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Stress Induced Damages in Psammophilic Bivalve Species: A Pilot Study on Wedge Clams from Black Sea Habitats in the Upper Subtidal Zone (Bulgaria)

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Abstract. The aim of the present pilot study was to estimate the presence of oxidative damages in wedge clams (*Donax trunculus* L.) from different sites in the upper subtidal zone along the Bulgarian Black Sea Coast. In response to this aim the levels of the oxidative stress biomarkers lipid peroxidation, protein oxidation and glutathione, together with the activity of the antioxidant enzymes superoxide dismutase, catalase and glutathione-S-transferase were measured. The obtained results indicated significant variations of the studied biomarkers, depending both on the sampling site and the specific marker. Increased concentrations of lipid peroxidation and protein oxidation, along with glutathione decrease, indicated the presence of stress and tissue damages in the wedge clams. For most of the studied sites a moderate level of oxidative stress in wedge clams was found. Significant oxidative stress was present in the wedge clams from Kabakum Bay. Here, highest values of lipid peroxidation, superoxide dismutase and catalase, along with low glutathione, which was probably depleted by the action of glutathione-S-transferase, were found and this clearly indicated the presence of environmental stress and cell damage. The analysis of samples taken from different seasons, allowed some preliminary conclusions which suggested that the oxidative stress in the wedge clams seemed to be higher in spring, somewhat lower in summer and then somewhat rising in autumn. In conclusion, *D. trunculus* is a suitable bio-indicator of the stressfulness of the environment in the sandy habitats of the subtidal Black Sea zone and can thus present an "early-warning" signal in biomonitoring studies.

Key words: Black Sea, *Donax trunculus*, antioxidant enzymes, lipid peroxidation, protein oxidation, glutathione.

Introduction

Human activity exerts increasing pressure on the marine environment through the deposition of a huge variety of xenobiotics, eutrophication, habitat changes, overfishing, introduction of alien species etc., resulting in ecosystem degradation, which in turn negatively affects important marine ecosystem services. This urgently requires research and systematic monitoring using

reliable environmental indicators coupled with informative biomarkers to adequately assess the state of marine biodiversity and ecosystems. The use of bivalve marine species in monitoring programs as a tool to assess impacts and resilience of ecosystems is supported by the Water Framework Directive of the EU (EC, 2000).

Bivalves, as filter feeders, have the capacity to accumulate various xenobiotics and to concentrate them in their tissues. It is considered that the analysis of the substances accumulated in their soft tissues and shells brings more information on the effect of environmental pollution than the analysis only of abiotic environmental components (Baumard et al., 1999). In addition to their accumulation capacity, bivalves respond to contaminants through a number of physiological and biochemical reactions, which underline the biomarker concept (Hook et al., 2014).

One of the biomarkers recently most widely used in the study of effects of the aquatic environments on organisms is the oxidative stress (OS). OS occurs when pro-oxidant processes intensify as a result of overproduction of reactive oxygen species (ROS) and/or a decrease in the antioxidant defense, resulting in cell and tissue damage (Sies, 2015). A wide range of chemical pollutants can upset the redox balance and cause OS in aquatic organisms (Belcheva et al., 2015). OS in marine bivalves can be also caused by changes in environmental factors such as temperature, salinity, oxygen saturation etc. (Soldatov et al., 2014). The response of organisms to pro-oxidants is complex and causes a cascade of interrelated cellular responses with possible significant effect on higher levels of ecological organization (Tlili et al., 2013).

In addition, marine bivalves, in particular clams, are beginning to gain increasing commercial and economic importance as food resource. OS changes in bivalve tissues not only impair their taste and nutritional value (Amaral et al., 2018), but can have direct negative effect on human health (Estevez & Luna, 2017).

Studies of shellfish as bioindicators of the state of the Black Sea ecosystems mainly concerned the black mussel (*Mytilus galloprovincialis* Lam.). This studies are related to assessment of the effect of petroleum products on lipid composition (Nechev et al., 2002), comparisons of antioxidant activity between mussels from clean and contaminated habitats (Gorinstein et al., 2003; Moncheva et al., 2004), metal accumulation, oxidative and genetic status (Yakimov et al., 2017, 2018; Ivanov et al., 2019). Regarding the biological responses to pollution and changes in the habitats of clams in the Black Sea area, the available data are limited and concern mainly species of the *Anadara* genus (Gostyukhina & Andreenko, 2020).

The present study aimed to carry out an initial pilot assessment of the presence of oxidative stress damages in the tissues of *Donax trunculus* L. in response to the different environmental conditions of several typical sublittoral sandy habitats along the Bulgarian Black Sea Coast.

Materials and Methods

Specimens: The clams *D. trunculus* (length 23-40 mm) were gathered manually in different seasons (spring, summer, autumn) from their typical natural habitats in the upper subtidal zone along the Bulgarian Black Sea Coast (Fig. 1).

Tissue preparation. The soft tissues of 6-10 mussels for each site were excised and homogenized separately in 100 mM potassium phosphate buffer (pH 7.4). To obtain a post nuclear fraction for determination of lipid peroxidation and glutathione levels, the homogenates were centrifuged at 3000 g for 10 min. 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 enzyme activity. All work was carried out at 4°C.

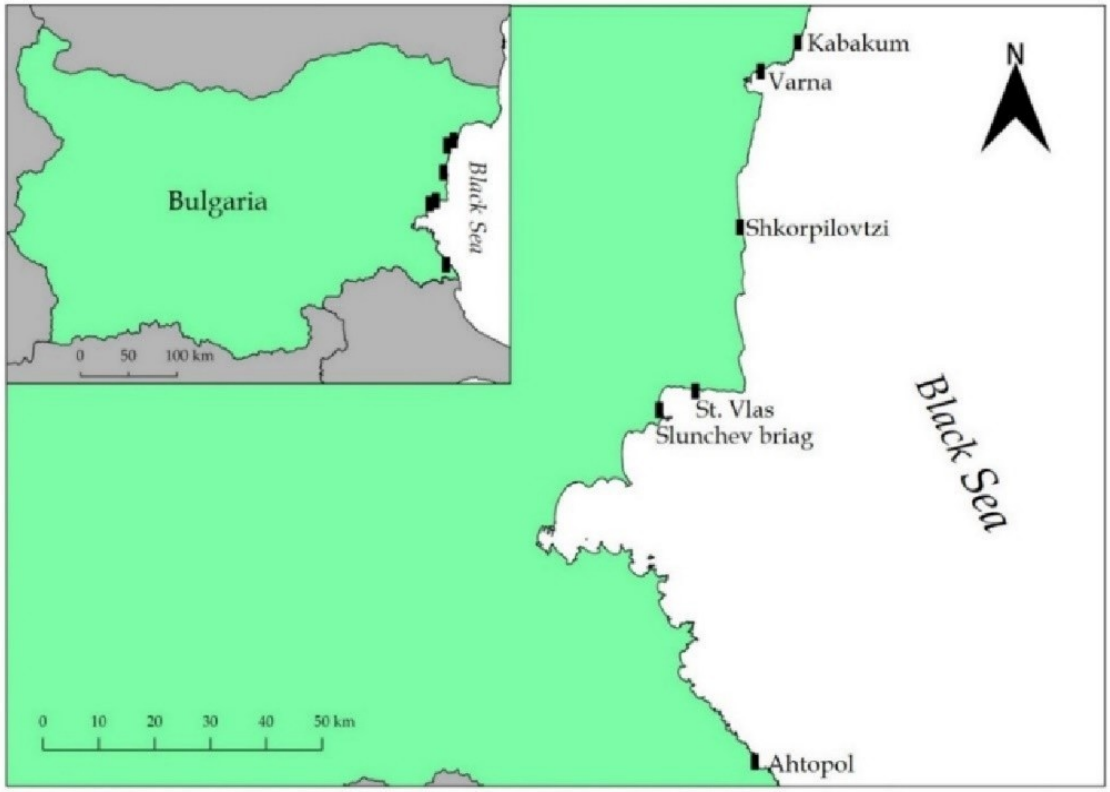

Fig. 1. Wedge clam sampling sites location along the Bulgarian Black Sea Coast.

Biochemical analysis. All biomarkers were measured spectrophotometrically using commercially available kits from Sigma-Aldrich Co. LLC, USA, in accordance to the manufacturer instructions.

Lipid peroxidation (LPO) was estimated by the content of thiobarbituric acid reactive substances (TBARS), by using Lipid peroxidation (MDA) assay kit MAK085. The amount of TBARS was expressed in nmoles malondialdehyde (MDA)/mg protein.

Protein oxidation (PO) was measured by using Protein Carbonyl Content Assay Kit MAK094. The method is based on the reaction of the protein carbonyls (PC) with 2,4-dinitrophenylhydrazine (2,4-DNPH). The carbonyl content was expressed in nmoles PC/mg protein.

Glutathione content (GSH) were measured by the absorption of the color product from reaction of reduced glutathione with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in presence of glutathione reductase and NADPH by using Glutathione Assay Kit CS0260. The amount of glutathione was calculated from the reference standard and expressed as ng/mg protein.

Superoxide dismutase (SOD) activity was determined by using SOD Assay Kit-WST 19160 and was expressed as U/mg protein. As the unit of activity is considered the amount of the enzyme needed for 50 % inhibition of nitroblue tetrazolium (NBT) reduction.

Catalase (CAT) activity was measured by the absorption decrease at 240 nm in result of H_2O_2 decomposition by using Catalase Assay Kit CAT100. Enzyme activity was expressed as U/mg protein.

Glutathione-S-transferase (GST) activity against 1-Chloro-2,4-dinitrobenzene (CDNB) was determined by using Glutathione-S-Transferase Assay Kit, CS0410. Enzyme activity was expressed as U/mg protein.

The protein content was measured according to Lowry et al. (1959) with bovine serum albumin as a standard. The absorbance was measured at 700 nm.

Statistical analyses. Data on the measured oxidative stress markers were subjected to statistical analysis by the Generalized Linear Model Procedure present in the statistical environment of the package STATISTICA 10 (StatSoft Inc., 2007). The factorial ANOVA design was applied. Principle component analysis (PCA) was used to study underlying gradients and grouping of sample sites by oxidative status.

Results

The OS indicators measured in the wedge clams showed significant variations among the studied sites (Table 1). The changes in the pro-oxidants markers LPO and PO indicated the oxidation potential (stressfulness) of the marine environment of the studied wedge clam habitats. In particular, the levels of LPO were lowest (0.36±0.04 nmoles MDA/mg protein) in the specimens from Shkorpilovzti Bay in summer and the highest (1.65±0.36 nmoles MDA/mg protein) were present in the sample from Kabakum Bay in Autumn (Table 1). The levels of PO also varied significantly being the lowest (3.07±0.87 nmoles MDA/mg protein) in clams from Varna Bay in autumn and highest (11.22±1.57 nmoles MDA/mg protein) at Shkorpilovtzi Bay in spring (Table 1).

The measured concentration of the antioxidant GSH was lowest (330.67±63.30 ng/mg protein) in the samples from Slunchev Briag Bay in spring and reached a maximum value (1457.00±184.20 ng/mg protein) in the wedge clams gathered from Ahtopol Bay in summer.

The study of the antioxidant enzyme system biomarkers in the wedge clams also showed that their values varied among the studied sites (Table 1).

The enzyme activity variations among habitats however, seemed to follow a very similar pattern. The lowest activity of the antioxidant enzymes SOD and CAT was present in wedge clams gathered in summer from the sandy sublittoral at Shkorpilovtsi Bay and Ahtopol Bay. The highest activity of these enzymes was registered in wedge clams sampled from Kabakum Bay in autumn. The GST also showed similar variation to the other antioxidant enzymes with lowest values measured in wedge clams gathered from Ahtopol Bay and Shkorpilovtsi Bay in summer and highest activity in the wedge clam samples from Kabakum Bay.

The interrelations between the measured values of the most important biomarkers LPO, PO and GST, indicating the level of OS in the wedge clams from the studied sites are presented in Fig. 2.

The data showed that the wedge clams sampled from Kabakum Bay (in autumn) were the most affected by OS. They had the highest LPO and also showed highly activated antioxidant enzyme defense compared to all others sites (Table 1). Here, the highest activity of GST together with low values of the non-enzyme antioxidant GSH was also present. However, the oxidative damage of the proteins (PO) was relatively low.

In wedge clams from the sites Shkorpilovtzi, Sveti Vlas, Slunchev Briag and Ahtopol (in spring) there was an almost identical and moderate degree of OS stress indicated by the intermediate values of LPO, GSH, SOD, CAT and GST. Similar results were obtained for wedge clams sampled in autumn from the region of Varna Bay, but here somewhat higher SOD activities were registered. In the wedge clams sampled in autumn from both Kabakum Bay and Varna Bay low levels of PO were present. In an attempt to study the significance of the observed variations in the examined OS biomarkers in the wedge clams from the different habitats and the responsible factors we applied factorial ANOVA analysis (Table 2).

The analysis showed that effects of both the site and the particular OS marker

separately, as well as their interaction, were statistically significant. These results confirmed that the measured OS markers varied significantly among the studied sites and that the differences significantly depended both on the site (i.e. the local environmental conditions of the habitat) and on the specific interrelation of the OS markers (i.e. how the oxidative process develops in the wedge clams at the particular site).

In our study there were samples taken in different seasons from the studied sites, although not all sites were sampled in each season. Nevertheless, this allowed us to make some very preliminary conclusions on the presence of some seasonal differences. In general, OS in the studied wedge clams seemed to be higher in spring, as indicated by the measured markers, being somewhat lower in summer and then somewhat higher in autumn. This general observation was partly confirmed by the results from the two sites, i.e. Shkorpilovtzi and Ahtopol, where samples were taken both in spring and in summer (Table 1).

In the summer samples from Shkorpilovtzi Bay and Ahtopol Bay the lowest levels of LPO were measured against the background of very high values of the non-enzymatic antioxidant GSH. In the same samples, the antioxidant enzymes were not activated and showed the lowest activities compared to the wedge clams from the other sites, studied in this research. In contrast, in spring, higher values of LPO, SOD and CAT were present in the wedge clams from the same sites (Table 1). The most pronounced difference between the spring samples and the summer samples was the threefold increase in the concentration of the antioxidant GSH in the summer samples of wedge clams. Hence, it can be assumed that in summer the OS of the wedge clams from these sites was lower compared to the OS of the wedge clams in the spring samples from the same sites.

In order to study the overall pattern of the OS changes in the wedge clams from the different sites, which are indicative of tissue damages, a PCA analysis of changes in the values of the pro-oxidant markers LPO and PO was carried out (Fig. 3).

Table 1. Oxidative stress markers (mean ± SD) in wedge clams gathered from different sites and seasons. *Legend: Shk* – Shkorpilovtzi; *Aht* – Achtopol; *Kab* – Kabakum; *Var* –Varna; *Svlas* - Sveti Vlas; Slb – S*lunchev Briag* (*Spr* – Spring; *Sum* – Summer; *Aut* – Autumn).

Site (Season) / OS marker	**Shk (Spr)**	**Slb (Spr)**	**SVlas (Spr)**	**Aht (Spr)**	**Shk (Sum)**	**Aht (Sum)**	**Kab (Aut)**	**Var (Aut)**
LPO nmoles MDA/mg protein	0.84 ±0.08	0.89 ±0.12	0.99 ±0.15	0.71 ±0.07	0.36 ±0.04	0.43 ±0.11	1.65 ±0.36	0.89 ±0.11
PO nmoles PC/mg protein	11.22 ±1.57	11.17 ±2.49	9.07 ±1.14	10.40 ±1.65	7.43 ±0.79	8.98 ±1.14	4.72 ±1.58	3.07 ±0.87
GSH ng/mg protein	345.10 ±64.50	330.70 ±63.30	370.80 ±87.10	385.30 ±54.80	1436.80 ±152.90	1457.10 ±184.20	393.10 ±92.00	460.70 ±53.30
SOD U/mg protein	3.02 ±1.34	2.75 ±0.54	5.18 ±1.32	1.99 ±0.71	0.59 ±0.09	0.87 ±0.14	12.54 ±1.84	8.85 ±1.34
CAT U/mg protein	2.56 ±0.40	2.83 ±0.56	2.59 ±0.37	2.53 ±0.25	1.17 ±0.27	1.33 ±0.32	3.16 ±1.32	2.44 ±0.91
GST U/mg protein	126.90 ±22.70	197.30 ±60.60	153.30 ±17.50	119.50 ±15.30	114.90 ±10.30	112.10 ±21.80	303.90 ±31.90	162.10 ±31.60

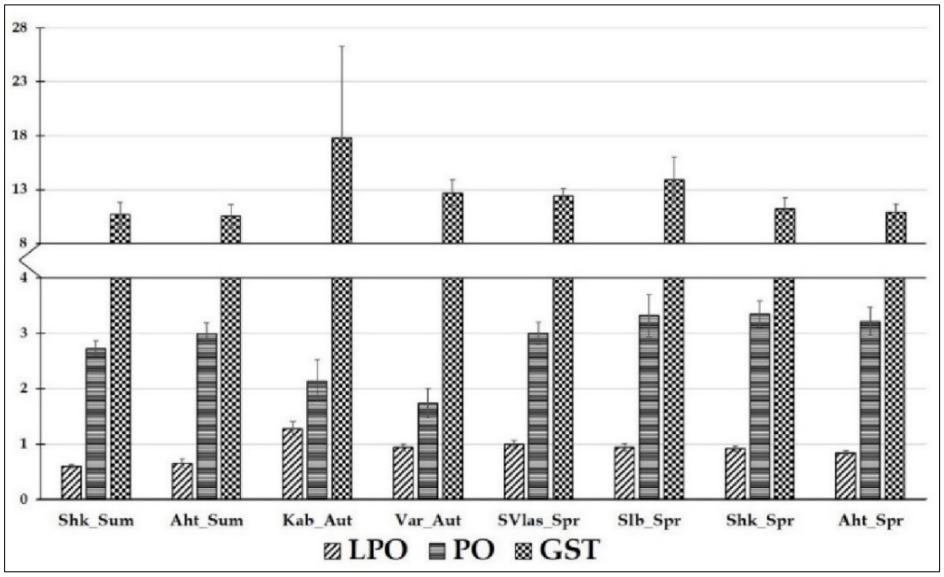

Fig. 2. Mean levels (±SD) of lipid peroxidation (LPO), protein oxidation (PO) and glutathione-s-transferase (GST) activity of *D. trunculus* from different locations (for abbreviations see Table 1). The data was square root transformed (n=6-10).

Table 2. Analysis of variance (ANOVA – factorial design) of the studied oxidative stress biomarkers in wedge clams from different sites.

	SS	DF	MS	F	P
Intercept	5649386	1	5649386	644.81	<0.0001
Site	1522310	7	217473	24.82	<0.001
Biomarker	16555132	5	3311026	377.91	<0.0001
Site*Biomarker	9372483	35	267785	30.56	<0.0001
Error	2304231	263	8761		

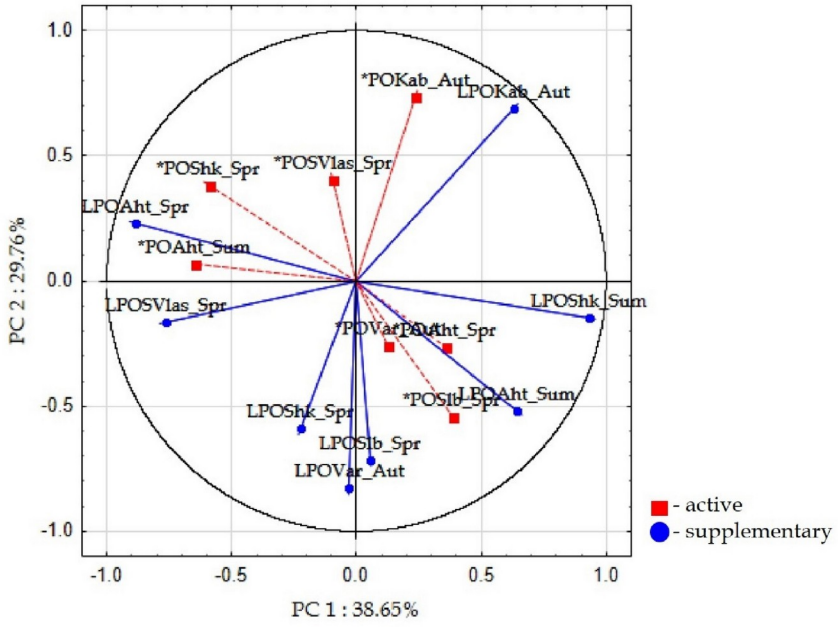

Fig. 3. Ordination graph of PCA analysis of LPO (active variable) and PO (supplementary variable).

The first two principle components (PC) explained 68.41% of the total variation (PC1 - 38.65% of the variation and PC2 -29.76% of the variation). The analysis demonstrated the presence of a gradient along PC1 from sites with relatively high LPO levels in wedge clams together with relatively high PO levels (on the left) towards sites with low LPO levels and relatively high PO levels in the wedge clams (on the right of the diagram). Along the second main PC axis the sites with intermediate LPO and high values of PO in the wedge clams (bottom of figure) are separated from the site with very low PO and with relatively high LPO (top of figure), i.e. the Kabakum Bay.

Discussion

The present paper reports result of the first preliminary comparative study of the level of OS in the wedge clams from several representative sites of the sandy sublittoral habitats along the Bulgarian Black Sea coast.

The wedge clam *Donax trunculus* L. is common in the Black Sea (Petrova & Stoykov, 2010) and is also widely spread in the Mediterranean Sea and the Atlantic coasts of western Europe. The wedge clams are used in monitoring programs for assessment of marine environmental pollution through direct measurement of several OS biomarkers in them (Amira et al., 2011; Sifi et al., 2013). OS is the result of misbalance of the pro-oxidant and antioxidant processes in organisms which is indicated by specific biomarkers. Oxidative processes in marine bivalves can be induced by different environmental pressures on their habitats and can result in OS cell damage. The ratio of the levels of markers of pro-oxidant processes and the antioxidant system activity can indicate the severity of the OS in the wedge clam tissues and hence the potential damages. One of the most sensitive OS markers is considered to be the lipid peroxidation (LPO), especially in bivalves, because of the high content of polyunsaturated fatty acids (Rudneva, 1999). Among the various products resulting from the peroxidation of lipids, the aldehydes (MDA) and hydroxynonenal (HNE) are the most significant and also most studied (Ayala et al., 2014). In the present study, highest rates of MDA were found in the tissues of wedge clams from Kabakum Bay. The oxidative modification of lipids leads to impairment of membrane fluidity and permeability causing damage of cellular metabolism and ultimately disruption of membrane and cell dead (Ayala et al., 2014). Increased LPO in *D. trunculus* has been specifically associated with urban, harbor, agricultural and industrial pollution of sea waters (Amira et al., 2011; Sifi et al., 2013).

Overproduction of ROS in response to environmental stress can lead not only to high LPO in cell membranes, but also to increased protein oxidation (PO). As a marker of PO, protein carbonyls (PC) are widely used, also in aquatic organisms (Merad & Soltani, 2015). Carbonylation of proteins is the most commonly occurring oxidative protein modification. A significant increase in PC has been demonstrated in clams after exposure to metals (Merad et al., 2016) and organic compounds (Xiu et al., 2016). Since the carbonyl groups are chemically stable, the carbonylation is irreversible and unrepairable. Carbonylation of proteins leads to alteration of protein functions, incl. inhibition of enzyme activities or increase of their susceptibility to proteolytic attack. Interestingly, in our study low PC values were found in the samples from Kabakum Bay and Varna Bay. On the other hand, our results showed high level of OS in the clams from Kabakum Bay. It could be assumed that the reduction of PC levels in the wedge clams from this site could have been the result of activation of metabolic pathways to remove the damaged proteins (Grune et al., 2011). In support of this assumption is the reported observation that during the depuration period after Cd intoxication, lower levels of PO were present in *D. trunculus* (Merad et al., 2016).

The induction and development of OS depends not only on the activity of pro-

oxidants but also on the strength and activity of the antioxidant cell defense system, which includes enzymatic and non-enzymatic components. A major cellular non-enzymatic antioxidant is accepted to be GSH and its depletion is considered as a marker of OS development. Reduction of this tripeptide has been found in a number of bivalves exposed to pollutants and toxicants (Sifi et al., 2013). The importance of GSH for the antioxidant protection of cells is essential as it is involved not only in the direct scavenging of ROS, but is also a co-substrate of important enzymes. One such enzyme is GST, which, in addition to being involved in the elimination of OS products, is an important enzyme in phase II of xenobiotic detoxification (Wojtal-Frankiewicz et al., 2017). Induction of GST activity in different mussels has been established after exposure to PAHs, PCBs, dioxins (Van der Oost et al., 2003) and metal pollution (Vidal-Liñán et al., 2014).

Our results suggested that the very low GSH content observed in wedge clams from Kabakum Bay was probably related to its depletion upon the action of GST, which in turn showed excessively high activity in these samples. On the other hand, the high GSH levels measured during the summer were probably a prerequisite for the detected low LPO levels. Similar reciprocal relationship between these two OS markers has been often reported (Gostyukhina & Andreenko, 2020). High levels of GSH in different bivalves in summer have been established also in earlier studies (Power & Sheehan, 1996).

The antioxidant enzymes SOD and CAT are also recognized indicators of OS. They are being activated as an adaptive response to toxicants effects, allowing partial or complete overcoming of the OS in a polluted environment. Such effects have been reported in *M. galloprovincialis* (Serafim et al., 2011), *Chamelea gallina* (Rodríguez-Ortega et al., 2002), *Perna perna* (Jourmi et al., 2015) and *D. trunculus* (Amira et al., 2011). However, prolonged exposure to toxicants can lead to inhibition of the antioxidant enzymes due to deterioration in the state of organisms caused by chronic stress (Regoli et al., 2003). High doses of xenobiotics have a similar inhibitory effect (Trevisan et al., 2014). Thus, the antioxidant enzymes exhibit a "bell-shaped" response curve with increasing the dose or the exposure time (hormesis effect) (Marigomez et al., 2013). The presence of such hormesis effects has been reported in *M. galloprovincialis* (Tsangaris et al., 2008) and *Mytilus edulis* (Yaqin et al., 2008). This dose-response phenomenon is recently recognized as the basis of adaptive mechanisms and resistance to environmental stress in organisms.

Conclusions

The results of this study strongly indicated that the wedge clam *D. trunculus* is a suitable biological model which can be used to assess the stressfulness of the marine environment of its habitats and can thus present an "early-warning" signal in ecological biomonitoring.

Acknowledgements

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