with the lipid peroxidation (LPO) level and concentrations of selected trace metals (Cd, Cu, Pb, Ni, Zn) were measured in each organ. Significant, although not high, correlations were established between different tissue biomarkers and Cu, Pb and Cd. In gills the highest degree of oxidative stress (OS) was present, compared to the other tissues, as evidenced by the significantly higher LPO levels along with activated antioxidant enzymes CAT, GPX, GST, SOD and extremely high G6PDH. Among the organs, the lowest levels of OS were detected in the foot, indicated by the lowest LPO and the highest GSH concentrations. The digestive gland showed a relatively low degree of OS, indicated by the significantly lower LPO and antioxidant enzyme activities, with exception of the high CAT activity. In conclusion, the established significant variability in the tested OS parameters indicated their different sensitivity towards environmental pro-oxidants and reaction of the antioxidant defense system in different organs of the mussels. The gills seemed to be most suitable for biomonitoring of oxidative stress in the mussels.

Key words: lipid peroxidation, antioxidants, heavy metals, *Mytilus galloprovincialis*.

Introduction

The Black Sea makes no exception of the increasing anthropogenic pressures on the marine ecosystems due to its geographical position and limited water exchange with other seas and the ocean. In addition, it accepts large amounts of river waters from the territory of more than 20 countries of Europe and Asia Minor, which is added to the local coastal pollution effects (MIRINCHEV *et al.*, 1999; Todorova & Moncheva, 2013; ROBU *et al.*, 2015; BAT *et al.*, 2015; MONCHEVA *et al.*, 2016). One of the dangerous types of water pollution is with trace metals (As, Cu, Cd, Hg, Pb, Zn etc.) due to their toxicity and ability to accumulate or even biomagnify in aquatic organisms.

The risk assessment of pollution and changes in the Black Sea ecosystem were, until recently, mainly based on physical-chemical analysis of environmental samples, concentrations of xenobiotics in sentinel organisms or shifts in species communities (YANCHEVA *et al.*, 2018) while the individual biological effects and responses of the organisms themselves remained underestimated. More recently, multi-biomarker approaches (VLAHOGIANNI *et al.*, 2007; SANCHEZ *et al.*, 2013) were used to evaluate effects of exposure to contaminants and the responses of marine biota to environmental stress. Being the primary consumers in the water food chain in the Black Sea and filter feeders, the black mussels *Mytilus galloprovincialis* Lam. play a highly important role for the resilience of the whole seawater ecosystem and therefore is among the primary objects of research and monitoring.

Numerous studies indicated that a variety of natural factors of the sea water environment, as well as anthropogenic contamination, provoke excess generation of reactive oxygen species (ROS) or decrease of antioxidant defense and thus induce oxidative stress (OS) in bivalves (SOLDATOV *et al.*, 2014; TSANGARIS *et al.*, 2016; MAISANO *et al.*, 2017; GIANNETTO *et al.*, 2017; YANCHEVA *et al.*, 2018). As the environmental effect is multifactorial, different mechanisms may be involved in the production of ROS, and the interactions between them in combination with the antioxidant response rate, contribute to the degree of the oxidative processes in cells. Although OS in marine bivalves is studied and explored in various (biological, ecological, evolutionary) aspects, the response of the different organs (tissues) to the ROS and their contribution to the overall protection and/or adaptation of the whole organism to the adverse effects in the Black Sea environment remains underestimated in Bulgaria.

The present study aims to assess the oxidative stress in different organs (foot, gills and digestive gland) of black mussels from the Black Sea coastal area of Bulgaria by using multi-biochemical biomarkers (glutathione (GSH), catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH), glutathione-S-transferase (GST), lipid peroxidation (LPO)).

Materials and Methods

The mussel sampling was performed in June and September during two consequent years (2017 and 2018). The mussels (45–60 mm) were collected from 11 different sites (Fig. 1) along the southern Bulgarian Black Sea coast, including ports, aquaculture and protected areas in order to cover various environmental conditions. Mussels were hand-collected from their natural habitats at a depth of 1-6 m and from artificial structures in the harbors. They were cleaned on the spot and immediately transferred to the laboratory in clean thermostable containers with sea water.

Measurement of oxidative stress parameters

Tissue preparation. For the biochemical analyses the mussels (n=6 for each site) were immediately dissected on ice and the gills, foot and digestive gland were excised. Each individual organ was frozen in liquid nitrogen and stored at –80°C until the analyses began. Thereafter, the organs were homogenized in 100 mM potassium phosphate buffer (pH 7.4) using Potter Elvehjem homogenizer fitted with a Teflon pestle (Thomas Scientific, USA). The homogenates were centrifuged (Janetzky K24 refrigerated centrifuge, Germany) for 10 min at 3000 g to obtain a post nuclear fraction for determination of lipid peroxidation and glutathione levels. A portion of the fraction was re-centrifuged at 12 000 g for 20 min to obtain a post mitochondrial supernatant used for measurement of the antioxidant enzymes activity. All work was carried out at 4°C.

Biochemical analysis. All tested OS biomarkers were measured spectrophotometrically using commercially available kits (Sigma-Aldrich Co. LLC, USA): Catalase

Assay Kit CAT100; Glucose-6-phosphate dehydrogenase Assay Kit MAK015; Glutathione Assay Kit CS0260; SOD Assay Kit-WST 19160; Glutathione Peroxidase Cellular Activity Assay CGP1; Glutathione Reductase Assay Kit GRSA; Glutathione-S-Transferase Assay Kit, CS0410; Lipid peroxidation (MDA) assay kit MAK085. The protein concentrations were measured according to LOWRY *et al.* (1951) by using a standard curve of bovine serum albumin as standard.

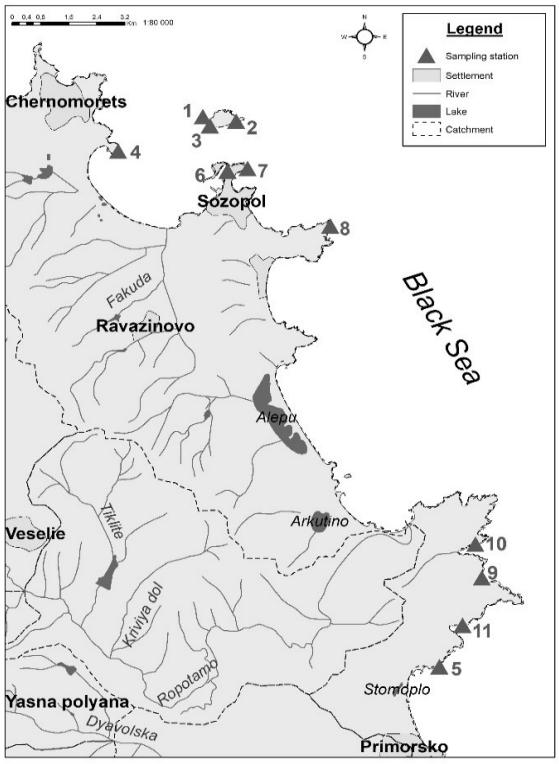

Fig. 1. Mussel sample sites along the Bulgarian Black Sea coast

Measurement of metal concentration

Tissue preparation. The mussels used for trace metal bioaccumulation analyses were kept alive for 48 hours in sea water, which is considered sufficient to clean their digestive system. The gills, foot and digestive gland (n=35-45 specimens from each sampling site) were excised and washed with distilled water. After drying at 110°C to an air-dry state, weighed and pooled samples of 2 g of dry weight were prepared and stored in desiccator until further analyses.

Metal analyses. The air-dry samples were homogenized in a laboratory mortar and 1 g of each sample was subjected to a wet mineralization with 15 ml of a 2:1 mixture of concentrated perchloric ($HClO_4$) and nitric (HNO_3) acids. The samples were heated and evaporated on a sand bath to a moist residue. Using the same procedure, a blank sample was prepared with bi-distilled water and a mixture of acids. Tissue concentrations of Cd, Cu, Ni, Pb, and Zn were quantified by using Perkin Elmer Optima 5300 DV/ICP-OES (USA). The data measurements obtained from the analysis were in mg/L, therefore they were recalculated to mg/kg of air-dry sample.

Statistical analyses

Statistical analyses of raw data were carried out using STATISTICA 10 package (StatSoft Inc., 2010).

Results

To analyze the overall patterns of interdependence between the values of the studied OS biomarkers in the different organs we applied factorial ANOVA (Table 1). The analysis indicated that they differed significantly with high degree of reliability. Hence, the reaction of the organism to pro-oxidative factors appeared to be specific for the target organ and the type of biomarker reactions.

In order to compare the intensity and direction of the pro-oxidative/antioxidative balance in the different studied organs, we compared the mean values of the OS biomarkers among them applying the Student's t-test (Table 2).

In gills the highest concentration of MDA (as LPO indicator) and high activities of the antioxidant enzymes SOD, CAT, GPX and GST were found (Table 2). Although the SOD and GST activities in gills did not differ significantly from those in the foot, they were significantly higher than in the digestive gland. Twice higher activity of G6PDH was measured in the gills in comparison to the foot and digestive gland.

Table 1. Analysis of variance (ANOVA – factorial design) of the overall pro/antioxidant biomarker reactions in organs.

	SS	Degree of freedom	MS	F	P
Intercept	58268385	1	58268385	270.21	0.000000
Organ	13323600	2	6661800	30.89	0.000000
Indicator	206860753	4	51715188	239.82	0.000000
Organ*Indicator	85684530	8	10710566	49.66	0.000000
Error	283999213	1317	215641		

Table 2. Mean values of biomarkers (± error of mean) and their significance of difference between organs (Student's t-test; $p \leq 0.05$). *Legend:* $^{+}$- significant difference from foot; *- significant differences of digestive gland from gills**.**

Organ\ Indicator	Gills	Foot	Digestive gland
LPO (nmoles MDA/mg protein)	$5.58 \pm 0.25^{+}$	1.87 ± 0.09	3.49 ± 0.16*
GSH (ng/mg protein)	$999.09 \pm 117.8^{+}$	1735.5 ± 125.0	$451.41 \pm 46.19^{+}$*
SOD (U/mg protein)	18.63 ± 1.70	19.35 ± 1.79	$4.13 \pm 0.39^{+}$*
CAT (U/mg protein)	$2.13 \pm 0.43^{+}$	0.86 ± 0.14	$3.90 \pm 0.67^{+}$*
GPX (U/mg protein)	$15.01 \pm 2.83^{+}$	13.22 ± 1.37	$8.08 \pm 0.69^{+*}$
GR (U/mg protein)	7.04 ± 0.67	6.93 ± 1.09	$6.23 \pm 1.20^{+}$*
GST (U/mg protein)	104.23 ± 10.07	112.66 ± 10.22	$48.51 \pm 5.02^{+}$*
G6PDH (U/mg protein)	$40.16 \pm 3.39^{+}$	19.58 ± 1.14	20.94 ± 1.07*

The GSH level, as well as the SOD, GPX and GST activities were found to be lowest in the digestive gland in comparison to that in the other tested organs (Table 2), whereas the CAT activity was the highest. The LPO levels and G6PDH activity did not differ significantly between the digestive gland and the foot.

In the foot the highest GSH level and low MDA concentration were observed. As regards the antioxidant enzymes – the lowest CAT activity among the tested organs was measured in the foot. The SOD and GST activities in the foot did not differ significantly from those in the gills. The GPX activity was significantly lower in the

foot than in the gills, but higher than in the digestive gland (Table 2).

The studied OS biomarkers were also analyzed for correlations with the accumulated in the same tissue metals (Table 3). Not all metals studied were found to have significant correlation with one or more biomarkers.

The concentration of Cu in the gills (i.e. branchial tissue) was significantly and positively correlated with the LPO level (r = 0.57) and SOD activity (r = 0.53). Further, a significant although not high, correlation between the Cu concentration and the GSH level was present (r = 0.47) in the digestive gland. The G6PDH activity was correlated significantly with concentrations of Cu (r = 0.51) in the foot.

The concentrations of Pb had significant positive correlations with the LPO level (r = 0.58) and GST activity (r = 0.67) in the foot and a negative correlation with the GPX activity (r = - 0.72) in the digestive gland.

The only significant correlation of Cd concentrations was found to be with the GR activity (r = 0.52) in the gills.

In an attempt to search for relationship between the complex interactions of OS biomarkers in the different organs and metal concentration, principle component analysis (PCA) on correlation matrices was used to reveal the underlying structure of the data (Fig. 2). The studied organs of the mussels were analysed separately.

In the 2D graphical representations (Fig. 2) the underlying variables (PC coordinates) are situated according to their correlation to the two main principle component (PC) axes. Heavy metal concentrations were used as secondary variables and were also visualized on the factor plane.

Table 3. Pearson's Product Moment Correlations between biomarkers and concentrations of trace metals in organs (underlined correlations significant at $p \leq 0.05$).

Tissue/		Biomarker response							
	Metal	**LPO**	**SOD**	**CAT**	**GSH**	**GPX**	**GR**	**GST**	**G6PDH**
Gills	**Cu**	**0.57**	**0.53**	0.02	-0.19	-0.10	-0.12	-0.00	0.32
	Pb	-0.09	0.09	0.16	-0.11	-0.31	-0.12	0.27	0.22
	Zn	0.31	0.35	-0.18	0.03	-0.04	0.11	-0.31	0.05
	Cd	0.01	0.34	-0.19	-0.33	-0.20	**0.52**	-0.31	0.16
	Ni	0.18	-0.09	-0.18	-0.26	-0.17	0.05	-0.06	-0.46
Foot	**Cu**	0.22	0.19	0.21	0.02	0.22	0.06	0.07	**0.51**
	Pb	**0.58**	-0.36	0.33	0.10	-0.36	0.29	**0.67**	0.01
	Zn	0.04	0.18	0.13	0.08	0.13	0.09	-0.12	0.31
	Cd	-0.15	0.28	0.07	0.00	0.16	-0.07	-0.15	0.16
	Ni	0.34	-0.23	-0.04	-0.12	-0.26	-0.03	0.35	-0.22
Digestive gland	**Cu**	0.33	-0.05	0.12	**0.47**	-0.07	-0.32	-0.17	-0.21
	Pb	-0.18	-0.26	-0.01	-0.06	**-0.72**	0.40	0.38	-0.22
	Zn	-0.14	0.16	-0.23	0.04	0.32	-0.35	-0.42	-0.03
	Cd	0.09	0.04	-0.31	-0.20	0.29	-0.03	-0.34	-0.21
	Ni	0.33	-0.32	-0.17	0.27	0.23	-0.17	-0.07	-0.40

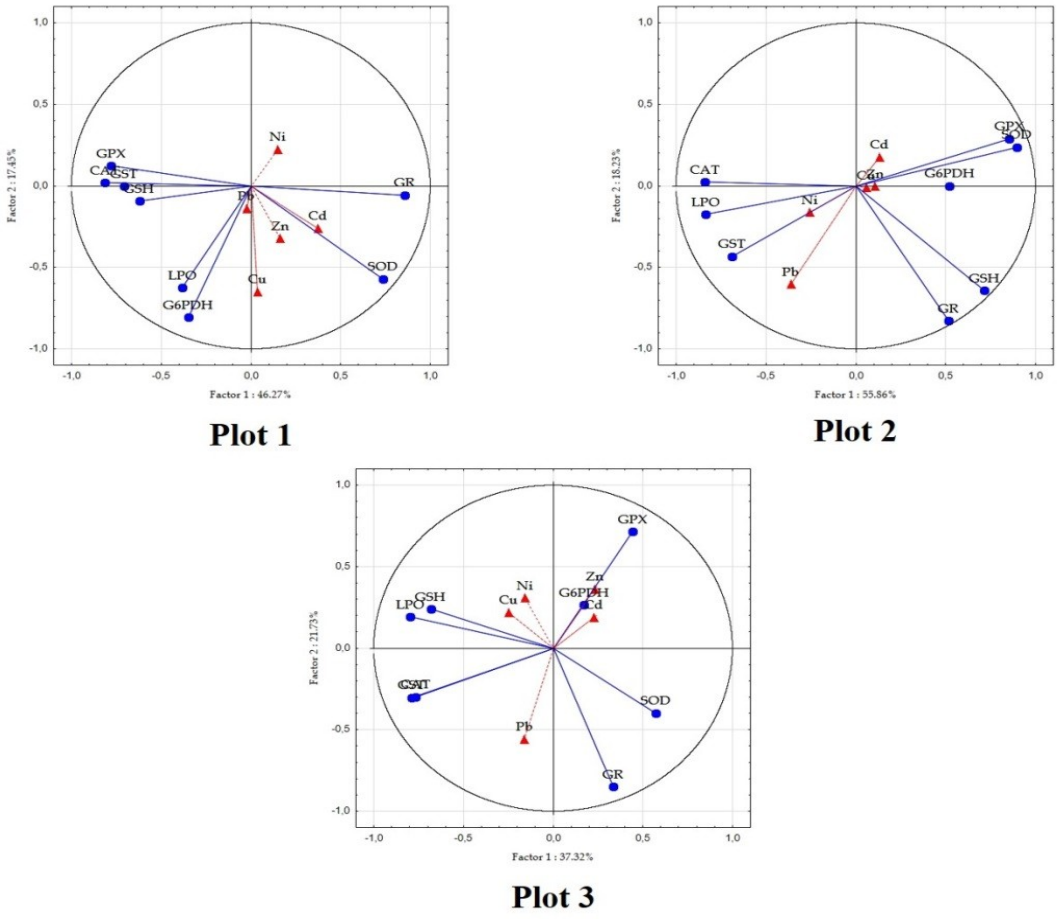

Fig. 2. PCA analysis: projections on factor plane of the first two PC (*circles* - oxidative stress indicators; *triangles* - metal concentrations as supplementary variables) (*Plot 1* – gills; *Plot 2* – foot; *Plot 3* – digestive gland).

The PCA analysis of OS biomarkers data in the gills indicated that the first two extracted PC (factors) explained 63.72% of the total variance. (Fig. 2, Plot 1). The first PC (Factor 1) explained 46.27% of the total variance and showed a gradient from significant negative loads of the activity of SOD, CAT, GPX and GR, towards positive loads of GR activity and to some extend of SOD. The second PC (Factor 2) accounted for 21.17% of the variance with higher loadings of LPO level and G6PDH activity. The Cu concentration showed also significant correlation with the second PC. The Ni concentration was not significantly related to neither of the OS variables and was separated in the positive part of the plot. The concentration of Cd was related to some extend with the SOD activity.

The PCA analysis of OS biomarkers data in the foot indicated that the first two principle components explain 74.09% of the total variance (Fig. 2, Plot 2). The first PC (Factor 1) explained 55.86% of variance. This PC characterized a gradient of OS biomarkers - from CAT, LPO and GST (with negative loadings) towards SOD, GPX and G6PDH (with positive loadings). The GST activity had lower and similar loadings on Factor 1 and Factor 2. The second PC (Factor 2) explained 18.23% of the total variance and was characterized by relatively lower loads of GSH and GR. The Pb concentration had significant loads both on PC1 and PC2, but

having somewhat greater load on the second.

In the digestive gland the first two PC (factors) extracted from the OS biomarkers data explained 59.05% of the total variance (Fig. 2, Plot 3). The first PC (Factor 1) explained 37.32% of variance and indicated a gradient from both GSH and LPO levels and CAT activity (negative loads) towards SOD, although this indicator had a relatively low weight. The second PC (Factor 2) explained 21.73% and was characterized by a gradient from GR towards G6PDH activities. Both indicators however had relatively low loads. The relatively low total variance explained indicated that in the digestive gland more factors affecting the OS biomarkers were present, each with a low individual effect.

Discussion

Our data demonstrated the presence of significant variability in the pro-oxidative processes and antioxidant defense in the different organs of *M. galloprovincialis* from the Bulgarian costal area of the Black Sea. As far as the studied mussel individuals were exposed to the same variability of environmental conditions during the study, the observed differences in OS biomarkers among the tested organs strongly indicated the presence of specificity in both the reaction towards pro-oxidants and the antioxidant system functioning.

It was found that the gills were the most affected organ by oxidative stress compared to the others, as evidenced by the significantly higher LPO. The gas exchange and filtration function of the gills is a prerequisite to increased intensity of pro-oxidative pressure, characterized with high background levels of LPO, which was also shown by previous studies (VLAHOGIANNI *et al.*, 2007; FERNÁNDEZ *et al.*, 2010). We observed a high CAT and GPX activity in the gills, probably as a consequence of the effort for eliminating the formed lipid peroxides. The established twofold higher activity of G6PDH in the gills, in comparison to the other organs studied, could explain the relatively high levels of GSH, since G6PDH is involved indirectly in its recovery mechanism (HALLIWELL & GUTTERIDGE, 2015). ROS formation can occur not only by the continuous interaction of gill cells with molecular oxygen in seawater and/or maximal exposure to high dissolved oxygen (SANTOVITO *et al.*, 2005), but also as the result of exposure to different pollutants, including metals. The observed positive correlation between GR induction and Cd concentration in the gills could be the result of overlapping processes related to Cd elimination through GSH utilization and compensatory activation of mechanisms for its recovery. Further, we found a significant positive correlation between LPO and Cu concentrations in gills. In addition, the activity of SOD was also positively correlated with the concentration of Cu in gills. The PCA analysis showed that LPO level in gills, G6PDH and SOD activities together with Cu concentration had significant loads on the second significant factor (PC2) and hence seemed to be involved in the neutralization of pollutants in the gills. This was also in line with findings that SOD in the gills can be induced directly by water pollutants (VLAHOGIANNI *et al.*, 2007; FERNÁNDEZ *et al.*, 2010).

Our results demonstrated that the foot had the lowest levels of pro-oxidants (compared to the other organs) as indicated by the lowest MDA and the highest GSH concentrations, measured. High GST activity in the foot, which is in direct contact with the environment matrixes (water, substrate), is related to the antioxidant defense system of cells eliminating diverse oxidative stress products such as lipid hydroperoxides, quinones, epoxides and α, β-unsaturated aldehydes (Belabed & Soltani, 2013). On the other hand, highest activities of SOD and GPX were also established in the foot. Highest activity of SOD in the foot mussel (compared to other organs) was previously also reported (SOLDATOV *et al.*, 2008a). In general, the activities of the measured antioxidants in the foot were close to those in gills.

In our study the antioxidants' activities in the digestive gland showed the lowest values (in comparison to foot and gills) with the exception of CAT. The lowest activity of GPX and GST, observed in the digestive gland, could be related to the lowest concentration of GSH (which is the co-substrate of the enzymes' action) compared to the values in the foot and gills. Since GSH is involved actively in the neutralization of both the LPO products and ingested toxic substances this could lead to depletion of its resources (GOSTYUKHINA & ANDREENKO, 2015). The high CAT activity in digestive gland was likely to be related to presence of increased H_2O_2 concentrations in this organ (SOLDATOV *et al.*, 2008a). CAT has a leading role in degradation of hydrogen peroxide in high concentrations, because the enzyme has low affinity to H_2O_2 (HALLIWELL & GUTERIDGE, 2015). In the same time high H_2O_2 concentrations inhibit the GPX and make turn it low efficient for hydrogen peroxide neutralization. This seems to be a characteristic of the mollusc digestive gland, which was indicated by the high activity of catalase in this organ (SOLDATOV *et al.*, 2008b).

In our study correlations were present between oxidative stress biomarkers and metal concentrations in the organs. The high activity of CAT and low levels of GSH could be probably due to the level of Cu accumulation. It has been reported, that the cellular levels of glutathione are markedly decreased in Cu-exposed mussels and the accumulated Cu can affect at different extent the GSH metabolism in mussels and it has been also suggested that the rate of bioaccumulation of essential heavy metals is faster than nonessential metals in bivalves (KAMARUZZAAN *et al.*, 2011). Laboratory data have shown that the exposition of bivalves to Cu, Cd and Pb, resulted in a significant increase of CAT activity (RAJKUMAR & MILTON, 2011; BOUDJEMA *et al.*, 2014).

Conclusions

In conclusion, the established significant variability in the tested OS biomarkers among the organs of Black Sea mussel *M. galloprovincialis* indicated the different sensitivity towards environmental pro-oxidants and the reaction of the antioxidant defense system. In general, our results strongly indicated that the variation of antioxidant enzymes activities in the mussel foot and gills seemed to be directly related to the presence of different pollutants in the marine environment. The biomarkers of OS in the mussels can present useful bioindication of the Black Sea environmental health. However, the specificity of the pro- and antioxidant processes in the mussel organs to be used for this purpose should be taken into account. In this respect the gills seemed to be the better bioindicator. Obviously, further studies are needed in order to develop reliable marine environmental assessment scales, based on OS biomarkers in mussel gills.

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