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Adaptive Responses of Key Species from Sozopol Bay to Multiple Environmental Stressors as Indicators of the Ecological State of Habitats

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Abstract: Sozopol Bay was a dry land area with a system of lakes/marshes until 7.000 years ago, before the sea transgression. Now, a submarine "petrified forest" from this time is exposed on the bottom of the bay, representing a new specific habitat. This study aimed to assess the health condition and adaptive capacity of bivalves and fish species as bioindicators of the state of the marine environment in these habitats. The studied bivalves were *Donax trunculus* (Linnaeus, 1758), *Mytilus galloprovincialis* (Lamarck, 1819), *Chamelea gallina* (Linnaeus, 1758), *Flexopecten glaber* (Linnaeus, 1758) and *Crassostrea gigas* (Thunberg, 1793). The demersal fish studied were *Neogobius melanostomus* (Pallas, 1814), *Mullus barbatus* (Linnaeus, 1758), *Merlangius merlangus* (Linnaeus, 1758), *Platichthys flesus* (Linnaeus, 1758), *Scorpaena porcus* (Linnaeus, 1758) and *Trachinus draco* (Linnaeus, 1758). Oxidative stress (OS) biomarkers were measured to estimate the species' health status and stress tolerance. The bivalves from infralittoral sandy habitats were in good health, indicating a good state of the habitats. OS was higher in the bivalve and fish species associated with infralittoral rock habitats, indicating less favourable ecological conditions. In conclusion, the studied species can tolerate the present environmental conditions of the habitats in Sozopol Bay and maintain normal health status.

Key words: Black Sea, bivalves, demersal fish, oxidative stress, petrified forest

Introduction

Sozopol Bay is a semi-enclosed bay in the Southern shelf region of the Bulgarian Black Sea with a max depth of 20m, situated between the towns of Sozopol and Chernomorez (Fig. 1A). The bay has a complex geomorphologic structure. According to Preisinger et al. (2000–2001), the present-day area of Sozopol Bay was not covered by seawater during the Last Glacial Maximum. In the period till ~7500 years BP, the Bay Area was an arid landscape with a lake/swamp system (Fig. 1B). A specific and rare natural geological phenomenon is the submarine coals exposed as a "soil" bed of a rooted "petrified forest", situated on the bottom in the strait between Sozopol peninsula and St. Ivan Island (Šiškov et al. 1988, Yossifova et al. 2011) (Fig. 2).

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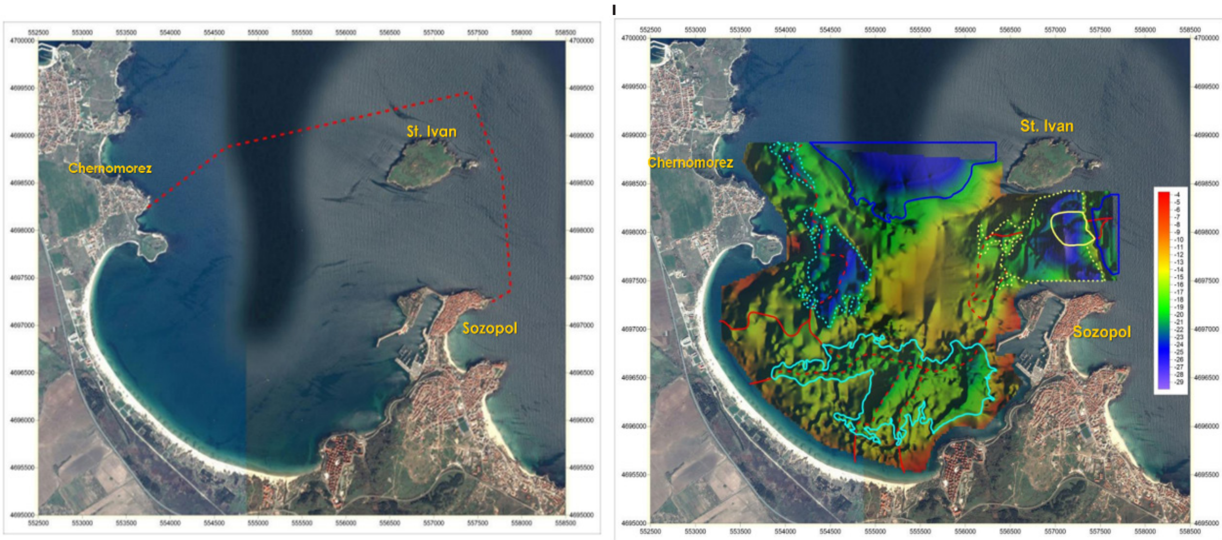

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Fig. 1. Sozopol Bay with the NATURA 2000 site area (dashed line) (A) and field model of the main bed of the bay (B) (after Velkovsky, 2021): "...of the bay (B) (after Velkovsky, 2021)".

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Fig. 2. Silicified fossil logs from the underwater forest with attached *M. galloprovincialis* (A) and other macrobenthic species (B).

The area of Sozopol Bay comprises a variety of marine habitats, including sandbanks, rocky reefs, seagrass meadows, and biogenic reefs. Part of the region is also a NATURA 2000 site (Fig. 1A). The underwater forest with exposed silicified trunks rooted in submarine coals presents a specific and still not sufficiently characterised habitat (Fig. 2).

The evaluation of the status of Black Sea marine habitats is traditionally based mainly on the measurement of physical parameters of seawater and sediments in compliance with the Marine Strategy Framework Directive of the EU (https://eur-lex.europa.eu/eli/dir/2008/56/oj). However, this approach alone cannot be considered objective enough to determine the habitat's environmental status locally because the environmental and anthropogenic stressors can form complex mixtures, and their interactions can affect local habitats and their biodiversity differently (Steinberg 2012, Heys et al. 2016). In addition, the health status of the organisms in local habitats is never or rarely considered. A more objective assessment of the multifactorial stressogenicity of the environment and the tolerance of marine organisms to stress can be obtained by the stress-response approach using the analysis of suitable markers of biological effects, also known as stress ecology (Van Straalen 2003, Van der Oost et al. 2003, Steinberg 2012, Hook et al. 2014, Lemos 2021). With the development of stress ecology, it is accepted that lower levels of biological organisation (molecular and cellular) are more sensitive to the stressful effects of the marine environment and that they can also be used as early warning indicators for changes in populations, communities, and ecosystems (Lemos 2021). It is already well established that in aerobic organisms, multiple environmental stressors can induce oxidative stress (OS), which consists of a disruption of the balance between the pro-oxidative and anti-oxidative processes in the cells and results in a redox imbalance (Halliwell & Gutteridge 2007, Betteridge 2000). Thus, the shifts in the redox balance and the resulting OS have the potential to be used as indicators in the research and monitoring of the marine environment and ecosystems (Hermes-Lima & Zenteno-Savín 2002, Hook et al. 2014).

This study aimed to assess the health condition and tolerance capacity using OS biomarkers of key bivalve and fish species as bioindicators of the environmental status of the habitats in Sozopol Bay.

Materials and Methods

Sampling

Specimens of *Donax trunculus* (Linnaeus, 1758) *Mytilus galloprovincialis* (Lamarck, 1819), *Chamelea gallina* (Linnaeus, 1758), *Flexopecten glaber* (Linnaeus, 1758), and *Crassostrea gigas* (Thunberg, 1793) were collected from natural habitats in Sozopol Bay (2018-2022) (Fig. 1A), including the NATURA site, to cover the diversity of local environmental conditions. The bivalves were gathered manually by diving at 1-7 m depth. Additional samples of *M. galloprovincialis* from logs on the sea floor of the underwater petrified forest (13-15 m) were also gathered (Fig. 2). The bivalve samples were cleaned on the spot, shock frozen in styrofoam boxes with dry ice pellets and transferred to the laboratory.

For the study of demersal fish, samples of *Neogobius melanostomus* (Pallas, 1814), *Mullus barbatus* (Linnaeus, 1758), *Merlangius merlangus* (Linnaeus, 1758), *Platichthys flesus* (Linnaeus, 1758), *Scorpaena porcus* (Linnaeus, 1758), and *Trachinus draco* (Linnaeus, 1758) were gathered. Fish were also obtained from monitor trawling in the region of the wider Sozopol Bay. The fish were shock-frozen and transferred to the laboratory.

Tissue preparation

The bivalves and fish were thawed and dissected on ice for biochemical analysis. The bivalves' soft body and the liver and gills of the fish were excised. The extracted tissues were homogenised in 100 mM potassium phosphate buffer (pH 7.4). The homogenates were centrifuged for 10 min at 3000 g to obtain a post-nuclear fraction to determine lipid peroxidation and glutathione levels. A portion of the fraction was re-centrifuged at 12 000 g for 20 min to get a post-mitochondrial supernatant to measure the antioxidant enzyme activity. All work was carried out at 4°C.

Measurement of oxidative stress biomarkers

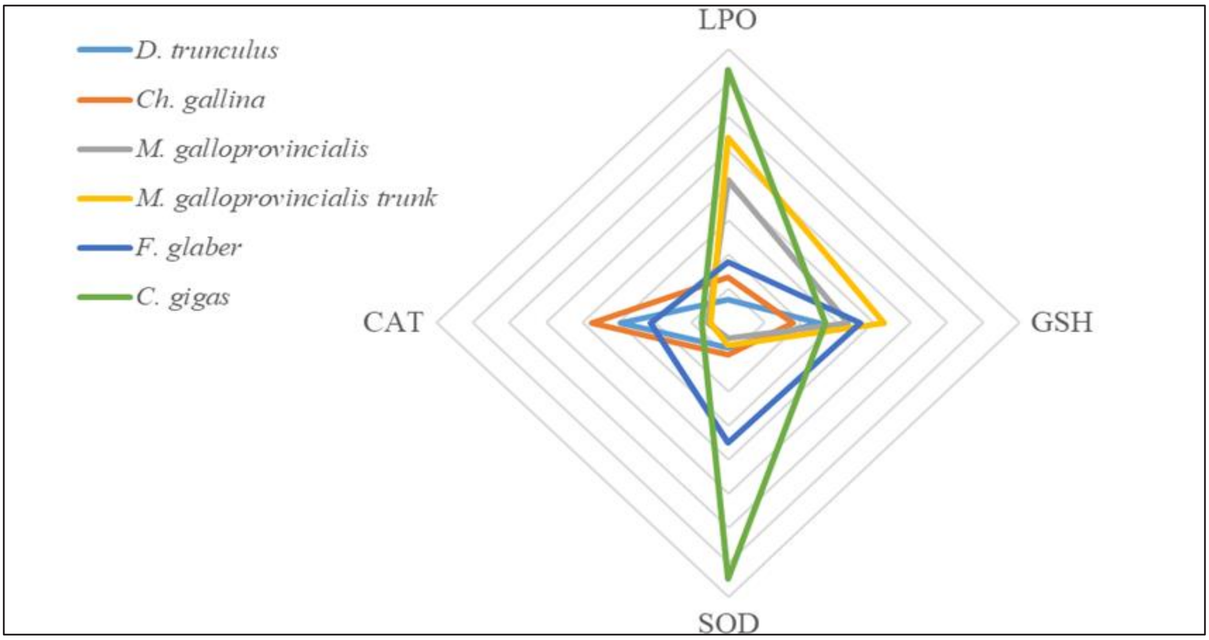
Kits purchased from Sigma-Aldrich Co. LLC, USA, were used for spectrophotometrical measurement of Lipid Peroxidation (LPO) (MAK085), Glutathione (GSH) (CS0260), and the activities of Superoxide dismutase (SOD) (19160), Catalase (CAT100), Glutathione peroxidase (GPx) (CGP 1), Glutathione reductase (GR) (GRSA), Glutathione-S-Transferase (GST) (CS0410) and Acetylcholinesterase (AChE) (CS0003).

Protein concentration was measured according to LOWRY et al. (1951) and calculated from a standard curve obtained using bovine serum albumin as a standard.

Results

The results of the measured OS markers in the studied bivalve species are presented in Table 1. The bivalve species studied showed apparent differences in the markers of OS, which were obviously induced by environmental factors in their habitats. The comparisons of the level of basic OS markers among bivalve species are presented graphically (Fig. 3). The two species typical for the infralittoral sands, *D. trunculus* and *C. gallina*, showed low levels of OS. In *D. trunculus*, the LPO were low, and the average values of the antioxidant protection indicators were moderate. In *C. gallina*, medium levels of LPO were present, but there was low GSH concentration and CAT activation. The early phase stress in this species with mobilisation of the non-enzymatic antioxidant and activation of the enzymatic reaction can be tolerated.

In *M. galloprovincialis* from infralittoral rocks distributed in Sozopol Bay, relatively high LPO and high concentration of glutathione, combined with low activities of antioxidant enzymes, were present (Table 1, Fig. 3), indicating a presence of OS, probably due to probably due to suppression of the enzymatic antioxidant protection. For the first time,



	D. trunculus	*Ch. gallina*	*M. galloprovincialis (infralittoral rocks)*	*M. galloprovincialis (silicified trunks)*	*F. glaber*	*C. gigas*
LPO	0,66	1,34	4,18	5,40	1,76	7,38
GSH	248,21	177,79	330,22	428,28	364,03	266,55
SOD	7,19	9,36	4,59	6,47	35,09	74,60
CAT	2,93	3,75	0,54	0,49	2,14	0,72
OS status	**very good**	**good**	**satisfactory**	**satisfactory**	**adaptation**	**compensation**
OS tolerance capacity	**high**	**high**	**moderate**	**moderate**	**active adaptation**	**active adaptation**

Fig. 3. Star diagram of the measured values of the basic oxidative stress markers in bivalves and a comparative table of their stress tolerance capacity.

M. galloprovincialis OS marker measurements were conducted on specimens attached to silicified logs of the underwater forest. These specimens also had relatively high LPO and low activity of antioxidant enzymes. Still, their GSH concentration was higher, showing somewhat greater protection than the mussels from the infralittoral rock habitat.

In the studied specimens of *M. glaber*, intermediate levels of OS were established and characterised by the medium level of LPO, high GSH, and high activity of antioxidant enzymes. This activation of the enzymatic and non-enzymatic defence systems in the cells of *M. glaber* can sufficiently compensate for the stress impact of the marine environment of Sozopol Bay (Table 1, Fig. 3).

The Pacific oyster (*C. gigas*) is a non-indigenous species introduced on the Bulgarian Black Sea shelf. It is now present as a single individual, forming wild micro populations on infralittoral rocks and hard bottom substrate. In the studied oysters, very high LPO values were present, accompanied by very high activity of SOD and the lowest recorded activity of CAT (Table 1, Fig. 3), indicating the activation of the stress-protective system of cells allowing the successful adaptation of this species.

The demersal indicator species of fish studied showed different responses and sensitivity to the multiple stressor effects of the marine environment in the Sozopol Bay area, expressed by changes in the measured OS markers. In general, in all studied

Table 1. Values of measured oxidative stress markers (mean±SD) in bivalves from Sozopol Bay**.**

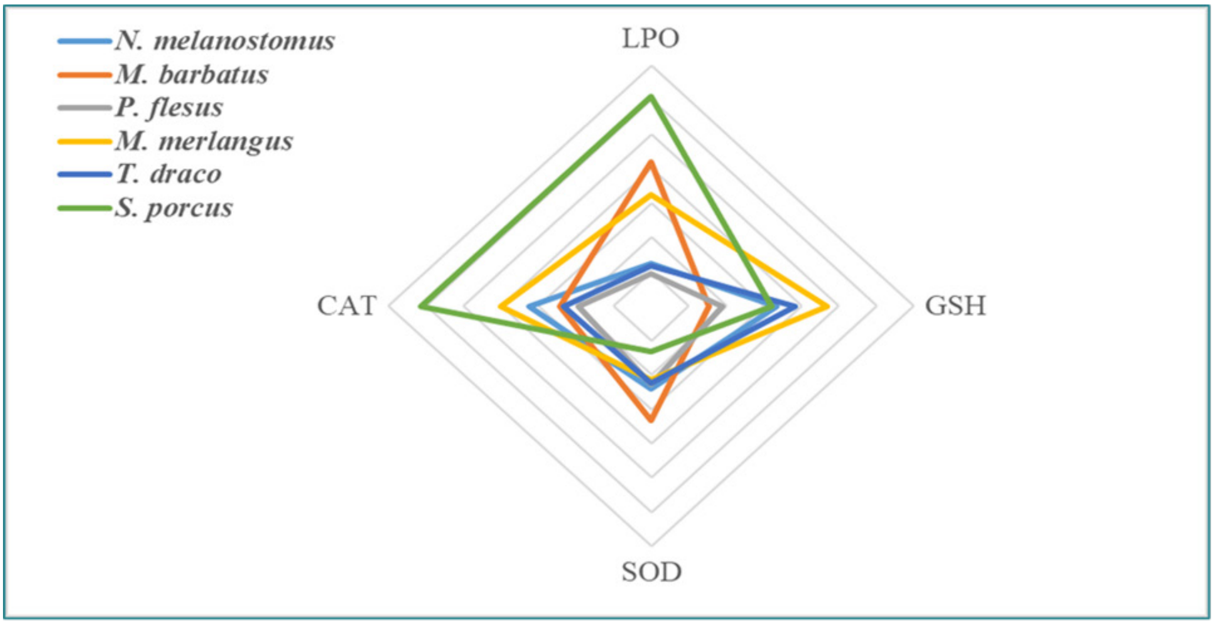
	LPO	GSH	SOD	CAT	GPx	GST
	nM MDA/ mg prot	ng/mg prot	U/mg prot	U/mg prot	U/mg prot	U/mg prot
D. trunculus	0.664	248.21	7.188	2.934	2.078	178.69
	±0.54	±69.87	±2.95	±0.54	±1.34	±47.79
Ch. gallina	1.33	177.79	9.36	3.75	2.15	42.87
	±0.33	±58.28	±5.76	±0.72	±1.81	±3.62
M. galloprovincialis **infralittoral rocks**	4.182	330.21	4.588	0.536	4.137	4.436
	±1.49	±117.99	±2.58	±0.26	±0.95	±0.42
M. galloprovincialis **silicified trunk**	5.363	428.28	6.472	0.492	9.645	53.494
	±3.041	±114.12	±0.81	±0.10	±2.15	±8.77
F. glaber	1.75	364.03	35.08	2.140	4.38	152.54
	±0.349	±70.23	±6.25	±0.396	±1.039	±14.41
C. gigas	7.38	266.54	74.63	0.720	14.03	21.71
	±2.81	±88.12	±15.8	±0.19	±2.16	±2.22

Table 2. Values of measured oxidative stress markers (mean±SD) in demersal fish from Sozopol Bay region.

	LPO	GSH	SOD	CAT	GPx	GST	AChE
	nM MDA/ mg prot	ng/mg prot	U/mg prot	U/mg prot	U/mg prot	U/mg prot	U/mg prot
				Liver			
Neogobius melanostomus	1.22	402.52	13.76	12.25	10.76	80.97	8.10
	±0.79	±253.8	±12.01	±4.98	±8.21	±33.3	±5.5
Mullus barbatus	1.19	467.2	59.9	7.72	3.29	336.8	114,4
	±0.33	±71.7	±9.74	±0.57	±1.12	±72.7	±62.6
Merlangius merlangus	5.42	958.5	25.26	14.6	9.21	37.2	46.1
	±6.7	±372.8	±12.9	±16.7	±7.5	±33.5	±13.4
Platichthys flesus	0.86	443.6	24.4	7.10	30.14	47.96	48.37
	±0.31	±174.2	±16.9	±5.85	±19.05	±14.79	±4.23
Trachinus draco	0.695	634.579	4.915	6.500	23.76	27.05	75.01
	±0.035	±9.236	±0.290	±0.679	±0.49	±0.36	±1.73
Scorpaena porcus	0,64	582.36	26.99	9.02	20.64	27.27	81.34
	±1.27	±54.94	±0.32	±0.27	±2.90	±1.68	±1.27
				Gills			
Neogobius melanostomus	3.66	929.69	34.49	0.84	10.84	27.73	10.25
	±4.01	±244.51	±3.86	±0.45	±0.72	±25.05	±1.96
Mullus barbatus	15.57	147.54	6.76	1.99	14.34	39.61	104.5
	±4.113	±44.453	±1.759	±0.285	±6.139	±7.802	±17,29
Merlangius merlangus	7.58	910.6	17.57	1.38	7.61	14.45	10.67
	±7.38	±209.41	±16.20	±0.31	±5.01	±3.66	±1.92
Platichthys flesus	2.90	313.62	21.12	0.71	7.17	20.22	37.59
	±1.04	±177.34	±9.59	±0.43	±6.04	±6.56	±12.74
Trachinus draco	3.93	904.56	40.15	2.81	21.30	97.29	20.83
	±0.219	±9.274	±1.315	±0.424	±1.159	±1.301	±2.36
Scorpaena porcus	13.23	411.76	18.84	0.45	14.29	18.70	52.58
	±1.24	±45.10	±3.23	±0.12	±1.17	±3.44	±4.38

fish species, clear differences were present between the values of OS markers in the liver and gills (Table 2, Fig. 4), which could be explained by the direct contact of gills with the surrounding marine environment.

The benthic species *N. melanostomus* showed intermediate values for all measured OS markers in the liver, except for very low AChE activity (Table 2). In the gills, high concentrations of GSH and SOD activity were present. This finding suggested that the



marker	organ	*N. melanostomus*	*M. barbatus*	*P. flesus*	*M. merlangus*	*T. draco*	*S. porcus*
LPO	**Liver**	1,22	1,19	0,86	5,42	0,69	11,20
	Gills	3,66	15,57	2,90	7,58	3,93	13,23
GSH	**Liver**	402,52	467,22	443,65	958,53	634,57	871,55
	Gills	929,69	147,54	313,62	910,69	904,56	411,76
SOD	**Liver**	13,76	59,92	24,44	25,26	4,92	7,93
	Gills	34,49	6,76	21,12	17,57	40,15	18,83
CAT	**Liver**	12,25	7,72	7,10	14,63	6,50	24,12
	Gills	0,84	1,99	0,71	1,38	2,81	0,45
OS status		**active OS compensation**	**stressed**	**good (low stress)**	**active OS compensation**	**good (low stress)**	**stressed**
OS tolerance capacity		**moderate**	**limited**	**high**	**moderate**	**high**	**limited**

Fig. 4. Star diagram of the measured values of the basic oxidative stress markers in the liver and gills of fish, and a comparative table of their stress tolerance capacity.

fish were under pressure from environmental stressors in their habitats in Sozopol Bay but were compensating for these effects by activating their antioxidant system. The very low AChE activity in the liver indicated organic pollution and/or eutrophication. In general, the gobies can tolerate variations in the environmental conditions of their habitats.

Among the other benthic species studied, the lowest levels of LPO were measured in the liver and gills of *P. flesus*, and average values of the other OS markers were present. This species seemed to successfully tolerate the variations in the Sozopol Bay environment (Table 2, Fig. 4).

In the other benthic species, *S. porcus,* high values of LPO in both the liver and gills were measured, along with high GSH concentrations and CAT activities in the liver. These results suggested that *S. porcus* had a high level of induced OS, which is compensated by the activated liver antioxidant system. Thus, the species seemed to have limited ability to tolerate the large variations in the effect of environmental factors in the Sozopol Bay area. (Table 2, Fig. 4).

In contrast, the venomous benthic and demersal species *T. draco* showed increased levels of antioxidant protection in the gills, where the highest values of the enzymes SOD, CAT, GPx and GST were measured, as well as high concentration of the non-enzymatic antioxidant GSH. In the liver, however, average values of the measured markers were present, except for a low activity of SOD. The activated antioxidant defence in gills suggested that *T. draco* had a high potential to tolerate variations in the environmental factors in the region of Sozopol Bay (Table 2, Fig. 4).

High activities of SOD and GST were measured in the liver of *M. barbatus*, together with low GPx activity. Very high LPO and low concentrations of GSH and SOD were measured in the gills. A high activity of AChE was found in both the liver and gills. This indicated the presence of OS in gills and some limitation of the capacity to tolerate the significant variations in the environmental factors in the species' habitats (Table 2, Fig. 4).

In the liver of *M. merlangus,* high LPO values were measured, and high concentrations of GSH were present in both the liver and gills. Low GPx activity was also present in both the gills and the liver. The measured AChE in gills was relatively low, which may indicate the effects of organic pollution or eutrophication of the environment on this benthopelagic fish species in the region of Sozopol Bay (Table 2, Fig. 4).

Discussion

The measured OS markers were used to estimate shifts in the balance of the pro-oxidant and antioxidant processes in the indicator bivalves and show their adaptive potential to the environmental conditions of the local habitats.

Taking into account the main indicators of OS (LPO as a marker of pro-oxidant processes, GSH as a non-enzymatic antioxidant and the two main antioxidant enzymes SOD and CAT), we can conclude that *M. galloprovincialis,* which was dominating the infralittoral rock habitats, displayed shifts in the pro-antioxidant balance and were subjected to strong OS (high LPO and low activity/inhibition of antioxidant enzymes). This suggested the possible presence of xenobiotics and indicated that the environmental status of this species' habitat in Sozopol Bay was not sufficiently good.

In the current study, mussels growing on fossil logs of the underwater petrified forest rooted in the bottom of Sozopol Bay were studied for the first time. They had higher concentrations of the essential antioxidant GSH, which indicated that they were better protected against environmental stress. Specifically, GSH levels are defined as one of the important indicators of the state of an antioxidant system, since this tripeptide, in addition to direct antioxidant activity, also functions as a hydrogen donor for antioxidant enzyme systems and maintains sulfhydryl groups of functionally important proteins in a reduced state (Kenya et al. 1993).

The mussels growing on rocky habitats (including fossilised logs) in Sozopol Bay seemed capable of tolerating environmental variations, and their health condition could be described as satisfactory. However, it may be suspected that their OS tolerance is at the limit, and future significant environmental pollution or other changes, including temperature rise, may have negative consequences on their health condition.

The other two examined bivalve species, *Ch. gallina* and *D. trunculus*, indicative of the sandy habitats, were characterised by relatively low LPO and activated antioxidants to varying degrees, indicating good health and adaptation to the environmental conditions of their habitat in Sozopol Bay, which is also included in the NATURA protected area. Of these species, the healthiest was *D. trunculus,* with low LPO and medium levels of antioxidant defence, indicating high tolerance to the environmental conditions of the sandy habitat. The known physiological and behavioural adaptations of *D. trunculus*, in particular their ability to burrow (Gumus et al. 2020) and actively move (swim actively by water jets from the mantle cavity, generated by shell adductions) (Gaspar et al. 2002), probably helped them to avoid unfavourable conditions. The environment had a more negligible negative influence on them. These results confirmed the capacity of the two clam species to be good sandy habitat condition indicators, as shown in an earlier study (Georgieva et al. 2022).

Relatively high antioxidant protection was present in the studied *F. glaber* individuals. This likely contributes to the successful recovery and adaptation of the smooth scallop populations to its habitats in the Bulgarian Black Sea, along with improved environmental conditions such as reduced levels of eutrophication, warming waters and a decline in the rapana whelk (*Rapana venosa* Valenciennes, 1846) population (Todorova et al. 2022).

The Pacific Oyster (*C. gigas)* is a non-indigenous species, reported to be present in the Bulgarian Black Sea region since 2010, which can now be found forming wild micro populations in different sites along the coast and not just as single scattered individuals (Mitov et al. 2020). The *C. gigas* from the rocky habitats of Sozopol Bay showed similarity to the black mussels in the high pro-oxidant LPO levels, but this was partially compensated by the activation of SOD and indicated the presence of active adaptation of the species to the local environmental conditions. Other studies have also shown that oyster survival correlates positively with SOD activity (Fuhrmann et al. 2018).

Overall, our study's analyses of the demersal fish confirmed the bivalve species' results. The marine environmental conditions in the region of Sozopol Bay induced different levels of OS in the different fish species studied. Specifically, there were differences in the OS in fish gills and liver. Intensive detoxification processes were present in the liver, which usually exhibits the highest antioxidant levels in fish (Aksnes & Njaa 1981; Wilhelm Filho et al. 1993). Taken as a whole, fish livers exhibited a somewhat better control of OS effects than the gills, allowing minimisation of intracellular damage when exposed to environmental stress conditions (Lattuca et al., 2009). It is proven that in benthic fishes, the high activity of detoxifying processes is triggered by habitat stressors, namely the accumulation of pollutants in bottom sediments and shallow water layers (Rudneva et al., 2010, Alexandrova et al., 2021). On the other hand, our study indicated that gills are more sensitive to OS induced by pollutants in the environment compared to the liver, which was also presented in previous studies (Ahmad et al. 2004, Guilherme et al. 2012, Alexandrova et al. 2021). The reason for this may be the direct contact of the gills with the marine environment and the pollutants in it, as well as the active metabolism in this organ (de Castro Sachi et al. 2021).

The summary analysis of the fish studied clearly showed that *S. porcus* was most stressed (with high LPO in both liver and gills), followed by *M. barbatus*. This indicated the presence of OS in the gills and some limitation of the capacity of the fish to tolerate significant variations in the environmental factors of the region of Sozopol Bay, probably due to their way of life. They are benthic fish in constant contact with sediments, where various pollutants are deposited, and are predatory. It is possible that biomagnification of xenobiotics from the trophic chain can occur in them. On the other side were *P. flesus* and *T. draco,* which had the lowest OS. In *T. draco*, this was most likely due to the activated antioxidant defences in their gills.

In conclusion, the environmental conditions in marine habitats in different localities can exercise different stressogenic effects on the organisms living there, whose response to stress can vary, depending on the species and the locality. Our study showed that the key bivalve and fish species of the Sozopol Bay habitats can tolerate the existing ecological conditions of their habitats and maintain a normal health status.

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